Analysis of Cosmetic Products

Editors: Amparo Salvador and Alberto Chisvert

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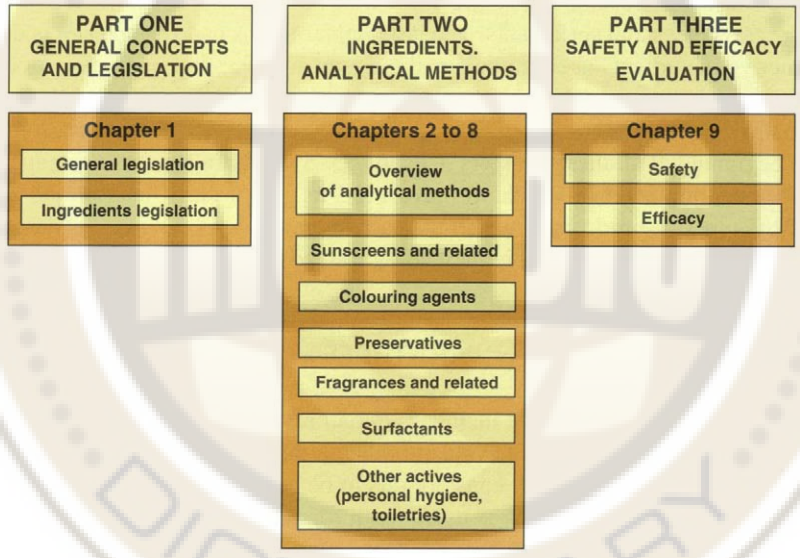
Preface

Both editors of this book are analytical chemists who have been working together for more than eight years on the development and validation of analytical methods for cosmetic products, and particularly in the field of sunscreen products.

In this multi-author book, we would like to share our experience in this field with our readers and give them advice, with the help of the other authors participating in this book, on the choice, which is often difficult, of suitable analytical methods for production monitoring and quality control of cosmetic products according to their composition.

To do this, we have tried to give the reader extensive and varied information on the topic, so that the reader can gain insight into the aspects related to the world of cosmetics from the viewpoint of Analytical Chemistry. We have divided the book into three parts.



PART ONE

This part (Chapter 1) sets out the definitions and general concepts regarding cosmetic products, current legislation in different countries is discussed as well as specific legislation on ingredients.

PART TWO

The central body of this book is dedicated to analytical methods for monitoring and quality control of cosmetic products.

The fundamental objective of this part of the book is to put at the reader's disposal scientific reviews, carried out by experts in Analytical Chemistry, about existing methods to be found in the bibliography for different types of analytes and/or cosmetic samples, their use, potential, etc. also indicating the sources where one can find the original article, with the idea that the reader him/herself will be the one to choose the method that he/she considers most suitable to tackle a specific analytical problem.

To begin with, Chapter 2 offers the reader information about the official methods for cosmetic product analysis and how to access the corresponding reference documents, and also gives a global vision of published analytical methods as a whole, which are later dealt in greater depth in subsequent chapters.

Later, a detailed revision is given of the published analytical procedures specific to the different analytes and cosmetic samples, including sample preparation, analytical techniques to be used, particular methodologies, etc. The first chapters (Chapters 3–5) are devoted to those ingredients to which specific legislation applies in some countries, i.e. UV filters, colouring agents and preservatives. We have dedicated two chapters (Chapters 6 and 7) to other types of ingredients that, like the former, are of great interest in cosmetics and are fragrances and surfactants. The last chapter in this part (Chapter 8) is dedicated to other active ingredients used for personal hygiene and face and body care products. In these chapters not only usual ingredients are considered but also forbidden ones and contaminants.

At the beginning of all these chapters (Chapters 3–8) either introductions or special sections have been included in which we have tried to give a classification and/or global vision of the types of products in which the corresponding active ingredients are used, to introduce readers to the topic before they go on to read the chemical–analytical part.

PART THREE

To conclude, in Chapter 9 we have included a small review of the alternative methods by using animals for cosmetic product evaluation that, although they do not refer to chemical analysis of these products, we still consider this to be of interest to our readers.

We sincerely hope that this book is useful both for scientists and technologists specializing in cosmetic research, manufacturing and quality control as well as for students of cosmetic science and related topics and other people too, who are connected with the field of cosmetics (practitioners, consultants, etc.).

We are at the entire disposal of our readers, and will be delighted to answer any questions about the topics dealt with here, insofar as we are able, via electronic mail.

Alberto Chisvert Sanía
University of Alicante, Spain

Amparo Salvador Carreño
University of Valencia, Spain

Foreword

Everyone is aware of the unstoppable growth in the use of cosmetics worldwide. In recent years, men have come to use them almost as much as women. Cosmetic uses among babies and children have also expanded at an increasing pace and now provide easy solutions for needs which could not even be envisaged only a few years ago. Everybody, of course, is aware of the exorbitant amounts of money handled in the world of cosmetics.

Few people other than dedicated scientists are aware of the extremely wide range of general and specific cosmetics currently available for purposes such as (a) facial treatment (lips, eyes and hair included) and body care (hands, nails and feet included), which is provided by creams, emulsions, lotions, gels, oils, lipsticks, face masks and antiwrinkle products, among others; (b) personal hygiene products for which include toilet and deodorant soaps, bath and shower preparations, deodorants and antiperspirants, depilatories, shaving creams and gels, after-bath powders, hygienic powders, make-up cleansers, teeth and mouth care products, external intimate hygiene products or hair cleansers; and (c) sunscreens and related products (e.g. sunbathing lotions, products for tanning without sun, skin whiteners).

In addition, these products vary in composition according to skin type (normal, oily, dry, mixed or sensitive), age (baby, child, young, adult, elderly) and ethnic group (white, eastern, black). Also, each cosmetic manufacturer uses their own traditional ingredients, and there is a growing trend of adding vitamins and a wide range of other compounds within nutraceuticals.

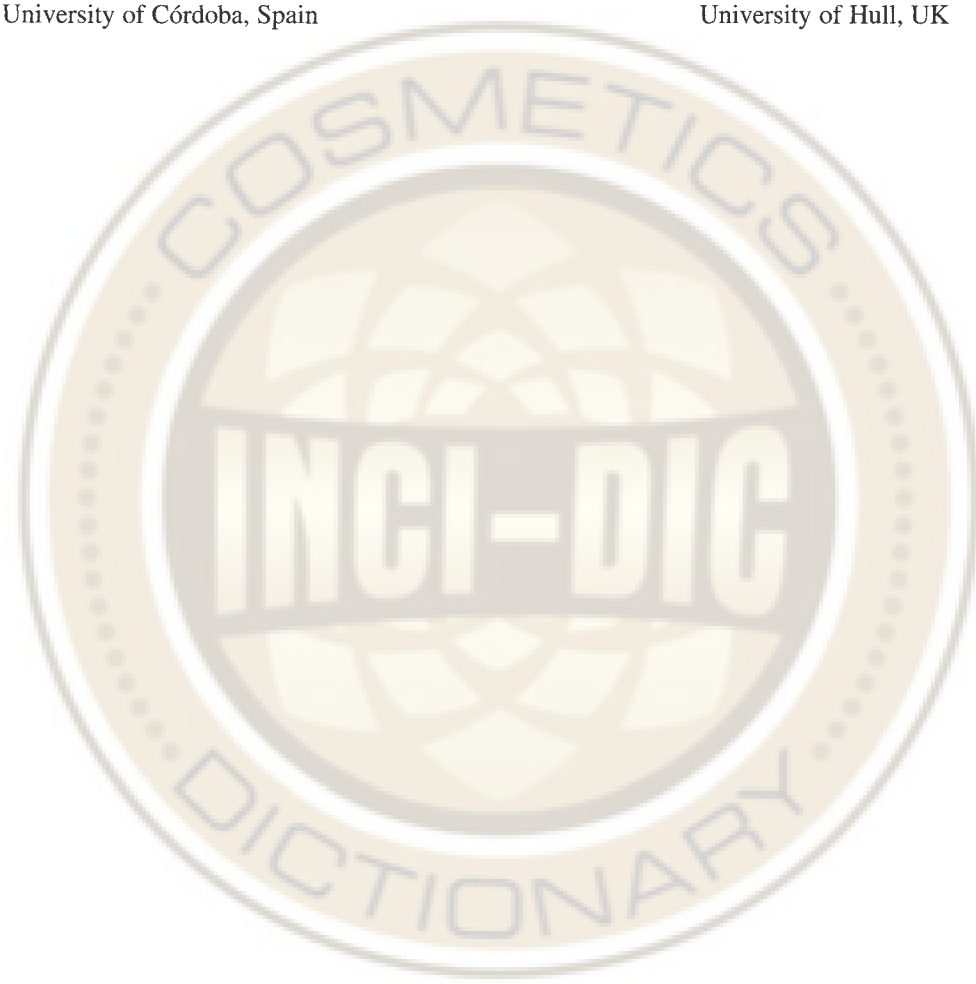
The wide range of products and the complexity of their composition present a formidable challenge to the analytical chemist, as well as to the toxicologist and the formulation scientist. A glance at the very complicated mixture of ingredients listed on the label of a popular sunscreen liquid gives a good indication of how challenging is the analysis of such a product and how important it is to employ officially validated methods. This book presents a thorough description of the analytical methodology applicable to cosmetic products. The scarcity or even absence of officially endorsed analytical methods for the control of cosmetics and their ingredients, however, together with the dispersion and poor documentation of available methods, is an important reason for producing a book such as this. Edited by analytical chemists well known for their expertise in cosmetics analysis, the book is extremely timely for cosmetics specialists, and also for non-specialists with some scientific curiosity about this topic.

The book includes updated legislation on cosmetics and an assortment of tables that illustrate the state-of-the-art analysis for individual cosmetics, ingredients, surfactants, etc. Particularly important is the inclusion of articles by industrial professionals involved in such analyses. Most of the authors are from France, Italy and Spain, with other from Switzerland, the USA and Argentina.

The Editors' engagement with the world of cosmetics is clearly reflected in the large number of references to their own publications — which is now expanded and diversified with the release of this book. This has facilitated careful selection of its contents, which will no doubt bridge existing gaps in this area.

Maria Dolores Luque de Castro
University of Córdoba, Spain

Alan Townshend
University of Hull, UK



Acknowledgements

While preparing the second edition of the *Encyclopaedia of Analytical Chemistry* (published by Elsevier in 2005), its editors Prof. Dr. Paul Worsforld, Prof. Dr. Alan Townshend and Prof. Dr. Colin Poole, placed their trust in our research group and gave us the opportunity to participate by writing the articles "Cosmetics and Toiletries" and "Perfumes". On the basis of this participation, Dr. Gilles Jonker (Publisher of Elsevier), proposed that we should prepare a book dealing with perfume analysis, which we finally decided to extend to encompass analytical methods for cosmetic products in general, with the Editor first assigned to this project being Mr. Derek Coleman (Development Editor), and later the contact persons becoming Ms. Joan Anuels (Administrative Editor), Drs. Anita Koch (Manager Editorial Services, Books), Andy Gent (Acquisition Editor), Paul Penman (Production Editor) and the production and editorial team of Macmillan India Limited. We would like to express our most sincere thanks to all of them for placing their trust in us, for their help and their contribution to the satisfaction we now feel on seeing this project finished.

We thank the authors from the different universities and international institutions for their participation. They have done a splendid job in all the sections—those with analytical–chemical content, those concerning legislation, alternative methods, etc., and we would like to give them special thanks for their patience in bearing with our many revisions and adaptations of the texts until finally managing to complete the book, with a uniform structure, an up-to-date content and of interest for all our readers.

We wish to thank equally the enthusiastic collaboration of the authors belonging to the different companies in the cosmetic sector, who have participated in writing the non-analytical sections of the different chapters, dedicated to introducing the reader to the different types of cosmetic products and ingredients. Their professional vision of cosmetic products, as chemists that carry out their work within this industry has, without doubt, enriched the contents of this book.

The coordination of the different "analytical" and "cosmetic" sections of this book has been a somewhat complicated task, though always interesting and we are happy with the result.

Without the valuable help of our proofreader, Ms. Fabiola Barraclough, director of Interglobe Language Links and efficient collaborator of Mètode (the University of Valencia's research review) and of our research group for many years, this project would not have been possible. She has worked, for us and with us, to improve the texts, making as great an effort as the rest of us, for which we thank her wholeheartedly.

We would like to thank the Spanish *Ministerio de Educación y Ciencia* (Ministry of Education and Science) for financing our research projects in the field of cosmetic product analysis, which has enabled us to continue this line of research for so many years.

We have also appreciated the support shown in our research work, as well as in the preparation of this book, by our many colleagues, who belong to either of the two institutions of which we form a part: The *Sociedad Española de Químicos Cosméticos* (SEQC) (Spanish Society of Cosmetic Chemists), and the *Sociedad Española de Química Analítica* (SEQA)

(Spanish Society of Analytical Chemists) to whom we wish to express our most sincere thanks. We specially wish to mention the support by Prof. Dr. Maria-Dolores Luque de Castro as well as her example and tireless work in Analytical Chemistry.

We would like to thank the support to Consellería de Sanitat de Valencia (Generalitat Valenciana) and specially Mr. Eliseo González Abellán, President of the working group on "Normas de correcta fabricación de cosméticos".

We would like specially mention to our colleague and friend Mr. Pascual Cuadrado Escamilla, R&D Director of RNB S.L.-Cosméticos, his large experience in the cosmetic field has been a great help for us.

Last but not least, we would like to thank our respective families, and also our friends, and ask them to forgive us for the lost leisure time that we should have spent with them and that was "stolen" by the preparation of this book, but we hope they will share the happiness we feel when at last we hold the finished project in our hands. We specially wish to mention and welcome Alberto Chisvert Jr. who came into this world during the final days of revision.

We wish to dedicate this book to the memory of Paquita Carreño Nogueroles† and Alberto Chisvert Marco†.

Alberto Chisvert Sanía
University of Alicante, Spain

Amparo Salvador Carreño
University of Valencia, Spain

List of Contributors

Balaguer A.	Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain
Benaiges A.	R&D Department. Provital S. A., Barcelona, Spain
Capellier R.	Laboratoires Clarins, Pontoise, France
Casas C.	DSM Nutritional Products, Tarragona, Spain
Cháfer A.	Department of Chemical Engineering, School of Engineering, University of Valencia, Valencia, Spain
Chaintreau A.	Firmenich S.A., Corporate R&D Division, Geneva, Switzerland
Chisvert A.	Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Alicante, Spain
Cuadrado P.	RNB S.L.-Cosméticos, Paterna, Valencia, Spain
De Orsi D.	Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Roma, Italy
del Cañizo Gómez C.	L'Oreal España, Madrid, Spain
del Pozo A.	Unitat de Tecnologia Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain
Diez Sales O.	Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Valencia, Valencia, Spain
Dorato S.	Department of Pharmaceutical Sciences, Facoltà di Farmacia, Università di Genova, Italy
Dormoy M.	Laboratoires Clarins, Pontoise, France
Fernández de Córdova Manent B.	Health Department, Regional Government of Comunidad Valenciana, Valencia, Spain
Fitoussi F.	Laboratoires Clarins, Pontoise, France
Gagliardi L.	Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Roma, Italy
Gennaro M.C.	Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro", Alessandria, Italy

Ginestar J.	Laboratoires Clarins, Pontoise, France
González Abellán E.F.	Health Department, Regional Government of Comunidad Valenciana, Valencia, Spain
Gosetti F.	Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro", Alessandria, Italy
Guillén P.	Quality Control Department, Provital S. A., Barcelona, Spain
Herráez Dominguez M.	Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Valencia, Valencia, Spain
León González Z.	Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain
López-Mahía P.	Departament of Analytical Chemistry, University of A Coruña, A Coruña, Spain and Institute of Environment, University of A Coruña, Pazo de Lóngora, A Coruña, Spain
López Nogueroles M.	Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain
March J.G.	Department of Chemistry, Faculty of Sciences, University of Islas Baleares, Palma de Mallorca, Spain
March Roselló G.A.	Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain
Mayor M.	Laboratoires Clarins, Pontoise, France
Milstein S.R.	Office of Cosmetics and Colors, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD, USA
Montoro-Martínez P.	Corporacion Dermoestética, S.A., Valencia, Spain
Muniategui-Lorenzo S.	Departament of Analytical Chemistry, University of A Coruña, A Coruña, Spain
Negri R.M.	Institute of Chemical Physics of Materials, Environment and Energy (INQUIMAE). Department of Inorganic, Analytical and Chemical Physics, School of Sciences, University of Buenos Aires, Buenos Aires, Argentina
Pla M.D.	Departamento I&D, Germaine de Capuccini S.A., Alcoi, Spain
Polati S.	Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro", Alessandria, Italy

Prada Rodríguez D. Departament of Analytical Chemistry, University of A Coruña. A Coruña, Spain and Institute of Environment, University of A Coruña, Pazo de Lóngora, A Coruña, Spain

Prieto-Blanco M.C. Departament of Analytical Chemistry, University of A Coruña, A Coruña, Spain

Salvador A. Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain

Scalia S. Department of Pharmaceutical Sciences, Facoltà di Farmacia, University of Ferrara, Ferrara, Italy

Scher A.L. Office of Cosmetics and Colors, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD, USA

Sisternes J. Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain

Torréns-Tomás A. Corporacion Dermoestética, S.A., Valencia, Spain

Valet B. Laboratoires Clarins, Pontoise, France

Viscasillas A. Unitat de Tecnologia Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

Vidal Gandía M.T. Department of Chemistry, Universidad Politécnica de Valencia, Valencia, Spain

Vivó-Sesé I. Departamento I&D, Germaine de Capuccini S.A., Alcoi, Spain

Weisz A. Office of Cosmetics and Colors, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD, USA

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General Concepts and Cosmetic Legislation

1.1. General Concepts. Current Legislation on Cosmetics in Different Countries

L. Gagliardi[1*] and S. Dorato[2]

[1]Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy
[2]Department of Pharmaceutical Sciences, Facoltà di Farmacia, Università di Genova, Italy

GENERAL ASPECTS

Cosmetics represent a global industry with their main markets to be found in the European Union (EU), the Unites States (US) and Japan with a value of 34.3, 25.7 and 11.9 billion euros, respectively, in 2004, according to data compiled by COLIPA, i.e. the European Cosmetic, Toiletry and Perfumery Association (COLIPA, 2005).

To ensure safety and efficacy, cosmetic products are regulated and controlled worldwide. However, harmonisation of laws dealing with cosmetics is far from being achieved and regulatory frameworks vary greatly between countries making it practically impossible for a global industry to sell the same product on all markets.

In the last decade of the 20th century, the three main markets were regulated by different models: a liberal one in the US based on regulations dating back to the late-1930s; in Japan administrative regulations restricted even the choice of common ingredients; while in the EU

*Corresponding author. E-mail address: luigi.gagliardi@iss.it

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a modern approach applying a vertical, risk-based directive. In recent years the latter has become an international model, which is easy to understand and more or less easy to apply.

The different national approaches to regulating cosmetics are connected to divergent cosmetic definitions, labelling requirements and lists of ingredients that are permitted, restricted or banned. Apart from these divergences, at the international level an easier distinction can be made by considering the so-called in-market control and pre-market approval approaches.

The key features of the in-market control system are the following:

1. Avoidance of time-consuming and expensive pre-market licensing/registration of products.
2. The company responsible for placing the cosmetic on the market is also responsible for the regulatory compliance, for the safety and efficacy of the product, and must be able to justify this on authority investigation.
3. Notifying the appointed competent authority of data concerning the company.
4. Effective in-market control by competent national authorities monitoring the market to check compliance with regulations. Market surveillance is performed through random control on the market (in retail shops), by routine checks on compliance with regulations or by systematic control of given product categories, in the event of a problem arising, like a consumer complaint.
5. In case of non-compliance there can be penalties of varying strength and if the manufacturer fails to prove the cosmetic is safe for use the product will be withdrawn from the market.
6. It encourages companies to ensure product safety and therefore it offers better health protection benefits.
7. Administrations must be aligned and compatible, not identical.
8. One safety standard for consumers.
9. It increases the competitiveness of the market.
10. It ensures that new products will be available to consumers more quickly.
11. It guarantees equal treatment of all companies.

On the other hand, the key features of pre-market approval are:

1. Registration time delays the launch of cosmetics that change quickly in line with developments in technology and/or fashion.
2. It does not prevent fraud or stop illegal products reaching the market.
3. In any event, in-market surveillance is needed to monitor the system, implying a duplication of enforcement procedures.
4. It restricts the availability of new products on the market.
5. Time-consuming procedures and high-registration costs imply higher cost for companies and consequently more expensive products.

THE EUROPEAN UNION COSMETICS DIRECTIVE

The EU Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products, known as the EU Cosmetics Directive, was adopted on

27 July 1976 and published in the Official Journal of the European Communities L 262 on 27 September 1976, and has, to date, undergone seven Council and Parliament amendments (i.e. changes in articles) and more than 30 Commission adaptations to technical progress (i.e. changes in the different Annexes regulating banned or restricted substances, cosmetic colourants, preservatives and UV filters).

The European Commission has overall responsibility for cosmetics legislation within the EU, while a competent authority that enforces the legislation is designated in each of the 25 Member States (Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxemburg, Malta, Netherlands, Poland, Portugal, Slovak Republic, Slovenia, Spain, Sweden, United Kingdom).

The EU Cosmetics Directive and its amendments represent an autonomous sectorial legislation, based on the principle of risk assessment, having a clear scope based on a clear definition. The main objective of the EU Cosmetics Directive is to ensure a high level of consumer protection, requiring, among other provisions, a safety assessment of each cosmetic by a qualified professional as part of product development. Free circulation of cosmetic products throughout the EU single market is another key point addressed by the EU Cosmetics Directive: a complying cosmetic product can be sold anywhere in the EU without any other national barrier to intra-EU trade, as Article 7.1 states: "Member States may not, for reasons related to the requirements laid down in this Directive and the Annexes thereto, refuse, prohibit or restrict the marketing of any cosmetic products which comply with the requirements (…)".

In recent years the EU Cosmetics Directive has become the model of modern cosmetic regulations worldwide, representing a dynamic legislation that allows for continuous adaptation to technical progress, leading to relevant restrictions and/or warnings and to rapid inclusion of new regulated molecules (e.g. preservatives, colours, UV filters).

Article 1 of the EU Cosmetics Directive defines a cosmetic product as, "(…) any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition".

Annex 1 of the EU Cosmetics Directive contains a non-exhaustive, illustrative list of the products to be considered as cosmetics within the meaning of the definition in Article 1, which are shown below

- creams, emulsions, lotions, gels and oils for the skin (hands, face, feet, etc.);
- face masks (with the exception of chemical peeling products);
- tinted bases (liquids, pastes, powders);
- make-up powders, after-bath powders, hygienic powders, etc.;
- toilet soaps, deodorant soaps, etc.;
- perfumes, toilet waters and eau de Cologne;
- bath and shower preparations (salts, foams, oils, gels, etc.);
- depilatories;
- deodorants and anti-perspirants;

- hair care products (hair tints and bleaches, products for waving, straightening and fixing, setting products, cleansing products (lotions, powders, shampoos), conditioning products (lotions, creams, oils), hairdressing products (lotions, lacquers, brilliantines));
- shaving products (creams, foams, lotions, etc.);
- products for making-up and removing make-up from the face and the eyes;
- products intended for application to the lips;
- products for care of the teeth and the mouth;
- products for nail care and make-up;
- products for external intimate hygiene;
- sunbathing products;
- products for tanning without sun;
- skin-whitening products; and
- anti-wrinkle products.

The definition of a cosmetic identifies the site of application (epidermis, hair system, nails, lips, external genital organs, teeth, oral mucous membranes) and the intended functions (cleaning, perfuming, changing the appearance, correcting body odours, protecting, keeping in good condition). By using the sentence *exclusively or mainly* the EU Cosmetics Directive foresees that cosmetics may have some functions other than those specified in Article 1, as long as one of the six above-mentioned functions is predominant.

Conversely to the US and Japan, within the EU regulatory frameworks products are mutually exclusive; therefore, there are no intermediate product categories among cosmetics and pharmaceutical or foodstuffs or medical devices (RPA, 2004). The European Court of Justice (ECJ) repeatedly states, in several case laws, that a product can only be either a cosmetic or another type of product but it cannot be both at the same time (the so-called non-cumulation rule) (ECJ, 1991a, 1991b, 2005).

Safety of cosmetics and consumer protection are primarily addressed in Article 2 of the EU Cosmetics Directive, "A cosmetic product put on the market within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use, taking account in particular, of the product's presentation, its labelling, any instructions for its use and disposal as well as any other indication or information provided by the manufacturer or his authorized agent or by any other person responsible for placing the product on the Community market. The provision of such warnings shall not, in any event, exempt any person from compliance with the other requirements laid down in this Directive".

The manufacturer, the first importer into the EU or the responsible person for placing a product on the market has the primary responsibility to ensure compliance with the requirements of the EU Cosmetics Directive and, of course, the safety of cosmetics. The EU Cosmetics Directive does not require information on the safety of cosmetics to be submitted to Member State competent authorities before a product is placed on the market, however "The manufacturer or his agent, or the person to whose order a cosmetic product is manufactured, or the person responsible for placing imported cosmetic products on the Community market, shall notify the competent authority of the Member State of the place of manufacture or of the initial importation of the address of the place of manufacture or of initial importation into the Community of the cosmetic product before the latter are placed on the Community market" (Article 7a.4).

Meanwhile, the Member States designate the competent authorities that will cooperate in areas where such cooperation is necessary to ensure proper application of this Directive.

Each Member State appoints national inspectors, responsible for the in-market surveillance system, who may monitor compliance, visit local manufacturing sites and any retail outlet to see what is sold, take products from the marketplace to official laboratories for compliance testing, act against those companies that do not comply and that may present a threat to consumers and the market (e.g. counterfeit cosmetics), have access to Product Information via direct consultation or through another Member State authority under the principle of mutual recognition. The cooperation system among competent national authorities focuses on mutual recognition, enforcement in the event of non-compliant products and administrative understanding of different national systems.

According to Articles 3, 4 and 5, Member States will have to take all necessary measures to ensure that only cosmetic products that conform to the provisions of the Cosmetics Directive are put on the market. Member States will therefore prohibit the marketing of cosmetics containing,

- banned substances listed in Annex II;
- substances listed in Annex III, beyond the limits and outside the conditions laid down;
- colouring agents other than those listed in Annex IV with the exception of cosmetic products containing colouring agents intended solely to colour hair;
- colouring agents listed in Annex IV, used outside the conditions laid down, with the exception of cosmetic products containing colouring agents intended solely to colour hair;
- preservatives other than those listed in Annex VI;
- preservatives listed in Annex VI, beyond the limits and outside the conditions laid down, unless other concentrations are used for specific purposes apparent from the presentation of the product;
- UV filters other than those listed in Annex VII; and
- UV filters listed in Annex VII, beyond the limits and outside the conditions laid down therein.

Article 4a, which was inserted in Directive 2003/15/EC (7th amendment to the EU Cosmetics Directive), stipulates that Member States, according to the timetable established by the law, will prohibit the marketing of "(…) cosmetic products where the final formulation, in order to meet the requirements of this Directive, has been the subject of animal testing using a method other than an alternative method after such alternative method has been validated and adopted at Community level with due regard to the development of validation within the OECD", and also, "(…) cosmetic products containing ingredients or combinations of ingredients (…) subject of animal testing using a method other than an alternative method after such alternative method has been validated and adopted at Community level with due regard to the development of validation within the OECD", where OECD is the Organisation for Economic Co-operation and Development. A marketing ban on cosmetics tested on animals and/or containing ingredients tested on animals will take into effect 6 years after the entry into force of this directive, i.e. on 11 March 2009, and a deadline of 10 years after the entry into force of

this directive, i.e. on 11 March 2013, for toxicological tests concerning repeated-dose toxicity, reproductive toxicity and toxicokinetics, for which there are no alternatives yet under consideration.

Concerning the labelling of cosmetics (Article 6), "Member States shall take all measures necessary to ensure that cosmetic products may be marketed only if the container and packaging bear the following information (...)":

(a) the name or style and the address or registered office of the manufacturer or the person responsible for marketing the cosmetic product inside the EU. Member States may require the country of origin to be specified for goods manufactured outside the Community;
(b) the nominal content at the time of packaging, given by weight or by volume, except in the case of packaging containing less than 5 g or 5 ml, free samples and single application packs; for pre-packages normally sold as a number of items for which details of weight or volume are not significant, the content need not be given provided the number of items appears on the packaging. This information need not be given if the number of items is easy to see from the outside or if the product is only normally sold individually;
(c) the date of minimum durability needs to be indicated by the words *best used before the end of* followed by either the date itself (month and year or day, month and year) or details of where it appears on the packaging. If necessary, this information should be supplemented by an indication of the conditions, which must be satisfied to guarantee the stated durability. Indication of the date of durability should not be mandatory for cosmetic products with a minimum durability of more than 30 months. For such products, there needs to be an indication of the period of time after opening (Period after Opening—PaO) for which the product can be used without any harm to the consumer. This information is indicated by the symbol shown below, which appears in the EU Cosmetics Directive Annex VIIIa followed by the period (in months and/or years);

(d) particular precautions to be observed in use, especially those listed in the column "Conditions of use and warnings which must be printed on the label" in Annexes III, IV, VI and VII, which must appear on the container and packaging, as well as any special precautionary information on cosmetic products for professional use, in particular in hairdressing. Where this is impossible for practical reasons, an enclosed leaflet, label, tape or card must contain that information to which the consumer is referred either by abbreviated information or the symbol shown below, given in Annex VIII, which must appear on the container and the packaging;

(e) the batch number of manufacture;
(f) the function of the product, unless it is clear from its presentation; and
(g) a list of ingredients preceded by the word *ingredients* (A more detailed explanation about ingredients labelling is shown in Section 1.2).

According to Article 7, Member States may "(…) require that the particulars provided for in Article 6 (1) (b), (c), (d) and (f) be expressed at least in their own national or official language or languages (…)".

Article 7a indicates further obligations by manufacturers and importers towards safety and efficacy: "The manufacturer or his agent or the person to whose order a cosmetic product is manufactured or the person responsible for placing an imported cosmetic product on the Community market shall for control purposes keep the following information readily accessible to the competent authorities of the Member State concerned at the address specified on the label in accordance with Article 6 (1) (a)", which should be available in the national language of the Member State concerned, or in a language readily understood by the competent authorities,

(a) The qualitative and quantitative composition of the product.
(b) The physicochemical and microbiological specifications of the raw materials and the finished product and the purity and microbiological control criteria of the cosmetic product.
(c) The manufacturing method must comply with the good manufacturing practices (GMP) laid down by Community law or, failing that, laid down by the law of the Member State concerned. To date no definition of GMP has been provided in the regulations, even though the Commission is currently preparing EU guidelines. Voluntary GMP guidelines have been published by COLIPA (2003). The person responsible for manufacture or first importation into the Community must possess an appropriate level of professional qualification or experience in accordance with the legislation and practice of the Member State, which is the place of manufacture or first importation.
(d) Assessment of the safety for human health of the finished product. To that end the manufacturer shall take into consideration the general toxicological profile of the ingredients, their chemical structure and their level of exposure. There shall be *inter alia* a specific assessment for cosmetic products intended for use on children under the age of three and for cosmetic products intended exclusively for use in external intimate hygiene. Guidelines for safety testing have been prepared by the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) (as was formerly named the present Scientific Committee on Consumer Products (SCCP) operating inside the EU Commission), and COLIPA has also published guidelines on safety assessment (SCCNFP, 1999; COLIPA, 1997).

(e) The name and address of the qualified person or persons responsible for the assessment referred to in (d). That person must hold a diploma in the field of pharmacy, toxicology, dermatology, medicine or a similar discipline.
(f) Existing data on undesirable effects on human health resulting from use of the cosmetic product.
(g) Proof of the effect claimed for the cosmetic product, were justified by the nature of the effect or product.
(h) Data on any animal testing performed by the manufacturers, their agents or suppliers, related to the development or safety evaluation of the product or its ingredients, including any animal testing performed to meet the legislative or regulatory requirements of non-member countries. Without prejudicing protection, in particular, of commercial secrecy and intellectual property rights, Member States shall ensure that the information required under (a) and (f) shall be made easily accessible to the public by any appropriate means, including electronic means. The quantitative information required under (a) to be made publicly accessible shall be limited to dangerous substances covered by Council Directive 67/548/EEC (1967).

Moreover, according to Article 12, "If a Member State notes, on the basis of a substantiated justification, that a cosmetic product, although complying with the requirements of the Directive, represents a hazard to health, it may provisionally prohibit the marketing of that product in its territory or subject it to special conditions. (…)". The other Member States and the Commission must be immediately informed about this decision and the Commission, if assuming that technical adaptations to the Directive are necessary, should adopt them in accordance with the procedure laid down in the Cosmetics Directive.

THE US REGULATORY APPROACH TO THE SAFETY AND EFFICACY OF COSMETICS

Cosmetics in the US are defined according to the Food, Drugs & Cosmetics Act (FD&C Act) dating back to 1938 and, in practice unchanged, excluding the amendments to colour additives. Cosmetics manufactured or imported in the US must comply with the provisions of the FD&C Act, the Fair Packaging and Labelling Act (FPLA) dated 1967 and the regulations published under the authority of these laws. The complete regulations published by the Food and Drug Administration (FDA) can be found in Title 21 of the Code of Federal Regulations (21 CFR).

The FD&C Act defines cosmetics as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body (…) for cleansing, beautifying, promoting attractiveness, or altering the appearance". Included in this definition are skin care products, fragrance preparations, manicuring products, lipsticks, eye and facial make-up preparations, bath preparations, hair preparations (non-colouring), hair colouring preparations, oral hygiene products, baby products, personal cleanliness, shaving products, tanning products and deodorants. Soaps consisting primarily of an alkali salt of fatty acid and making no label claim other than cleansing of the human body are not considered cosmetics under the law.

The same FD&C Act defines drugs as "(…) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease, and articles (other than food) intended to affect the structure or any function of the body of man or other animals (…)". Over-the-counter (OTC) drugs are those that can be purchased without a doctor's prescription.

In the US, some products considered as cosmetics in the EU are classified as OTC drugs. FDA reviews these products to establish single monographs under which the drugs are generally recognised as safe and effective, and not misbranded. This reviewing process involves three steps: issuance of a panel report (or advance notice of proposed rulemaking (ANPR)); followed by a tentative final monograph (TFM), which has the legal status of a proposed rule; and finally the establishment of the final monograph (FM). After considering the comments on the panel report, the FDA publishes in the Federal Register (http://www.gpoaccess.gov/fr/index.html) the tentative final monograph, and finally, when an OTC drug monograph is made final, it is published as a final rule or new regulations in 21 CFR. A product covered by a final monograph must conform to the terms of the monograph. In this sense, specific regulations of different OTC drugs were published, e.g. anti-caries (21 CFR Part 355) and anti-plaque (21 CFR Part 356) toothpastes (fluoride-containing), anti-perspirants (21 CFR Part 350), anti-dandruff and anti-seborrheic products (21 CFR Part 358), skin bleaching products (21 CFR Part 310), sunscreens (21 CFR Part 352) and skin protectant products (21 CFR Part 347 and 358). The OTC monograph process in an ongoing one, also for final monographs, therefore manufacturers should periodically consult either the Federal Register or "The Rulemaking History for Drug Products: Drug Category List" at FDA's webpage (see references).

Many products may qualify both as cosmetics and as OTC drugs when they have two intended uses. For instance, a shampoo is a cosmetic, since its intended use is to cleanse the hair. An anti-dandruff treatment is a drug, since its intended use is to treat dandruff. Consequently, an anti-dandruff shampoo is both a cosmetic and a drug. Fluoride-containing toothpastes, deodorants that are also anti-perspirants, moisturizers and make-up products with sunscreen claims are other examples of cosmetic/drug combinations (FDA, 2002).

In many cases, assigning a product to the category of cosmetic or drug depends on the concept of "intended use". Claims stated on the product label, in advertising, on the Internet or in any promotional material, consumer perception and some ingredients may cause a product to qualify as a drug, even if marketed as if it were a cosmetic. Claims regarding treatment or prevention of disease or implying it otherwise affects the structure or functions of the human body, makes a cosmetic into a drug.

The FDA regulates the safety of cosmetics, establishes labelling requirements and is also responsible for enforcing laws about cosmetics and the specific department is the Office of Colours and Cosmetics within the Centre for Food Safety and Applied Nutrition (CFSAN). Issues related to drugs are handled by the Center for Drug Evaluation and Research (CDER).

Such laws are enforced by the FDA by means of checking and investigating products, inspecting establishments where products are manufactured or held, determining whether and what toxicological and/or other testing the firm has conducted to substantiate product safety, and seizure of adulterated or misbranded cosmetics.

FDA does not require pre-market approval of cosmetics, neither does it ask firms to register manufacturing premises nor make available safety data or other information before

marketing. Manufacturers or distributors of cosmetics may submit information about establishment and cosmetic product ingredients to the agency voluntarily under the Voluntary Cosmetic Registration Program. However, cosmetic firms are responsible for marketing safe, properly labelled products; without using banned ingredients and adhering to limits on restricted ingredients (see further on). If the safety of an ingredient or cosmetic is not adequately substantiated prior to marketing, the label must bear the following conspicuous statement on the principal display panel: "Warning–The safety of this product has not been determined".

Other than government agencies, safety of cosmetic ingredients is evaluated and reviewed by the Cosmetic Ingredient Review (CIR), an independent panel of scientific experts setup by the Cosmetic, Toiletry and Fragrance Association (CTFA), looking at all available data and assessing the safety of ingredients used in cosmetics.

The regulatory requirements for OTC drugs are more extensive as, for example, the FD&C Act requires that drug manufacturers register with the FDA at the beginning of the activity, then every year and update their lists of all manufactured drugs twice annually. Additionally, OTC drugs must be manufactured in accordance with current GMP regulations.

Cosmetic manufacturers may use essentially any raw material as a cosmetic ingredient insofar as it is not considered as only a drug active ingredient, and market the product without approval. The law regulates only colour additives specifically approved for cosmetics and a few prohibited and restricted ingredients (e.g. bithionol, mercury compounds, vinyl chloride, halogenated salicylanilides). Cosmetics should not be contaminated with nitrosamines, 1,4-dioxane and pesticide residues, whose potential presence is regularly checked by the FDA (see Section 2.1).

On the other hand, OTC drugs active ingredients are strictly regulated: only the substances present in the positive list of each monograph can be used, and they must conform to the maximum and minimum permitted concentrations. It is extremely difficult to add a new active ingredient to these positive lists, because it must first be used in a therapeutic formula authorised by the cumbersome procedure called "New Drug Application".

Cosmetics marketed in the US must comply with the labelling regulations under the FD&C Act and the FPLA. The principal display panel, i.e. "the part of a label that is most likely to be displayed, presented, shown or examined under customary conditions of display for retail sale", shall bear a statement identifying the product and an accurate statement of the net quantity, using US measures and additionally the metric system. The latter declaration must be distinct, placed at the bottom of the panel in line, generally parallel to the base on which the package rests.

Other information required on the label are: the name and place of business of the manufacturer, packer or distributor (if the distributor is not the manufacturer or packer, this fact must be stated on the label by the qualifying phrase *Manufactured for (…)* or *Distributed by (…)* or similar wording); the country of origin; appropriate warnings and directions for use (warnings for coal-tar hair dyeing products, aerosols, feminine deodorant sprays, foam baths and products whose safety has not been proven, are compulsory); a declaration of the name of each ingredient in descending order of predominance. Cosmetics, which are also drugs, must first identify the drug ingredient(s) as *active ingredients(s)* before listing the cosmetic ingredients (see Section 1.2 for a detailed explanation about ingredients labelling). They must also mention a use-by date if their validity is less than three years,

and warnings or instructions for use as required by each specific monograph. It is worth noting that the OTC product labelling was standardized to make it easy to read for consumers. The so-called drug facts box has been applied since May 2002 except for OTC products, which are not yet regulated by a final ruling.

When sold retail, some products like liquid oral hygiene products (e.g. mouthwashes, fresheners) and all cosmetic vaginal products (e.g. douches, tablets) must be packaged in tamper-resistant packages, which if breached or missing, alert a consumer that tampering has occurred.

JAPANESE DEREGULATION: THE EFFECTS OF MARKETING COSMETICS IN JAPAN

The Pharmaceutical and Food Safety Bureau (2000) inside the Ministry of Health, Labour and Welfare (MHLW), formerly known as Ministry of Health and Welfare (MHW), is the regulatory body in charge of cosmetics monitoring compliance to the Pharmaceutical Affairs Law (PAL), which was first adopted in 1943, with subsequent amendments in 1948, 1960 and 1979. Nevertheless, new regulations revising and drastically changing the PAL were implemented in 2001 (see further on). The PAL regulates drugs, quasi-drugs, cosmetics and medical devices to guarantee their quality, safety and efficacy. The PAL, notwithstanding the fact that it contains provisions affecting all categories, specifies that a product can only fall within, and comply with, the definition and requirements of one category (MHW, 1992).

Cosmetics are defined in Article 2, paragraph 3 of the PAL as "(…) any article intended to be used by means of rubbing, sprinkling or by similar application to the human body for cleaning, beautifying, promoting attractiveness, altering the appearance of the human body and for keeping the skin and hair healthy provided that the action of the article on the human body is mild". Types of cosmetics are: hair cosmetics; washing products; skin lotions, creams and packs; make-up products; face and toilet powders; lip colours; nail make-up; perfumes; bath preparations; cosmetic oils; face cleansing products; sunscreens; soaps; and oral care products.

Under the PAL, quasi-drugs (Article 2, paragraph 2) are defined as

> "(…) articles which have the following purposes and exert mild actions on the human body:
> – prevention of nausea or other discomfort, or prevention of foul breath or body odour;
> – prevention of prickly heat, sore and the like;
> – prevention of hair loss, promotion of hair growth, or depilation;
> – killing or repellence of rats, flies, mosquitoes, fleas, etc. for maintaining the health of man or animals, and exert mild action on the human body but are not intended for use in the diagnosis, cure or prevention of disease or to affect the structure or any function of the human or animal body and are not equipment or instruments".

In this sense, the products designated as quasi-drugs by the MHW are cotton products intended for sanitary purposes (including paper cotton); and the following products with a mild action on the human body: hair dyes; permanent waving products; hair-growers;

depilatories; deodorants; medicated cosmetics (including soaps, bath preparations, anti-dandruff shampoos and rinses, anti-acne, anti-chapping, anti-frostbite, prickly heat, diaper rash lotions, creams, powders and packs, whitening and anti-bacterial products); medicated toothpastes; insect repellents; sanitary cotton products; products used to disinfect or protect abrasions, cuts, puncture wounds, scratches and wound surfaces; products to alleviate discomfort of the throat; products to alleviate discomfort of the stomach; diet supplements; rodenticides and others.

Effective 1 April 2001, new regulations, such as Ordinance No. 125/2000 and Notifications Nos. 330/2000, 331/2000 and 332/2000 from MHW and Notification No. 990/2000 from Pharmaceutical and Medical Safety Bureau, partially revising the PAL changed the cosmetic system in Japan. This so-called "deregulation" implied a drastic change from the past: the abolition of the pre-market approval or licensing system for each cosmetic product, meaning that the manufacturer/seller is now responsible for ensuring that any marketed cosmetic is safe and to substantiate its harmlessness. Primary distributors are held responsible for the quality and safety of the cosmetic products concerned, and are required to indicate their names and addresses on the label, i.e. the label must show the name of the company which has the licence to market the cosmetics. The primary distributors are those who obtain products from manufacturers for resale and are fully responsible for the safety and quality of the cosmetic product.

Following the abolition of the former licensing system, manufacturers and/or importers are required to notify the name of a product before it is actually manufactured or imported, thus allowing the competent authorities to identify each cosmetic product.

The following are the main points of the "deregulation":

(1) The separation of manufacture and sales activities (shipment and launch) from manufacturers, and the setting up of a licensing system for manufacturers that do not assume tenancy of the manufacturing site.
(2) It is necessary to obtain approval to manufacture/sell cosmetics and the licence must be renewed every five years; moreover, it is issued by the head of the local prefecture.
(3) Effective 1 April 2005, a Licence for the Marketing Business of Cosmetics has been implemented and must be obtained by a company, which intends to market cosmetics. In order to obtain a licence to market cosmetics, a company must observe good quality practice (GQP) and good vigilance practice (GVP) in accordance with the standards specified by MHLW Ordinances in terms of quality control and post-marketing safety management. In order to comply with GQP, the company must have a product quality manager responsible for keeping records on product quality, for proper manufacturing as well as shipment, and, if necessary, for product recall. Even if there are no mandatory GMPs, voluntary guidelines have been issued by the Japanese cosmetic trade association (JCIA—Japanese Cosmetic Industry Association). In order to comply with GVP, a safety control manager is necessary to monitor post-marketing product safety control and keep all necessary records. Furthermore, the company must have a general marketing business controller, who oversees proper marketing practices and supervises the GQP and GVP managers.
(4) Post-manufacture and sales safety control standards, and manufacture and sales quality assurance standards have been established.

Moreover a full-ingredient labelling policy was introduced, adopting the INCI names translated into Japanese, thus enabling consumers to easily select and check a product. Conversely, ingredient labelling of quasi-drugs is a voluntary regulation of the industry (see Section 1.2 for a detailed explanation).

Besides ingredient labelling, cosmetics must fulfil the following requirements (in Japanese): name and address of the approved and licensed company; product name and function; batch code; full-ingredient list in descending order (only on outer container); nominal content using metric units of weight or volume; country of origin; expiry date only for cosmetics designated by the MHLW, in particular those having a minimum durability below three years (MHW, 1980); warning statements (prescribed by law or voluntary); instructions for use and instructions for storage (if required).

Regarding the scope and efficacy of cosmetics, reference has to be made to Notification No. 1339/2000 which lists the following functions for a cosmetic product:

(1) Clean the scalp and hair
(2) Mask unpleasant odours
(3) Keep the scalp and hair healthy
(4) Make hair strong and resilient
(5) Moisturize the scalp and hair
(6) Keep moisture in the scalp and hair
(7) Make hair supple
(8) Make hair easier to comb
(9) Maintain the lustre of hair
(10) Make hair lustrous
(11) Treat dandruff and scalp itch
(12) Suppress dandruff and scalp itch
(13) Give and maintain hair moisture and oil
(14) Prevent hair from breaking, severing or splitting
(15) Set and keep hairstyle
(16) Prevent static electricity build-up in hair
(17) Clean the skin (by removing dirt)
(18) Prevent pimple and heat rash (by cleaning) (face cleaning products)
(19) Improve the skin
(20) Improve skin texture
(21) Keep the skin healthy
(22) Prevent skin roughness
(23) Tighten the skin
(24) Moisturize the skin
(25) Give and maintain skin moisture and oil
(26) Maintain skin elasticity
(27) Protect the skin
(28) Prevent dry skin
(29) Make the skin soft
(30) Make the skin strong
(31) Give the skin lustre
(32) Make the skin smooth
(33) Make shaving easier
(34) Improve the skin after shaving
(35) Prevent heat rash
(36) Prevent sunburn
(37) Prevent sun-induced spots and freckles
(38) Give a pleasant fragrance
(39) Protect nails
(40) Keep nails healthy
(41) Moisturize nails
(42) Prevent lip roughness
(43) Improve lip texture
(44) Moisturize the lips
(45) Make the lips healthy
(46) Protect the lips. Prevent dry lips
(47) Prevent dryness-induced chapped lips
(48) Make the lips smooth
(49) Prevent cavities (toothpastes used in brushing)
(50) Whiten teeth (toothpastes used in brushing)
(51) Remove dental plaque (toothpastes used in brushing)
(52) Clean the oral cavity (toothpastes)
(53) Prevent bad breath (toothpastes)
(54) Remove teeth stains (toothpastes used in brushing)
(55) Prevent tartar build-up (toothpastes used in brushing)

Table 1.1.1

Comparison of the main features of the EU, the US and Japan cosmetic regulations

Item	EU	US	Japan
Product notification	Yes	No (voluntary)	Yes
In-market control	Yes	Yes	Yes
Product safety: manufacturer responsibility	Yes	Yes	Yes
List of banned/restricted ingredients	Yes	Yes	Yes
Positive list for colours	Yes	Yes	Yes
Positive lists for preservatives	Yes	No	Yes
Positive lists for UV filters	Yes	Yes (but as OTC)	Yes
Use of INCI names	Yes	Yes	Yes
Product categories	Cosmetics or drugs	Cosmetics or OTCs or drugs	Cosmetics or quasi-drugs or drugs

Following deregulation, ingredients are no longer regulated by a global list of ingredients (the Comprehesive Licensing Standards or CLS), but are only limited by positive and negative lists as in the EU: banned substances; restricted cosmetic ingredients; positive lists of preservatives and UV filters for skin protection. The use of coal-tar colouring agents (which correspond to synthetic colourants) has been regulated since 1966 by means of MHW Ordinance No. 30, successively amended in 1972 and 2003 (CTFA, 2003).
On the other hand, all active ingredients used in quasi-drugs, as well as excipients, must first be authorized by the Japanese authorities.

Table 1.1.1 above gives a brief summary showing a comparison of the three main cosmetic regulations.

SAFETY AND EFFICACY OF COSMETICS: A BRIEF SURVEY OF OTHER SIGNIFICANT WORLDWIDE MARKETS

Australia

According to the Australian Trade Practices on Consumer Product Information Standard (1991), a cosmetic product means "a substance or preparation intended for placement in contact with any external part of the human body, including: the mucous membranes of the oral cavity; and the teeth; with a view to: altering the odours of the body; or changing its appearance; or cleansing it; or maintaining it in good condition; or perfuming it; or protecting it". A non-exhaustive list of cosmetics, only intended for illustrative purposes, reflects Annex I of the EU Cosmetics Directive.

While cosmetics are controlled by the Department of Health and Ageing within the Australian Government and the State and Territory Governments; the Therapeutic Goods Administration (TGA, 1990) regulates therapeutic products within the definition outlined by the Therapeutic Goods Act 1989. The delineation between therapeutic goods and

cosmetics is not always clear. The National Coordinating Committee on Therapeutic Goods (NCCTG) issued a Cosmetic Claims Guidelines document on 9 May 1997 to make the distinction between cosmetics and therapeutic goods. In general a therapeutic good can be described as a product intended for "therapeutic use" which includes modifying a physiological process or to prevent, diagnose, cure or alleviate a disease, ailment or defect. Examples of therapeutic goods are anti-perspirants, sun protection lotions and creams with a stated SPF or similar claim (conversely lotions and creams providing sun protection as a secondary function without stating an SPF rating or making a sun protection claim other than "contains sunscreen"; tinted facial make-up other than moisturisers and tinted unmedicated lip preparations stating the actual SPF are classified as cosmetics); skin disinfectants and hygienic hand washes; antiseptic mouth washes; toothpaste with therapeutic claims or a fluoride content of more than 1000 mg/kg.

Currently, this regulatory system is being reviewed and certain therapeutic goods could be reclassified as cosmetics, for example, skincare products with secondary sun protection claims.

Cosmetics and their manufacturers are not subject to licensing or audit requirements while therapeutic goods and their manufacturers must be included in the Australian Register of Therapeutic Goods (ARTG) and manufacturers must be licensed to demonstrate GMP compliance.

Regarding cosmetic ingredients, these must all be included in the Australian Inventory of Chemical Substances (AICS), if not they have to be assessed under the National Industrial Chemicals Notification and Assessment Scheme (NICNAS). Ingredients used for therapeutic goods must be listed in the Australian Approved Name (AAN) list.

The Trade Practices Regulations establish the labelling of cosmetics under the authority of the Australian Competition and Consumer Commission (ACCC). The ACCC is responsible for enforcing mandatory consumer product safety and information standards.

Moreover, the Trade Practices Regulations and other regulations such as the Trade Measurement Act of 1990 require the following to be mentioned, in English: list of ingredients; name and address of the packer or distributor; manufacturer or the person responsible for the placing the product on the market; product name and function; net quantity of contents; warnings; instructions for use and country of origin.

The Standard for the Uniform Scheduling of Drugs and Poisons provides for mandatory "warning" labelling of containers when certain scheduled substances have been added to the cosmetic.

Labelling of therapeutic goods includes information such as the name of the active ingredients according to the AAN nomenclature and their concentration, name and Australian address of the product sponsor, batch number, expiry date, storage conditions, warnings and the TGA listing or registration number.

The ACCC conducts random surveys of retail outlets throughout Australia to detect products that do not comply with the Trade Practices and Consumer Affairs legislation. It also investigates allegations by consumers and suppliers about non-complying goods and frequently seeks the immediate withdrawal of defective goods from sale, as well as the recall of the goods for corrective advertising.

A cosmetic will be considered defective *if its safety is not such as persons generally are entitled to expect*. Generally it is the manufacturers or importers or the person responsible

for placing the products on the Australian market that are liable. However, in instances where other suppliers, such as retailers, cannot identify the manufacturer, they may be deemed liable for damages.

The Uniform Recall Procedure for Therapeutic Goods (URPTG) defines the action to be taken by health authorities and sponsors when therapeutic goods for human use, for reasons relating to their quality, safety and efficacy, are to be removed from supply or use, or subjected to corrective action.

While most recalls are initiated by sponsors, recall action is underpinned by the Therapeutic Goods Act 1989 and, in accordance with the provisions of the Act, the Secretary to the Department of Health and Ageing may demand therapeutic goods to be withdrawn.

Brazil

The Agência Nacional de Vigilância Sanitária (ANVISA, 2000—National Health Agency) enforces regulations concerning cosmetics, which are defined according to the EU Cosmetics Directive, but are divided into four categories (hygiene products, cosmetics, fragrances and baby products) and two risk groups (Level 1 and Level 2).

Level 1 cosmetics include those with a minimal safety risk, while Level 2 cosmetics might present potential risk and include, for example, anti-dandruff preparations, anti-caries toothpastes, deodorants, anti-perspirants, anti-wrinkle products, sunscreens, hair dyes, hair bleaching, permanent waving products, nail hardeners and all baby products.

Registration procedure differs for the two risk groups. Level 1 cosmetics may be simply notified (which involves nevertheless sending a small file to ANVISA) at least 30 days before marketing. By contrast, Level 2 cosmetics must be registered, are subject to a specific tax and the registration process may require up to 60 days. Procedures could change in the future since the MERCOSUR (Mercado Común del Sur) (constituted by Argentina, Brazil, Paraguay and Uruguay) health authorities are negotiating a system of mutual recognition in MERCOSUR so that products registered in one country do not need to be re-registered in the other countries.

Ingredients are regulated according to the Annexes of the EU Cosmetics Directive with some discrepancies; however, a local scientific body, the Câmara Técnica de Cosméticos (CATEC—Technical Group on Cosmetics) may introduce different restrictions or bans.

The following information, in Portuguese, must be labelled: name and function of the product; trademark; registration number; batch number; expiry date; net content; country of origin; name and address of the manufacturer, importer or person responsible for putting the product on the market; specific instructions for use and warnings required for certain categories of cosmetics; ingredient list, INCI name need not be translated into Portuguese anymore.

Manufacturers must ensure safety and efficacy of cosmetics. Safety of Level 2 cosmetics is scrutinised by ANVISA during the registration procedure. A complete file on the finished product must be kept at the disposal of the controlling authorities for both notified and registered cosmetic products.

Canada

The Food and Drugs Act, which includes the Cosmetic Regulations, defines a cosmetic as "any substance or mixture of substances manufactured, sold or represented for use in cleansing, improving or altering the complexion, skin, hair or teeth, and include deodorants and perfumes". Cosmetics therefore include, for example, lipsticks, mascara, eye shadows, nail polish, shampoos, non-fluoride toothpaste, conditioners, soaps, moisturizers, cleansers, hair dyes, hair permanents and depilatories.

However, products such as sunscreens, acne treatments, anti-dandruff shampoos, anti-caries toothpastes and anti-perspirants are considered to be non-prescription drugs subject to Category IV Monographs. These regulatory instruments indicate, notably, the definition of the non-prescription drug, the approved active ingredients and permitted combinations, their maximum and minimum permitted concentrations and the labelling requirements (statement of identity, indications for use, warnings and directions). Any product that has a therapeutic claim or that contains certain ingredients not permitted in cosmetics is considered to be an OTC drug and is handled by the Therapeutic Products Program. Products containing natural therapeutic ingredients will be regulated by the office of Natural Health Products.

Cosmetics must not imply actions that are therapeutic in nature, as these are considered to be drug claims. If a manufacturer wishes to retain such a claim it will be required to apply for a Drug Identification Number (DIN) and cannot sell the product until a DIN has been issued.

Drug and cosmetic claims are acceptable for non-prescription drugs that possess both drug and cosmetic properties and are regulated under Category IV Monographs.

Whereas the Therapeutic Product Directorate deals with products regulated under Category IV Monographs, the Cosmetic Division of the Consumer Health Safety Bureau inside Health Canada is the authority responsible for cosmetics, subject to the provisions of the Food and Drugs Act and its Regulations (regarding composition, safety, labelling, advertising) and to the provisions of the Consumer Packaging and Labelling Act and its Regulations (regarding bilingual labelling, deceptive packaging, net quantity declaration in metric units).

Manufacturers and importers are required to submit a notification to Health Canada within 10 days of the first sale of a cosmetic in Canada.

Cosmetic notification is not a product evaluation or approval procedure and acceptance by the authorities, but rather the manufacturer has the responsibility of ensuring that a cosmetic meets the requirements of Act and Regulations. Products falling into Category IV Monographs are subject to pre-market approval and registration.

A list of restricted or banned ingredients is to be found in the Cosmetic Regulations. Excluding colours used in the area of the eye, there is no approved or banned list of colours. Health Canada also makes available an ingredients "Hot List", a non-exhaustive list of restricted raw materials. If an ingredient on the notification form also appears in this "Hot List" the manufacturer will be warned, depending on the ingredient, to take action, including possible removal of the ingredient or concentration reduction or registration of the product as a drug, etc. Category IV Monographs identify the ingredients recognised as safe and effective for that specific purpose, i.e. list of UV filters.

The labelling of cosmetics, with the exception of the manufacturer's name and address, must be in both English and French. Special labelling is required under the Quebec Official Languages Act for products sold in that province.

Manufacturers of cosmetics must print certain information on the labels of each product, that include: the identity and function of the product; the net quantity in metric units; the name of the manufacturer or distributor and the address of the principal place of business; country of origin for imported products; any warnings or cautions necessary for the safe use of the product, especially mandatory ones such as those required for coal-tar hair dyes, etc.

On 1 December 2004, Health Canada published an amendment to the law-making ingredient labelling mandatory for all cosmetics sold in Canada with a 16 November 2006 compliance deadline. Health Canada has identified the INCI system required in Europe as the reference standard for ingredient labelling on the basis that it is applied in most countries and that the European system made more use of Latin, the international scientific language.

Non-prescription drugs must follow stricter mandatory labelling including, for example, batch number, expiry date, registration number, percentage of active ingredients; claims, instructions of use and warnings according to the relevant monograph.

The Act and the Regulations also set out the safety requisites for all cosmetics sold in Canada, prohibiting the sale of a cosmetic that is either prepared under unsanitary conditions or is unsafe when used as directed. It is the manufacturer's responsibility to ensure that the cosmetics are safe for their intended use. Health Canada is the authority responsible for testing and analysing all marketed products. The Ministry may request in writing that a manufacturer submits, on or before a specified day, evidence to establish the safety of a cosmetic under the recommended or normal conditions of use.

Recently, Health Canada has published the Guidelines for Cosmetics Manufacturers, Importers and Distributors 2005 that include all main areas of cosmetic regulations to be followed.

Kingdom of Saudi Arabia

Cosmetics in the Kingdom of Saudi Arabia (KSA) are regulated by the Ministry of Health and the Ministry of Commerce and Industry whereas the Saudi Arabian Standards Organization (SASO) is responsible for establishing standards, labelling and testing.

Based on the definition of the EU Cosmetics Directive, perfumes and cosmetics are defined as "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition". Products that possess a therapeutic effect and claim medical properties or contain pharmaceutical substances are not considered as cosmetics.

Basically, the KSA authorities consider Annex 1 to the EU Cosmetics Directive an illustrative list of cosmetics. Conversely to the EU law, perfumery products based on ethanol are classified according to the essential oil content as follows:

Category A: essential oil content is between 2–2.999% by volume
Category B: essential oil content is between 3–7.999% by volume
Category C: essential oil content is 8% or greater by volume

Cosmetics are not subject to registration or notification, there are pre-shipment testing procedures in force, based on mandatory SASO standards applied equally to both imported and domestically produced products. These encompass the protection of health, safety, national security, public morals, the environment and prevention of deceptive practices. For imported cosmetics, procedures to ensure conformity to the applicable Saudi standards are enforced by the Ministry of Commerce and Industry, which implements the International Conformity Certification Program (ICCP) as a combined conformity assessment, inspection and certification scheme, on the basis of which goods are allowed entry into the KSA. Compliance with the relevant Saudi standards, or approved equivalent international alternatives, leads to the issue of a Certificate of Conformity (CoC) prior to shipment by Country Offices or Regional Licensing Centres. The ICCP applies to all consumer products exported to the KSA (KSA, 2004). Samples may be selected for minimum verification tests as a part of annual routine inspections. Periodic pre-shipment inspection will be performed.

Banned, restricted and regulated ingredient lists are based on the relevant annexes to the EU Cosmetics Directive. In compliance with Islamic law, cosmetics shall contain no pork, pork fat or pork derivatives, ethanol must be denatured and should not exceed 90%, containers containing ethanol should not exceed 250 ml and a non-removable pump is mandatory.

Labelling requirements may differ by product type according to each applicable standard, however the following information should be legibly and indelibly marked: description name or function of the product; name of the manufacturer or registered trademark; name and address of the person (company) responsible for marketing the product if different from the manufacturer; date of production or batch number; expiry date (applicable to these cosmetics only: hair creams, shampoos and oils; liquid oxidative hair dyes; chemical depilatories; toothpastes; lipsticks; deodorants; talcum powders; skin creams or if minimum durability is less than 30 months); net content in volume or weight; country of origin; ingredients list (using INCI names); instructions for use and storage (if needed); warnings concerning restricted substances or voluntary ones.

All products shall be marked in either Arabic or English, but instructions for use and storage, voluntary and mandatory warnings must be in Arabic.

Manufacturers or exporters are requested to provide evidence that their finished products are safe for consumer's use based upon the toxicological profile of the ingredients, their chemical structure and exposure level. The toxicological evaluation needs to be signed by an authorised/recognised safety assessor and should contain a series of data similar to that included in the EU product information.

People's Republic of China

Several agencies are involved in the regulation and control of cosmetics: the Ministry of Health, the State Food and Drug Administration (SFDA), the Administration for Quality Supervision, Inspection and Quarantine (AQSIQ), the State Administration for Industry

and Commerce and the Ministry of Commerce. Cosmetic regulations have been implemented during the 1990s following the fast development of the cosmetic industry and several different laws, regulations, ordinances and standards apply.

The definition of a cosmetic is referable to that outlined in the EU Cosmetics Directive, but they are divided into two categories: common cosmetics (skin care, make-up, hair care, fragrances) and special-function cosmetics (e.g. anti-perspirants, hair dyes, sunscreens).

A time-consuming pre-market approval from Chinese authorities is compulsory. The first step is an authorisation/registration for sale by the Ministry of Health for which a huge amount of technical and safety documents, plus tens of samples need to be presented for each cosmetic. Following registration, an imported cosmetic must obtain authorisation for import via the AQSIQ, which includes the presentation of specific Bovine Spongiform Encephalopathy (BSE) certificates, declaring the status of ingredients of bovine, ovine or caprine origin, signed by the appropriate national body of the exporting country.

Ingredients are mainly regulated through lists of banned, allowed and restricted ingredients like in the EU Cosmetics Directive, except that a positive list of hair dyeing ingredients has been recently published and that all cosmetic ingredients must be included in the PRC's Inventory of Existing Chemical Substances (IESCS) or be approved as new chemicals after submission of a technical and safety dossier to the State Environmental Protection Administration's Chemical Registration Center (SEPA-CRC).

General labelling of cosmetics is also addressed by a specific Chinese standard. Information required on the label, in Chinese, include: the identity and function of the product; the net quantity in metric units; the name of the manufacturer or importer distributor and the address of the principal place of business; date of manufacture plus shelf-life or batch number plus expiry date; health licence number (for imported cosmetics); any warnings or cautions necessary for the safe use of the product; instructions for storage (if applicable); and country of origin. Full ingredient labelling is not required.

Safety is assessed during the registration process by means of both the documents presented by the manufacturer/importer and local chemical, physical, microbiological, toxicological and clinical testing.

Republic of Korea

The Bureau of Pharmaceutical Affairs of the Ministry of Health and Social Affairs (MOHSA) and the Korean Food and Drug Administration (KFDA) are the agencies responsible for the laws ruling cosmetic production and marketing in Republic of Korea (ROK). The Cosmetic Law, which came into force on 1 July 2000, is the reference text and it identifies two categories of cosmetics: cosmetics as such and functional cosmetics or cosmeceuticals.

Cosmetics are defined as "items with mild action on the human body for the purpose of cleaning, beautifying, adding to the attractiveness, altering the appearance, or keeping or promoting the skin or hair in good condition" while functional cosmetics, even if falling under the cosmetic definition, are designated as "items fulfilling specific actions like skin whitening, minimizing the appearance of lines in the face and body, protecting from the sun and sun tanning".

As in Japan, there are also quasi-drugs, such as oxidation hair dyes, deodorants, hair loss products, etc.

Cosmetics containing ingredients found in the approved compendia by the MOHSA need only to be notified to the KFDA, no pre-marketing approval is requested. Conversely data on the safety and efficacy of functional cosmetics must be submitted to the KFDA, which must assess the safety and the efficacy of cosmeceuticals before they are put on the market. A less complicated procedure is in place for functional cosmetics containing the ingredients listed and allowed by KFDA.

Ingredients are regulated according to positive, restrictive and negative lists whose content is quite different from the Annexes of the EU Cosmetics Directive. Moreover a cosmetic ingredient needs to be approved for cosmetic use, i.e. it must be listed in one of the officially approved compendia by the MOHSA. Positive lists of active ingredients are available for cosmeceuticals as well as for quasi-drugs.

Korean is the language for labelling requirements which include: product name or function of the product; name and address of the manufacturer/importer or of the company responsible for marketing the product; date of manufacture; batch number; expiry date (applicable only to specific cosmetic types); net content in volume or weight; country of origin; ingredients designated by the MOHSA (in some cases also the percentage of the ingredient must be stated); statement "functional cosmetic" (if applicable); retail price; instructions for use and storage (if needed); warnings concerning restricted substances or voluntary ones.

The manufacturer or the importer is responsible for the safety and efficacy of cosmetics marketed in ROK. Cosmetics must not present a risk for human health due to inadequate ingredients, microbial or heavy metal contamination, unhealthy manufacturing practices or insufficient packaging.

Republic of South Africa

South African cosmetic regulations represent the only example of cosmetic self-regulation in the world. Nevertheless, the Codes of Practices published in the Cosmetic Compendium by the South African Cosmetics, Toiletry, and Fragrance Association (CTFA-SA) must comply with the requirements of the Standards Act 29 of 1993. This provides an appropriate regulatory system for cosmetics in South Africa.

The definition of a cosmetic product is close to the EU definition except for the last sentence which has been added to this definition: "A cosmetic product shall mean any substance or preparation to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs), or with the teeth and the mucous membranes of the oral cavity, with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours or keeping them in good condition, except where such cleaning, perfuming, protection, changing, keeping or correcting is for the purpose of treating or preventing disease".

No pre-market notification is required but cosmetics must be in compliance with the aforesaid guidelines.

Making reference to the EU Cosmetics Directive, various positive and negative lists control the use of cosmetic ingredients.

The following information must be on the label in English: name of the manufacturer or of the person responsible for placing it on the market; product name and function if not obvious from the presentation; net quantity of contents; list of ingredients (INCI names allowed; the inclusion of the 26 perfumery allergens has been recently adopted); warnings and instructions for use, notably those listed in the "substances" annexes; storage (if needed); date of minimum durability if less than 30 months; and batch number.

Special labelling is required for sunscreens according to the Sunscreen Product Standard 2001 published by the CTFA-SA and the Standard 1557–2002 published by the South African Bureau of Standard (SABS). This last standard is being amended to take into consideration the publication of the International Harmonisation Committee Sun Group, of which South Africa is a member.

Manufacturers must ensure safety and efficacy of cosmetics marketed in the RSA. An in-market control system is in place but the cosmetic industry has no inspectors to check compliance to national standards. The Advertising Standards Authority (ASA), which receives complaints from the public or companies from time to time on misleading or false advertising, can check cosmetics. In this case, the non-compliant companies have been asked to withdraw their products.

Russian Federation

Cosmetics are regulated by several acts promulgated in the second part of the 1990s, i.e. Consumers Protection Rights Law of the Russian Federation (1996), of the Committee of the Russian Federation for Standardisation, Metrology and Certification, Resolution n°14 (1996), SanPin 1.2.676-97 (1998), Ministry of Health Act N 217 (1998), Gosstandart P 51391-99 (1999) (Russian Committee on Standardisation, Metrology and Certification).

A new draft cosmetic regulations is currently under discussion at the Russian Parliament, the Duma. These technical regulations will greatly harmonize with the EU Cosmetics Directive and will introduce, among other issues, post-marketing control by government and a reduced registration system.

Cosmetic products are defined as "products that are designed to be applied to several parts of the human body (epidermis, hair system, nails, lips, external genital organs, teeth and mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition".

The following eight categories requiring certification are identified: products for face and body skin care; hair care products; decorative cosmetics; products for intimate hygiene; special cosmetic products (sunscreens, suntans, whiteners, etc.); products for hygiene care and odours; dental and oral care; products for protecting the skin against harmful factors.

Registration and approval by Gosstandart prior to sale is compulsory for cosmetics. First a hygiene certificate must be released by RosPotrebNadzor (formerly known as GosSanEpidNadzor—Russian Committee for Sanitary and Epidemiological Surveillance), which will allow a Certificate of Compliance to be issued by Gosstandart. This process,

aiming to substantiate product safety, requires the manufacturer to present of a wide range of documents and samples. A Conformity Mark has to be put on each individual cosmetic packaging as proof that the product is certified.

Generally speaking, ingredients management is linked to the EU annexes, FDA and Japanese provisions. Special attention is given to ethanol-containing cosmetics that must comply with a number of stringent rules concerning denaturation and packaging. Alcohol-containing perfumery and cosmetic products must also follow specific registration by the Ministry of Economy.

Labelling requirements are listed in different areas of regulation. In summary, the following information, mostly in Russian, should appear: name, brand name and function of the product; name and address of the manufacturer and person responsible for marketing the product; date of production or batch number; net content; expiry date or durability date plus date of manufacture; country of origin; ingredients list (INCI names allowed); batch number; conformity mark; registration number and date; instructions for use and storage (if needed); warnings; and ethyl alcohol content (if applicable).

Switzerland

The definition of a cosmetic product is close to the EU Cosmetics Directive one, with the following appendix: "Cosmetics have a local action on healthy skin and appendages, on mucous membranes, on the external genital organs, or on the teeth. Ingredients contained in cosmetics if absorbed must not exert an internal action". The illustrative list of cosmetics in the Swiss ordinance is referable to the Annex 1 of the EU Cosmetics Directive (Département fédéral de l'intérieur, 2005).

The Federal Office for Public Health does not require a mandatory notification of cosmetics.

Ingredients are basically allowed, restricted or banned according to the annexes of the EU Cosmetics Directive but some discrepancies are in force (i.e. restrictions for alpha-hydroxy acids or AHAs, essential oils, arbutin, etc.) (Département fédéral de l'intérieur, 2004). To date Switzerland is the only country in Western Europe imposing a tax on the volatile organic compounds (VOCs) content of cosmetics.

Labelling is also in line with the provisions of the EU Cosmetics Directive and must be in at least one the official languages, except warnings that must be in all official languages.

As in the EU, manufacturers or importers or the person responsible for putting the product on the market must assure the safety and efficacy of the cosmetic concerned. Authorities may ask companies for proof of safety assessment.

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1.2. Quality Control of Cosmetic Products. Specific Legislation on Ingredients

B. Fernández de Córdova Manent and E.F. González Abellán*

Health Department, Regional Government of Comunidad Valenciana,
Guardia Civil St. 21, 46020-Valencia, Spain

QUALITY CONTROL OF COSMETIC PRODUCTS

Legislation concerning cosmetic products in the main markets worldwide, such as the European Union (EU), the Unites States (US) and Japan, demand the assurance of three very important features, namely safety, efficacy and quality of cosmetic products, as is the case for pharmaceuticals or foods.

General aspects of current legislation on safety and efficacy in the different countries, including label information requirements were dealt with in Section 1.1.

As indicated in Section 1.1, manufacturers must have enough data available to assure cosmetic product safety under the normal conditions of use. Data can be obtained either specifically on the finished products or be deduced from the properties of their ingredients. Moreover, data can be obtained through different studies (toxicology, sensitivity, allergic reactions, etc.), some of which are commented in Section 9.1. Sometimes, surveillance of cosmetics in use can be requested to detect possible side effects.

Likewise, as mentioned in Section 1.1, manufacturers must have enough data available to demonstrate cosmetic efficacy (fulfilling that claimed on the label). These can be obtained through different studies (moisturized state, elasticity, etc.), some of which are commented in Section 9.2.

Both safety and efficacy have to be considered under the following conditions:

- The final product must accord with the composition designed by the manufacturer and be in a perfect state.
- The cosmetic has to be applied by the user under the normal given conditions.

Another very important feature of cosmetic products is their quality and this requires thorough control.

Sometimes it is not easy to differentiate between quality and safety problems. Both could cause adverse effects on users, however the origin is different. Experience shows that quality problems affect specific batches which have to be withdrawn from the market, whereas if there is a safety problem it affects all the batches. This is because in the latter case, product

*Corresponding author. E-mail address: gonzalez_eli@gva.es

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use has proven to have toxicological effects on users, thus it is a design failure of the product in question, which must then be completely withdrawn from the market.

Often, quality failures are so evident (like, for example, separation of phases, rarefaction of the fat phases, etc.) that users will realise that they should not apply the product. On the other hand, some quality failures do not cause adverse effects on users. For example, an error in the label does not usually cause adverse effects, although it may in certain cases; like for instance the wrong sun protection factor labelled on a sunscreen product could give rise to solar erythema in users who would trustingly overexpose themselves to the sun.

Difference between quality and safety failures are shown in Figure 1.2.1 with the following example: Let us suppose that some dermatological infections have occurred and the authorities are searching for the origin. Several cases could be given that could have caused adverse effects on the user, but the origin is different

Case 1: The amount of anti-microbial preservatives in the cosmetic formulation has not been calculated properly in the product design, and the cosmetic is not preserved well enough. This is an example of a safety problem; the manufacturer must modify the formulation, and all the batches that were put onto the market will have to be withdrawn.

Case 2: The product should contain a specific amount of anti-microbial preservatives (according to its formulation), but due to a production failure several batches were produced without the correct dose. This is an example of a quality problem; the cosmetic product is well formulated but the operator in charge made a mistake and added an insufficient amount of preservatives in several batches. Only the affected batches will have to be withdrawn from the market.

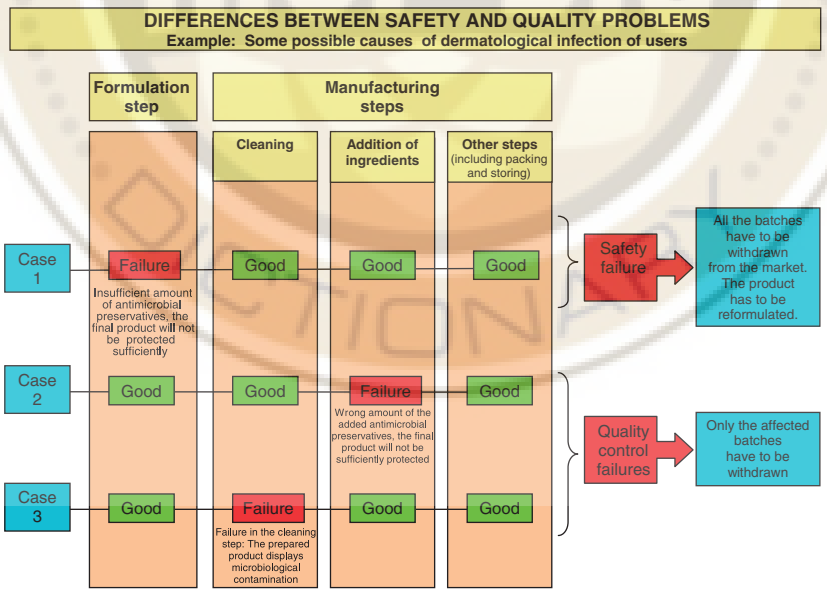

Figure 1.2.1 Diagram where differences between safety and quality problems are exemplified.

Case 3: The product has the correct amount of preservatives but there was a failure during the cleaning steps of production and several batches were damaged. This is also a quality problem; failure to maintain proper standards of cleanliness in the factory plant gave rise to a microbiological increase, causing contamination. Only the contaminated batches have to be withdrawn.

Proper quality control of the manufacturing process or of the final product would avoid the quality problems described above. To this end, quality control requires the manufacturing laboratory (or an external laboratory) to:

- Use appropriate chemical, physicochemical, biological or microbiological analytical procedures to control production. Precision and accuracy of the applied procedures have to be known. These methods must be modified according to new scientific research and advances.
- Assure stability and good preservation of the final product through the necessary assays. Expiry date of the product must also be considered.

Some countries have established specific practices for the manufacture of the cosmetic products, usually named Good Manufacturing Practices (GMP) in order to avoid possible problems or errors in each and every step of the manufacturing process (COLIPA, 2003). By following these rules, one will obtain a final product with the expected quality. The final product must have a constant and specific qualitative and quantitative composition.

Moreover, authorities can carry out analytical controls of commercial products or raw materials, packing, preservation conditions, etc. to assure the quality of the finished product.

SPECIFIC LEGISLATION ON INGREDIENTS

One of the main aspects to be considered in quality control of cosmetic products concerns the substances they contain, i.e. cosmetic ingredients. Different authorities have used the ingredients as a way to perform more direct or indirect controls of cosmetic products, thus enabling them to regulate and manage the market.

In more developed countries, different strategies have been adopted in order to classify cosmetic products, thus enabling gradual requirements to be established in terms of different aspects such as:

- Legal requirements, like for example to prohibit narcotic and/or psychotropic substances, whose trade is regulated by international treaties, or for example, to classify new or toxic substances that could be allowed/restricted in the formulation of cosmetic products.
- Public health requirements, such as sunscreen products to prevent sunburn, pediculicide products, etc.
- Products, considered in principle as cosmetics, but which could exert a marked pharmacological effect.
- Toxicological aspects.

Moreover, the competent authorities also establish requirements with regard to labelling the ingredients composition, warnings about the presence of certain ingredients, etc.

The aim of this section is to give a specific overview of the requirements imposed by the three main legislations on cosmetics (EU, US and Japan) in terms of allowed, restricted or prohibited ingredients and their labelling requirements.

INTERNATIONAL NOMENCLATURE OF COSMETIC INGREDIENTS

The use of cosmetic products is on the increase around the world. As described in Section 1.1, there are different organisms regulating the manufacture of this type of products in different countries. An increasing number of new cosmetic products appear on the market, with new or improved properties, implying the use of new chemical substances. This means that a great many substances are employed in cosmetic products worldwide. In order to avoid language barriers that may promote problems of free trade, and may also confuse consumers, it is necessary to harmonize the nomenclature of the substances employed in cosmetics.

The Cosmetic, Toiletry and Fragrance Association (CTFA) was pioneer in trying to harmonize cosmetic nomenclature following the guidelines of the United States Food and Drug Administration (FDA). In a survey carried out in cosmetic companies in the late 1960s, they realized that the same chemical was named by different trade and chemical names. They then created a committee comprised of industrial experts in the fields of chemistry, cosmetic science and technology, as well as members of the American Medical Association, the United States Adopted Names Council and the FDA. In 1973, the CTFA published the First Edition of the CTFA Cosmetic Ingredient Dictionary where the substances employed in cosmetic products were described by their CTFA Adopted Names. Afterwards, the FDA cited this dictionary as the primary source of nomenclature for cosmetic-product labelling. Later, in 1993, because of the strong repercussion it had in different countries around the world, the designation was changed from CTFA Adopted Names to International Nomenclature of Cosmetic Ingredients (INCI), as it is known nowadays (Gottschalck and McEwen, 2006).

This nomenclature was officially adopted by the other two main legislations on cosmetic products over the world, i.e. in the EU and Japan frameworks, in 1996 and 2001, respectively, although a few discrepancies can be observed in case of colouring agents (see Section 4.1), botanical extracts, and a few trivial names. Nevertheless, the CTFA is working closely with the European Cosmetic, Toiletry and Perfumery Association (COLIPA) and with Japan's Cosmetic Industry Association (JCIA) in order to harmonise these final discrepancies.

The INCI names may only be assigned by CTFA's International Nomenclature Committee (INC). In order to insert a new substance in the dictionary, an application needs to be addressed to CTFA, which, after a preliminary review, will be submitted to the INC. Then an INCI name is assigned based on chemical structure and composition and is published in the next edition of the dictionary.

Now, the International Cosmetic Ingredient Dictionary and Handbook is in its eleventh edition (Gottschalck and McEwen, 2006), and incorporates more than 13,000 ingredients.

Nevertheless, it should be emphasized that this dictionary does not represent a positive list of the cosmetic ingredients that appear here. The inclusion of any chemical means only that this chemical is or was sold for use in cosmetic products, and does not imply that the substance is safe for use as a cosmetic ingredient, nor does it indicate that its use as a cosmetic ingredient complies with the laws concerning cosmetic products. On the other hand, the absence of a chemical substance from this list does not imply that this substance may not be used in cosmetic products. In this sense, when a cosmetic product is going to be marketed in a certain domain, manufacturers have to consult the specific legislation in force on cosmetic products of the country.

Next, we will summarize the legislation concerning cosmetic ingredients set out by the three main legislations worldwide.

As already mentioned, the use of a harmonized nomenclature not only helps free trade, but makes it easier for consumers and the medical community to act when a dermatological problem arises.

COSMETIC INGREDIENTS IN THE EUROPEAN UNION FRAMEWORK

Article 5a of the EU Cosmetics Directive (Council Directive 76/768/CEE), established a deadline on 14 December 1994 for the Commission to compile an inventory of ingredients employed in cosmetic products, which should be updated periodically. It must contain information on the identity of each ingredient, its function in the cosmetic product, and any restriction and condition of use and/or warning which must be printed on the label. In addition, the same article, defines cosmetic ingredient as *"any chemical substance or preparation of synthetic or natural origin, except for perfume and aromatic compositions, used in the composition of cosmetic products"*. Nevertheless, according to Article 6.1, the impurities in the raw materials used are not considered as ingredients, nor are the subsidiary technical materials used in the preparation but not present in the final product or the materials used in strictly necessary quantities as solvents or as carriers for perfume and aromatic compositions.

However, in 1996, in the Commission Decision 96/335/EC, the Commission first publishes an inventory containing around 6400 substances (or families of substances), divided into two sections, i.e. Section 1 concerned all those ingredients other than perfume and aromatic raw materials and Section 2 contained only the latter. This Decision stated that INCI names were to be adopted as the common nomenclature to refer to cosmetic ingredients. Recently, Section 1 has been updated (Commission Decision 2006/257/EC) and includes around 1400 new entries.

Nevertheless, it should be emphasized that this list does not constitute a positive list of the substances authorized for use in cosmetic products, but has only indicative purposes.

The lists that reflect regulatory aspects in the EU framework are, as already mentioned in Section 1.1, the different annexes of the EU Cosmetics Directive, where Annex II is a negative list of over 1200 substances (or families of substances) that are banned for use in the composition of cosmetic products. Annex III gives over 150 ingredients which cosmetic products may only contain subject to the restrictions and conditions established therein. Finally, Annexes IV, VI and VII are positive lists of over 150 colouring agents,

almost 60 preservatives and around 30 UV filters, respectively, permitted for use in cosmetic products within the limits and under the conditions therein. These lists are not closed, and are permanently updated according to the data provided by the Scientific Committee on Consumer Products (SCCP), formerly known as the Scientific Committee of Cosmetic and Non-Food Products intended for Consumers (SCCNFP), in response to technical progress and/or concerns about the impact of particular ingredients on safety, taking the final decision on the addition (or removal) of substances from the lists by the Commission and the Member States. In fact, Annexes III, IV, VI and VII are divided into two parts, where Part 1 lists the corresponding permitted substances (or families of substances) which cosmetic products may currently contain, while Part 2 lists those provisionally allowed until a given date. After this date, these provisionally authorized ingredients may be further maintained in an updated Part 2 for a given period of time if there are insufficient data, or may be definitively allowed (and will then be moved to the corresponding Part 1), or on the contrary, they may be definitively prohibited if considered harmful to human health, (and then will be moved to Annex II of the EU Cosmetics Directive), or they may simply be deleted from these annexes on the basis of available scientific information or because they are no longer used.

Moreover, the EU Cosmetics Directive, in its Article 4b, prohibits the use in cosmetic products of substances classified as carcinogenic, mutagenic or toxic for reproduction (CMR), of categories 1, 2 and 3 under Annex I to Council Directive 67/548/EEC, which regulates the classification, packaging and labelling of dangerous substances placed on the market in the Member States of the EU. Nevertheless, a substance classified in category 3 may be used in cosmetics if the substance has been evaluated by the SCCNFP and found acceptable for use in cosmetic products. Also, in Council Directive 76/769/EEC there is a list of substances (including those listed in the previously mentioned Directive) for which marketing and use in the EU is limited, and thus this includes cosmetic products. A large number of these substances (or families of substances) have recently been included in Annex II of the EU Cosmetics Directive.

Nevertheless, according to Article 8a of the EU Cosmetics Directive, "(…) a Member State may authorize the use within its territory of other substances not contained in the lists of substances allowed, for certain cosmetic products specified in its national authorization (…)". In this case, the authorization must not exceed a maximum period of three years, and the Member State must carry out an official check on cosmetic products containing the substance or preparation, which it has authorized. Moreover, cosmetic products containing the new authorized substance or preparation must bear a distinctive indication, which will be defined in the authorization.

On the other hand, despite all these lists regulating the substances prohibited or restricted in cosmetics, it is worth mentioning that certain confusion could arise among manufacturers, since there are substances that are not listed as such, but are included in any one of the listed families of substances. This is the case, for example, of cocaine (a narcotic drug), which is not listed as such but is included under entry number 306 of Annex II which lists "Narcotics, natural and synthetic: All substances listed in Tables I and II of the single Convention on narcotic drugs signed in New York on 30 March 1961". This example shows the importance of knowing legislation concerning cosmetics appropriately.

On the other hand, considerations on packaging and labelling of cosmetics products were discussed in Section 1.1; however, those concerning cosmetic ingredients are described here in depth.

Taking into account Article 6 of the EU Cosmetics Directive, Member States have to take all measures necessary to ensure that the cosmetic products marketed in their territory are labelled with a list, preceded by the word *Ingredients*, of ingredients (by using INCI names, according to Decision 96/335/EEC) in descending order of the weight at the time they were added. Those ingredients whose concentration is less than 1%, may be listed in any order after those in concentrations of more than 1%, and colouring agents may be listed in any order after all the other ingredients, in accordance with the colour index (CI) number or denomination adopted in Annex IV of the EU Cosmetics Directive. Moreover, in the event of decorative cosmetic products marketed in several colour shades, all colouring agents used in the range may be listed, provided that the words *may contain* or the symbol "+/–" are added. In addition, all warnings for any ingredients used described in Annexes III, IV, VI and VII of the EU Cosmetics Directive, like for example *Contains oxybenzone*, must also appear on the label of the product. Where that is impossible for practical reasons, an enclosed leaflet, label, tape or card must contain the ingredients to which the consumer is referred either by abbreviated information or the symbol given in Annex VIII, which must appear on the packaging.

In the event of perfume and aromatic compositions and their raw materials, they have to be referred to by the word *perfume* or *aroma*. However, the presence of aromatic substances, for which there is a special mention according to Annex III of the EU Cosmetics Directive, must be declared in the labelling list irrespective of their function in the product. This is the case of the 26 potentially allergenic fragrance compounds, which according to the above mentioned annex must be indicated when their content in the finished product is higher than 0.001% in leave-on products and 0.01% in rinse-off products.

Nevertheless, a manufacturer may, for reasons of trade secrecy, apply not to include one or more ingredients on the above mentioned list, according to the procedure established by the Commission Directive 95/17/EC. In event of approval by the competent authority, the name of the ingredient will be removed from the ingredient list, and replaced by a seven-digit code number, with the first two corresponding to the year of confidentiality approval, the second two to the code assigned to each Member State, and the three final digits being assigned by the competent authority. The approval granting the right to confidentiality is valid for a five-year period, with a maximum three-year extension period; however, it could be cancelled by the competent authority if there is any evidence putting into question the safety of the concerning ingredient.

Despite the concessions regarding trade secrecy, for control purposes and according to Article 7a of the EU Cosmetics Directive, the manufacturer, agent or person to whose order a cosmetic product is manufactured or the person responsible for placing an imported cosmetic product on the Community market, must have readily available, among other documents, the qualitative and quantitative composition of the product on request by the competent authorities of the Member States concerned at the address specified on the product label.

COSMETIC INGREDIENTS IN UNITED STATES

In the United States (US) framework, regulations published by the Food and Drug Administration (FDA) concerning cosmetic products can be found in Title 21 of the Code of Federal Regulations (21 CFR) Parts 700–740, which states that "(…) 'ingredient' means any single chemical entity or mixture used as a component in the manufacture of a cosmetic product".

21 CFR Part 701.3 about cosmetic labelling establishes that each cosmetic package has to bear a list, in descending order of predominance, naming each ingredient, except that fragrance or flavor may be listed as *fragrance* or *flavour*. If it is not possible to declare this on the package for practical reasons, the declaration may appear on a firmly affixed tag, tape or card. Similarly to the EU Cosmetics Directive, a permitted alternative is to list those ingredients, other than colouring agents, present at a concentration greater than 1%, in descending order of predominance, followed by those (other than colouring agents) present at a concentration of not more than 1% without respect to order of predominance. However, all these could also be joined together and listed in order of predominance, and finally followed by colouring agents, without respecting the order of predominance. In the event of shaded products or products with similar composition and intended for the same use, colouring agents may be included in the label even they are not in the cosmetic, provided the phrase *may contain* followed by the colouring agent name is written. The term *and other ingredients* at the end of the ingredient declaration will replace the name of the ingredients that the FDA have authorized the company to exclude from the label for confidentiality purposes, according to 21 CFR Part 720.8.

In the event that there is a current or anticipated shortage of a cosmetic ingredient, alternative ingredients may be used. These must be declared either immediately after the normally used ingredient it substitutes with the word *or*, or following the declaration of all normally used ingredients after the sentence *may also contain*.

The incidental ingredients that could be present in a cosmetic product at insignificant levels and that have no technical or functional effect in the cosmetic need not be declared on the label. This is the case of substances that have no technical or functional effect in the cosmetic but are present because they have been incorporated into the cosmetic as an ingredient of another cosmetic ingredient. Nor is it necessary to declare on the label substances that are added to a cosmetic during manufacture for technical and functional effects, but are removed before the cosmetic product is packaged in its finished form; or, are converted into the same substances as those constituents of declared ingredients, without significantly increasing the concentration of these constituents; or, are present in the finished cosmetic at insignificant levels and do not have any technical or functional effect on the cosmetic.

When a cosmetic product is also considered as an over-the-counter (OTC) drug product (see Section 1.1), the active ingredients are to be listed first of all, after the sentence *Active Ingredients*, and the quantity of each one must also be declared. The rest of ingredients will be listed next, after the sentence *Inactive ingredients* according the rules listed above.

The cosmetic ingredients have to be identified in the declaration of ingredients by the name specified in 21 CFR Part 701.30, where only eight ingredients are listed (a few chlorofluorocarbon derivatives and ethyl esters of hydrolyzed animal protein). In the event of absence, which is most likely, the source to be employed will be the CTFA's

International Cosmetic Ingredient Dictionary and Handbook. In the unusual case that an ingredient does not appear in this database, other sources such as the US Pharmacopeia, the National Formulary, the Food Chemicals Codex and finally the United States Adopted Name (USAN) will be consulted in this order of preference. If the ingredient does not appear in any of the aforementioned databases, the name generally recognized by consumers will be used, or finally the chemical or other technical name or description.

However, none of the above mentioned sources constitutes a list of substances allowed for use in cosmetic formulations. As described previously in Section 1.1, FDA only lists a small number of strictly regulated or prohibited ingredients, which are summarized in Table 1.2.1, and also have positive lists for colouring ingredients. One of these colouring

Table 1.2.1

Prohibited or restricted substances in cosmetic products by FDA (in alphabetical order)

Substance	Restriction/prohibition	Cause of the prohibition/restriction
Bithionol	Prohibited	Photocontact sensitization
Cattle material	Prohibited	
Chlorofluorocarbon propellants	Prohibited	
Chloroform	Prohibited	Animal carcinogenicity and likely hazard to human health
Halogenated salicylanilides (dibromsalan, tribromsalan, metabromsalan and tetrachlorosalicylanilide)	Prohibited	Photocontact sensitization
Hexachlorophene	<0.1% and when an alternative preservative has not been shown to be as effective and it may not be used in cosmetics to be applied to mucous membranes	Neurotoxic effect and ability to penetrate human skin
Mercury compounds	65 mg/kg of metallic mercury in eye area cosmetics when no other effective and safe preservative is available for use	Absorption through the skin on topical application and tendency to accumulate in the body. May cause allergic reactions, skin irritation or neurotoxic manifestations
	<1 mg/kg of metallic mercury in other area cosmetics when unavoidable under conditions of good manufacturing pratice	
Methylene chloride	Prohibited	Animal carcinogenicity and likely hazard to human health
Vinyl chloride	Prohibited in aerosol products	Carcinogenicity
Zirconium-containing complexes	Prohibited in aerosol products	Toxic effect on lungs, including the formation of granulomas

ingredients list (21 CFR Part 74) shows those colouring agents subject to batch certification of composition and purity by FDA. These colouring agents are synthetic organic chemicals, and are usually referred to as "coal-tar" colouring agents, due to their original source in the 19th century. On the other hand, the other positive list (21 CFR Part 73) is comprised by colourants obtained primarily from mineral, plant or animal sources. These last colour additives are exempt from batch certification and although they are free of such testing, manufacturers must assure that each colouring agent complies with the identity, specifications, labelling requirements, use and restrictions of colouring-agent regulations. Nevertheless, with the exception of coal-tar hair dyes, all colour additives, whether they are subject to certification or not, must be approved by the FDA for their intended use, otherwise the cosmetics containing them will be considered as adulterated. So manufacturers need to check the above mentioned lists to determine whether a colouring agent is approved for an intended use and whether it is subject to certification requirements.

Moreover, as mentioned in Section 1.1, different products considered as cosmetics in the EU are considered as OTC drugs in US. These include anti-perspirants, sunscreens, anti-caries toothpastes, anti-dandruff and anti-seborrheic products among others. For each of these product types, FDA has published in 21 CFR parts from 350 to 360 a monograph containing positive lists of the active ingredients that can be employed.

Thus, regarding cosmetic products as such, excepting colouring agents, any substance could be used as a cosmetic ingredient except those few listed in Table 1.2.1, but under the responsibility of the manufacturer. Qualitative and quantitative formulas have to be available in case of the FDA inspection. Nevertheless, manufacturers can send the FDA data about cosmetic product ingredients voluntarily under the Voluntary Cosmetic Registration Program.

Nevertheless, there are different cosmetic and fragrance trade associations that have recommended eliminating or limiting maximum levels of different ingredients taking into account the health issue. For example, the Cosmetic Ingredient Review (CIR) Expert Panel, an independent panel of scientific experts established by the CTFA with support of the FDA, thoroughly reviews and assesses the safety of numerous ingredients used in cosmetics and publishes their results yearly in open, peer-reviewed scientific literature. According to its Annual Reports, CIR has classified around 1300 ingredients according to their safety profile. So, it lists almost 800 substances as safe at certain concentrations of use, more than 400 substances as safe with certain restrictions, around 120 substances whose safety is not well documented, and only 9 substances are classified as unsafe. Table 1.2.2 lists those substances found unsafe by CIR, and its webpage (http://www.cir-safety.org) gives a detailed list of all findings. The output of the CIR has no legal weight, since the final decision corresponds to the FDA.

In similar way, the International Fragrance Association (IFRA) establishes usage guidelines for cosmetic ingredients related to fragrance products. In its Code of Practice, available on line (http://www.ifraorg.org) can be found recommendations for avoiding many ingredients, but once again the final decision corresponds to the FDA.

Other substances to be considered are those not added intentionally, but formed by the reaction between different ingredients whether during manufacture or storage, as is the case of nitrosamines. These hazardous substances can be formed by reaction of amines with nitrosating agents (such as sodium nitrite or preservatives like 2-bromo-2-nitropropane-1,3-diol,

Table 1.2.2

Ingredients found unsafe for use in cosmetics by CIR

Substance	Function	Safety concern
Chloroacetamide	Preservative	Skin sensitization
Ethoxyethanol and its acetate	Solvent	Reproductive and developmental toxicity
HC Blue No. 1	Hair colouring ingredient	Carcinogenicity
p-Hydroxyanisole	Antioxidant	Skin bleaching
4-Methoxy-*m*-phenylenediamine and its hydrochloride and its sulphate	Hair dye ingredients	Carcinogenicity
Pyrocatechol	Used in hair dyes and skin care preparations	Carcinogenicity in leave-on products

5-bromo-5-nitro-1,3-dioxane or tris(hydroxymethyl) nitromethane). The FDA expressed its concern about the contamination of cosmetics with nitrosamines in a Federal Register notice dated 10 April 1979, which stated that cosmetics containing nitrosamines may be considered adulterated and subject to enforcement action.

JAPAN

Until recently, Japan had a positive list (the Comprehensive Licensing Standards or CLS) system under which each ingredient used in a cosmetic formulation had to be pre-approved by Ministry of Health, Labour and Welfare (MHLW) formerly know as Ministry of Health and Welfare (MHW). Since a deregulation process in April 2001 (see Section 1.1), and with regard to cosmetic ingredients, the establishment of negative and positive lists like in EU was carried out by the MHW in the Standards for Cosmetics notification (MHW, 2000). In this sense, Japan adopted a list of around 30 prohibited ingredients (Appendix 1 of the aforementioned notification), a list containing approximately 20 restricted ingredients (Appendix 2), a positive list of more than 40 preservatives (Appendix 3) and a positive list of nearly 30 UV filters (Appendix 4).

Nevertheless, a positive list of colouring agents has been regulated since 1966 (MHW, 1966).

According to the Standards for Cosmetics, "cosmetics shall not contain any medical drug ingredients (excluding those used only as additives and those listed in Appendix 2 through 4), or any ingredients that do not meet the Standards for Biological Materials (Ministry of Health, Labour and Welfare Notification No.210 of 2003), or any of the materials listed in Appendix 1". Moreover, cosmetic products will not be able to contain any of the ingredients listed in Appendix 2 at amounts higher than those specified in the aforementioned list, and no other preservative included in Appendix 3 nor other UV filters included in Appendix 4 under the conditions established therein.

Moreover full ingredient labelling must be provided for cosmetics using the INCI names translated or transliterated into Japanese, in descending order of predominance.

On the other hand, as has been seen in Section 1.1, different products considered as cosmetics in the EU are considered as quasi-drugs in Japan, like for example, hair dyes, permanent waving products, depilatories, deodorants and anti-dandruff shampoos among others. For this type of products, the deregulation process of cosmetics in 2001 and the changes it involved were not applied, and a pre-market approval is needed by MHLW. Moreover, registration of all ingredients used in product manufacture, as well as product safety data which specify the active ingredients, usage and dosage, indications or effects is also required. Full lists of approved quasi-drug ingredients are not published, although the MHLW has published lists of ingredients approved for use in certain categories, such as hair dyes, permanent waving agents, medicated toothpaste and bath preparations. Full ingredient listing is not required for quasi-drugs; however, the MHLW has listed 138 ingredients that must be indicated on the label.

SUMMARY

The main similarity between the three main legislations regarding cosmetic products, i.e. those in force in the EU, the US and Japan, concerning cosmetic ingredients lies in the fact that pre-market approval is unnecessary, and thus, full responsibility for the safety of products, which depends mainly on the ingredients, falls on the manufacturer. Meanwhile, authorities carry out in-market surveillance in order to check the compliance with each one of the legislations.

Another similar point is that INCI nomenclature is widely used in all three regulatory systems, avoiding consumer confusion and improving the marketing of the products between different domains.

In terms of the major difference between these three regulatory systems we can see that while both the EU and Japan maintain lists of prohibited and restricted substances, together with positive lists for colouring agents, preservatives and UV filters, in the US there are no positive lists for cosmetic ingredients (except for colouring agents) and the list for prohibited ingredients is somewhat shorter than in the other two legislations. Moreover, the European and Japanese lists are not identical and some ingredients that are prohibited or restricted on one market are permitted on the other. Furthermore, in some cases the authorized content for the same permitted ingredient differs enough from one legislation to the other.

Finally, different products considered as cosmetics in the EU, are considered as OTC drugs in the US, and similarly, in Japan there is another category that is named quasi-drugs. These product categories need pre-market approval of the competent authority and obey different regulations to cosmetics. Besides, in case of OTC drugs in the US, the FDA has published different monographs that compile specific data regarding this type of products with regard to the ingredients allowed and their maximum contents. On the contrary, the Japanese MHLW only discloses ingredients for hair dyes, permanent waving agents, medicated toothpaste and bath products. Consequently, it may be difficult for manufacturers to identify which ingredients are approved for which use.

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– 2 –

General Overview on Analytical Methods for Cosmetic Ingredients

2.1. General Review of Official Methods of Analysis for Cosmetics in Different Countries

L. Gagliardi,[1*] D. De Orsi[1] and S. Dorato[2]

[1]Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Roma, Italy
[2]Department of Pharmaceutical Sciences, Facoltà di Farmacia, Università di Genova, Italy

GENERAL OVERVIEW OF COSMETIC REGULATIONS

As described in Section 1.1, three main regulatory systems exist at a global level in the domain of cosmetics: those of the European Union (EU), the United States (US) and Japan. These regulatory models have been used by many other countries or communities to prepare their own cosmetic legislation.

Since cosmetics are widely used consumer goods, all regulations concerning them have, as their prime and principal purpose, the guaranteeing of consumer safety. Thus, Article 2 of the European Union Cosmetics Directive 76/768/EEC expressly states: *"A cosmetic product put on the market within the Community must not cause damage to human health under normal or reasonably foreseeable conditions of use (…)"*. Article 3 states that *"Member States shall take all necessary measures to ensure that only cosmetic products which conform to the*

*Corresponding author. E-mail: luigi.gagliardi@iss.it

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Amparo Salvador and Alberto Chisvert

provisions of this Directive and its annexes may be put on the market". Thus, it is the responsibility of national authorities to verify the conformity of cosmetic products sold in their respective countries.

Another concern of the responsible authorities, especially in the numerous countries where legislation forbids misleading advertising, is to verify the veracity of claims and the efficacy of products. Efficacy and claims may be linked to the presence of an ingredient in the finished product, whose presence and/or content must be verified, if necessary.

As described in Section 1.1, depending on the local regulatory system, the tests are performed before and/or after placing the cosmetic products on the market. The regulations applicable to cosmetics can, indeed, be classed in two main categories: regulations imposing an obligation to submit a registration dossier to the authorities prior to placing the finished product on the market (pre-market approval), and those which impose responsibility on the manufacturer selling the product, without the need for prior approval from the authorities, but with control, if necessary, once the product is on the market (in-market control).

Whether applying a pre-market or an in-market control system, the authorities need official analysis methods to verify the regulatory compliance of cosmetics *vis-à-vis* the requirements applicable to substances which may be included in the formulations undergoing testing. Thus, the competent authorities can verify whether the qualitative–quantitative formula submitted by the manufacturer does, in fact, correspond to the analysis of the finished cosmetic. The possible presence of banned substances in the finished product may also be tested. Similarly, the authorities responsible for in-market control may verify whether the cosmetic complies with the concentration restrictions applicable to certain ingredients, or whether they contain substances other than those listed on the ingredient label (see Section 1.2 for a detailed explanation on ingredients labelling).

It should be noted that physical/chemical tests performed on the finished product prior to sale do not prevent a second round of testing once the product is placed on the market. The authorities will then check whether the information supplied at the time of registration corresponds to the product sampled at random when on the market, by controlling, for example, whether the stated concentrations vary over time. This type of control is performed mostly in countries where a shelf life or expiry date is required on the finished product label. In such cases the authorities can check whether the quantity of active substances remains within the permitted limits throughout the shelf life, during that time the product is assumed to remain effective.

For their part, manufacturers must, at all times, be able to guarantee their products conform to legislation through a quality-control system (see Section 1.2). The analytical methods used by manufacturers must enable them to control the manufacturing process and the conformity of each batch of raw materials it intends to use, and also, the final cosmetics before sale.

SUBSTANCES SUBJECT TO BE CONTROLLED BY THE AUTHORITIES

As illustrated in Section 1.1, most cosmetic regulations control the use of cosmetics ingredients by publishing three types of substance lists:

(a) Substances banned in cosmetics:
These substances must not be found in the finished product, except as traces. Indeed, according to the Article 4.2 of the EU Cosmetics Directive, the presence of

traces of a banned substance (i.e. those listed in Annex II of the EU Cosmetics Directive) is allowed provided that such presence is technically unavoidable when using good manufacturing practice and if they do not pose any risk to consumer health.

(b) Substances restricted in cosmetics:
These substances may not be used in cosmetic products, except subject to the restrictions and conditions laid down by the different legislations (e.g. EU Cosmetics Directive Annex III). The restrictions may concern maximum concentrations, pH limits in the finished product, warning labels or purity criteria, depending on the product in which they are formulated. For example, see Table 2.1.1 for the restrictions specific to thioglycolic acid and its salts in the EU Cosmetics Directive Annex III.

A specific case concerns the 26 potentially allergenic fragrance ingredients listed in EU Cosmetics Directive Annex III: these substances must be shown on the ingredient list on the label if their content in the final product exceeds 0.001% in leave-on cosmetics and 0.01% in rinse-off cosmetics.

The authorities may verify whether a restricted ingredient is contained in a cosmetic product at a concentration higher than that permitted for the field of application under consideration.

(c) Substances subjected to positive listing:
These substances are used in cosmetics for specific functions: for example cosmetic colouring agents, preservatives or UV filters for skin protection in the EU and in many other countries. In the context of cosmetic regulations, China has also published a list of hair dyes.

However, the positive lists may also concern functions which are considered to correspond to a cosmetic in the EU, but whose ingredients are considered as active ingredients of over-the-counter (OTC) drugs in the US, quasi-drugs in Japan, functional cosmetics in Korea or medicated products in Taiwan (see Section 1.1). Only substances included on a specific positive list are allowed for the function concerned in the country issuing the list. However, a substance that is not included in a specific positive list (e.g. a preservative not listed in EU Cosmetics Directive Annex VI) may nevertheless be used in a cosmetic insofar as it is used for a function other than that taken into account in the positive list concerned (preservation in this example).

These substances are also, in most cases, subject to concentration limits, and the authorities will make every effort to verify they are not used at concentrations higher than those authorized.

In contrast, the authorities may also control substances that are not necessarily regulated in cosmetic products:

(a) Substances that are claimed to be present in the final product or for which an exact concentration is claimed.

These tests are performed by the authorities responsible for advertising control, for example the Direction Générale de la Consommation, de la Concurrence et de la Répression des Fraudes (DGCCRF) in France. In fact, these tests are generally performed with a view to control fraud and misleading advertising.

Table 2.1.1

Restrictions on thioglycolic acid and its salts according to EU Cosmetics Directive Annex III

Substance	Field of application and/or use	Maximum concentration authorized in finished product	Other limitations and requirements	Conditions for use and warnings to be included mandatory on the label
Thioglycolic acid and its salts	(a) Hair waving or straightening products – General use – Professional use (b) Depilatories (c) Other hair care products that are removed after application	– 8% ready for use, pH 7 to 9.5 – 11% ready for use, pH 7 to 9.5 – 5% ready for use, pH 7 to 12.7 – 2% ready for use, pH 7–9.5 (as thioglycolic acid)	(a), (b) and (c): The directions for use drawn up in the national or official language(s) must obligatorily incorporate the following sentences: – Avoid contact with eyes – In the event of contact with eyes, rinse immediately with plenty of water and seek medical advice – Wear suitable gloves [only for (a) and (c)]	(a): – Contains thioglycolate – Follow the instructions – Keep out of reach of children – For professional use only (b) and (c): – Contains thioglycolate – Follow the instructions – Keep out of reach of children

(b) Ethanol concentration.

Some Authorities verify the alcohol content for fiscal or religious reasons. For example, Saudi Arabia has published a standard concerning alcohol-containing cosmetics which limits the ethanol content of finished products to 90%, and a standard on the test methods to be applied to alcoholic products which specifies, notably, how to check the essential oil content, ethanol, methanol, 1-propanol and denaturant contents.

In contrast, Sri Lanka, in its Regulation No. 12 of 17 March 1993, requires submission of a product analysis certificate and information on the analysis protocol or on the other controls for verifying the alcohol content in the dossier required for registration of a new cosmetic.

(c) Substances used in cosmetic products but subject to non-cosmetic regulations.

For example, volatile organic compounds (VOCs) present in specific consumer goods are taxed in some countries (Switzerland) or restricted (in several US States such as California, New Jersey or New York among others) in the context of environmental regulations. The authorities responsible for environmental problems may possibly verify the presence and/or content of VOCs in the products concerned.

Hence one can consider that the presence and/or content of practically any substance contained in a cosmetic product may be controlled for one reason or another. We intend to verify, with reference to the zones corresponding to the three cosmetic regulation models (the European Union and other countries following EU legislation; US and the countries it has influenced; Japan and other Asian countries), which official physical/chemical analysis methods exist for cosmetic ingredients.

ANALYTICAL METHODS IN THE EUROPEAN UNION

Article 8.1 of the European Union Cosmetics Directive 76/768/EEC stipulates that *"the methods of analysis necessary for checking the composition of cosmetic products"* and *"the criteria of microbiological and chemical purity of cosmetic products and methods for checking compliance with those criteria"* shall be determined.

The Commission, the Member States and the European Cosmetic Industry, represented by COLIPA (the European Cosmetic, Toiletry and Perfumery Association), worked on these physical/chemical methods of analysis between 1980 and 1996. The first European directive on cosmetic methods of analysis (80/1335/EEC) included analytical methods for only a few substances used in cosmetic products; however, it also described aspects related to sampling and sample pre-treatment according the type and physical state of the cosmetic preparation. The number of methods developed for cosmetic analysis has increased in successive directives, and to date, six other directives have been published (82/434/EEC, 83/514/EEC, 85/490/EEC, 93/73/EEC, 95/32/EC, 96/45/EC); of which two have been modified to improve some of the methods (87/143/EEC, 90/207/EEC). The European Commission edited a document (European Commission, 1999) in which all the aforementioned directives are compiled and described.

As shown in Table 2.1.2, the 38 officially published methods in the European directives regarding methods of analysis, concern approximately 60 ingredients or families of

Table 2.1.2

Substances with an official method of analysis in the European Union

EU annexes	Number of substances listed in the annex	Substances or families of substances for which there exists an Official Method of Analysis in the EU			
		Annex Reference number	Name	Total	EU directive
Annex II (banned substances)	1132	167	– Glyceryl PABA	4	85/490/EEC
		178	– Hydroquinone benzylether and ethylether		95/32/EEC
		366	– Chloroform		80/1135/EEC
		371	– Hexachlorophene		83/514/EEC
Annex III (restricted substances)	157	2a and 2b	– Thioglycolic acid and its salts and esters	31	83/514/EEC
		3	– Oxalic acid and its esters and alkali salts		80/1335/EEC
		4	– Ammonia		83/514/EEC
		5	– Chloramine T		83/514/EEC
		6	– Alkali chlorates		85/490/EEC
		7	– Dichloromethane		83/514/EEC
		8	– m-and p-phenylenediamine and certain derivatives		82/434/EEC
		9	– Toluenediamines and certain derivatives		82/434/EEC
		10	– Diaminophenols and certain derivatives		82/434/EEC
		12	– Hydrogen peroxide		82/434/EEC
		13	– (Free) formaldehyde		90/207/EEC
		14	– Hydroquinone		95/32/EC
		15a	– (Free) potassium and sodim hydroxides		80/1335/EEC
		16	– 1-Naphthol		82/434/EEC
		17	– Sodium nitrite		82/434/EEC
		18	– Nitromethane		83/514/EEC
		19	– Phenol and its alkali salts		82/434/EEC
		21	– Quinine and its salts		85/490/EEC
		22	– Resorcinol		82/434/EEC

		23	– Alkali and alkaline-earth sulfides		83/514/EEC
		24	– Zinc derivatives		80/1335/EEC
		25	– Zinc phenolsulfonate		80/1335/EEC
		26 to 43, 47, and 56	– Fluoride derivatives		83/514/EEC
		45			93/73/EEC
		48	– Benzyl alcohol		93/73/EEC
		49	– Silver nitrate		93/73/EEC
		50	– Selenium sulfide		93/73/EEC
		51	– Aluminium zirconium chloride hydroxyde complexes		83/514/EEC
		52	– Oxyquinoline and its sulphate		82/434/EEC
		54	– Methyl alcohol		96/45/EC
		95	– Phenoxypropanol		95/32/EC
			– Hydroquinone methylether		
Annex IV (cosmetic dyes)	157	Barium and Strontium soluble in pigments and lakes		1	93/73/EC
Annex VI (preservatives)	55	1	– Benzoic acid and its salts	17	95/32/EC
		2	– Propionic acid and its salts		95/32/EC
		3	– Salicylic acid and its salts		95/32/EC
		4	– Sorbic acid and its salts		95/32/EC
		5	– (Free) Formaldehyde		90/207/EC
		9	– Sulfites and bisulfites		85/490/EEC
		10	– Sodium iodate		85/490/EEC
		11	– Chlorobutanol		85/490/EEC
		12	– 4-Hydrobenzoic acid and its salts and esters		95/32/EC, 96/45/EC
		15	– Dibromohexamidine		93/73/EEC
		16	– Thimerosal		83/514/EEC
			– Phenylmercuric acid and salts		83/514/EEC
		17	–Phenoxyethanol		96/45/EEC
		29	–Benzyl alcohol		93/73/EEC
		34	–Chlorhexidine		93/73/EEC
		42	–Phenoxypropanol		96/45/EC
		43	–Hexamidine		93/73/EEC
		47			

(*Continued*)

Table 2.1.2 (cont.)

EU annexes	Number of substances listed in the annex	Substances or families of substances for which there exists an Official Method of Analysis in the EU			
		Annex Reference number	Name	Total	EU directive
Annex VII (UV filters)	27		—		
Substances not listed in an EU annex			– Dibromopropamidine	3	93/73/EEC
			– Ammonium, potassium and sodium persulfates		82/434/EEC
			– Potassium and sodium bromates		82/434/EEC
Total	1132 banned substances and 396 restricted substances			55	

substances, primarily restricted ingredients and preservatives. This is a relatively small number, considering how many substances are actually regulated by EU Cosmetics Directive Annexes III, IV, VI and VII, i.e., almost 400 ingredients. This number is even lower if one takes into account how many ingredients used in the European Union for cosmetics according to the EU Inventory of Cosmetic Ingredients (Commission Decision 96/335/EC): approximately 6400 ingredients. Moreover, to this we must add the 1400 ingredients incorporated in the first update of the inventory (Commission Decision 2006/257/EC); giving a total of 7800 ingredients. What is more, this does not take into account the 1132 substances that are banned in cosmetics, which have been listed in Annex II since the addition of almost new 680 substances in 2005, considered as carcinogenic, mutagenic or toxic for reproduction (CMR) under Annex I to Council Directive 67/548/EEC, which regulates the classification, packaging and labelling of dangerous substances placed on the market in the member states of the EU. Nevertheless, all those substances listed in Council Directive 67/548/EEC are listed in a later directive (Council Directive 76/769/EEC), which regulates the marketing and use of certain substances and preparations within the EU, and thus including cosmetics products.

Therefore, it should be pointed out that the number of official methods of analysis can be considered largely inadequate, thus making it difficult for authorities to control the presence and/or contents of regulated ingredients and the presence of banned substances in cosmetics. We should recall, in fact, that official methods of analysis are mandatory for official laboratories, but not for industry, which remains free to use other more suitable methods if deemed necessary. It should be noted that many ingredients banned in cosmetics concern pharmaceutical substances that, in the context of the European Pharmacopoeia, are covered by specifications and analysis methods used to verify conformity to these specifications.

For its part, COLIPA, in collaboration with the Institute for Reference Materials and Measurements (IRMM), the Rome Istituto Superiore di Sanità (ISS), the Tuscany Regional Health Authorities (ARPAT) and the University of Siena (Italy), has published a compilation of analytical methods aimed specifically at cosmetic products (Anselmi *et al.*, 2004). These methods are intended to identify and quantitatively determine the substances listed in the various annexes of the EU Cosmetics Directive. Although most of these methods are "not official", they do, at least, exist.

Note, moreover, that some member states have published official analysis methods that do not always correspond to the official EU methods. This is the case of France, which has published two methods for thioglycolic acid and its disulphide in depilatories and for Chloramine T in cosmetics, respectively.

ANALYTICAL METHODS IN COUNTRIES WHICH HAVE ADOPTED OR FOLLOWED EUROPEAN UNION COSMETIC REGULATIONS

The EU lists of regulated cosmetic ingredients are being acknowledged by an increasing number of countries worldwide, either because these countries are bound to incorporate them into their national regulations, or because they have decided to adopt them without any such obligation.

Countries in the European free trade area (Iceland, Liechtenstein and Norway) and EU candidate countries (Bulgaria, Croatia, Romania and Turkey) must incorporate the European cosmetic directives—including those concerning analytical methods—into their national regulations, even if some may have retained certain differences when compared to the EU model, by regulating various substances which are not regulated in the EU, or by regulating them in a different way.

Asian countries in which cosmetic regulations concerning substances have been based on the EU Cosmetics Directive will be considered later on. These are countries such as China and India, and also the Association of South East Asia Nations (ASEAN) which integers other 10 Asian countries.

Some countries in South or Central America have incorporated the EU list of regulated substances with a few modifications. This is the case of Chile and the countries forming MERCOSUR (Mercado Común del Sur) (i.e. Argentina, Brazil, Paraguay and Uruguay). Other South or Central American countries, like those which signed the Pacto Andino (i.e. Bolivia, Colombia, Ecuador, Perú and Venezuela) and those part of the Central American Common Market (i.e. Costa-Rica, El Salvador, Guatemala, Honduras and Nicaragua), have globally adopted the lists of regulated substances published by the EU and the US, considering that in the case of a discrepancy, it is the least restrictive legislation that applies. No official method of analysis for cosmetic products has been published to our knowledge.

Concerning Africa and Middle Eastern countries, note that some countries such as Morocco have also incorporated the analysis methods published in France.

In the case of the Kingdom of Saudi Arabia, it has issued many "finished products" standards and test methods for controlling these (see Table 2.1.3), including various methods based on European analysis methods, but also on various Indian standards for finished cosmetics, to check the presence or concentration of regulated ingredients. For example, in the standard of the Saudi Arabia Standard Organization (SASO) 776/1994 concerning liquid oxidizing hair dyes, the methods for analyzing various hair dyes and hydrogen peroxide are described. In principle, the Saudi standards are also accepted in the Gulf countries (Bahrain, United Arab Emirates, Kuwait, Qatar) and several have been published as Gulf Standards (GS).

ANALYTICAL METHODS IN THE UNITED STATES

In the US there are no official analytical methods for cosmetics or OTC drugs.

As mentioned in Section 1.1, concerning cosmetics, few substances are banned or restricted in the framework of US cosmetics regulations according to Title 21 of the Code of Federal Regulations (known as 21 CFR). It is not obvious whether the Food and Drug Administration (FDA), the body in charge of cosmetic products, controls the presence of these substances in cosmetics. According to *the Guide to Inspections of Cosmetic Product Manufacturers*, published by the FDA – Food and Drug Administration (1995), the Agency conducts periodic testing for the presence of three contaminants in cosmetics: nitrosamines, dioxane and pesticide residues. It is probable that analytical methods do exist and are used by the agency to carry out tests, but they are not published officially.

It should also be mentioned that there are two positive lists of cosmetic colorants. One of them, which lists synthetic colours, is subject to certification. This means that each

Table 2.1.3

Saudi Arabia and Gulf standards

Standards	SASO Number[a]	GS and/or ISO Number[b]
Analysis of soaps—determination of ethanol-insoluble matter	298/1998	ISO 673/1990
Soaps—determination of moisture and volatile matter content—oven method	299/1994	GS 200/1994; ISO 672
Surface active agents: analysis of soaps—determination of free caustic alkali	300/1994	GS 201/1994; ISO 456
Analysis of soaps—determination of total alkali content and total fatty matter	302/1994	GS 203/1994; ISO 685
Soaps—determination of chloride content—titrimetric method	304/2000	ISO 457/1983
Surface active agents—determination of pH of aqueous solutions—potentiometric method	306/1994	GS 206/1994; ISO 4316
Surface active agents and soaps—determination of water content-azeotropic distillation method	309/1998	ISO 4318/1989
Methods of testing toilet soaps	492/1987	—
Toilet soaps	493/1987	—
Determination of the biodegradability of anionic surfactants	494/1987	—
Surface active agents and detergents—methods of sample division	495/1994	GS 256/1994; ISO 607
Regulations for perfumery products based on ethanol	585/2000	GS 1046/2000
Essential oils—evaluation of miscibility in ethanol	591/1994	GS 303/1994; ISO 875
White spirit and its test methods	657/1994	GS 349/1994
Cosmetic products—substances added to cosmetic products—coloring substances	723/1994	GS 394
Cosmetic products—Hair shampoos—methods of test	724/2000	GS 395
Cosmetic products—Hair shampoo	725/2000	GS 396
Cosmetic products—liquid oxidative hair dyes—methods of test	776/1994	GS 427/1994
Cosmetic products—liquid oxidative hair dyes	777/1994	GS 428/1994
Cosmetic products—toothpaste—methods of test	801/1994	GS 691
Cosmetic products—toothpaste	802/1994	GS 692
Shaving soap	1196/1996	GS 885
Test methods for shaving soap	1197/1996	GS 886
Metal aerosol dispensers	1228/1997	GS 917
Methods of testing metal aerosol dispensers	1229/1997	GS 918
Aerosol air fresheners	1326/1997	GS 659/1997
Methods of testing aerosol air fresheners	1327/1998	GS 660/1997
Shaving cream	1387/1998	—
Methods of testing shaving cream	1388/1998	—
Vaseline (petroleum jelly)	1389/1998	—
Methods of test for vaseline (petroleum jelly)	1390/1998	—
Determination of the content of ethanol in methanol	1453/1998	—
Skin cream	1512/1999	—
Methods of test for skin cream	1513/1999	—
Hair cream	1514/1999	—
Methods of testing hair cream	1515/1999	—
Cosmetic products—chemical depilatories	1744/2000	—
Cosmetic Products—methods of test for chemical depilatories	1745/2000	—

(*Continued*)

Table 2.1.3 (cont.)

Standards	SASO Number[a]	GS and/or ISO Number[b]
Cosmetic products—talcum powder	1750/2000	—
Cosmetic products—methods of test for talcum powder	1751/2000	—
Cosmetic products—hair oil	1788/2000	—
Cosmetic products—methods of testing hair oils	1789/2000	—
Cosmetic products—deodorants	1794/2000	—
Cosmetic products—deodorants methods of test	1795/2000	—
Cosmetic products—lipsticks	1871/2001	—
Cosmetic products—methods of testing for lipstick	1872/2001	—
Liquid toilet soap for hand	1873/2001	—
Cosmetic products—the cosmetic products (safety) Regulations	1953/2001	—
The cosmetic products (safety) regulations—test methods	2185/2003	—
Cosmetic products—nail polish (nail enamel)	2186/2003	—

[a]SASO: Saudi Arabia Standard Organization.
[b]GS: Gulf Standard; ISO: International Standard Organization.

batch of the latest dyes must be certified as conforming to the specifications published by the FDA, according to a paid-for procedure described in 21 CFR Part 80 (form to fill in, limitations of the certificate, etc.). However, the analysis methods used to verify the purity criteria of these dyes are not specified in 21 CFR. For the record, the use of non-certified batches for a cosmetic product containing a dye subject to the certification procedure, or the use of an unauthorized colorant results in the product being considered "adulterated", which can be penalized by the courts, except in the case of a coal-tar hair dye, provided the precautions for use imposed by law are shown on the cosmetic label (see the Federal Food, Drug and Cosmetic Act, Sec. 601). Yet again, no official method exists for controlling the presence of an illegal cosmetic colour.

Some products considered as cosmetics in the EU have the status of OTC drugs in the US (see Section 1.1); these are topical acne treatments, anticaries and antiplaque toothpastes, antidandruff products, antiperspirants, skin lighteners, products for the protection of chapped skin or mucous membranes, sunscreens (including all products claiming a sun protection factor, even if this is not its main purpose). For all these products, the FDA has published OTC monographs, which are regulatory instruments for each specific category of OTC products. These monographs mainly indicate the definition of the OTC product, the approved active ingredients and permitted combinations, their maximum and minimum permitted concentrations, the labelling requirements (statement of identity, indications for use, warnings and directions); however, they do not specify analytical methods to control the ingredients.

Nevertheless, there is an individual monograph for each active ingredient published in the United States Pharmacopoeia (USP) under its United States Adopted Name (USAN); this includes specifications and verification methods. However, the USP does not outline the methods necessary to detect OTC drug ingredients in the finished product. It is the responsibility of each supplier to define such methods. The FDA simply indicates that these products must be open to analysis and states the acceptance criteria to be taken into

account. Consequently, when manufacturers develop a new OTC drug product, they must define a suitable analytical method at the same time, as well as providing the necessary data to prove the efficacy of the method, and then submit all these information.

However, it has to be noted that, according to 21 CFR Part 2.19, the FDA states that *"Where the method of analysis is not prescribed in a regulation (…) utilize the methods of analysis of the AOAC (…)"*, where AOAC stands for the Association of Analytical Communities. These methods will be treated later on.

Finally, it should be noted that some non-cosmetic regulations may restrict the use of ingredients in cosmetic products. This is the case notably for VOCs as regulated by various states (such as California, New Jersey or New York among others) and by the Environmental Protection Agency (EPA). The most longstanding and severe regulations are those published by California (see Table 2.1.4). The EPA has also published standards on VOCs, which must be applied by States that have not yet published their own regulations on the presence of such substances in consumer goods. The environmental agencies responsible for the regulatory requirements, such as the California Air Resource Board (CARB), can control the exact content of VOCs in certain products. To this end, CARB has published an official method to measure the total VOC content of consumer products (CARB, 2003). This document is currently the subject of new discussions.

In summary, official analytical methods do exist in the US, notably those of the FDA, but apart from information in the USP, those published by the AOAC and the method for VOCs published by CARB in California, most of them have not been published officially. In addition, concerning OTC products, it is the responsibility of the suppliers to develop and formulate the analytical methods to control their final products individually.

Table 2.1.4

California Air Resource Board (CARB) regulations on VOCs as amended

Cosmetics concerned	Maximum VOCs (%)
Hair sprays	55
Hair foams	6
Hair shine	55
Hair styling gels	6
Hair styling products	6% by end of 2006 (aerosol or spray) 2% by end of 2006 (all other forms)
Nail varnish removers	0%—acetone not concerned
Shaving creams	5
Shaving gels	7% by end of 2006 4% by end of 2009
Aerosol antiperspirants and deodorants	40% HVOC[a]—10% MVOC[b] Ethanol exempted
Non-aerosol antiperspirants and deodorants	0% HVOC and MVOC Ethanol exempted
Personal fragrances: ⩽20% of fragrance	70
Personal fragrances: >20% of fragrance	65

[a]HVOC: VOC with high volatility.
[b]MVOC: VOC with medium volatility.

ANALYTICAL METHODS IN COUNTRIES ADOPTING OR FOLLOWING US COSMETIC REGULATIONS

Canada and Mexico, together with the US, constitute the North American Free Trade Association (NAFTA), which explains why the cosmetic regulations of Canada and Mexico have been influenced by those of the US. However, the situation is changing.

The positive list of cosmetic colorants used in OTC drugs in Canada (Health Canada, Food and Drugs Act, Chapter F-27) is identical to that published by the US FDA. The lists of active ingredients permitted for OTC drugs (topical acne, anticaries, antidandruff, antiperspirants, sunscreens, skin-care products, etc.) are also very similar to the lists in the corresponding US monographs. In contrast, Health Canada has, for years, published a "hot list" on the internet incorporating approximately 500 substances banned or restricted in cosmetic products. This list includes typically Canadian legislation (e.g. ban of methyl methacrylate in cosmetics) as well as US restrictions; moreover, it also includes almost all substances banned by the EU Cosmetics Directive Annex II together with many restrictions from EU Cosmetics Directive Annex III.

The same situation applied in Mexico, which published lists of cosmetic dyes (29 colorants and mineral pigments, 7 natural organic colorants and 70 synthetic organic colorants) with their purity criteria which were sometimes more stringent than those in the US. However, these lists were repealed in October 2004. The list of substances regulated in cosmetics published in July 1999 is no longer in force, although it has never been repealed. The latest draft list, which should replace it, dating from April 2005, is largely aligned with European requirements rather than the US lists.

To the best of our knowledge, neither of these countries has published any official analytical methods for cosmetic products.

As mentioned before, the countries composing the Central American Common Market (i.e. Costa-Rica, El Salvador, Guatemala, Honduras and Nicaragua) have also globally adopted the US-regulated substances.

ANALYTICAL METHODS IN JAPAN

In Japan, since deregulation on 1 April 2001 (see Section 1.1), cosmetics are no longer subjected to a global list of permitted ingredients. Lists of regulated substances have been published: negative (i.e. banned substances), restrictive and positive lists, the latter includes coal-tar cosmetic colorants (i.e. of synthetic dyes as opposed to those of animal, plant or mineral origin), preservatives and UV filters for skin protection. The Japanese lists do not correspond to the EU lists.

Prior to deregulation, all ingredients used in cosmetics had to appear in the Japanese Comprehensive Licensing Standards (JCLS) incorporating approximately 2700 substances. Each of these substances required a monograph establishing its precise specifications, published in the Japanese Standards of Cosmetic Ingredients (JSCI) (see Table 2.1.5 for the points covered by the monograph for a cosmetic ingredient, and Table 2.1.6 for the description of tests to verify the specifications). However no official cosmetic-specific analytical method has been published. Since 1 April 2001, these monographs have no longer applied to cosmetic ingredients, but they remain applicable to the excipients of quasi-drugs.

Table 2.1.5

Monograph of a cosmetic ingredient before deregulation of 1 April 2001

Batch monograph consists of the following items put in the order indicated below, except that unnecessary items are omitted depending on the nature of the ingredient:

(1) English title
(2) Commonly used name(s)
(3) Structural formula or empirical formula
(4) Molecular formula and molecular weight
(5) Origin
(6) Limit of the content of the ingredient
(7) Potency of the ingredient
(8) Method of preparation
(9) Description
(10) Identification
(11) Specific physical and/or chemical values
(12) Purity
(13) Loss on drying, loss on ignition and/or water
(14) Residue on ignition
(15) Special tests
(16) Assay on the content of the ingredient(s)
(17) Miscellaneous items

Table 2.1.6

General tests described in the Japanese Standards of Cosmetic Ingredients

(1) Acid-soluble substances
(2) Acid-insoluble substances
(3) Acid value
(4) Ammonium limit test
(5) Anionic surfactants
(6) Arsenic limit test
(7) Boiling point and distilling range
(8) Chloride limit test
(9) Clouding point
(10) Freezing point
(11) Ester value
(12) Fluorine limit test
(13) Gas chromatography
(14) Heavy metals limit test
(15) Hydroxyl value
(16) Infrared spectrophotometry
(17) Iodine value
(18) Iron limit test
(19) Lead limit test
(20) Liquefied gases
(21) Loss on drying
(22) Loss on ignition
(23) Matching fluids for colour
(24) Measuring instruments, appliances
(25) Melting point
(26) Methanol and acetone tests
(27) Methoxyl determination
(28) Nitrogen determination
(29) Non-volatile residue
(30) Optical rotation
(31) Oxygen flask combustion method
(32) Paper chromatography
(33) Perfume tests
(34) pH determination
(35) Qualitative tests
(36) Readily carbonizable substances
(37) Reagents, test solutions
(38) Refraction index
(39) Residue on ignition
(40) Saponification value
(41) Softening point
(42) Specific gravity
(43) Specific volume
(44) Spectrophotometry
(45) Standard solutions
(46) Standard solutions for volumetric analysis
(47) Sulfate limit test
(48) Thin-layer chromatography
(49) Unsaponifiable matter
(50) Viscosity
(51) Vitamin assay
(52) Water determination (Karl–Fisher method)
(53) Water-soluble substances

Indeed, in Japan there is an intermediate category between cosmetics and drugs: quasi-drugs. The uses of quasi-drugs have been specified by the authorities (see Section 1.1): hair growth products, oxidation hair dyeing and bleaching products, hair perms and straighteners, depilatories, skin whiteners, deodorants and antiperspirants, treatment toothpastes and treatment bath products, insecticides, etc. Medicated cosmetic products (e.g. antidandruff shampoos, antiacne creams, products for oily skin or cracked skin, etc.) are also generally considered quasi-drugs.

Only approved active ingredients can be used in quasi-drugs but all the lists are not officially published. Whereas there are standards for 55 active ingredients in oxidation hair dyeing products and 7 active ingredients in hair perms, such information is not available for antiperspirants, antidandruff shampoos, etc. It is only by obtaining information on a case-by-case basis from the Ministry of Health and Welfare that it is possible to establish whether an active ingredient has already been accepted for another company, and the answer will be positive only if the provided formula contains exactly the same substance with the same specification and the same concentration.

Similarly, only excipients listed in the old JCLS for cosmetics are officially permitted in quasi-drugs by the authorities. This list has not been updated since April 2001, so it does not include recently authorized excipients.

For active ingredients and excipients that are not yet authorized, dossiers must be submitted by the company wishing to formulate them in a new quasi-drug. The dossier must mainly include information on the specifications of the new ingredient and a method of analysis for the ingredient itself as a raw material to ensure it complies with the specification (purity) as well as a method to analyse the ingredient in the finished product.

So, methods of analysis for the final product do exist and are in the possession of the Japanese authorities for all active ingredients in quasi-drugs, but they are not officially published.

Note that some substances (very few) in the Japanese Pharmacopoeia can be used in cosmetic products, and the Pharmacopoeia presents specifications for these substances as well as a method for titration of the substance as a raw material. Of course, this method does not permit titration of a given substance in a finished cosmetic product.

In the event that a control is carried out by the authorities, for all quasi-drug active ingredients, the concentration notified by the manufacturer must be found within an accepted tolerance of $\pm 10\%$. Beyond these limits, the finished product is considered not to conform. This requirement is applicable, in particular, to quasi-drugs with a shelf life expiry date on the label because the stability of the finished product is less than three years. This requires the manufacturer to carry out accelerated stability tests (6 months at 40 °C and 75% humidity on products from three different batches, with verification of the concentration of active ingredients at the end of the test) and real time stability tests as well.

ANALYTICAL METHODS IN COUNTRIES ADOPTING OR FOLLOWING JAPAN COSMETIC REGULATIONS

The Republic of Korea (ROK) has adopted Japanese regulations insofar as quasi-drug regulations exist in this country for all products with a minor treatment effect, for example

body deodorants and oral hygiene; toothpastes and mouthwashes; antihair loss products; oxidation hair dyeing and bleaching products; treatment baths and skin treatment products. The list is similar to the Japanese one.

The ROK Ministry of Health and Welfare (MOHW) has published a positive list of active ingredients authorized in oxidation dyes, semi-permanent dyes and bleaching products (MOHW Decree 1995/68). It is fairly similar to the Japanese list for oxidation dyes, but 19 hair dyes and 3 bleaching active ingredients have been added by modification of the Decree on 23 August 2004.

Quasi-drugs are subjected to strict regulations. When a registration dossier is submitted, the specifications of the finished product and of the raw materials must be stated and an analysis certificate supplied. The authorities regularly conduct tests to verify the analyses supplied by the declarer (first series of verifications at the time of registration, followed by a challenge test 3 months later).

ROK has created a new category of cosmetics, known as cosmeceuticals or functional cosmetics, which does not exist in Japan. To date, it has incorporated three categories of products (see Table 2.1.7). The regulations do not provide for systematic updating of the list of functional active ingredients, even if a new active ingredient is authorized in a finished product at the request of a company.

Since 1 January 2001, this product category has been subject to a quasi-pharmaceutical registration procedure. The dossier to be submitted to the Korean authorities (i.e. Korean Food and Drug Administration, KFDA) must include, notably, specifications of the final product, titration of active ingredients in the cosmetic, a stability test performed according to the Korean protocol: 3 batches over 6 months at 40 °C and 75% humidity, with analysis of active ingredients and preservatives each two months during these 6 months.

Before 2003, when a cosmeceutical active ingredient was not included on the lists approved by KFDA, it was necessary to forward specifications of all ingredients in the final product, including fragrance, and to submit comprehensive data on the laboratory performing the analyses. The revision of 2003-1 eliminated this obligation, replacing it by the International Nomenclature Cosmetic Ingredient (INCI) name and commercial name of ingredients not included in Korean dictionaries (Korean Cosmetic Ingredient Dictionary (KCID), Korean Standards on Cosmetic Ingredients (KSCI), Korean Food Code) or Japanese dictionaries (Japanese Cosmetic Ingredients Dictionary (JCID), Japanese Standards on Cosmetic Ingredients (JSCI), JCLS).

Table 2.1.7

Functional cosmetics and active ingredients in Republic of Korea

Categories	Text	Active ingredients
Sun protection products	UV filter listed in Notification 2003–23, modified in 2004	27 ingredients (list similar to the US list + various substances accepted in the EU and Japan)
Skin whiteners	Listed in Notification 2003–23, modified in 2004	6 ingredients
Anti-wrinkle products	Listed in Notification 2003–23, modified in 2004	4 ingredients

As for normal cosmetics, there are many regulations that again require the availability of analytical methods. Registration is no longer required for ordinary cosmetics, except for cosmetics containing a "new" substance (an ingredient not yet approved by the authorities or which is not included in the approved lists, such as KSCI, KCID, JSCI, JCID or INCI dictionary). The manufacturer or the importer must submit a substantial dossier concerning the new ingredient and containing, notably, specifications of the latter and analysis methods for its verification.

ROK has published several lists of substances regulated in cosmetics (see Table 2.1.8).

It should be noted that Annex V of Notification 2003-23 requires the heavy metal (lead, arsenic, mercury) content and the pH to be determined for various cosmetic products, as well as the presence of methanol in some alcoholic products (see Table 2.1.9). The same

Table 2.1.8

Substances regulated in cosmetics in Republic of Korea

Lists	Texts	Substances regulated
Banned substances	Annex 4 of Notification 93-57 + Notification 2003–23	List fairly similar to EU Cosmetics Directive Annex II
Restricted substances	Annex "Others" of Notification 2003–23	11 ingredients or families of substances
Preservatives (positive list)	Annex 3 of Notification 2003–23	71 preservatives (list similar to EU Cosmetics Directive Annex VI + a few preservatives authorised in Japan)
Cosmetic colorants (positive list)	Notification 87-42 as amended	List similar to the US list

Table 2.1.9

Limits in heavy metals, methanol, pH value in certain cosmetics to be verified according to the official analysis protocols in Republic of Korea

Tests to be performed	Cosmetic concerned	Limitations
Lead and arsenic	Make-up products (unless the concentration in lead and in arsenic has been verified in the raw materials), eye makeup, shampoos, rinses, hair sprays	Lead: ⩽20 mg/kg Arsenic: ⩽5 mg/kg for shampoos, rinses, hair sprays; and ⩽10 mg/kg for makeup
Mercury	Basic skin care products, baby care products	⩽1 mg/kg
Methanol	Products containing ⩾4% of ethanol	0.2% v/v max.
pH value	Liquid products (including foundations), eye makeup, lipsticks, creams	between 3.0 and 9.0

Annex V describes the protocols to be used for calculating the heavy metal and methanol content, the pH value, etc.

KFDA controls the analysis certificates every two years (including pH value, heavy metal and methanol content) for each batch of products placed on the market by Korean cosmetics manufacturers and envisages the same procedure for importers.

It should be noted in this context that imported cosmetics are subject to many controls. The Cosmetics Control Act required the Authorities to systematically conduct analyses of all products imported into ROK for the first time, before they could be put on the market; this requirement has been repealed since 1 January 2000. Systematic auto-control of the quality of imported products has not been eliminated, except for those European companies, which have agreed to an audit of their European manufacturing sites by Korean inspectors. Nevertheless, the authorities consider that a certificate of analysis must still be submitted with each batch of goods imported, and inspections have been instigated on several occasions since May 2002 to verify whether the foreign companies are complying with this requirement.

In summary, in the ROK there are official specifications for cosmetic ingredients found in the Japanese or Korean dictionaries (JSCI and JCID, KSCI and JSCI, INCI dictionary) and official analytical methods for some heavy metals, methanol and pH. The manufacturers must have analytical methods available to check the content of active ingredients in quasi-drugs or cosmeceuticals in the finished product, and submit these to the authorities, which will use them to control products at the time of registration and once the product is on the market. These methods are not officially published.

Taiwan is another country that has been influenced by Japanese regulations. In this country, there are “medicated cosmetics” subject to registration, contrary to “general cosmetics”; these are fairly similar to Japanese quasi-drugs.

The maximum content of some heavy metals is also regulated for medicated cosmetics: 10 mg/kg for arsenic (5 mg/kg in permanents), 20 mg/kg for cadmium and 20 mg/kg for lead (5 mg/kg in permanents).

At the time of registration of medicated cosmetics, the manufacturer or the importer must supply authorities with, among other things, methods for titration of active ingredients and an analysis certificate for the final product. It is possible to register a product range under the same license, but in this case, the analysis certificates and control methods must be supplied for each shade. The authorities tolerate a difference of $\pm 10\%$ between the stated percentage of active ingredients and the concentrations detected during the analysis. Companies that have successfully undergone government analysis for two years need no longer undergo the official analysis tests for new products they wish to register.

It should also be noted that lists of regulated substances also exist for general cosmetics: substances banned or restricted (e.g. the maximum concentration in released formaldehyde must not exceed 100 mg/kg for authorized formaldehyde donors, such as the dimethylol dimethyl hydantoin (DMDM hydantoin), imidazolidinyl urea and Quaternium-15), positive lists for cosmetic colorants and preservatives.

To our knowledge, there are no official methods of analysis published by the Taiwanese authorities for these substances.

ANALYTICAL METHODS ON COSMETICS IN OTHER SIGNIFICANT WORLDWIDE MARKETS

People's Republic of China

The Chinese Ministry of Health (MOH) has *inter alia* regulated cosmetics by the Hygiene Standard for Cosmetics 2002, which incorporates all lists of substances regulated by the EU Cosmetics Directive up to its 25th adaptation to technical progress. It should be noted, however, that the People's Republic of China (PRC) has also just published a positive list of hair dyes, whereas such a list does not exist in the EU yet.

But the Chinese 2002 standard also subjects cosmetics to non-EU requirements: it indicates, for example, the permitted contents of various heavy metals in cosmetics: 1 mg/kg for mercury (except mercury compounds authorized as preservatives in eye products), 40 mg/kg for lead (except for lead acetate in hair dye) and 10 mg/kg for arsenic.

Moreover, the 3rd part of the same standard describes 23 analytical methods used by the official laboratories in Beijing or Shanghai to control cosmetics prior to their placement on the market (see list of methods in Table 2.1.10).

In fact, to obtain authorization to market a new cosmetic, the manufacturer or the importer must submit a substantial dossier to a committee of experts, which meets every three months. This dossier must contain, notably, the results of these analytical tests and of the safety and efficacy tests performed on the finished products. It is important to note that the results obtained by the official Chinese laboratories are not open to dispute and tests performed outside China are not accepted.

Association of South East Asia Nations

The ASEAN, an association with 10 member states from South East Asia (Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand, Vietnam) published

Table 2.1.10

Methods of analysis published officially in People's Republic of China

(1) General principles	(13) Selenium disulfide
(2) Mercury	(14) Formaldehyde
(3) Arsenic	(15) Thioglycolic acid
(4) Lead	(16) Phenol and hydroquinone
(5) Methanol	(17) Sexual hormones
(6) Microwave digestion method	(18) Ultra-violet absorbents
(7) pH	(19) Preservatives
(8) Cadmium	(20) Oxidative hair dyes
(9) Strontium	(21) Chlormethine
(10) Total fluorine	(22) Cantharidin
(11) Total selenium	(23) α-Hydroxy acid
(12) Boric acid and borate	

an ASEAN Cosmetics Directive in 2003 based largely on the EU Cosmetics Directive. The EU lists of substances regulated in cosmetics were incorporated up to the 25th adaptation to technical progress of the EU Cosmetics Directive. Note, however, that a list of differences specific to certain ASEAN member states has been published under the title *ASEAN Handbook of Cosmetic Ingredients*.

Concerning analytical methods, Article 9 of the ASEAN Directive specifies that companies or those responsible for the sale of cosmetic products on national markets, must make available to the authorities in charge of these products, the analytical methods to control the ingredients contained in the formulation and to verify the purity of the cosmetic ingredients.

Member states must incorporate the ASEAN Directive by 2008 at the latest. Most will retain their specific cosmetic regulations up to this limit date, even if they have already largely incorporated the EU substance lists.

Note that in Thailand, the authorities tolerate a difference of 18% more, or 15% less, between the percentages stated for a regulated substance and the concentration detected during analysis.

Analytical methods in India

Since 2001, the Indian authorities have largely adopted the EU lists of regulated substances, up to the 25th adaptation to technical progress of the EU Cosmetics Directive. Nevertheless, a few differences remain. Thus, the concept of traces of banned substances, which must be "non-detectable" using standardized analytical methods, is inappropriate in view of technological changes.

Alongside this recent regulation, there are a number of cosmetic standards issued by the Bureau of Indian Standards (BIS). These standards, frequently old and not updated to take into account modern technology, concern either cosmetic ingredient (e.g. standards IS 4653:1985 and IS 6333:1985 concerning methylparaben and propylparaben used in cosmetics, respectively) or finished product (see list in Table 2.1.11). They describe the specifications to which ingredients or finished cosmetics must conform, as well as the tests needed to control conformity to standards, including some physical/chemical analysis methods.

INTERNATIONAL METHODS FOR ANALYSIS OF COSMETICS

Before concluding, it should be noted that the development of methods recognized at an international level could become widespread. In the context of the Comité Européen de Normalisation (CEN), a project for European standardization of methods to analyse allergenic substances (CEN/BT/WG 132) began in December 2001. The objective of this project is to develop analytical methods to evaluate the presence of known allergenic substances in products for public or professional use. In January 2003, the EU Working Group on Sensitisation recommended setting up a technical committee responsible for developing methods to determine the percentage of allergens contained in metals, plastics, preservatives, dye additives, fragrances, etc. Eventually, this project could have substantial repercussions for cosmetic products.

Table 2.1.11

Categories of finished products subject to Bureau of Indian Standards (BIS), including the control tests

– Skin powders	IS 3959, revised in 2004
– Skin powders for children	IS 5339, revised in 2004
– Toothpaste	IS 6356, revised in September 2001
– Skin creams	IS 6608, revised in 2004
– Hair oils	IS 7123, revised in October 1998
– Soap-based shampoos	IS 7669, revised in February 2001
– Hair creams	IS 7679, revised in February 2001
– Synthetic shampoos	IS 7884, revised in 2004
– Liquid oxidation dyes, gels and creams	IS 8481, revised in May 2005
– Eau de cologne	IS 8482, revised in March 2001
– Nail varnish	IS 9245, revised in February 2001
– After shave lotions	IS 9255, revised in March 2001
– Hair pomades and brilliantine	IS 9339, revised in April 2001
– Chemical depilatories	IS 9636, revised in April 2001
– Shaving creams	IS 9740, revised in April 2001
– Cosmetic crayons	IS 9832, revised in August 2002
– Lipstick	IS 9875, revised in September 2000
– Care products for lips	IS 10284, revised in August 1998
– Powder hair dyes	IS 10350, revised in September 1999
– Liquid foundation	IS 14318, revised in April 2001
– Hair dyes for bleaching	IS 15202 of 2002

In contrast, the International Standard Organization (ISO) Technical Committee on Cosmetics Products is developing suitable methods for the quantitative analysis of nitrosamines (ISO/WD 15819), retinol (ISO/NP 24413) and arbutin (ISO/NP 24414) in cosmetics.

Moreover, the international AOAC could constitute another source of official methods. This association develops or evaluates methods for substances or products that could be used by a large number of manufacturers. This group is supervising a validation programme intended to evaluate a method for use in various fields. Although most of these methods are used primarily in the area of foodstuffs, different analytical methods for cosmetics can be found in the official methods of analysis of AOAC International (Horwitz, 2005). The AOAC analytical methods regarding cosmetic products are listed in Table 2.1.12.

SUMMARY

All countries with cosmetic regulations have published lists of substances regulated in such products. The rapid world review we have just concluded concerning the physical/chemical analytical methods necessary to verify the presence or content of regulated substances in a cosmetic product reveals that very few of these methods have been published officially. Moreover, in many cases manufacturers must develop the analytical methods necessary to control a cosmetic or OTC product.

Table 2.1.12

Analytical methods published by the AOAC for different types of cosmetics

Cosmetic matrix	Analytes
Cosmetics in general	Water and ethanol Propylene glycol
Deodorants and antiperspirants	Aluminium and zinc Zirconium (soluble) Boric acid Chlorides Sulfates Hexachlorophene Methenamine Phenosulfonates Urea
Depilatory powders	Sulfides
Face powders	Fat and fatty acids Boric acid Zinc Calcium (acid soluble) Calcium (acid insoluble) Magnesium (acid soluble) Magnesium (acid insoluble) Barium sulfate Titanium and iron Oxides of iron, titanium and aluminium Aluminium Silica Starch
Hair dyes and rinses	Toluene-2,5-diamine p-phenylenediamine Pyrogallol
Hair lotions	Resorcinol Salicylic acid
Cold permanent waves	Thioglycolate Dithiodiglycolic acid
Cold wave neutralizers	Potassium bromate and sodium perborate (qualitative assay)
Suntan preparations	Pentyl dimethyl PABA
Vanishing creams	Water Ash Chloroform soluble material Glycerin

Therefore we conclude that the number of analytical methods officially published for cosmetics can be considered inadequate. This situation could present real problems, particularly in the case of disputes brought about by the presence—real or supposed—in cosmetic products of substances called into question. Such disputes could arise notably due to non-governmental organizations (NGOs), since the methods used by industry, authorities and/or NGOs are not necessarily the same.

It should be recalled, however, that some methods developed for medications, food or consumer goods in general could be applied to cosmetics. Hence, the method published by Californian CARB to detect the VOCs content in consumer goods is applicable to this category of products.

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2.2. General Review of Published Analytical Methods for Cosmetics

A. Salvador,[1*] J.G. March,[2] M.T. Vidal,[3] A. Chisvert[4] and A. Balaguer[1]

[1]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Doctor Moliner Street 50, 46100-Burjassot, Valencia, Spain
[2]Department of Chemistry, Faculty of Sciences, University of Islas Baleares, Palma de Mallorca, Spain
[3]Department of Chemistry, Universidad Politécnica de Valencia, Valencia, Spain
[4]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Alicante, Spain

ANALYTICAL METHODS FOR COSMETIC ANALYSIS

Section 2.1 was devoted to official methods for cosmetic analysis. Unfortunately, these official methods do not cover the analytical methods necessary to control ingredients that are prohibited/restricted in cosmetics by different legislations (see Sections 1.1 and 1.2).

Cosmetic enterprises worldwide act responsibly, therefore voluntary addition of forbidden ingredients is very scarce. However, methods to detect and/or determine them are necessary because public administrations have to assure that forbidden ingredients are not contained in the finished product, either through voluntary addition or as a by-product; in fact, health problems in such cases can derive from this type of unexpected compound.

Moreover, the analytical control of ingredients in the batches of finished products on the market is necessary to guarantee that the contents are the same as those of the designed and developed formulation. Thus, the efficacy claimed on the label will be that desired and evaluated by the manufacturer.

An even more interesting problem is the need for analytical methods to determine the content of the ingredients whose maximum concentration is limited by legislation. It is necessary to assure that the content of each ingredient in the commercialized batches of finished product is that same as the designed formulation, because they must ensure that there are no batches with a higher content than that authorized (which may occur accidentally in the manufacturing process).

For instance, if a sunscreen product is labelled with a specified sun protection factor (SPF), the quality control will assure that the batches of finished product on the market

*Corresponding author. E-mail: amparo.salvador@uv.es

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contain the same amount of UV filter as the formulation that has been designed and developed. Thus, both solar protection efficacy and legislation requirements will be endorsed (for details on the SPF concept see Section 3.1)

As official methods do not cover the analytical methods that are necessary to determine all the prohibited/restricted ingredients in cosmetics, new analytical method have to be developed. Some of them, with time, can be adopted as official methods, after the corresponding validation process.

Analytical methods must encompass a series of properties, with the most important being

- Accuracy: The result obtained for the analyte must be close to the true concentration of the analyte in the sample.
- Precision: Several results obtained for the same analyte and sample must be close.
- Sensitivity: A suitable analytical technique must be used and the method must allow the detection of low concentrations of the analyte (this is particularly important when traces of toxic or contaminant compounds must be detected).
- Selectivity: The method must allow the analyte to be determined without interference from other components of the sample, which could affect the accuracy.
- Robustness: A slight (undesired) difference in the experimental conditions must not affect the accuracy of the result.
- Other properties: Simplicity and rapidity in sample preparation, low consumption of reagents, use of low-toxicity reagents (safety of reagents for both, laboratory workers and environment) and high level of automation.

More detailed definitions and explanations about these and other analytical properties can be consulted in analytical literature (Valcárcel, 2000).

In the particular case of cosmetic analysis (see Figure 2.2.1) the following features must be taken into account: (1) cosmetic samples are complexes containing a high number of components; (2) for quality control, many analytes have to be determined in the same sample; and (3) many samples have to be processed (intra and inter batches). The first two

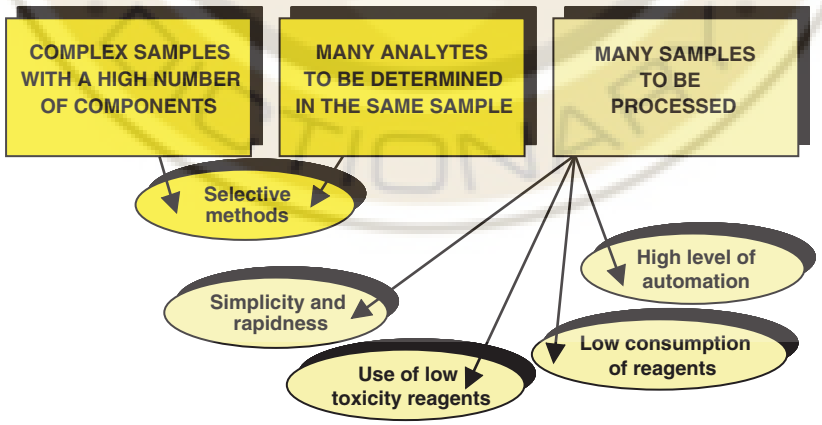

Figure 2.2.1 Cosmetic analysis. Features and most suitable properties of the analytical methods.

points require selective analytical methods to be available. The third one requires a high level of feasibility.

A substantial number of research articles can be found in analytical literature about cosmetic analysis in which different analytical procedures are proposed to determine a great many ingredients in cosmetics. Despite this, more research in this field is necessary because the proposed methods are still insufficient.

Consequently, researchers in the field of analytical chemistry have to make an effort to extend and modernize the existing methods and develop new analytical methods, which can fulfil the requirements of legislation. Therefore

- Some pretreatments should be improved to gain greater simplicity and rapidity.
- Modern and automated analytical techniques should be also proposed for official methods, to improve the analytical properties of procedures and give them a boost (Salvador *et al.*, 2000).
- The society in general and analytical chemists in particular focus on safety assurance. Thus, green analytical methods that avoid or reduce the use of toxic reagents in analytical procedures should be implemented. This is particularly important in industrial quality control where a great many samples are processed (Salvador *et al.*, 2002).

The development of new analytical methods will allow cosmetic enterprises to carry out a suitable monitoring and control of their own production by periodical analysis of their finished products. This will allow them to ensure that they contain the predetermined amount of active ingredients (in accordance with the developed formulation). This could be carried out at either their own laboratories or by specialized analytical enterprises.

PUBLISHED SCIENTIFIC ARTICLES ON COSMETIC ANALYSIS

Official methods employed in different countries are discussed in Section 2.1. Detailed procedures approved as official methods of analysis in the EU (European Commission, 1999).

The present section gives an overview of the other analytical procedures for cosmetic analysis proposed by different researchers working on this subject. Only general data are given here. For details on the procedures to be used to determine different cosmetic ingredients, readers should refer Chapters 3–8 of this book.

An exhaustive study has been carried out based on the abstracts given by Analytical Abstracts (Royal Society of Chemistry) database January 1980–June 2006. A great many keywords covering all types of cosmetic products has been used to carry out the search. It is necessary to indicate that the total number of articles recovered in this search was nearly 1500 and consequently only the information given in the abstracts was processed.

Types of samples

Reported procedures in the reviewed papers have been grouped according to the analysed cosmetic product (if indicated in the abstract) and classified according of the

Table 2.2.1

Categories of cosmetic products

Category of cosmetic products	Section of the book dealing with properties, types, etc.
Hair products (excluding hair dyes)	8.3
Products for care of the teeth and mouth	8.2, 8.4
Specific products for skin care	8.1, 8.5, 8.6, 8.7
Sunbathing and related products	3.1, 3.2, 3.3
Products intended for application to the lips	8.1, 4.1
Decorative products (including hair dyes)	4.1, 4.3
Personal hygiene and other toiletry products	8.2
Perfumes, eau-de-toilette, etc.	6.1

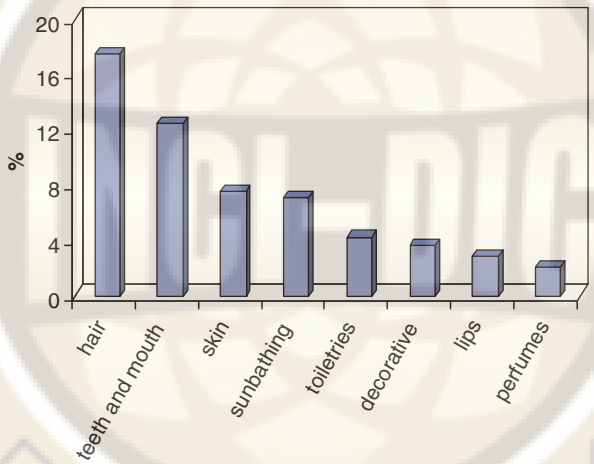

Figure 2.2.2 Percentages of analysed categories of cosmetic products in reviewed literature.

categories proposed in Annex I of the EU Cosmetics Directive (Council Directive 76/768/EEC).

Established categories, products included in each category and the sections of the book where details and analytical procedures can be found are indicated in Table 2.2.1.

Figure 2.2.2 shows the percentage of published procedures for each cosmetic product category. As can be seen, the highest percentage of papers focuses on the analysis of hair-care products, especially hair-cleansing products, which constitute 77% of the procedures included in this category. Much less information can be found on other toiletry and personal-hygiene products, decorative preparations, lip products, perfumes and eau-de-toilette. Intermediate attention has been paid to products for teeth or mouth and skin care as well as sunbathing and related products.

Types of analytes

The substances determined in the published procedures have been classified in different families, shown in Table 2.2.2, which also indicates the section of the book that deals with them.

UV filters

Most of the UV filters (inorganic and organic compounds) authorized by the legislation of different countries have been determined in the published procedures. A relatively high number of analytical methods have been proposed covering the determination of UV filters in cosmetics. The most commonly studied UV filters are benzophenone-3, ethylhexyl methoxycinnamate, ethylhexyl dimethyl PABA and butyl methoxydibenzoylmethane, among others.

Analytical methods for related substances, such as tanning products containing dihydroxyacetone or bergapten, are less abundant. Moreover, analytical methods to determine whitening actives such as hydroquinone and/or some of its esters, ascorbic acid derivatives and kojic acid have also been proposed.

Colouring agents

Analytical methods to determine different types of colouring agents, i.e. monoazo-, disazo-, triphenylmethane-, fluoran-, xanthene-, quinoline- and anthraquinone-based colouring agents, can be found in the literature. Also for diaminobenzenes, aminophenols and polyphenols used as hair dyes.

Preservatives

Both types, antimicrobial and antioxidant preservatives are determined. The most frequently studied are the different parabens and formaldehyde and formaldehyde-releasing preservatives.

Table 2.2.2

Classification of analytes

Classification of analytes	Section of the book dealing with analytical methods
UV filters, tanning and whitening agents	3.1, 3.2, 3.3
Colouring agents	4.2, 4.3
Preservatives	5.1
Fragrance ingredients	6.1, 6.2, 6.3
Surfactants	7.1
Other actives	8.8

Fragrance ingredients

Some fragrance ingredients have been determined in perfumes or other cosmetics. The most commonly studied are the potentially allergenic substances recently classified by the EU Cosmetics Directive.

Surfactants

This type of ingredient (cationic, anionic, non-ionic and amphoteric) has been widely studied in analytical literature.

Other organic compounds

Botanical extracts such as lanolin, *Aloe vera*, *Centella asiatica* or glycyrrhetic acid are studied. Vitamins such as A, C, B5 and E have also been reported. Studies on the following compounds used as ingredients can also be found: heterocyclic compounds, organic acids, thiocompounds, alkanolamines, amines, amino acids, alcohols, peroxides, amides, polymers, peptides and ceramides. Analytical methods to determine toxic compounds present as impurities or as by-products such as: nitrosamines, phenols, pesticides, polychlorinated biphenyls, etc. have also been proposed in literature.

Elements and inorganic anions

A great many of them have been studied. Some of them come from salts or organometallic compounds used as ingredients, but others can be toxic and are present as by-products or impurities coming from raw materials. The most commonly studied are Se, Pb, Hg, Cd and Zn.

Analytical methods

Sample preparation

Matrix of cosmetic samples is not simple, they usually contain a high number of ingredients, and often the analysis of formulation requires extensive treatments, such as solubilization, purification and/or preconcentration. Next, the analytical signal is measured with or without previous analytical reaction.

Dissolution or lixiviation of analytes can be carried out using either suitable chemicals and/or solvents assisted by physical complementary treatments like heating, or exposure to ultrasound or microwave radiation. In some cases strong acid or basic digestion is necessary. You can see the relative use of the aforementioned treatments obtained from literature depicted in Figure 2.2.3.

Sometimes, analytical procedure requires a purification/preconcentration step of the analytes of interest. To do this either solid phase or liquid–liquid extraction are employed. Distillation and headspace volatilization are seldom used. See comparative use in Figure 2.2.4.

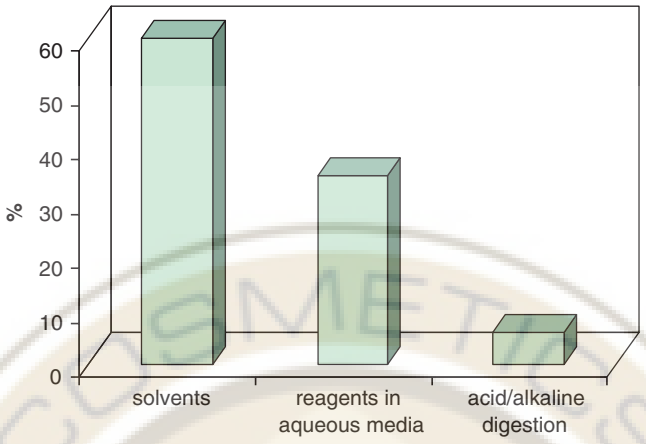

Figure 2.2.3 Relative frequency of treatments for solubilization of samples and/or analytes.

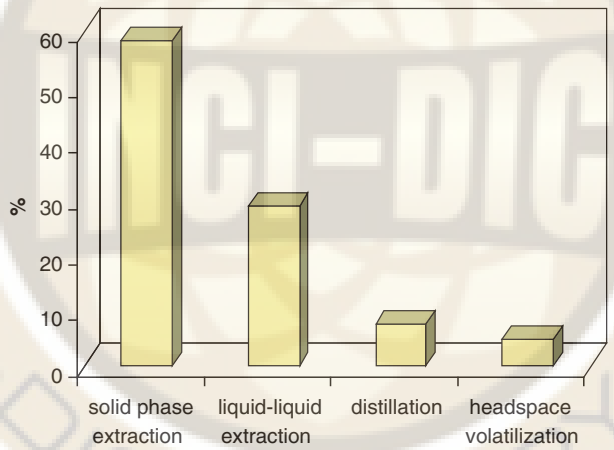

Figure 2.2.4 Relative frequency of treatments for purification/preconcentration of analytes.

In some cases, analytes should be transformed (by means of derivatization) to compounds with better analytical features for the analytical technique to be used, for instance, gas chromatography requires that low-volatile analytes be transformed into volatile derivatives. Also, the formation of a complex sometimes enables coloured or fluorescent derivatives to be obtained before determination with spectrophotometric or fluorimetric detectors. Other reactions, like hydrolysis, saponification or redox are seldom applied (Figure 2.2.5).

Details for the preparation of samples before the determination of different analytes can be found in the corresponding sections of this book.

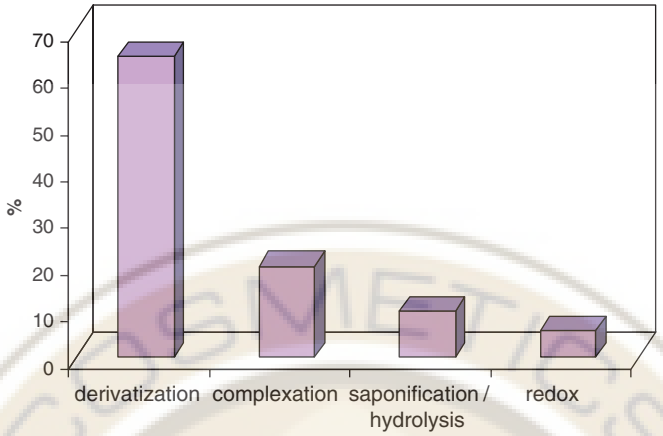

Figure 2.2.5 Relative frequency of chemical reactions of analytes to be determined.

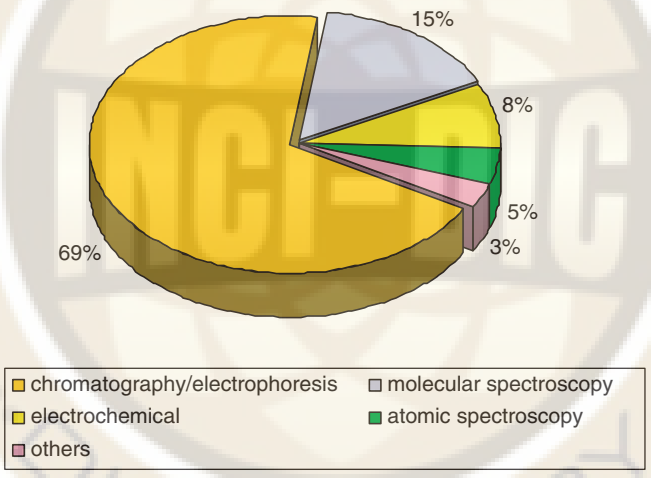

Figure 2.2.6 Percentages of analytical techniques used in reported methods for cosmetic analysis.

On the other hand, interested readers can find extensive literature on sample preparation in analytical chemistry (Cámara *et al.* 2002; Luque de Castro and Luque García, 2002; Mitra, 2003; Luque de Castro and Priego Capote, 2006).

Analytical techniques

Revised references on cosmetic analysis have been classified into five groups according to the analytical technique used (Figure 2.2.6), namely, chromatographic and related techniques (such as electrophoresis) (69%), molecular spectroscopy (15%), electrochemical measurements (8%), atomic spectroscopy (5%) and others (3%).

Methods based on a previous separation step of the analytes are used extensively, with liquid chromatography (38%) being more extensively applied than gas chromatography (16%), thin layer chromatography (7%), capillary electrophoresis (6%), supercritical fluid chromatography (0.2%) and others.

Spectral characteristics of molecular analytes have enabled useful spectroscopic methods to be developed, with UV-visible spectrometry as the most commonly used technique in this group (10%), followed by infrared spectrometry (2.2%).

Atomic spectrometric techniques have been successfully applied to determine elements, with atomic absorption techniques (2.3%) being most extensively applied.

Other reported procedures have been classified as "others", which include classical methods like gravimetry and titrimetry (1.5%), and other methods such as radiochemical methods, mass spectrometry and thermogravimetry.

Details of the different analytes and samples can be found in the corresponding sections of this book.

On the other hand, interested readers can find extensive literature on modern analytical techniques (Skoog *et al.*, 2001; Kellner *et al.*, 2004).

OTHER PUBLICATIONS

As discussed in the Preface, this book attempts to give the reader a complete revision of all the published articles dealing with analytical procedures for cosmetics.

Readers can extract enough information from the different chapters of this book to select the most useful analytical procedure to tackle the analytical problem in question. Details are given in the text and also in the corresponding tables. Journals where full articles can be consulted are also indicated. Full papers in electronic format are often available via internet from journals or scientific societies' websites. Some articles are free, others can be purchased. It is also very usual among colleagues to ask the corresponding author for a reprint, which will be sent free of charge.

Other books where procedures for cosmetic analysis are given have already been published. From our point of view, three books are of special interest for readers involved in cosmetic analysis. They are

Bore (1985): This book is divided into several chapters where different instrumental techniques in cosmetic analysis are described giving some examples based on interesting analytical procedures developed by the Research Division of L´Oreal.

Mitsui (1997): This is a general book with detailed information on all types of cosmetics, manufacturing, legislation, etc. One of the chapters is devoted to cosmetic analysis, summarizing the different instrumental techniques that are useful in cosmetic analysis and providing interesting examples.

Anselmi *et al.* (2004): This book is divided into several chapters, each of which concerns the different Annexes to the EU Cosmetic Directive where cosmetic ingredients are classified. The book considers a representative selection of these ingredients. Bibliographic references are given so officially published methods can be consulted. A very practical and representative collection of unofficial analytical procedures is also provided.

Other books dealing with general cosmetic science can be also interesting for readers (Diez-Sales, 1998; Barel *et al.*, 2001; Philippsborn, 2000).

Moreover, in the recently published *Encyclopedia of Analytical Sciences*, readers can find a short revision on perfumes (Salvador and Chisvert, 2005) and another one on cosmetics and toiletries (Salvador and Pascual-Martí, 2005).

SUMMARY

The official methods approved by the different legislations are not enough to carry out the necessary analytical control of cosmetics.

There are many analytical methods that are available in published analytical literature and can be applied to cosmetic samples.

The development and validation of new useful methods with suitable analytical features is still a field under development. Researchers in analytical chemistry hope to improve cosmetic quality control feasibility in the near future.

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– 3 –

UV Filters in Sunscreens and other Cosmetics. Tanning and Whitening Agents. Analytical Methods

3.1. UV Filters in Sunscreens and other Cosmetics. Regulatory Aspects and Analytical Methods

A. Chisvert[1] and A. Salvador[2*]

[1]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Alicante, Spain
[2]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Doctor Moliner Street 50, Burjassot-46100, Valencia, Spain

INTRODUCTION

It is well-known that, approximately, the visible (VIS) radiation (400 760 nm) constitutes 44.3% of the solar radiation reaching Earth, while 49.5% is due to infrared (IR) radiation ($760 - 1 \times 10^6$ nm), and only 6.2% is attributed to ultraviolet (UV) radiation (100 400 nm), since the ozone layer attenuates it. Within UV radiation, 98% is comprised by ultraviolet A (UVA) (320 400 nm) and the other 2% corresponds to ultraviolet B (UVB) (290 320 nm); meanwhile ultraviolet C (UVC) (100 290 nm), which has the highest energy values and is thus the most harmful, fortunately does not reach the Earth s surface.

*Corresponding author. E-mail: amparo.salvador@uv.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

Exposure to UV radiation in small amounts has a therapeutic effect on different pathologies since it improves the endogenous vitamin D production by the human body. This, in turn, increases calcium absorption and thus prevents osteoporosis and rickets, and also has beneficial effects on arthritis, blood-pressure regulation, diabetes and muscle strength, and also improves mood (Grant and de Gruijl, 2003). On the other hand, tanned skin has been associated to a beauty standard by Caucasian people. However, because of damage caused to the ozone layer, the intensity of solar UV radiation reaching Earth has increased in recent years, which could explain the increased incidence of skin tumours. Furthermore, it is well-documented that over-exposure to sunlight can promote other harmful effects on human health, such as skin inflammation or sunburn, UV-induced immune-suppression, hyperqueratosis or skin photo-ageing and photo-induced allergenic reactions. Because of all this, the use of sunscreen products on the skin quenching the harmful solar radiation may prevent or minimize the aforementioned effects on the human body.

Sunscreen products incorporate different chemicals that have high UV-light-absorbing properties, which are commonly referred to as UV filters.

These products are classified into different categories by different countries, which depend on the legislation in force in each country. As mentioned in Section 1.1, the three main regulatory systems on cosmetic products are the European Union (EU) Cosmetics Directive, the United States (US) Food and Drug Administration (FDA) rules and the Japanese legislation. Both EU and Japan consider sunscreen products as cosmetics, whereas the US considers them as over-the-counter (OTC) drugs (see further on).

The aim of this section is to familiarize the reader with sunscreen products and their active ingredients, that is. UV filters, relevant legislation, and also to review the analytical methods for UV filters determination in cosmetic products.

TYPES OF SUNSCREEN PRODUCTS

To begin with, sunscreen products were designed to protect users from sunlight when they went to the beach. Later, the same products were used for practising snow sports, since sunlight affects people s skin more strongly as it is reflected off snowy surfaces. However, nowadays, UV filters are being incorporated into daily-use cosmetics, such as moisturizing day creams and other hair-care products, aftershave products, lipsticks or makeup products, etc. Also, special products originally intended just for snow sports, such as high moisturizing lipsticks to prevent lips from drying, are being formulated with UV filters for normal use. Moreover, in line with consumer needs, new sunscreen products with different characteristics intended for the beach have appeared on the market. So, apart from the traditional creams and milks, which are water-in-oil (W/O) or oil-in-water (O/W) emulsions, with different viscosity degrees, and oils, we can find a wide range of products, among which are water-based and hydroalcoholic lotions and microemulsions, which are called easy to use sunscreens, since they can be sprayed on.

Moreover, UV filters can also be added to cosmetics to protect the cosmetic from sunlight. Therefore, they can usually be added to all types of cosmetics, and also to all kinds of cosmetic matrices.

All this has led to a great variety of UV filters being designed, with different solubility properties, since samples with different fat/water ratio content are available on the market.

So, we can find fat-soluble and water-soluble UV filters. Thus, usually, lipsticks, oils, hydroalcoholic lotions and foundations (which have high fat content) are formulated with fat-soluble UV filters; watery lotions and gels incorporate water-soluble UV filters; and finally, creams, milks and microemulsions are formulated with both water- and fat-soluble UV filters, where both water- and fat-soluble UV filters remain in the aqueous or fatty phase of the emulsion, respectively.

SUNSCREEN PRODUCTS IN THE EUROPEAN UNION

Within the EU framework, sunscreens are considered as cosmetics, and are regulated under Annex VII of the EU Cosmetics Directive (Council Directive 76/768/EEC), which specifies the chemicals permitted as UV filters, the maximum authorized contents and the conditions under which they can be used. Under this annex, UV filters are defined as *"(...) substances which, contained in the cosmetic sunscreen products, are specifically intended to filter certain UV rays in order to protect the skin from certain harmful effects of these rays"*, although adds that *"(...) may be added to other cosmetic products under the conditions laid down in this Annex"*.

However, in some cases, these compounds used as UV filters may be added to certain cosmetics to protect the product instead of the consumer, and in this event the chemical is designated as UV absorber. Obviously, all the UV filters can act as UV absorbers, but Annex VII of the EU Cosmetics Directive emphasizes that other chemicals used in cosmetic products solely for the purpose of protecting the product against UV rays are not included in the aforementioned annex.

Therefore, a sunscreen cosmetic product could be defined as *"any cosmetic product containing UV filters in its formulation in order to protect the human body from the solar deleterious UV light, avoiding or minimizing the damage that this radiation might cause on human health"* (Salvador and Chisvert, 2005a), and thus, cosmetics containing only UV absorbers are not considered sunscreen cosmetics as such.

Nevertheless, in the EU Cosmetics Directive, nothing is said about the threshold concentration under which a chemical with UV-absorbent properties is considered to be a UV absorber or over which the same chemical is considered as a UV filter. This can create a loophole in the EU legislation, since manufacturers could use non-prohibited chemical substances by claiming that they are not used as UV filters but as UV absorbers, even though they are not allowed as UV filters in Annex VII.

On the other hand, as mentioned in Section 1.1, Annex VII is divided into two parts: Part 1 lists the permitted UV filters which cosmetic products may contain at present, whereas Part 2 lists those provisionally allowed. The allowed compounds are reviewed periodically by the Scientific Committee on Consumer Products (SCCP), formerly known as Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP), which is composed of scientists from the different member states, who study and review the data of the target compounds provided by different sources, such as hospitals, industries and research centres. After reporting the study, the European Commission together with the member states, adopt the appropriate actions. In this way, some compounds have been eliminated that were provisionally allowed, and

even some permitted UV filters have been banned (e.g. urocanic acid), as a consequence of data showing them not to be safe for human health or because of their low capacity to afford protection from sunlight or poor photostability. On the contrary, some provisionally allowed UV filters have been allowed definitively because they have been shown safe for people and efficient against sunlight, and also new substances have been approved for use, which have higher UV-light absorption potential, greater stability and fewer side-effects.

Today there are 27 substances forming Part 1 of the Annex VII, whereas there are no compounds in Part 2. These UV filters and their authorized contents are listed in Table 3.1.1.

SUNSCREEN PRODUCTS IN THE UNITED STATES

As opposed to the EU Cosmetics Directive, and as mentioned in Section 1.1, in Title 21 of the Code of Federal Regulations (21 CFR) published by the FDA (see references), sunscreen products are classified as OTC drug products instead of cosmetic products, and they fall under the regulations described in 21 CFR Part 352, where different aspects of use, labelling, etc. are stated, and moreover, nearly 20 substances are listed as sunscreen active ingredients , which are defined as substances *"(...) that absorbs, reflects, or scatters radiation in the UV range at wavelengths from 290 to 400 nanometers"*.

FDA does not allow free combination of all these UV filters, but only permits those combinations that appear in 21 CFR Part 352.

Table 3.1.1 shows all those sunscreen-active ingredients, that is, UV filters, allowed in the US framework at present, as well as the authorized contents.

SUNSCREEN PRODUCTS IN JAPAN

As mentioned in Section 1.1, in Japan, under the Pharmaceutical Affairs Law (PAL), sunscreen products are considered as cosmetic products, as in the EU. From the deregulation process on April 2001 (see Section 1.1), according to the Ministry of Health and Welfare (MHW, 2000), UV filters are designated as UV absorbers , which are defined as *"(...) materials that specifically absorb ultraviolet rays and that are incorporated in cosmetics for the purpose of protecting skin or hair from adverse effects of ultraviolet rays"*. No specifications are made concerning the chemicals incorporated to protect the product itself. The UV filters to be used in cosmetics currently, are listed under Appendix 4 of the Standards for Cosmetics (MHW, 2000), which contains a positive list of nearly 30 UV filters. Appendix 4 distinguishes those UV filters restricted in all types of cosmetics from those whose restriction depends on the type of cosmetic product.

The UV filters allowed in Japan, and their authorized contents, are also listed in Table 3.1.1.

With reference to Table 3.1.1, we must point out the differences in the permitted compounds and also in their maximum concentration that is allowed depending on the different legislations. As can be seen, there are only ten of these compounds that are simultaneously authorized for these three main legislations on cosmetic products.

Table 3.1.1

Updated list until June 2006 of UV filters that can be employed in sunscreen products according to legislations in force in European Union (EU), United States (US) and Japan (JP), and their maximum authorized contents (alphabetical order)

Key[a]	INCI name	EU	US	JP[b]
3BC	3-benzylidene camphor	2		
BCS	Benzylidene camphor sulphonic acid	6[c]		
BDM	Butyl methoxydibenzoylmethane	5	3	10
BZ1	Benzophenone-1			10
BZ2	Benzophenone-2			10
BZ3	Benzophenone-3	10	6	5
BZ4	Benzophenone-4[d]	5	10	10
BZ6	Benzophenone-6			10
BZ8	Benzophenone-8		3	
BZ9	Benzophenone-9			10
CBM	Camphor benzalkonium methosulfate	6		
CX	Cinoxate		3	5
DBT	Diethylhexyl butamido triazone	10		
DDP	1-(3,4-Dimethoxyphenyl)-4,4-dimethyl-1,3-pentanedione			7
DHH	Diethylamino hydroxybenzoyl hexyl benzoate	10		
DMC	Diisopropyl methyl cinnamate			10
DRT	Drometrizole trisiloxane	15		15
EDP	Ethylhexyl dimethyl PABA	8	8	10
EDDP	Ethylhexyl dimethoxybenzylidene dioxoimidazolidine propionate			3
EMC	Ethylhexyl methoxycinnamate	10	7.5	20
EMT	bis-Ethylhexyloxyphenol methoxyphenyl triazine	10		
ES	Ethylhexyl salicylate	5	5	10
ET	Ethylhexyl triazone	5		5
FA	Ferulic acid			10
GED	Glyceryl ethylhexanoate dimethoxycinnamate			10
GPH	4-(2-beta-Glucopyranosiloxy) propoxy-2-hydroxybenzophenone			5
HS	Homosalate	10	15	10
IMC	Isoamyl *p*-methoxycinnamate	10		
IPM	Isopropyl methoxycinnamate[e]			10
ITT	Isopentyl trimethoxycinnamate trisiloxane			7.5
MA	Menthyl anthranilate		5	
MBC	4-Methylbenzylidene camphor	4		
MBT	Methylene bis-benzotriazolyl tetramethylbutylphenol	10		10
OCR	Octocrylene	10 (as acid)	10	10
P15	Polysilicone-15	10		
P25	PEG-25 PABA	10		
PAB	PABA	5	15	4[f]
PBC	Polyacrylamidomethyl benzylidene camphor	6		
PBS	Phenylbenzimidazole sulphonic acid	8[b]	4	3
PDP	Pentyl dimethyl PABA (mixed isomers)			10
PDT	Disodium phenyl dibenzimidazole tetrasulfonate	10 (as acid)		
TDS	Terephthalylidene dicamphor sulphonic acid	10[c]	10	

(*Continued*)

Table 3.1.1 (Cont.)

Key[a]	INCI name	EU	US	JP[b]
TiO_2	Titanium dioxide	25	25	
TS	TEA-salicylate		12	
ZnO	Zinc oxide		25	

[a]Key system adopted by authors of this section.
[b]These values are for cosmetics not used for mucosa and not to be washed away. For others see the specific legislation.
[c]Potassium, sodium and TEA salts (expressed as acid) are also allowed.
[d]Benzophenone-5 (BZ5) is the sodium salt of benzophenone-4 (BZ4).
[e]It contains 3 9% methyl diisopropyl cinnamate (MDC) and 15 21% ethyl diisopropyl cinnamate (EDC).
[f]As total, including its esters: ethyl PABA (EP), butyl PABA (BP) and glyceryl PABA (GP).
INCI, International Nomenclature for Cosmetic Ingredients.

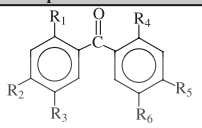
THE NATURE OF UV FILTERS

UV filters can be classified into two groups according to their nature. The inorganic UV filters, or also so-called physical UV filters, principally work by reflecting and scattering the UV radiation, while the organic UV filters, or also called chemical UV filters, absorb the light.

The physical UV filters are generally metallic oxides, although silicates and talc have also been used. They provide higher protection than the chemical ones and they are non-soluble in water. By contrast, they are less well accepted by people because they usually form a blocking film on the skin that is uncomfortable. Moreover, the cosmetic preparations based on physical UV filters are more difficult to formulate as they can break the emulsion. As can be seen in Table 3.1.1, at present, only titanium dioxide is approved as a physical UV filter by the EU Cosmetics Directive, and zinc oxide is not considered as such; however, it is considered as a colouring agent under Annex IV without any restriction of use just like titanium dioxide. On the other hand, both titanium and zinc oxides are authorized by the FDA as UV filters, whereas in Japan neither of them is considered as a UV filter, although neither is restricted under this legislation.

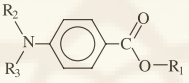
With regard to chemical UV filters, they are organic compounds with high molar absorptivity in the UV range. These compounds usually possess single or multiple aromatic structures, sometimes conjugated with carbon carbon double bonds and/or carbonyl moieties. Cosmetics containing these compounds are, however, better accepted by people than the physical ones, despite causing different dermatological side-effects (Berne and Ros, 1998; Darvay *et al.*, 2001; Goossens, 2004). In addition, different toxicological studies carried out on animals seems to indicate that some organic UV filters have significant estrogenic (Mueller *et al.*, 2003; Schlumpf *et al.*, 2004; Seidlova-Wuttke *et al.*, 2006) and antithyroid (Schmutzler *et al.*, 2004) effects, although a study on the change of hormonal levels in human volunteers after applying some sunscreens seem to indicate that these are not so high (Janjua *et al.*, 2004). They can be classified into different families according to their chemical structure (Figure 3.1.1): benzophenone derivatives, p-aminobenzoic acid and its derivatives, salicylates, cinnamates, camphor derivatives, triazine derivatives, benzotriazole derivatives, benzimidazole derivatives and others.

Benzophenone derivatives*



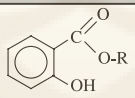
Name	R_1	R_2	R_3	R_4	R_5	R_6
BZ1	-OH	-OH	-H	-H	-H	-H
BZ2	-OH	-OH	-H	-OH	-OH	-H
BZ3	-OH	$-OCH_3$	-H	-H	-H	-H
BZ4	-OH	$-OCH_3$	$-SO_3H$	-H	-H	-H
BZ6	-OH	$-OCH_3$	-H	-OH	$-OCH_3$	-H
BZ8	-OH	$-OCH_3$	-H	-H	$-OCH_3$	-H
BZ9	-OH	$-OCH_3$	$-SO_3H$	-OH	$-OCH_3$	$-SO_3H$
GPH	-OH	$-O-(CH_2)_3-O-Si-Glucose$	-H	-H	-H	-H

p-aminobenzoic acid and its derivatives*



Name	R_1	R_2	R_3
EDP	$-CH_2-CH(-CH_2CH_3)-(CH_2)_3-CH_3$	$-CH_3$	$-CH_3$
EP	$-CH_2-CH_3$	-H	-H
GP	$-CH_2-CHOH-CH_2OH$	-H	-H
PAB	-H	-H	-H
PDP	$-C_5H_{11}$	$-CH_3$	$-CH_3$
P25	$-(CH_2-CH_2O)_x-CH_2-CH_3$ where x+y+z=25	$-(CH_2CH_2O)_y-CH_2-CH_3$	$-(CH_2CH_2O)_z-CH_2-CH_3$

Salicylates*



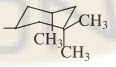
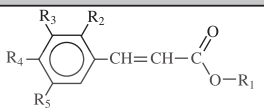
Name	R
ES	$-CH_2-CH(-CH_2CH_3)-(CH_2)_3-CH_3$
HS	
TS	$[NH(CH_2CH_2OH)_3]^+$

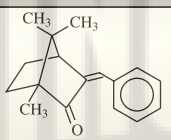
Figure 3.1.1 Classification of organic UV filters (*See Table 3.1.1 for key abbreviation).

Cinnamates*

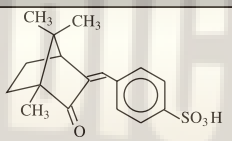


Name	R_1	R_2	R_3	R_4	R_5
CX	$-CH_2CH_2OCH_2CH_3$	-H	-H	$-OCH_3$	-H
DMC	$-CH_3$	CH_3-CH-CH_3	-H	CH_3-CH-CH_3	-H
EDC	$-CH_2-CH_3$	-H	CH_3-CH-CH_3	-H	CH_3-CH-CH_3
EMC	$-CH_2$-CH-$(CH_2)_3-CH_3$ CH_2-CH_3	-H	-H	$-OCH_3$	-H
FA	-H	-H	$-OCH_3$	-OH	-H
IMC	$-CH_2-CH_2$-CH-CH_3 CH_3	-H	-H	$-OCH_3$	-H
IPM	CH_3-CH-CH_3	-H	-H	$-OCH_3$	-H
ITT	O-Si-$(CH_3)_3$ $-(CH_2)_2$-CH-CH_2-Si-OCH_3 CH_3 O-Si-$(CH_3)_3$	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$
MDC	$-CH_3$	-H	CH_3-CH-CH_3	-H	CH_3-CH-CH_3

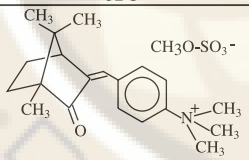
Camphor derivatives*



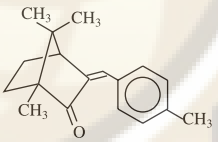
3BC



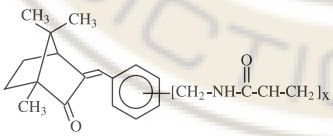
BCS



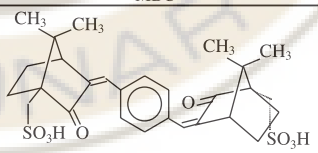
CBM



MBC



PBC



TDS

Figure 3.1.1 (*continued*)

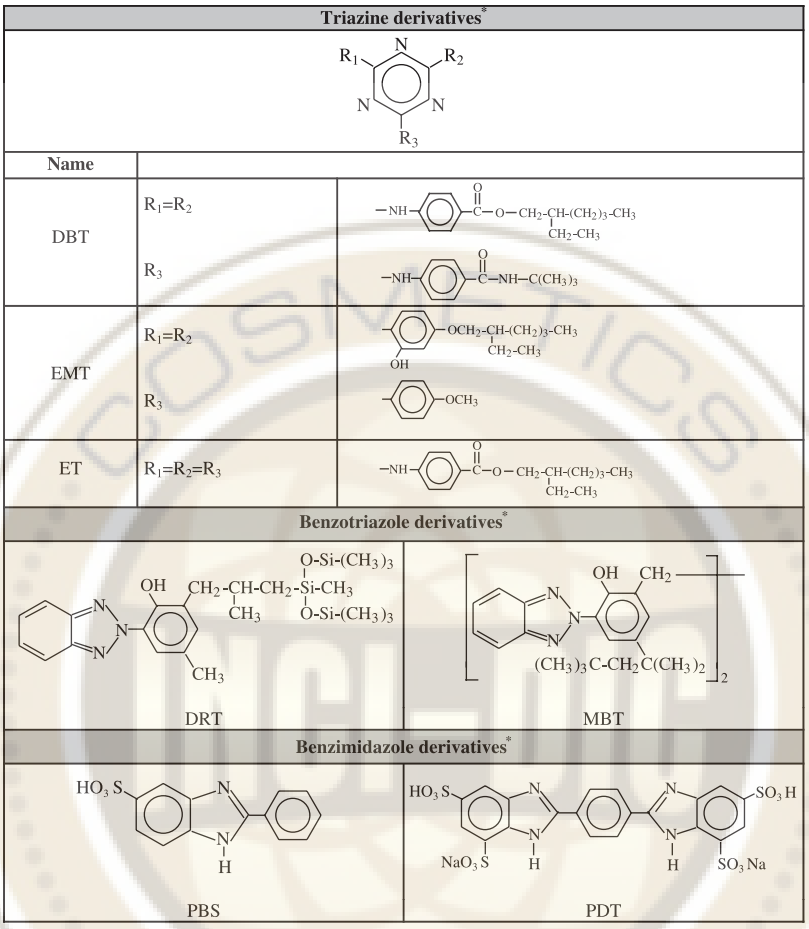


Figure 3.1.1 (*continued*)

As is shown in Figure 3.1.1, some of these organic UV filters have a structure with an ionizable moiety, such as sulphonic (SO_3H) or carboxylate (COOH), which affords their solubility in water.

Moreover, they can be classified as UVA or UVB filters, depending on the radiation that they attenuate. Figure 3.1.2 shows the spectra for a typical UVA, UVB and UVA + UVB filters, such as BDM, EDP and BZ3, respectively.

EFFICACY AND SAFETY OF SUNSCREEN PRODUCTS

The purpose of a sunscreen product is obviously to protect the user from solar radiation. To do this, the user must be properly informed about the protective capacity of the product used.

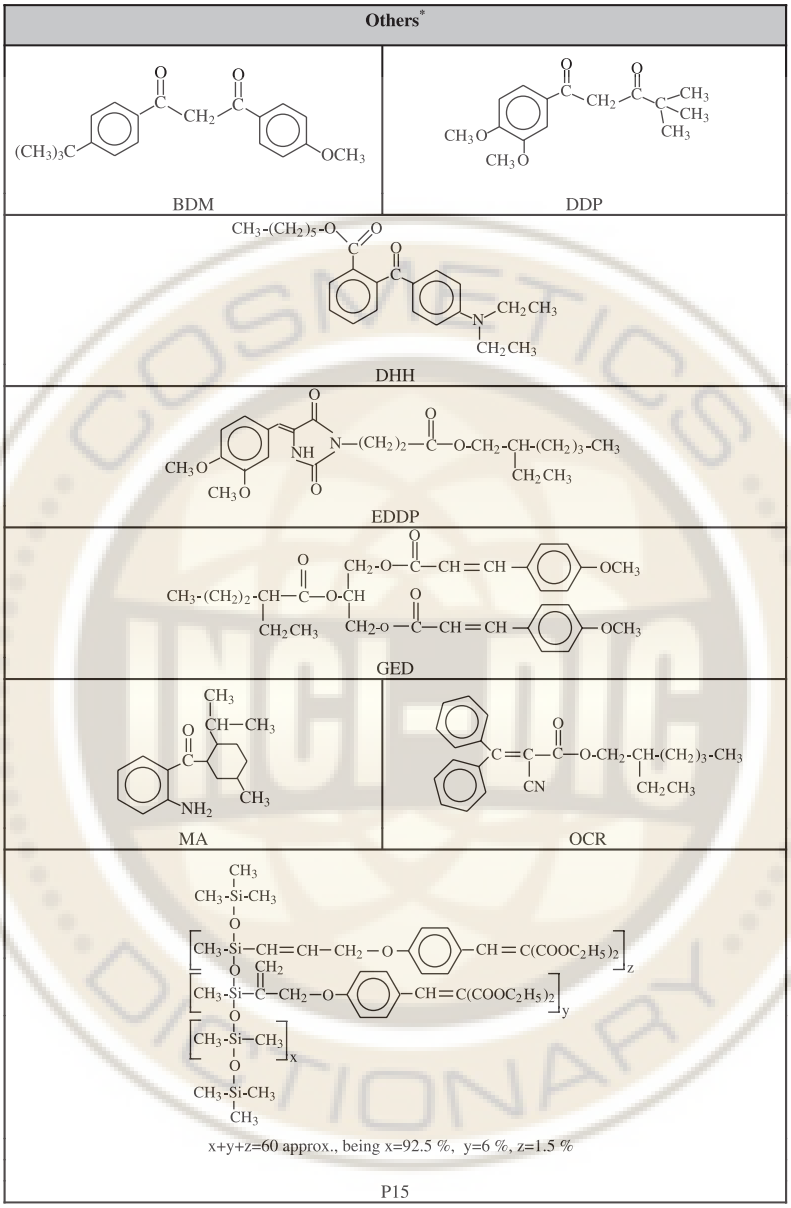


Figure 3.1.1 (*continued*).

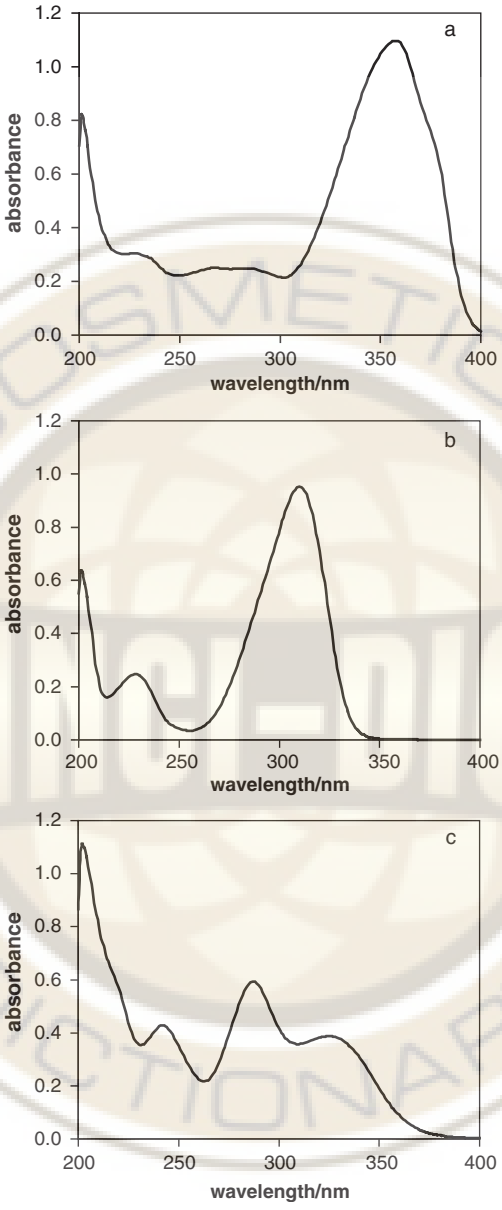


Figure 3.1.2 Spectra of some UV filters solutions. (a) BDM, (b) EDP (c) BZ3 (5 μg ml ^{1} in ethanol).

Different parameters have been used to evaluate the efficacy of sunscreen products and thus supply the user with the corresponding information on the product label. The most commonly used parameter is the so-called Sun Protection Factor (SPF) of a sunscreen product, which corresponds to the increase in UV dose that the protected skin can be subjected to without displaying erythema, as compared to unprotected skin.

The Cosmetics Industry of Europe, Japan, US and South Africa, represented by their associations, the European Cosmetic, Toiletry and Perfumery Association (COLIPA); the Japanese Cosmetic Industry Association (JCIA); the US Cosmetic, Toiletry and Fragrance Association (CTFA); and the South African CTFA), signed the International SPF Test Method in June 2006 (COLIPA, 2006).

This is an *in vivo* test (COLIPA, 1994) based on UV-light irradiation on the skin of several volunteers, under specified conditions and thus obtaining the time it takes for erythema to form. The SPF value is presented from the values of this time obtained with and without the previous application of the sunscreen product to be assayed. The method will be initially applicable throughout the EU, Japan and South Africa. In the US, FDA regulations prescribe a slightly different method.

Controversies about the use of this test, proposals for using alternative *in vitro* tests to estimate the SPF, and use of parameters other than SPF as an estimation of the sun protection are not dealt with in this book, but can be found in the extensive literature on the subject (Diffey, 2000; Diffey *et al.*, 2000; Zastrow *et al.*, 2004; Maier and Korthing, 2005; Nash *et al.*, 2006).

Obviously, the SPF of a product is related to the nature of the UV filters it contains and their concentration.

With regard to safety, as mentioned previously, some dermatological side-effects have been attributed to UV filters. Also, the number, nature and maximum allowed concentrations are restricted by different legislations.

Thus, it is clearly stated that the analytical control of UV filters is essential to assure the efficacy and safety of sunscreen products.

On the other hand, UV filters, such as p-aminobenzoic acid (PABA) and its derivatives supporting the amine function, could form nitrosamines, which are suspected of having carcinogenic properties (Meyer and Powell, 1991; Chou *et al.*, 1995). Therefore, a rigorous control of the finished product must be carried out.

Some relevant aspects of both the safety and efficacy of sunscreens are considered in different articles (Gasparro *et al.*, 1998; Nash, 2006).

ANALYSIS OF SUNSCREEN PRODUCTS

Although analytical control of sunscreen products is necessary, there are only few official analytical methods dealing with UV filters (see Section 2.1). According to the methods of analysis published by the EU Cosmetics Directive, there is only an official method to determine the UV filter named glyceryl PABA, which has been banned in cosmetic products to be marketed in the EU since 1992; however, it is still allowed in Japan. The analytical method is based on qualitative determination by means of thin layer chromatography and subsequent quantitative determination by liquid chromatography

(European Commission, 1999). Also, the international Association of Analytical Communities (AOAC) published an analytical method to determine another UV filter, named pentyl dimethyl PABA, which is based on a liquid liquid extraction procedure, followed by passing it through a chromatographic column, and finally the UV/VIS spectrum of the eluate is registered (Horwitz, 2005). Currently, this UV filter is only approved in Japan. To our knowledge, no other official methods regarding this type of ingredients have been published by the other two main legislations governing the cosmetic issue, that is, those in force in US and Japan.

Fortunately, a bibliographic search updated to June 2006, using analytical chemistry databases, revealed almost 90 publications on UV filters determination in cosmetic products. Bibliography concerning the analytical methods used for UV filters determination in cosmetic products and other types of samples has recently been revised by Salvador and Chisvert (2005a). Meanwhile, Granger and Brown (2001) have also published a revision article dealing with the chemistry of UV filters and related liquid chromatography determination methods. Another review, which covers the analysis of different cosmetics including sunscreens, was published previously by K nig (1985). It is also worth mentioning about the interesting book published by Lowe *et al.* (1997), which deals with the chemistry of UV filters, and also with the analysis and other aspects of sunscreen cosmetic products.

However, it should be emphasized that a detailed study of these published papers indicates the need for improvement in two aspects. First of all, although most of the published methods present good analytical properties, most of them do not deal with the high number of UV filters and mixtures currently used, and moreover, only some of the main types of cosmetic formulations, such as creams or lotions, are considered. On the other hand, most of them are not particularly suitable for periodical production control because either they require laborious sample preparation procedures, with an excessive amount of analysis time and/or use toxic organic solvents.

Table 3.1.2 gives a chronological summary of the experimental details and some interesting remarks of published papers dealing with UV filters determination in cosmetic products. Those papers which deal with standard solutions of UV filters are not included. It should be emphasized that the non-English publications have been reviewed on the basis of their respective abstracts, and thus, several data may be incomplete as shown in the aforementioned table. When the target UV filters are authorized by the three main current legislations on cosmetics (i.e. EU, US and Japan), they are named according to the abbreviation key in Table 3.1.1, and the rest are written between brackets and named as indicated in the footnote of Table 3.1.2.

It should be mentioned, however, that in bibliography different methods can be found dealing with UV filters determination in other type of samples, such as biological fluids, skin, waters, plastics, etc., but they escape the aim of this book and will not be considered here, however, they were included in Salvador and Chisvert s (2005a) review article. Moreover, those papers dealing with photostability studies of the UV filters are discussed in detail in Section 3.2.

On the other hand, as is deduced from Table 3.1.2, the number of published papers dealing with the determination of the different UV filters in cosmetics is very different depending on the UV filter. So, the number of published papers regarding those UV

Table 3.1.2

Published papers until June 2006 on UV filters determination in cosmetic products (chronological order)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Eiden *et al*. (1969)	(PMB)			TLC+(MP, UV/VIS, IR)	Qualitative purposes After isolation, quantification was done by gravimetry and photometry
Eiden and Tenczer (1971)	CX			TLC+(UV/VIS, NMR)	Qualitative purposes
Paulus (1972)	Cinnamates and salicylates			GC-FID	
Schmitz-Masse *et al*. (1979)	EP, GP			TLC+NMR GC-FID	NMR for qualitative analysis after TLC EP appeared as contaminant of GP
Liem and Hilderink (1979)	Screening of 45 UV filters	Not specified	Sample is mixed with MeOH and heated. Na_2SO_4 is added and left overnight. Supernatant is used for TLC where spots are scraped off and UV spectrum is measured	TLC+UV/VIS, Si plates. Three solvent systems are employed as eluents	Identification was done by comparison of Rf with standards, and characteristic colour when using anisaldehyde spray 24 UV filters were found in the 197 analysed samples
Mason (1980)	TiO_2			AAS	
Eiden and Tittel (1981)	Method 1: 3BC, BZ3, CX, EMC, IPM, MBC, + (4PB, BOR, BZ12, BZL, EPB, IDM, PMB) Method 2: BZ4, PBS + (CA, DPG)			LC-UV/VIS Method 1: Si column with hexane:EtAc or C_{18} column with MeCN:H_2O Method 2: C_{18} column with MeOH:H_2O as mobile phase	Methods 1 and 2 for fat and water-soluble UV filters determination, respectively

Cumpelik (1982)	BZ3, BZ8, CX, EDP, EMC, ES, GP, HS, PAB, PBS, PDP, TS + (DT, EHP)	Not specified	Sample is heated to eliminate water, and afterwards it is mixed with pyridine, derivatized with HMDS + TMS and centrifuged	GC-FID	Semiquantitave analysis
Masse *et al*. (1982)	(EHP)			LC-UV/VIS, C_{18} column with MeOH:0.02 M KH_2PO_4 as mobile phase	Fractions from LC were subjected to TLC, GC and NMR for qualitative purposes
K nig (1984)	Method 1: 3BC, BDM, BZ3, CX, EMC, IMC, MBC, PDP + (BOR, BZL, EPB) Method 2: BZ2, BZ4, BZ9, PBS + (DPG, CA)	Creams and oils		LC-UV/VIS Method 1: Si column with hexane:EtAc as mobile phase Method 2: C_{18} column with MeOH:H_2O as mobile phase	Methods 1 and 2 for fat and water-soluble UV filters determination, respectively
Tan *et al*. (1984)	BZ3, EDP	Lipsticks and lotions	Lotions are mixed with water and lipsticks with $CHCl_3$, and finally diluted with MeOH. Finally, they are filtered	LC-UV/VIS, C_{18} column with MeOH:MeCN as mobile phase	
Gagliardi *et al*. (1986)	EP, GP, PAB	Creams	Sample is mixed with NaCl, 2 M H_2SO_4 and MeOH, heated, diluted with MeOH and filtered	LC-UV/VIS, C_{18} column at 35 C with gradient MeCN:10 mM $NaClO_4$/ 5 mM TMAC at pH 3 as mobile phase	Only spiked analyte-free samples were analysed EP appeared as contaminant of GP
Gagliardi *et al*. (1987)	Group 1: BZ4, CBM, EDP, GP, PAB + (EHP) Group 2: BDM, BZ1, BZ3, EMC, 3BC + (BOR, CA, PS, SA)	Creams	Sample is mixed with 2 M H_2SO_4 and MeOH, sonicated and centrifuged. Supernatant is diluted with MeOH, acidified with 1 mM H_2SO_4 and extracted	LC-UV/VIS, C_{18} column at 30 C with gradient MeCN:1 mM $HClO_4$/ 50 mM $NaClO_4$ as mobile phase	Only spiked analyte-free samples were analysed Resolution problems made necessary to divide analytes into two groups by liquid liquid extraction

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
			with $CHCl_3$. Group 1 remains in the aqueous solution, and the organic extract contains the group 2, which is evaporated and dissolved in MeOH		Isocratic MeOH:H_2O (85:15) was necessary to achieve EMC BDM resolution
Ohshima and Saito (1987)	Group 1: BZ3, EDP + (DR) Group 2: BZ2, CX	Creams and oils	Sample is dissolved in THF	LC-UV/VIS with different MeCN:H_2O mixtures as mobile phases	UV filters were determined separately into two groups
Gagliardi *et al*. (1989)	Group 1: BZ4, BZ9, EDP, PBS Group 2: BZ1, BZ2, BZ3, BZ8, HS, IMC, MBC, EMC, + (CA, IBS)	Creams	Sample is mixed with 2 M H_2SO_4 and MeOH, sonicated and centrifuged. Supernatant is diluted with MeOH, and an aliquot is evaporated to dryness and group 1 is extracted with 2 M H_2SO_4. Group 2 remains in the residue, which is dissolved in MeOH	LC-UV/VIS, C_{18} column at 30 C with gradient MeCN:1 mM $HClO_4$/ 50 mM $NaClO_4$ as mobile phase	Resolution problems made necessary to divided analytes into two groups by liquid liquid extraction
Ikeda *et al*. (1989)	BDM, BZ3, CX, EDP, EMC + (IDM)	Creams, foundations, lipsticks, lotions and milks	Sample is diluted with THF, sonicated and filtered	LC-UV/VIS, C_{18} column at 40 C with MeOH:THF:H_2O as mobile phase	
Ikeda *et al*. (1990)	BDM, BZ3, CX, EDP, EMC + (IDM)	Foundations, lipsticks and lotions	Sample is diluted with THF, sonicated and filtered	GC-MS, 50% phenyl/50% dimethyl polysiloxane capillary column. Carrier gas: He	
DiNunzio and Gadde (1990)	BZ3, EDP, EMC, ES, MA, OCR	Not specified	Samples are dissolved in THF and diluted with	LC-UV/VIS, C_{18} column with THF:AcOH:H_2O as	

			MeCN. An aliquot is filtered and diluted with water and THF	mobile phase	
Gagliardi *et al*. (1990)	ET	Not specified	Sample is mixed with 2 M H_2SO_4 and THF, sonicated at 35 C and centrifuged. Supernatant is diluted with THF	LC-DAD, C_{18} column at 25 C with gradient MeOH:MeCN:1% AcOH as mobile phase	
Narayanan *et al*. (1991)	PAB	Lotions	Sample is dissolved in EtOH, and an aliquot is spotted on the substrate	SERS, Kr ion laser line at 647.1 nm, 150 mW of power	
Tomasella *et al*. (1991)	BZ3, EDP, EMC	Lotions	Sample is dissolved in *i*-PrOH and an aliquot is diluted with mobile phase and filtered	MLC-UV/VIS, C_8 column with 0.1 M SDS pH 3:*i*-PrOH as mobile phase	
Ohba *et al*. (1991)		Creams, lipsticks, lotions and oils	Sample is sonicated in acetone (or MeOH:$CHCl_3$ for oily suspensions), H_3PO_4 is added, and finally filtered	LC-UV/VIS, C_{18} column at 40 C with MeOH:H_2O containing 3 mM STAC as mobile phase	66% 1,4-dioxane containing 6 mM STAC as mobile phase is needed to separate OMC and BDM
Masse and Herpol-Borremans (1991)				LC-UV/VIS TLC	Only identification purposes
Herpol-Borremans and Masse (1992)	ET		Sample is treated with THF	LC-UV/VIS, C_{18} column with isocratic MeOH 100% as mobile phase	
Hild (1993)	BZ3, EDP, EMC, IMC, MBC, PBS + (IDM)			LC-UV/VIS, C_{18} or C_8 columns with 5 mM ammonium acetate buffer pH 3.8:MeCN as mobile phase GC-FID	UV, IR, critical film thickness and TLC are used for qualitative purposes

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Wallner (1993)	Method 1: BDM, BZ3, EMC, MBC + (IDM) Method 2: BZ4, PAB, PBS + (DPG)	Not specified	Method 1: Samples are dissolved in *i*-.PrOH, with agitation and heating, and diluted with mobile phase, and filtered Method 2: Samples are dissolved in mobile phase with agitation and heating, and filtered if necessary	LC-UV/VIS, C_{18} column, with the following mobile phases: Method 1: 10 mM phosphate buffer pH 2.5:MeOH Method 2: 5 mM ammonium acetate buffer pH 3.8:MeCN	Method 1 and 2 for fat- and water-soluble UV filters determination, respectively
Ro *et al*. (1994)	BZ1, BZ3, BZ2, BZ6, BZ8, ES, HS	Creams, foundations and lotions	Sample is dissolved in DMF by sonication An aliquot is filtered and mixed with BSTFA	GC-MS, 100% dimethylpolisiloxane	Only spiked analyte-free samples were analysed Silylation increases sensitivity and volatility
Pietta *et al*. (1995)	BDM, BZ1, BZ3, BZ4, PBS		Sample is mixed with 2 M H_2SO_4 and MeOH and centrifuged. Supernantant is extracted with CH_2Cl_2, dried, evaporated and re-dissolved in MeOH, and finally mixed with the buffer	MEKC-UV/VIS, fused-silica capillary, running buffer consisting in 18 mM phosphate pH 7 containing 30 mM SDS and 2.5% MeCN	
Meijer and Loden (1995)	BZ3, BDM, EMC	Lotions	Sample is extracted with EtOH at 60 C and agitation. Diluted with EtOH, centrifuged and supernatant analysed	LC-UV/VIS, C_8 column with gradient MeOH:1% AcOH as mobile phase	Method was applied to study the stability of the final product
De Orsi *et al*. (1995)	(UA)	Creams	Sample is mixed with MeOH:1 mM NaOH (1:3) and sonicated at 40 C After centrifugation, supernatant is filtered	LC-DAD, C_{18} column at 25 C with MeCN:0.1 M $HClO_4/NaClO_4$ pH 3 as mobile phase	Only spiked analyte-free samples were analysed Both Z and E isomers of UA are studied

Mori *et al*. (1996)	BDM, BZ1, BZ3, EDP, EMC		Samples are mixed with NaCl solution and extracted with $CHCl_3$, evaporated and re-dissolved in deuterated $CHCl_3$	NMR	Pyrazine is used as internal standard for quantitative purposes
Kawauchi *et al*. (1996)	TiO_2		Sample is dissolved in H_2O, sonicated and filtered. The filter is air-dried and used for the determination of titanium.	XRFS	
Schneider *et al*. (1996)	BDM, BZ3, BZ4, EDP, EMC, ES, ET, HS, MBC, OCR, PBS	Creams	Sample is dissolved in THF and sonicated. MeOH and 0.1% trifluoracetic acid are added, and sonicated again, and finally filtered	LC-DAD, C_{18} column with gradient 0.1% trifluoracetic acid:MeOH:MeCN as mobile phase	Only data for a synthetic sample are shown Gradient timetable is needed to be changed to achieve BDM-HS complete resolution
Jiang *et al*. (1996)	BDM, BZ3, EDP, EMC, ES	Creams and lotions	Sample is dissolved in MeOH, appropriately diluted with the same solvent, centrifuged and the supernatant analysed	LC-UV/VIS, C_{18} column with MeOH: H_2O as mobile phase	Studies on the stability of the UV filters in sunscreens after sun irradiation are also carried out
Scurei and Oprea (1996)	EMC, ES		UV filters are extracted, and first-order derivative spectra are measured	DUVS	
Cheng *et al*. (1997)	EMC	Lotions	Lotion is mixed with EtOH, sonicated at 50 60 C, stirred at room temperature and sonicated again. After centrifugation the supernatant is analysed	SERS, Ar ion laser line at 514.5 nm, 100 mW of power	
Musial and Sherma (1997)	EDP	Lotions and oils	Sample is dissolved in *i*-PrOH	TLC-UAD, C_{18} plates with MeOH:THF:H_2O as eluent	

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Yao *et al*. (1998)	BDM, BZ3, BZ4, EDP, EMC, ES, MBC, PAB + (IDM, PS, SA)		Sample is sonicated with mobile phase	LC-UV, C_{18} plates with MeOH:THF:H_2O:70% $HClO_4$ as mobile phase	
Musial and Sherma (1998)	EMC			TLC-UAD, C_{18} plates with MeOH:THF:H_2O as mobile phase	
Rastogi and Jensen (1998)	3BC, BCS, BDM, BZ3, BZ4, CBM, EDP, EMC, ES, ET, HS, IMC, MBC, OCR, PBS, P25, TDS + (IBS, IDM, UA)	Not specified	Sample is heated at 60 C with MeOH and 2 M H_2SO_4. After cooling it is diluted with MeOH and centrifuged, if necessary. An aliquot is diluted with mobile phase and analysed	LC-DAD, C_{18} column with gradient MeCN:THF:citrate buffer pH 9 as mobile phase	Identification purposes only, by matching the retention time and UV spectrum with a spectral library
Azevedo *et al*. (1999)	BZ4, PBS	Gels	Sample is dissolved in 95% EtOH or 0.1 M triethanol-amine solution for BZ4 or PBS determination respectively, and second-order derivative spectra are registered	DUVS	Two sample pretreatments are necessary for both UV filters determination, which are determined separately
Wang (1999)	BZ1, BZ2, BZ3, BZ4 BZ6, BZ8 + (BZ10)	Creams, foams, gels, lipsticks and lotions	Samples, except lipsticks, are dissolved in MeOH and H_2O. Lipsticks are dissolved in $CHCl_3$, extracted with MeOH by centrifugation, and diluted with MeOH. Filtered before analysis.	LC-UV/VIS, C_{18} column with MeCN:MeOH:H_2O as mobile phase	The analysed commercial samples only contained BZ1, BZ3 or BZ4
Scalia (2000)	BDM, BZ3, EDP, EMC, MBC	Creams, lipsticks and lotions	Sample is mixed with hydromatrix and loaded into the SFE cell. The extracts are collected in EtOH and analysed	SFE+LC-UV/VIS, Ph column with MeOH:MeCN:THF:0.5% AcOH as mobile phase	

Westgate and Sherma (2000a)	BZ3	Lotions		TLC-UAD, C_{18} plates	
Salvador *et al*. (2000)	Method 1: TiO_2 Method 2: ZnO	Creams	Method 1: Sample is introduced into PTFE reactor for microwave matrix digestion in acidic medium. The residue is transferred to porcelain crucible and $KHSO_4$ is added and fused. The molten is dissolved in H_2SO_4 and diluted with H_2O Method 2: Isobutyl methyl ketone and Nemol-K39 is added to sample, and diluted with H_2O	Method 1: ICP-AES Method 2: AAS	
Shih and Cheng (2000)	BZ3, MBC, EMC	Creams	MeOH is added to sample, and MAE procedure is applied. After cooling is filtered and analysed	MAE+LC-UV/VIS, C_{18} column with MeCN:H_2O as mobile phase	
Wang and Chen (2000)	EDP, EMC, EP, PAB + (PDP, TDP)	Creams	Sample is placed on filter paper and rolled and inserted into SFE cell. The extracts are collected in MeCN and diluted with buffer or with mobile phase for methods 1 or 2, respectively	Method 1: SFE+MEKC-UV/VIS, fused-silica capillary, and running buffer consisting in 10 mM CTAB, 0.20 M urea, 40 mM sodium tetraborate solution:MeCN Method 2: SFE+LC-DAD, C_{18} column with gradient MeCN:0.50% ammonium acetate buffer as mobile phase	The analysed commercial samples only contained EMC or EDP

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Westgate and Sherma (2000b)	ES	Lotions		TLC-UAD	
Westgate and Sherma (2000c)	ES	Lotions	Sample is dissolved in EtOH	TLC-UAD, C_{18} plates with MeOH:THF:H_2O as eluent	
Li *et al*. (2000)	BZ3, BZ4, EDP, EMC, ES + (PS)			LC-UV/VIS, C_{18} column with MeOH:THF:H_2O as mobile phase	
Fisher and Sherma (2000)	OCR	Lotions	Sample is extracted with EtOH	TLC-UAD, C_{18} plates, MeOH:THF:H_2O as eluent	
Chisvert *et al*. (2001a)	BZ3, EMC	Creams and milks	Sample is dissolved in EtOH, filtered and analysed by employing the second order derivative spectra	FI-DUVS Carrier stream: EtOH	
Chisvert *et al*. (2001b)	BZ3	Creams, lotions and milk	Sample is dissolved in EtOH, filtered and analysed by employing both manifolds	FI-UV/VIS, 20 mM Ni (II) in H_2O:EtOH and 1 M NH_4OH in H_2O:EtOH as merging carrier streams SI-UV/VIS, carrier: EtOH, sandwich arrangement: 10 mM Ni (II) in H_2O:EtOH-sample-0.5 M NH_4OH in H_2O:EtOH	Both manifold are compared in terms of analytical parameters
Chisvert *et al*. (2001c)	BZ3, BZ4, EDP, EMC, ES, HS	Creams, lotions, milks and oils	Sample is dissolved in EtOH and filtered	LC-UV/VIS, C_{18} column with EtOH:1.7% AcOH	
Chisvert *et al*. (2001d)	BDM, BZ3, BZ4, HS, EDP, EMC, ES	Creams, lotions, milks and waters	Sample is dissolved in EtOH, sonicated if necessary, and filtered	LC-UV/VIS, C_{18} column with EtOH:1.7 % AcOH as mobile phase, containing 65.4 mM HP-β-CD	

Salvador *et al.* (2001a)	BZ3	Lipsticks	Sample is suspended in EtOH into a Pyrex tube, heated in water bath and submitted to MAE. After cooling, EtOH and 25% NH_4OH are added, filtered and diluted with EtOH	MAE+SI-UV/VIS, carrier: EtOH, sandwich arrangement: 10 mM Ni (II) in H2O:EtOH-sample-10 nM Ni (II) in H_2O:EtOH	
Salvador *et al.* (2001b)	HS	Lipsticks	Siliceous earth is placed into SFE cell and sample is added. Extracts are collected in EtOH and filtered	LC-UV/VIS, C_{18} column with EtOH:0.4% AcOH as mobile phase	No real samples are analysed, only synthetic samples
Masse *et al.* (2001)	EDP, GP, PAB, P25 + (EHP)			GC-MS TLC+NMR	Identification purposes
Masse and Herpol-Borremans (2001)	EDP, ES, HS	Not specified	Sample is dissolved in MeOH and sonicated. After cooling is filtered or centrifuged	LC-UV/VIS, C_{18} column at 35 C with MeOH:H_2O as mobile phase	
Chang and Chang (2001)	BDM, BZ3, EMC, ES		Sample is agitated with MeOH, centrifuged and filtered. An aliquot is diluted in the support electrolyte	DPV, epoxy-carbon composite electrodes, 0.1 M TBAP in DMSO as support electrolyte	
Wang (2002)	BZ3, EMC, ES			DPV, glassy carbon and mercury film electrodes, 0.1 M TBAH as electrolyte	
Townshend *et al.* (2002)	EDP	Creams and milks	Sample is dissolved in EtOH:H_2O and filtered	FI-CL, carrier stream: EtOH:H_2O, reagent stream: 0.25 mM $KMnO_4$ in 2M H_2SO_4	
Chisvert *et al.* (2002a)	BZ4, PBS	Waters	Sample is dissolved in H_2O. A SPE clean-up procedure is carried out on line	SI-SPE-UV/VIS, carrier: H_2O, eluent: 0.1 and 1 M HCl	

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Dutra *et al.* (2002)	BZ3, EMC, ES	Creams and milks	Sample is dissolved with MeOH, sonicated and filtered. An aliquot is diluted with mobile phase	LC-UV/VIS, C_{18} column at 25 C with MeOH:H_2O	
Chisvert and Salvador (2002)	BZ4, PBS, TDS	Waters	Sample is dissolved and diluted with mobile phase	LC-UV/VIS, C_{18} column with EtOH:20 mM acetate buffer pH 4.6	
Klampfl *et al.* (2002)	BDM, BZ3, EDP, EMC, ES, HS, MBC, OCR, PBS	Lotions	Lotions are diluted with THF, sonicated and filtered. An aliquot is diluted with the microemulsion buffer	MEEKC-DAD, fused-silica capillary, microemulsion buffer consisting of 2.25 g SDS, 0.75 g Brij 35, 6,6 g BuOH, 0.8 g octane, 17.5 g *i*-PrOH and 72.1 g 10 mM borate buffer pH 9.2	Only one sample, containing BDM and MBC, was analysed
Chisvert *et al.* (2002b)	PAB	Creams and lotions	Sample is dissolved in EtOH and filtered. An aliquot is mixed with 0.1 M HCl and diluted with EtOH	SI-UV/VIS, carrier: EtOH, sandwich arrangement: 0.1 M nitrite-sample−0.1 M nitrite−0.1 M oxine−0.5 M NaOH	
Rastogi (2002)	3BC, BCS, BDM, BZ3, BZ4, CBM, DRT, EDP, EMC, ES, ET, HS, IMC, MBC, OCR, PBS, P25, TDS	Creams and lotions		LC-DAD	PAB was not considered in this study because it interfered in the analysis DRT could not be determined in one of the samples due to interferences
Wang and Lee (2003)	BZ1, BZ2, BZ3, BZ4, BZ6, BZ8 + (BZ12)	Not specified	Sample is rolled on a piece of filter paper, inserted into the SFE cell	Method 1: SFE+LC-UV/VIS, C_{18} column with gradient MeCN:H_2O	Bad recoveries were obtained for BZ2 by SFE Only BZ3 was found, in

			and filled with sea sand. Extract is collected in MeCN and filtered before its injection	Method 2: SFE+CZE-UV/VIS, buffer consisting in 0.2% Tween 20 in 20 mM borate buffer	three of ten analysed samples
Salvador *et al.* (2003)	PAB	Creams and lotions	Sample is dissolved in EtOH and filtered. An aliquot is diluted with 1 M HCl	SI-UV/VIS, carrier: water, sandwich arrangement: sample−0.2 mM hypochlorite H_2O−0.1 mM *o*-tolidine	
Klampfl and Leitner (2003)	BDM, BZ3, EDP, EMC, ES, HS, MBC, OCR, PBS	Lotions, milk and oils	Sample is dissolved in THF and sonicated. Finally, it is diluted with THF, filtered and diluted with the microemulsion buffer	MEEKC-DAD, fused silica capillary, microemulsion buffer consisting of 2.25 g SDS, 0.75 g Brij 35, 6,6 g BuOH, 0.8 g octane, 17.5 g i-PrOH and 72.1 g 10 mM borate buffer pH 9.2	
Hauri *et al.* (2003)	BDM, BZ2, BZ3, BZ4, CBM, DBT, DRT, EDP, EMC, EMT, ES, ET, HS, IMC, MBC, MA, MBT, OCR, PAB, PBS, TDS	Not specified	Sample is mixed with acetone:MeOH and sonicated by heating. After cooling is centrifuged, filtered and diluted with the same solvent. For TDS and for DBT, DRT, EMT, ET and MBP, 12.5 mM NaOH in MeOH and acetone:THF are used instead acetone:MeOH, respectively	LC-DAD, C_{18} column at 30 C with gradient 5 mM acetate buffer: MeOH:MeCN:THF as mobile phase	Identification purposes by measuring the UV spectra with a DAD P25 is not considered in this study because it interfered in the BZ3 determination Three different extraction processes, and consequently three chromatographic runs, according to the UV filters are to be analysed
Smyrniotakis and Archontaki (2004)	EMC, ES, MBT, OCR	Creams and lotions	Sample is dissolved in DMF, sonicated and diluted with mobile phase	LC-UV/VIS, C_{18} column with MeOH:MeCN as mobile phase	

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Hauri *et al.* (2004)	BDM, BZ3, DBT, DRT, EMC, EMT, ES, ET, IMC, MBC, MBT, OCR, PBS, TDS	Not specified	Sample is dissolved in acetone:THF, sonicated and heated	LC-DAD-MS, C_{18} column at 30 C with gradient H_2O:MeCN containing 0.1% formic acid as mobile phase	ES and HS are not detected by MS, and PBS and TDS give poor sensitivity Study of photodegradation of UV filters in the final product are also carried out
Schakel *et al.* (2004)	BDM, BZ3, BZ4, DBT, DRT, EDP, EMC, ES, ET, HS, IMC, MBC, OCR, PAB, PBS, TDS	Creams and oils	Sample in dissolved in EtOH containing Tween 80, heated and sonicated. Solution is diluted with EtOH (or EtOH:acetate buffer if contain PAB)	LC-UV/VIS, C_{18} column at 28 C with gradient EtOH:acetate buffer pH 2.5 as mobile phase, containing 0.2 mM of EDTA	Recovery data are shown only for 12 of 16 UV filters studied There is overlapping between the most polar UV filters, such as BZ4, PAB, PBS and TDS
Santoro *et al.* (2004)	BZ3, EMC	Creams		LC-UV/VIS	
Awatramani and Nucci (2005)	BDM, BZ3, EMC, ES	Creams		LC-UV/VIS	
Simeoni *et al.* (2005)	BDM, BZ3, EDP, EMC, ES, MBC, MBT, OCR, PBS	Creams and lotions	Sample is dispersed in MeOH (or 20% MeCN in THF, if contains MBP) and sonicated. Finally, it is filtered	LC-UV/VIS, CN column with MeOH:MeCN:THF: H_2O as mobile phase, containing 0.5% AcOH	Mobile phase slightly changed for determination of MBP Photodegradation studies are also carried out
Huang *et al.* (2005a)	BZ1, BZ2, BZ3, BZ6 + (other 4 benzophenones)	Lotions	Sample is mixed with MeCN, sonicated and centrifuged	CEC-DAD, methacrylate ester-based monolithic column, phosphate buffer:MeCN at pH 3 as eluent	Only BZ3 was found in the analysed samples

Salvador and Chisvert (2005b)	Run 1: BDM, BZ3, DBT, DRT, EDP, EMC, ES, ET, HS, IMC, MBC, OCR Run 2: BZ4, P25, PAB, PBS, PDT, TDS	Creams, lipsticks, lotions, foundations, milks, waters	Sample is dissolved in EtOH, and sonicated, if necessary On one hand, an aliquot is diluted with EtOH:AcOH to perform run 1, and on the other hand, other aliquot is diluted with EtOH:acetate buffer pH 4.75 to perform run 2. Both are filtered if necessary	LC-UV/VIS, C_{18} column with gradient EtOH:1% AcOH for run 1, and gradient EtOH:acetate buffer pH 4.75 for run 2	The 18 target analytes are determined by means of two runs.
Huang *et al.* (2005b)	BZ1, BZ2, BZ3, BZ6 + (other 4 benzophenones)	Lotions and waters	Sample is mixed with MeOH, sonicated and centrifuged. The supernatant is diluted with the microemulsion buffer.	MEECK-DAD, fused-silica capillary, microemulsion buffer consisting in 0.6% SDS, 0.5% EtAc, 1.2% BuOH, 5% EtOH and 92.7% Tris buffer pH 9.0 MECK-DAD, running buffer consisting in 0.9% SDS, 2% EtOH, 97.1% Tris buffer pH 9.0	Only BZ3 and BZ4 were found in the analysed samples
Salvador *et al.* (2005)	BDM, BZ3. EDP, EMC	Lipsticks and foundations	Siliceous earth is placed into SFE cell and sample is added. Extracts are collected in EtOH. Then AcOH and EtOH are added, filtered and analysed	SFE-LC-UV/VIS C_{18} column at 45 C with gradient EtOH:1% AcOH as mobile phase	
G mara *et al.* (2005)	MBC	Creams	Sample is dissolved in DMF, and diluted with MeOH:H_2O	EKC-DAD, fused-silica capillary set at 15 C, running buffer consisting of 15 mM CM-β-CD/ 120 mM α-CD in 100 mM borate buffer pH 9.0	Enantioselective separation and quantification of two pairs of enantiomers are carried out

(*Continued*)

Table 3.1.2 (Cont.)

Authors	Target UV filters[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]	Remarks
Da Silva *et al.* (2006)	EMC, MBC or BZ3, EMC	Creams and lotions	Sample is dissolved in MeOH, stirred and centrifuged. Then, the supernatant is diluted with MeOH, and measured	DPP, dropping mercury electrode, in micellar media provided by CTAC	

[a]See Table 3.1.1 for key abbreviation. Analytes into brackets are not currently permitted by the three main legislations regarding cosmetic products: 4PB, 4-phenylbenzophenone; BOR, bornelone; BZ10, benzophenone-10; BZ12, benzophenone-12; BZL, benzalazine; CA, cinnamic acid; DPG, dimethoxyphenylglyoxylate (sodium salt); DR, drometrizole; DT, digalloyl trioleate; EHP, ethyl hydroxypropyl PABA (mixture of isomers); EPB, ethylhexyl phenylbenzoylbenzoate; IBS, isopropylbenzyl salycilate; IDM, isopropyl dibenzoylmethane; PMB, phenyl methylbenzoxazole; PS, phenyl salicylate; SA, salicylic acid; TDP, ethyl dimethyl PABA; UA, urocanic acid.

[b]Symbol − means coupling between techniques, and symbol + means sequentially applied techniques. Key abbreviation: AAS, atomic absorption spectrometry; AcOH, acetic acid; AES, atomic emission spectrometry; BSTFA, bis-trimethylsilyltrifluoroacetamide; BuOH, butanol; $C_{8,}$ octylsilica; $C_{18,}$ octadecylsilica; α-CD, α-cyclodextrin; CEC, capillary electrochromatography; CL, chemiluminescence; CM-β-CD, carboxymethylated-β-cyclodextrin; CN, cyanopropyl silica; CTAB, cetyl trimethyl ammonium bromide; CTAC, cetyl trimethyl ammonium chloride; CZE, capillary zone electrophoresis; DAD, diode-array detector; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DPP, differential-pulse polarography; DPV, differential-pulse voltammetry; DUVS, derivative ultraviolet spectrometry; EDTA, ethylendiaminotetraacetic acid; EKC, electrokinetic chromatography; EtAc, ethyl acetate; FI, flow injection; FID, flame ionization detector; GC, gas chromatography; HMDS, hexamethyldisilazane; HP-β-CD, hydroxypropyl-β-cyclodextrin; ICP, inductive coupled plasma; IR, infrared spectrometry; LC, liquid chromatography; MAE, microwave assisted extraction; MeCN, acetonitrile; MEEKC, microemulsion electrokinetic chromatography; MEKC, micellar electrokinetic chromatography; MeOH, methanol; MLC, micellar liquid chromatography; MP, melting point; MS, mass spectrometry; NMR, nuclear magnetic resonance; Ph, phenylsilica; i-PrOH, isopropanol; PTFE, polytetrafluoroethylene; SDS, sodium dodecyl sulphate; SERS, surface-enhanced Raman scattering; SFE, supercritical fluid extraction; Si, silica gel; SI, sequential injection; STAC, stearyl trimethyl ammonium chloride; TBAH, tetrabutyl ammonium hydroxide; TBAP, tetrabutyl ammonium perchlorate; THF, tetrahydrofuran; TLC, thin layer chromatography; TMAC, tetramethyl ammonium chloride; TMS, trichloromethylsilane; UAD, ultraviolet absorption densitometry; UV/VIS, ultraviolet/visible spectrometry; XRFS, X-ray fluorescence spectrometry.

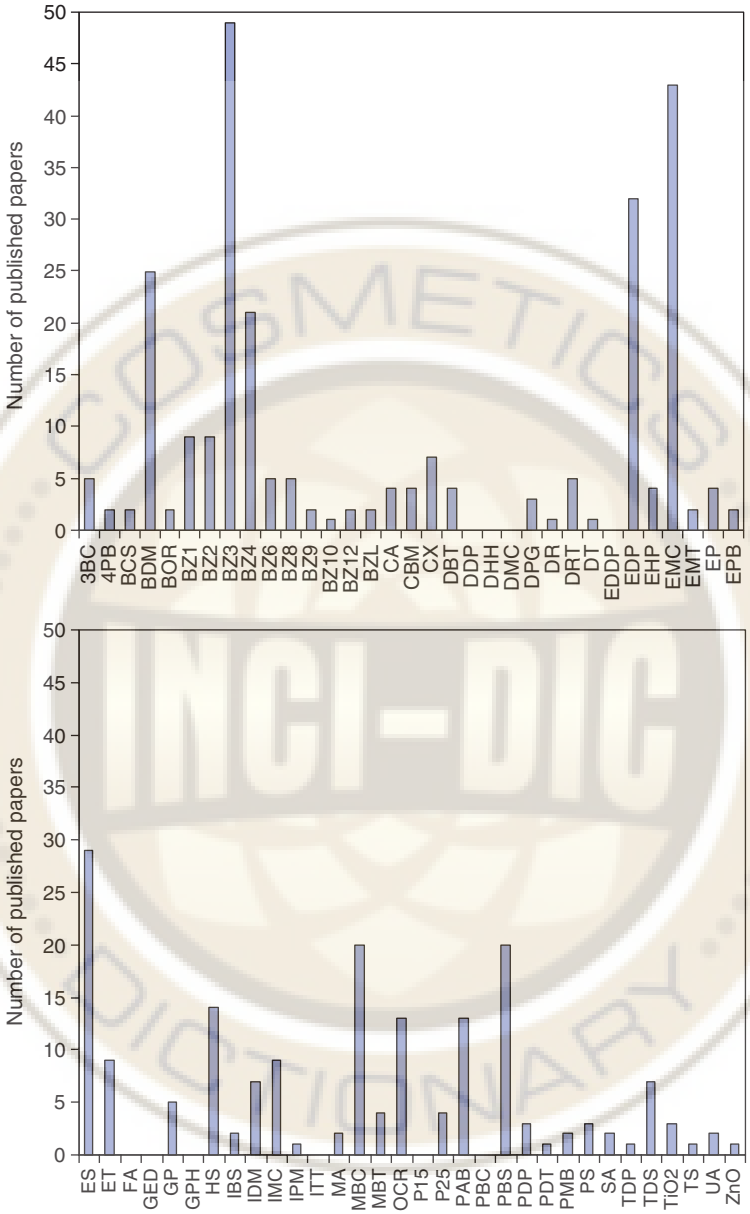


Figure 3.1.3 Published papers dealing with different UV filters.

filters which are commonly used worldwide, as is the case of BZ3 or EMC, is higher than those UV filters less used, or recently approved for its use in cosmetics. Figure 3.1.3 shows the number of published papers dealing with the determination of the different UV filters.

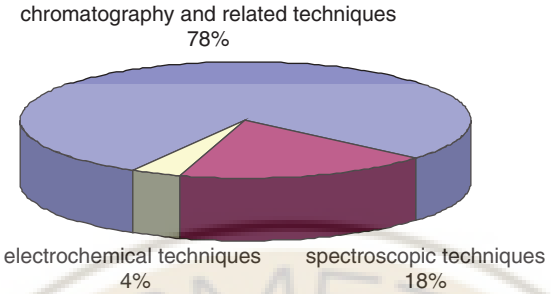


Figure 3.1.4 Percentage of publications grouped according to the analytical technique employed.

Analytical techniques employed for UV filters determination

As shown in Table 3.1.2, different analytical techniques are employed to determine UV filters, perhaps due to the different nature of the inorganic and organic UV filters, and also to the fact that the organic ones belong to different families, and thus have different physico-chemical properties. So, articles in which chromatographic, spectrometric and electrochemical techniques have been used can be found in the literature. In Figure 3.1.4, the percentage of publications is represented grouped according to the analytical technique employed.

As can be seen, the chromatographic techniques, such as thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), and other chromatography-related techniques, have been, by far, the most frequently employed techniques. This is because of the fact that there are more organic UV filters than inorganic ones, and also they are used more often than the latter. Moreover, they are usually combined in cosmetic formulations, and it is not an easy task to determine them by direct measurement without a previous separation step. Moreover, it is also necessary to take into account that matrix components might also interfere.

Next, we will discuss the different analytical techniques for UV filters determination in cosmetics in depth.

Chromatographic techniques

Among the chromatographic techniques, LC is the most commonly employed in the quantitative determination of UV filters, without doubt. The fact that LC can deal with low-volatile compounds makes it the technique of choice for UV filters determination, because most of these compounds have relatively high boiling points, especially the ionizable UV filters, and for this reason GC is less frequently used.

Nevertheless, as can be seen in Table 3.1.2, there are a few published papers in which GC has been employed. Derivatization with silylating reagents (Cumpelik, 1982; Ro *et al.*, 1994) can increase their volatility. Although the flame ionization detector (FID) has been the detector of choice for GC (Paulus, 1972; Schimtz-Masse *et al.*, 1979; Cumpelik, 1982; Hild, 1993), coupling GC with a mass spectrometry (MS) detector enables accurate on-line

identification of the UV filters in a cosmetic formulation (Ikeda *et al.*, 1990; Ro *et al.*, 1994; Masse *et al.*, 2001).

With regard to LC, the ultraviolet/visible (UV/VIS) spectrometry detector is the most commonly employed, because UV filters have significant absorbance in the UV range. The use of a diode-array detector (DAD) enables the whole UV spectrum to be obtained for each peak, and can be used for identification purposes (Rastogi and Jensen, 1998). Moreover, mathematical approaches have also been applied to quantify unresolved peaks, by measuring and processing the complete spectra using a DAD (Excoffier *et al.*, 1993; Fourneron, 2001).

In relation to the LC columns employed, the separations are usually carried out on reversed-phase columns using modified silica gel columns, like octadecylsilica (C_{18}) type. Octylsilica (C_8) is sometimes proposed (Tomasella *et al.*, 1991; Hild, 1993; Meijer and Loden, 1995), while other modified silica gel supports, such as phenylsilica (Ph) (Scalia, 2000) or cyanopropyl silica (CN) (Simeoni *et al.*, 2005) are rarely used. Determinations performed on normal-phase columns like silica gel (Si) are also very scarce (Eiden and Tittel; 1981; K nig, 1984).

Increasing the temperature of LC columns has proven to play a special role in the separation of some UV filters that co-elute at room temperature, since it affects the retention time of different compounds to a different extent (Salvador and Chisvert, 2005b).

Combinations of different solvents such as water, acetonitrile (MeCN), methanol (MeOH) and tetrahydrofuran (THF), either with isocratic or with gradient elution modes have been used for reversed-phase separations, while hexane, ethyl acetate (EtAc) and acetone are used when normal-phase mechanism is employed. It is worth mentioning that the authors of this section have published several methods in which mixtures of ethanol (EtOH) and water (or buffered solutions) are used as mobile phase (Chisvert *et al.*, 2001c; 2001d; Salvador *et al.*, 2001b; Chisvert and Salvador, 2002; Salvador and Chisvert, 2005b), thus avoiding the use of the aforementioned more hazardous organic solvents. Figure 3.1.5 shows an example in which 18 UV filters were determined.

As shown in Table 3.1.2, some authors have proposed adding different reagents to the mobile phase to reduce the peak tailing of some compounds, like for example acetic acid (AcOH) in the event of BZ3 (DiNunzio and Gadde, 1990), or ethylenediaminetetraacetic acid (EDTA) for BDM determination (Schakel *et al.*, 2004). Salts, such as citrate, phosphate and ammonium or sodium acetates, have often been used for buffering purposes. On the other hand, different authors have proposed the use of voluminous ions like tetramethylammonium chloride (TMAC) and/or sodium perchlorate (Gagliardi *et al.*, 1986, 1987, 1989; De Orsi *et al.*, 1995), or stearyltrimethylammonium chloride (STAC) (Ohba *et al.*, 1991) to establish an ion-pair partition process. Moreover, the use of cyclodextrines has helped to solve unresolved peaks, as performed by Chisvert *et al.* (2001d) who employed hydroxypropyl-β-cyclodextrine (HP-β-CD) as mobile-phase modifier.

The paper published by Hauri *et al.* (2004), who was the first to employ LC coupled to MS to determine UV filters, is worth mentioning. This method was also applied to the study of by-products coming to the photodegradation of the UV filters. However, MS conditions were optimized for cinnamates and camphor derivatives, and consequently salicylates (e.g. ES and HS) are not detected, and sulphonic acids like PBS and TDS give poor sensitivity.

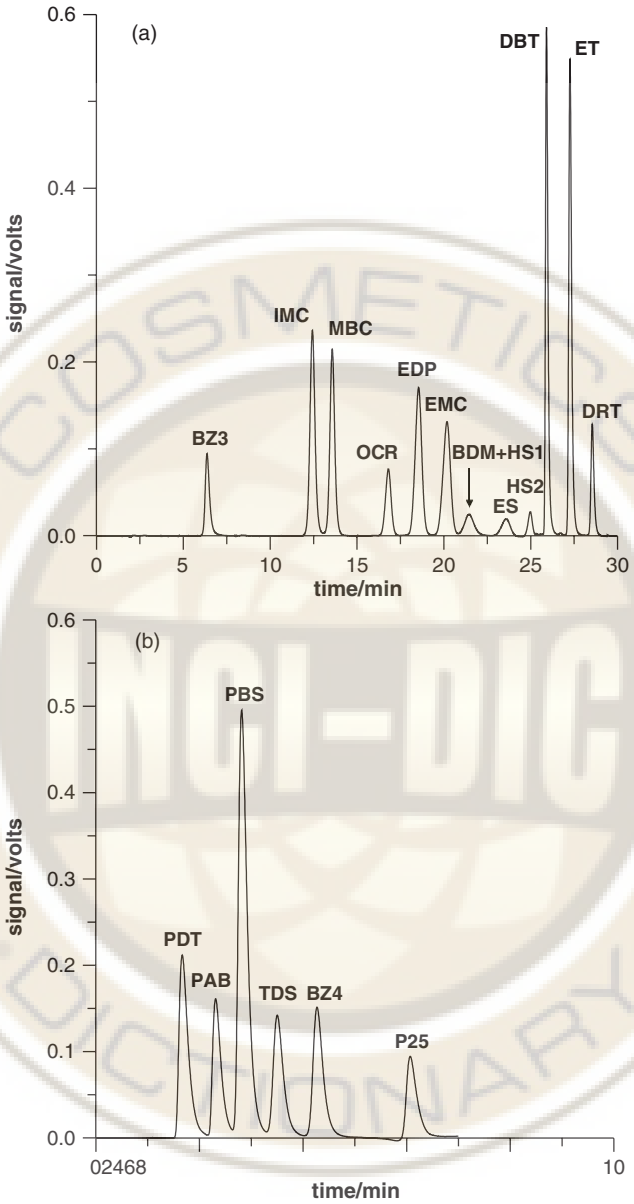


Figure 3.1.5 An example of LC chromatograms showing the separation of (a) 12 fat-soluble and (b) 6 water-soluble UV filters. Only environmentally friendly solvents were used (adapted from Salvador and Chisvert, 2005b).

Concerning TLC, this chromatographic technique has traditionally been employed for identification purposes, by scraping the spots from the plate and measuring off-line their UV, infrared (IR) or nuclear magnetic resonance (NMR) spectra (Eiden *et al.*, 1969; Eiden and Tecnzer, 1971, Schimtz-Masse *et al.*, 1979; Masse *et al.*, 1982, 2001; Masse and Herpol-Borremans, 1991; Hild, 1993). Quantification by gravimetry or UV spectrometry after scraping the spots has been also carried out (Eiden *et al.*, 1969; Liem and Hilderink, 1979). Moreover, several papers have been published, in which a particular UV filter has been determined on-line by employing a densitometric detector on the plate (Musial and Sherma, 1997, 1998; Westgate and Sherma, 2000a, 2000b, 2000c; Fisher and Sherma, 2000).

Finally, as shown in Table 3.1.2, other chromatography-related techniques, like micellar liquid chromatography (MLC) (Tomasella *et al.*, 1991) and electrophoretic techniques, such as capillary zone electrophoresis (CZE) (Wang and Lee, 2003), electrokinetic chromatography (EKC) (G mara *et al.*, 2005), micellar electrokinetic chromatography (MEKC) (Pietta *et al.*, 1995; Wang and Chen, 2000; Huang *et al.*, 2005b), microemulsion electrokinetic chromatography (MEEKC) (Klampf *et al.*, 2002; Klampf and Leitner, 2003; Huang *et al.*, 2005b) and capillary electrochromatography (CEC) (Huang *et al.*, 2005a) have also been employed for UV filters determination, but to a lesser extent.

Spectroscopic techniques

By contrast, this group of techniques have been used less often than chromatographic techniques, but they have been greatly used as detectors after the chromatographic separation of organic UV filters; the reason is that direct measurement is a difficult task due to the interference that each organic UV filter causes in the measurement of others, and also the interferences produced by matrix components, which make it necessary to perform a previous separation step. Nevertheless, different articles can be found in literature, in which different strategies are described for taking direct measurements. Strategies such as derivative ultraviolet spectrometry (DUVS) (Scurei and Oprea, 1996; Azevedo *et al.*, 1999; Chisvert *et al.*, 2001a), specific reactions forming (or consuming) coloured compounds easily measured in the visible range (Chisvert *et al.*, 2001b, 2002b; Salvador *et al.*, 2001a, 2003) or selective elution through microcolumns using solid phase extraction (SPE) strategies (Chisvert *et al.*, 2002a), have been used for UV/VIS spectrometry. Other spectrometric techniques, such as chemiluminescence (CL) (Townshend *et al.*, 2002), nuclear magnetic resonance (NMR) (Mori *et al.*, 1996) and surface-enhanced Raman scattering (SERS) (Narayanan *et al.*, 1991; Cheng *et al.*, 1997) have also been used for organic UV filters determination.

The numbers of papers focusing on the determination of inorganic UV filters is very scarce, perhaps due to the fact that only two compounds, TiO_2 and ZnO, are currently used as UV filters. Atomic spectroscopy techniques, such as atomic absorption spectromety (AAS) (Mason, 1980), inductive coupled plasma atomic emission spectrometry (ICP-AES) (Salvador *et al.*, 2000) and X-ray fluorescence spectrometry (XRFS) (Kawauchi *et al.*, 1996) have been used for titanium oxide determination, whereas to our knowledge zinc oxide has only been determined by AAS (Salvador *et al.*, 2000).

Using automated methods, such as flow injection (FI) (Chisvert *et al.*, 2001a, 2001b; Townshend *et al.*, 2002) or sequential injection (SI) (Chisvert *et al.*, 2001b, 2002a, 2002b; Salvador *et al.*, 2001a, 2003) manifolds, increase sample throughput.

Electrochemical techniques

To our knowledge, there are only three published papers dealing with the determination of UV filters in cosmetics products by means of electrochemical techniques. Differential-pulse voltammetry (DPV) using epoxy-carbon composite electrodes (Chang and Chang, 2001) or glassy carbon and mercury film electrodes (Wang, 2002) have been successfully applied to determine different UV filters in sunscreen products. Recently, Da Silva *et al.* (2006) proposed an analytical method based on differential-pulse polarography (DPP) in micellar media to determine EMC alone or in mixtures of EMC with MBC or with BZ3 in cosmetics.

Consideration on Sample Preparation

Sample preparation depends on different aspects, like type of sample, target analytes and analytical technique to be used. So, as mentioned previously, cosmetics, and particularly sunscreen cosmetics, can be very different in nature (creams, foundations, lipsticks, lotions, milks, oils, waters, etc.), and thus the sample preparation is expected to be different, since some of these preparations are difficult-to-solve (e.g. foundations and lipsticks). Moreover, the nature of the UV filters is very different, since there are inorganic (titanium and zinc oxides which are difficult-to-solve compounds) and organic UV filters, and also there are different families of organic ones. Indeed, within a specific family, UV filters have different moieties which change their physico-chemical properties considerably (e.g. BZ3 is a fat-soluble UV filter, whereas BZ4 is a water-soluble one), this obviously affects the sample preparation process. On the other hand, the analytical technique to be employed also plays a crucial role in sample preparation, since there are solvents or reagents that could be incompatible with the technique. Now we will go on to give a general overview of the published papers on UV filters determination in cosmetics from the sample preparation standpoint.

In general, sunscreen cosmetics do not require complex sample preparation methodologies, since solubilization of the most common preparations (i.e. creams, lotions, milks, waters or oils) is usually easy, by means of mixing them with the appropriate solvent, which also needs to solubilize the target analytes.

Nevertheless, although the determination of organic UV filters does not usually pose problems when they are going to be determined in easy-to-solve samples, due to the different properties of these chemicals, it is sometimes very difficult to propose an analytical methodology for the simultaneous determination of a large number of UV filters because it is difficult to find a single solvent (or mixtures of solvents) to be able to solve all water-soluble and fat-soluble UV filters which are also compatible with the technique to be used. Thus, analytical methods based on the separate determination of water-soluble and fat-soluble UV filters have been proposed, which enable a huge number of them to be determined (Eiden and Tittel, 1981; K nig, 1984; Wallner, 1993; Salvador and Chisvert, 2005b). This separate determination was seen in the example shown in Figure 3.1.5.

Sometimes, although complete solubilization of a sample is not possible, homogeneous slightly cloudy solutions are obtained because of the presence of few insoluble substances,

which can be removed by means of filtration or centrifugation. However, a different issue concerns difficult-to-solve samples, like lipsticks or foundations. In the case of a lipstick, the matrix is very fatty, and then it is not soluble enough in the usual solvents employed for reverse-phase LC (e.g. EtOH, MeOH, MeCN, THF, etc.). On the contrary, in the case of a foundation, it also contains metallic oxides and pigments in its formulation that are not easy-to-solve. Thus, as complete solubilization is not possible, leaching of analytes from the matrix is needed. On the other hand, leaching of target analytes could also be interesting in easy-to-solve samples to avoid interferences from the matrix. Sonication during different times, which can vary from the 5 min used by Dutra *et al.* (2002) to the 60 employed in two steps by Cheng *et al.* (1997), is the favourite technique for leaching UV filters from cosmetics, as can be seen in Table 3.1.2. Microwave-assisted extraction (MAE) (Shih and Cheng, 2000; Salvador *et al.*, 2001a) or supercritical fluid extraction (SFE) (Scalia, 2000; Wang and Chen, 2000; Salvador *et al.*, 2001b, 2005; Wang and Lee, 2003) have also been proposed. For SFE, which is less time consuming than the aforementioned extraction by ultrasounds, supercritical carbon dioxide is employed as an efficient extractant, although its extracting power, as well as extraction rate, can be increased by means of adding small amounts of other solvents, like 0.5% MeOH and 2% AcOH (Wang and Chen, 2000), 15% EtOH (Salvador *et al.*, 2001b) or 2.5% MeOH:10% aqueous phosphoric acid (1:1). Regarding MAE, it is claimed that it is faster than SFE, since it only requires a few minutes (or the order of 1 2 min) for irradiation, but one should bear in mind that after irradiation it needs to be left to reach ambient temperature, and this last step usually takes around 10 min. Finally, by sonication or MAE methodologies, the solution usually needs to be filtered or centrifuged to remove particles in suspension, which is not necessary in SFE because particles do not pass through the extraction cell.

Classical extraction techniques, such as liquid liquid extraction were used by Pietta *et al.* (1995) to avoid matrix interferences, and Gagliardi s research group (1987, 1989) proposed liquid liquid extraction to separate the target UV filters into two groups, avoiding the interferences that some UV filters caused on the others. Moreover, Wang (1999) employed MeOH as a liquid liquid extraction solvent to extract the target UV filters from chloroform, which was used to dissolve lipstick samples, to be injected into a reverse-phase LC system.

On the other hand, to determine inorganic UV filters, our group proposed a methodology (Salvador *et al.*, 2000) based on an acidic digestion in a microwave oven, and afterwards performed the fusion of the residue with $KHSO_4$ heating with a Bunsen flame, and dissolving the residue in concentrated sulphuric acid, for TiO_2 determination by ICP AES, and the use of surfactants to directly determine ZnO by AAS.

An additional sample preparation step was carried out by Cumpelik (1982) and Ro *et al.* (1994), who proposed a derivatization of the UV filters with different silylating agents to obtain more volatile compounds to be measured by GC.

SUMMARY

Everything discussed in this section points towards the need to perform analytical controls of the UV filters in cosmetic products to assure both sun protection and user safety.

There are positive lists for sunscreen agents in the three main legislations regarding cosmetic products, that is, those in force in EU, US and Japan (in the US they are considered as OTC drugs) where the maximum authorized contents are stipulated. However, despite this there are no official analytical methods.

There are almost 90 published papers proposing analytical methodologies for UV filter determination in cosmetic products, showing a wide interest in this subject.

An in-depth study of the proposed methods published in literature should be carried out, so as to select the most suitable methods to determine as many UV filters as possible in different cosmetic formulas, with the best analytical properties and highest safety for the operator and the environment. Such methods would be useful for production and quality control, and could be validated as official methods to determine UV filters.

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3.2. Monitoring and Quality Control of Sunscreen Photostability

S. Scalia*

Department of Pharmaceutical Sciences, Facoltà di Farmacia, University of Ferrara, Via Fossato di Mortara 17, 44100 Ferrara, Italy

THE PHOTOSTABILITY OF ORGANIC UV FILTERS

As mentioned previously in Section 3.1, as a consequence of the expanding knowledge about the harmful effects of solar UV rays, the use of topical sunscreen cosmetics has increased dramatically. The most common active constituents in sunscreen cosmetic products (about 80% of the total value of sunscreen ingredients) are organic chemicals, which attenuate the transmission of the solar UV rays to the skin by absorbing the radiation (Gasparro *et al*., 1998; Herzog *et al*., 2005), commonly referred to UV filters or sunscreen agents. The photoactivated sunscreen agent eventually dissipates the excitation energy in the form of heat, as light (fluorescence from the singlet state or phosphorescence from the triplet state) or by transferring it to the surrounding molecules. In addition, energy can be disposed off by chemical reactions that lead to degradation (e.g. fragmentation, cycloaddition, isomerization, adduct formation) of the UV filter (Schwack and Rudolph, 1995; Chatelain and Gabard, 2001; Bonda, 2005). The photoinduced decomposition of the sunscreen agent not only decreases the expected UV-protective power, but can also generate potentially harmful photolytic products (Tarras-Wahlberg *et al*., 1999; Chatelain and Gabard, 2001; Maier *et al*., 2001; Scalia *et al.*, 2002a). Consequently high photostability is an essential requirement for the efficacy and safety of UV filters. Thus, different published studies have demonstrated that some UV filters are unstable under sunlight exposure and, consequently, cannot maintain their initial absorptive capacity with the additional disadvantage of the safety concerns about photoproduct toxicology (Berset *et al*., 1996; Stokes and Diffey, 1999; Tarras-Wahlberg *et al.*, 1999; Scalia *et al*., 2002a). Thus, different strategies have been investigated to enhance the photostability of UV filters including sunscreen combinations, inclusion complexes with cyclodextrins, polymeric or lipid micro- and nano-particles (Alvarez-Roman *et al.*, 2001; Chatelain and Gabard, 2001; Scalia *et al.*, 2004; Iannuccelli *et al*., 2006). Therefore, evaluation of the photochemical stability of sunscreen preparations is of paramount importance for the development of UV absorbers and for the quality control of finished suncare products.

*Corresponding author. E-mail: sls@unife.it

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Table 3.2.1

Examples of the most commonly used components in the photostability tests

Irradiation source	Mercury lamp, fluorescent lamp, metal halide lamp, xenon arc
Sample support	Quartz cuvette, glass plate, Teflon membrane, Transpore™ tape, skin (reconstructed or excised), in vivo human volunteer s skin
Type of sample	Solution, liquid film, semisolid thin layer
Analytical technique	Absorption spectroscopy, transmission spectroscopy with integrating sphere, liquid chromatography, gas chromatography, supercritical fluid chromatography

PHOTOSTABILITY TESTING

The photostability of UV filters can be estimated by irradiating the sunscreen sample in the laboratory and by monitoring the photodegradation through different analytical techniques, such as spectroscopic or chromatographic techniques (Stokes and Diffey, 1999; Herzog *et al.*, 2005). Although the most common option is to perform the studies *in vitro* by using different sample supports, there are few papers in which the studies have been carried out *in vivo* on human volunteers. Such studies compare the amount of UV filters recovered from UV-irradiated and non-irradiated skin, using the tape-stripping technique (Marginean-Lazar *et al.*, 1997; Cambon *et al.*, 2001), which involves successive application and removal of adhesive tapes on the skin treated with the sunscreen product.

The basic components of the photostability test include the irradiation source, the sample support, the sample type and the analytical technique employed to evaluate the contents of the UV filter under study or byproducts formation. Table 3.2.1 shows different types of the aforementioned components, which are discussed below.

Irradiation sources

Although it is not practicable to use natural sunlight because of changing UV irradiance, there are a few studies that have used this natural irradiation source (Meijer and LodØn, 1995; Jiang *et al.*, 1996). Artificial sources of UV radiation designed to simulate the solar UV rays are usually employed, like those shown in Table 3.2.1. Moreover, a study by Stokes ad Diffey (1999) has shown that there is no significant difference in the photodegradation of sunscreen products exposed to a solar simulator or to natural sunlight.

Medium- and high-pressure mercury lamps have been used as an irradiation source by different authors (Roscher *et al.*, 1994; Broadbent *et al.*, 1996) because they are practical, but their spectrum lines do not mimic sunlight. Alternatively, metal halide bulbs (Maier *et al.*, 2001) or fluorescent lamps (Tarras-Wahlberg *et al.*, 1999; Diffey, 2005) have also been evaluated as laboratory sources, although the latter have been shown inappropriate as simulators of solar UV radiation (Brown *et al.*, 2000). Consequently, sunscreen photostability has been estimated almost exclusively with optically filtered xenon arc lamps as artificial sunlight sources (Schwack and Rudolf, 1995; Berset *et al.*, 1996; Serpone *et al.*, 2002). The lamp

Table 3.2.2

Photodegradation data for representative individual UV filters in solution or emulsion preparations

UV filter	Loss (%)
Benzophenone-3	>1% (Deflandre and Lang, 1988); 5 90% (Serpone *et al.*, 2002); 0 2% (Shaath *et al.*, 1990)
Butyl methoxydibenzoylmethane	36% (Deflandre and Lang, 1988); 14% (Schwack and Rudolph, 1995); 24 73% (Berset *et al.*, 1996); 56 70% (Chatelain and Gabard, 2001); 58 68% (Scalia *et al.*, 2002a)
Ethylhexyl methoxycinnamate	4.5% (Deflandre and Lang, 1988); 10 29% (Berset *et al.*, 1996); 65% (Chatelain and Gabard, 2001); 40 90% (Serpone *et al.*, 2002); 30 35% (Scalia *et al.*, 2002b); 19 39% (Shaath *et al.*, 1990)
Ethylhexyl dimethyl PABA	15.5% (Deflandre and Lang, 1988); 4 31% (Shaath *et al.*, 1990); 18 78 % (Berset *et al.*, 1996); 33 55% (Scalia *et al.*, 1999); 15 97% (Serpone *et al.*, 2002)
Phenylbenzimidazole sulphonic acid	<1% (Deflandre and Lang, 1988); 0 9% (Berset *et al.*, 1996); 50 90% (Serpone *et al.*, 2002); 7 11% (Scalia *et al.*, 2004)

output is filtered through optical filters to cut-out wavelengths shorter than 290 nm, corresponding to the portion of the sunlight spectrum screened out by the Earth s ozone layer, and by an infrared (IR)-blocking filter to avoid thermal effects. Consequently, this combination of lamp and filters produces a spectrum, which provides the best available copy of the spectral distribution of sunlight and, for this reason, is referred to as a solar simulator. Various models of such solar simulators are available with input power in the range 150 2500 W.

Moreover, to allow consistent radiation exposure of samples and the comparison of the results from different laboratories, spectroradiometers are used to measure and control the solar simulator irradiance output. However, accurate spectroradiometry requires careful attention to several parameters, such as wavelength calibration, calibration sources, linearity of spectroradiometer s response and stability (Saunders and Murthy, 1998). Besides these conditions, the technical differences in laboratory equipments (e.g. output of the light sources) together with the variability in irradiance values (Stokes and Diffey, 1999; Chatelain and Gabard, 2001) and test conditions (e.g. vehicle) represent one of the main causes for the observed dispersion of the results (Berset *et al.*, 1996; Gers-Barlag *et al.*, 2001) reported on light-induced degradation of sunscreen agents (Table 3.2.2).

On the other hand, to better simulate the conditions in which sunscreens are used in practice, the UV energy delivered to the sunscreen samples is often expressed in terms of minimal erythemal dose (MED) units, that is, the amount of solar UV radiation that will elicit a slight reddening (erythema) in the average person with light skin (Bonda, 2005). Samples exposed to the solar simulator are subjected to doses of radiant energy in the range 10 20 MED (where 1 MED contributes to 250 J/m^2), since these values are representative of daily solar emission close to the equator, and a sunscreen stable under these conditions will give sufficient protection (Schrader *et al.*, 1994; Berset *et al.*, 1996; Tarras-Wahlberg *et al.*, 1999; Chatelain and Gabard, 2001; Herzog *et al.*, 2002; Scalia *et al.*, 2002b).

Supports and types of samples

Photostability studies have been performed in diluted solutions of pure UV filters, in semi-solid formulations (i.e. emulsions) prepared in the laboratory and in commercial products (e.g. creams, gels, sticks and oils). It should be noted that the type of sample markedly influences the UV filter photodecomposition (Schwack and Rudolph, 1995; Berset *et al.*, 1996; Scalia *et al.*, 1999; Serpone *et al.*, 2002).

Sunscreen solutions of individual UV filters are transferred into quartz cuvettes (Schrader *et al.*, 1994; Schwack and Rudolph, 1995; Broadbent *et al.*, 1996; Scalia *et al.*, 1999; Vanquerp *et al.*, 1999), sandwiched between two quartz plates (Tarras-Wahlberg *et al.*, 1999) or spread on a glass surface (Berset *et al.*, 1996) and exposed to the solar simulator. Although these studies on individual UV filters in diluted solutions provide valuable information on their photochemistry (Roscher *et al.*, 1994; Schrader *et al.*, 1994; Schwack and Rudolph, 1995; Broadbent *et al.*, 1996; Scalia *et al.*, 1999; Tarras-Wahlberg *et al.*, 1999; Serpone *et al.*, 2002), they are not representative of the conditions in which sunscreen agents are used in practice, where they are always combined with other UV filters and numerous excipients. Surprisingly, the only standardized protocol to evaluate sunscreen photostability has been proposed and validated using UV filter solutions in cosmetically acceptable solvents (e.g. ethanol, glycerin, isopropylmyristate). This method requires sunscreens to be dissolved at their maximum authorized concentration in a mixture of volatile and non-volatile solvents (e.g. ethanol/isopropylmyristate, ethanol/glycerin) and applied as a liquid film on a glass surface. This system is considered to be a simple *in vitro* model simulating real conditions (Berset *et al.*, 1996).

Therefore, there are no official guidelines on photostability testing in finished products. This is a disadvantage, since the photochemical behaviour of sunscreen agents would be better determined under conditions that parallel those encountered in the real use of suncare formulations, namely in the finished products. Accordingly, the majority of research into the photolysis of UV filters has been performed on model emulsions (which represent by far the most common type of sunscreen preparation), although others have been carried out on commercial products. Generally, the emulsions are directly exposed to the solar simulator, although their dispersion in a solvent prior to irradiation has also been reported (Serpone *et al.*, 2002). In any event, the formulation is spread onto UV-transparent supports, such as roughened quartz plates (Deflandre and Lang, 1988; Diffey *et al.*, 1997; Chatelain and Gabard, 2001; Maier *et al.*, 2001; Herzog *et al.*, 2002), Transpore^{TM} tapes (a surgical tape fairly transparent to UV and able to simulate the texture of human skin) (Gers-Barlag *et al.*, 2001) or teflon membranes (Sayre and Dowdy, 1999). Alternatively, glass plates (Scalia *et al.*, 2004; Simeoni *et al.*, 2005; Gaspar and Maia Campos, 2006), reconstructed or excised human epidermis (Marginean-Lazar *et al.*, 1997; Stokes and Diffey, 1999) and *in vivo* human volunteer s skin (Marginean-Lazar *et al.*, 1997; Cambon *et al.*, 2001) have been used, as summarized in Table 3.2.1. The latter substrate has the advantage of approaching the *in vivo* conditions, making it possible to evaluate the photochemical behaviour of UV filters under their current conditions of use, which take into account the skin sunscreen interactions.

As no standardized protocol is available for photostability studies in finished products, the sunscreen amount indicated in the official guidelines for Sun Protection Factor (SPF)

determination is generally used for the stability tests. Thus, different amounts of products ranging from 0.75 to 2 mg/cm^2 (Stokes and Diffey, 1999; Chatelain and Gabard, 2001; Gers-Barlag *et al.*, 2001; Maier *et al.*, 2001, 2005; Herzog *et al.*, 2002) are distributed as uniformly as possible in a layer on the support. Then they are irradiated with simulated sunlight (DIN, 1985; COLIPA, 1994).

Protected samples (e.g. wrapped in aluminium foil) placed alongside the test samples, are used as dark controls to evaluate the possible contribution of temperature and the formulation/substrate interaction to the total observed changes (Stokes and Diffey, 1999; Chatelain and Gabard, 2001; Herzog *et al.*, 2002).

Sample pretreatment and analysis

At the end of the exposure period, the extent of decomposition has usually been assessed either by UV spectroscopy (Deflandre and Lang, 1988; Stokes and Diffey, 1999) or by chromatographic techniques (Roscher *et al.*, 1994; Scalia *et al.*, 1999; Vanquerp *et al.*, 1999).

In the case of the former technique, the spectral transmission or absorbance of the sunscreen sample is measured prior to and after exposure to the solar simulator and the results are expressed in terms of absorbance loss (Marginean-Lazar *et al.*, 1997; Maier *et al.*, 2001). In addition, changes in parameters derived from the spectra, such as the SPF (Stokes and Diffey, 1999), the critical wavelength (the wavelength at which 90% of the area under the 290 400 nm absorbance curve is reached) and the UV-A/UV-B ratio (the ratio of the mean absorbances in the UV-A (320 400 nm) and UV-B (290 320 nm) ranges) have also been considered (Chatelain and Gabard, 2001; Gers-Barlag *et al.*, 2001; Herzog *et al.*, 2002). This approach has the advantage of minimum sample manipulation because it simply involves applying the product on the support and determining the absorption spectra directly using spectrophotometers, which can be equipped with a light-collecting integration sphere for light-diffusing samples. However, the accuracy of this procedure can be affected by artifacts such as UV-absorbing photoproducts (Deflandre and Lang, 1988; Stokes and Diffey, 1999) and, therefore, should be paralleled by a separation technique, such as chromatography.

Alternatively, following irradiation, sunscreen solutions are directly subjected to analysis, whereas the emulsion samples are dispersed from the support in a suitable solvent (e.g. methanol, ethanol, dioxane) under mixing or sonication. The remaining UV filter concentration is quantified by liquid chromatography (LC) (Meijer and LodØn, 1995; Schwack and Rudolph, 1995; Berset *et al.*, 1996; Jiang *et al.*, 1996; Vanquerp *et al.*, 1999; Chatelain and Gabard, 2001; Scalia *et al.*, 2002b; Simeoni *et al.*, 2005; Gaspar and Maia Campos, 2006), by gas chromatography (GC) coupled to mass spectrometry (MS) detector (Schwack and Rudolph, 1995; Scalia *et al.*, 1999; Tarras-Wahlberg *et al.*, 1999), by supercritical fluid chromatography (SFC) coupled to MS detector (Broadbent *et al.*, 1996), or by LC coupled to MS detector (Hauri *et al.*, 2004).

The analytical method should be suitably validated and capable of resolving and detecting photolytic degradants. The analysis of the exposed product is performed concomitantly with that of any protected samples used as dark controls. The degree of photodegradation is obtained by determining the percentage of recovered sunscreen agent with respect to non-exposed samples.

The extent of sunscreen degradation is evaluated to assess whether or not acceptable changes have occurred at the end of the simulated sunlight exposure test. A product is considered photostable when the sunscreen agent loss after irradiation is <10%. (Herzog *et al.*, 2005).

It should be mentioned that, at first, most of the analytical methods described previously in Section 3.1 would be valid to perform photostability studies, provided that the forming byproducts do not interfere in the determination of the parent UV filters.

SUMMARY

The extensive use of sunscreens, which are incorporated in a multitude of skin products used in our daily lives, calls for additional, more effective testing procedures to ensure the safety and efficacy of UV filters at usage levels. The interest in sunscreen photostability has grown in the past years leading to a greater appreciation among major sunscreen manufacturers of the importance to assess the photochemical behaviour of UV filters for *in vitro* evaluation of their protection efficacy (Diffey *et al.*, 1997; Maier *et al.*, 2001). Standardized photostability tests of finished suncare products, which have been recommended by several researchers (Berset *et al.*, 1996; Sayre and Dowdy, 1999; Vanquerp *et al.*, 1999; Maier *et al.*, 2001), should become a general pre-marketing requirement.

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3.3. Tanning and Whitening Agents in Cosmetics. Regulatory Aspects and Analytical Methods

A. Chisvert[1*], A. Balaguer[2] and A. Salvador[2]

[1]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Ctra. San Vicente del Raspeig s/n, 03690 San Vicente del Raspeig, Alicante, Spain
[2]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain

TANNING COSMETIC PRODUCTS

Tanning products are cosmetic products closely related to sunscreen products described in Section 3.1, although their functions are not the same as the reader will realize further on.

Firstly, it is necessary to differentiate between sunless tanning products, which are products topically applied to skin producing a darkening effect without sun, and tanning accelerators, which are products that increase tanning when sunbathing. The former are becoming more popular because people can have tanned-looking skin without the harmful effects that solar radiation can cause, like those described in Section 3.1. They are considered as cosmetics by the European Union (EU) Cosmetics Directive (Council Directive 76/768/EEC), by the United States (US) Food and Drug Administration (FDA) and in Japan (see Section 1.1). Products intended to use for treating dermal diseases (e.g. vitiligo, psoriasis, etc.), even containing any of the tanning agents discussed in this section, will not be considered, since they fall outside the scope of this book, which focuses on cosmetic products.

These products are usually formulated as lotions or creams, which are spread on user s skin, producing a darkening effect. Moreover, in the event of sunless tanning cosmetics, there are sunless tanning booths where sunless tanning products are sprayed homogeneously onto all parts of the user s body.

As opposed to sunscreen products, there are no positive lists for active ingredients contained in tanning products. Nevertheless, the most popular active ingredient in sunless tanning cosmetics is dihydroxyacetone (DHA). DHA is considered as a general cosmetic ingredient in the EU and Japan frameworks, with tanning properties, whereas in US it is considered as a colour additive exempt from certification, and in fact, it is the only colour additive currently authorized by the FDA for use as a tanning agent (FDA, 2003).

*Corresponding author. Email: alberto.chisvert@ua.es

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DHA interacts with basic aminoacids found in the stratum corneum to form brown black compounds called melanoidins by means of Maillard reaction (Fu *et al.*, 2004). Since DHA does not promote the synthesis of melanin (i.e. the natural pigment responsible for pigmentation in human skin), the most important thing to bear in mind when using this type of cosmetic is that they do not provide an effective protection against the sun. Nevertheless, recent studies have revealed that DHA provides low protection against ultraviolet A (UVA) radiation, whereas it does not provide any protection against ultraviolet B (UVB) radiation (Monfrecola and Prizio, 2001). The tan provided by this type of product disappears a few days after applying the product. Dermatological side-effects from use of these products are infrequent, but some isolated cases of contact dermatitis have been reported (Morren *et al.*, 1991). Moreover, high DHA concentrations have been found to produce an uneven, streaky and unnatural yellow/orange tan and a strong drying out the skin that can be reduced or even eliminated by avoiding the use of DHA-based products and using erythrulose-based products (Jermann *et al.*, 2002). Erythrulose is a very similar compound to DHA that works in the same way. Similarly, other hydroxyaldehyde compounds have been proposed as an alternative to DHA, but to our knowledge, they are under different patents. The topical application of melanins has also been shown effective as sunless tanning agent (Pawelek, 1998).

There are also water-soluble dyes, which work by temporarily staining the skin. These products are essentially a form of make-up, since the tint only lasts until it is washed off. The tanned effect that they produce can easily be removed with soap and water (Fu *et al.*, 2004).

On the other hand, tanning accelerators usually contain tyrosine or tyrosine derivatives, which can affect the natural process of melanogenesis, that is, the process by which the natural pigment melanin is synthesized by specialized cells called melanocytes. By contrast to DHA-based products, tyrosine-based products need the presence of sun. Tyrosine participates in the melanogenesis by working as a substrate of the enzyme tyrosinase, which converts tyrosine to L-dopa, and then to dopaquinone (Brown, 2001), prior to transformation to melanin, which is responsible for tanning. It is assumed that topical-applied tyrosine can penetrate the skin, diffuse through body tissues, enter melanocytes, thus increase the substrate for tyrosinase and then increase production of melanin. FDA considers tyrosine as an unapproved drug (FDA, 2003).

Bergapten (5-methoxypsoralen) is a photosensitizing furocoumarine found in bergamot oil. Nowadays, EU legislation prohibits more than 1 mg/kg in cosmetic products, but in the past it was used as a tanning accelerator, since it increased skin sensitivity to UV light and thus stimulated melanocytes to produce melanin; however, it also intensified erythema formation and skin cancer (Ashwood-Smith *et al.*, 1980; Autier *et al.*, 1997).

The above-mentioned tanning agents and other compounds under development, such as dimethylsulfoxide, lysosomotropic agents (e.g. ammonium chloride), diacylglycerols, thymidine dinucleotides, DNA fragments, melanocyte-stimulating hormone analogs, 3-isobutyl-1-methylxanthine, nitric oxide donors and bicyclic monoterpene diols, all of which are attributed tanning accelerator properties, have been reviewed by Brown (2001) in an interesting article.

There are other ways of self-tanning, like for example UV-radiation booths, or tanning pills, which obviously fall outside the scope of this book and will not be reviewed here.

Determination of tanning agents

There are no official analytical methods for the determination of any of the aforementioned tanning agents. A bibliographic search updated to June 2006, using analytical chemistry databases, revealed that very few publications focused on the determination of tanning agents in cosmetic products, and they were published many years ago, which shows the low level of interest in this type of product nowadays, presumably due to the fact that most of the tanning agent used (i.e. DHA) do not cause severe safety problems, whereas the other tanning agents have either become obsolete or are under development. Regardless of this assumption, we think that the development of analytical methods to perform quality control of these products with a view to assuring their efficacy is necessary.

We will now provide an overview of the scarce bibliography on this topic; however it should be mentioned that most of these papers are not easily accessible, thus the review is made on the basis of their respective abstracts.

To our knowledge, the first published paper focusing on DHA determination in cosmetics dates from 1962 (Pollak and Lorant, 1962), where DHA was reacted with hydroxylamine hydrochloride, and the subsequent acid liberation was titrated. Almost 20 years later, a paper was published where DHA was determined by liquid chromatography (LC) with an ultraviolet/visible (UV/VIS) spectrometry detector (Baruffini *et al.*, 1981). Later, two other papers were published on LC determination of DHA. On the one hand, Galensa and Schuster (1985) proposed both normal-phase and reversed-phase LC methodologies, where DHA was previously derivatized by means of benzoylation. On the other hand, Ferioli *et al.* (1995) also derivatized DHA but by means of its bis-2,4-dinitrophenylhydrazone derivative, to carry out its determination by reversed-phase LC. Gas chromatography (GC) has been also applied to DHA determination. So, on the one hand Oberleithner and Wolff (1981), Matissek and Harper (1984) and Hild (1993) proposed GC methodologies where DHA was previously derivatized by means of an acetylation process carried out with acetic anhydride, to increase its volatility. On the other hand, other authors also proposed derivatization strategies, but by using benzoylating (Galensa and Schuster, 1985) or silylating (Cumpelik, 1982) reactions.

With regard to bergapten, most of the published papers deal with its determination by means of LC (Quercia *et al.* 1979; Bettero and Benassi, 1981, 1983; Verger, 1983), although GC has also been applied (Blaas *et al.*, 1985).

To our knowledge, there are no other published papers focusing on the determination of other tanning agents in cosmetic products.

WHITENING COSMETIC PRODUCTS

There is other type of product closely related to sunscreen products and to the aforementioned tanning products, but their function is completely contrary to the latter. Whitening products, also called skin-bleaching products, are commercial preparations containing chemicals that produce a whitening effect on the skin, by interfering in the biosynthesis of melanin by different mechanisms (Cabanes *et al.*, 1994; Briganti *et al.* 2003; Petit and PiØrard, 2003). In the EU, they are considered as cosmetics, whereas in US they are

considered as over-the-counter (OTC) drugs, and in Japan they are considered as quasi-drugs (see Section 1.1). These products are very popular in oriental countries, where people consider light skin as beautiful, as opposed to westerners, who prefer tanned skin.

Usually, skin-bleaching products may also contain peeling chemicals, such as α-hydroxyacids (glycolic, lactic or malic acids) or β-hydroxyacids (salicylic acid) to improve their effectiveness, since these chemicals remove the dead skin cells, making the task of the whitening agents easier (Ghadishah and Gorchynski, 2002; Bernett and Herderson, 2003; Monheit, 2004). Also sunscreen agents are added to protect users skin from sunlight to avoid tanning (Piamphongsant, 1998).

As in tanning products, there are no positive lists for the active ingredients they contain. Nevertheless, different chemicals with antioxidative properties have been attributed to cause a skin-bleaching phenomenon and they can be read about elsewhere. It is also worth mentioning that some of the whitening agents listed below may also have other functions than whitening the skin, such as antioxidant, emollient, chelating and/or buffering properties.

So, the most popular compounds used as whitening agents are arbutin (ARB), ascorbic acid (AA), azelaic acid (AZA), hydroquinone (HQ) (and its monomethyl (HQMM), monoethyl (HQME) and monobenzyl (HQMB) ethers), kojic acid (KA), phytic acid (PA) and retinoic acid (RA) among others (see Figure 3.3.1). Owing to its labile oxidative properties, KA is recently added to cosmetics by means of its dipalmitic ester, that is, as kojic dipalmitate (KDP). The same happens with AA, for which it is usual to find as ascorbyl palmitate (AP), ascorbyl dipalmitate (ADP), ascorbyl stearate (AS), magnesium (or sodium) ascorbyl phosphate (MAP, SAP), and more recently as ascorbyl glucoside (AG). Moreover, it should be mentioned that these derivatives change the solubility properties of the parent compound, which could be interesting in order to formulate new preparations. Plant extracts, like *Arctostaphylos uva ursi* and *Arbutus unedo* extracts that contain ARB have also been used. Mercury-containing cosmetic preparations have been used for years as skin-bleaching agents in US, but nowadays mercury is no longer permitted as such, because of the known hazards and its questionable efficacy as a skin-bleaching agent. Nor does the EU allow the use of mercury compounds for this purpose.

Special attention has to be paid to HQ, since it is the only FDA approved chemical in skin-bleaching OTC products (at concentration ranging from 1.5 to 2%) according to the

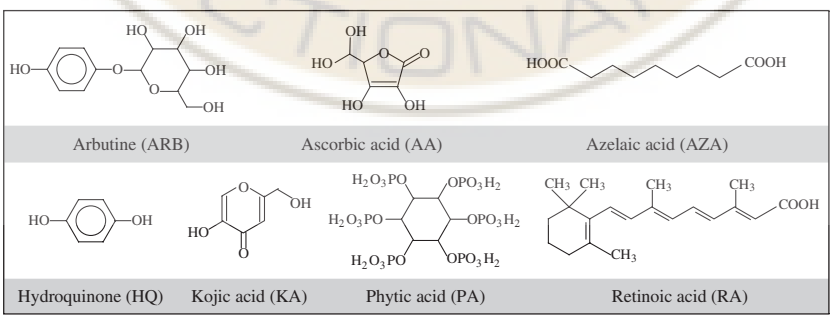

Figure 3.3.1 Some of the most commonly whitening agents used in cosmetics.

Tentative Final Monograph published for skin-bleaching products (FR, 1982), whereas this compound is not allowed in the EU framework as a bleaching agent because of its harmful side-effects (see further on) since the 24th adaptation (Commission Directive 2000/6/EC) of the EU Cosmetics Directive came into effect. Since then, under the EU Cosmetics Directive, it is allowed to be used only as hair-dying agent or in artificial nail systems under strict conditions of use. However, it may be used under medical prescription for treatment of skin spots, where these hydroquinone-based products are considered as pharmaceuticals instead of cosmetics. HQMM is also allowed by the EU Cosmetics Directive to be used in artificial nail systems under strict conditions of use, whereas HQME and HQMB are forbidden in cosmetic products. In Japan, the use of HQMB is also forbidden for cosmetic use (MHW, 2000).

RA is not allowed in the EU framework either.

Many dermatological side-effects and carcinogenic properties have been attributed to the use of HQ (Joseph *et al.*, 1998; Do Ceu Silva *et al.*, 2003; Gaskell *et al.*, 2005; Li *et al.*, 2006). Dermatitis has also been attributed to topical application of RA (Nordqvist and Mehr, 1977; Tosti *et al.*, 1992). KA has also been found to cause certain allergenic properties (Nakagawa *et al.*, 1995), and AZA as well (FernÆndez, 2000), although to a lesser extent than the aforementioned cases.

Those products intended to treat skin diseases such as brown spots called chloasma or melasma are excluded from the present book, although these products usually contain the same whitening agents at a higher concentration, since the aim of this book is to deal with cosmetic products.

Determination of whitening agents

Analytical methods to assure the compliance with different regulations are necessary. To our knowledge, there is only one official analytical method for controlling whitening agents in cosmetics in the EU framework (Commission Directive 95/32/EC). This method was compiled later by the European Commission in a compilation book (European Commission, 1999) and was validated by Borremans *et al.* (1999). The aforementioned method focuses on the determination of HQ and its methyl, ethyl and benzyl ethers (see Section 2.1). The method is based on their identification by means of thin-layer chromatography (TLC), followed by their quantitative determination using LC with UV/VIS detection, where the sample is extracted with a water/methanol mixture under heating. However, if a sample contains parabens, results obtained using this method could be inaccurate because of the interference that these compounds could cause. Nevertheless, according to the United States Pharmacopeia (2006), HQ can be determined by means of titration with cerium sulphate using diphenylamine as an indicator.

No other official methods have been published in either the EU framework or US and Japan frameworks. Thus, it is obvious that there is a need to develop analytical methods for controlling whitening agents in cosmetic products (or in OTC products or quasi-drugs, as are called in US and Japan, respectively). A bibliographic search updated to June 2006, using analytical chemistry databases, revealed almost 30 publications focusing on the analysis of these types of products. Table 3.3.1 shows a chronological summary of these

Table 3.3.1

Published papers until June 2006 on whitening agent s determination in cosmetic products (chronological order).

Authors	Target whitening agents[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
Herpol-Borremans and Masse (1986)	HQ, HQMB, HQME, HQMM			LC-UV/VIS, C_{18} column at 36 C with isocratic MeOH:H_2O as mobile phase
Firth and Rix (1986)	HQ	Creams		LC-UV/VIS, C_{18} column with isocratic MeOH:H_2O as mobile phase
Gagliardi *et al.* (1987)	HQ, HQDM, HQMB, HQMM, HQMP, HQMPh	Creams	Sample is dissolved in MeOH, heated at 50 C and centrifuged	LC-UV/VIS, C_{18} column with isocratic MeCN:H_2O as mobile phase
Teglia (1989)	ARB, HQ			LC-UV/VIS, C_{18} column
Luckewicz and Saccaro (1990)	ADP	Powders		DSC
Sakodiskaya *et al.* (1992)	HQ, HQDM, HQMB, HQMM, HQMP, HQMPh	Creams	Sample is mixed with MeOH:water mixture and vortexed by heating. Finally, filtered	MEKC
Lien *et al.* (1993)	ADP	Powders	Sample is dissolved in EtOH	LC-UV/VIS, C_{18} column with isocratic MeOH:40% acetic acid as mobile phase
Gatti *et al.* (1995)	AZA	Lotions and powders	Sample is dissolved in MeOH, centrifuged and filtered if necessary, and further diluted with H_2O. An aliquot is derivatized with 2-bromoacetyl-6-methoxynaphthalene	LC-FL, C_{18} column at 35 C with isocratic MeCN:MeOH:THF:H_2O as mobile phase
Semenzato *et al.* (1995)	MAP	Creams	Sample is diluted with THF:0.3 M phosphate buffer pH 4 mixture. After homogenization, dilutions are made with phosphate buffer	LC-UV/VIS, NH_2 column with MeCN:0.3 M phosphate buffer pH 4 as mobile phase

(*Continued*)

Table 3.3.1 (Cont.)

Authors	Target whitening agents[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
Wang (1995)	HQ, HQDM, HQMB	Creams		DPV, using carbon paste electrode
Scalia *et al.* (1997)	AZA		Sample is submitted to SPE with SAX column	SPE+LC-UV/VIS, C_{18} column with MeCN:phosphate buffer as mobile phase
Sottofattori *et al.* (1998)	AP, MAP (different preservatives were also determined)	Creams	Sample is mixed with THF:0.025 M phosphate buffer pH 3.5 mixture until a homogeneous suspension is obtained	LC-UV/VIS, CN column with gradient MeOH:0.025 M phosphate buffer pH 3.5 as mobile phase
Shih and Zen (1999)	KA	Creams and lotions	Creams are dissolved in water and filtered, next an aliquot is mixed with buffer pH 10. Lotions are directly mixed with buffer	DPV, using screen-printed carbon electrode
Shih and Zen (2000)	ARB	Creams	Cream sample is dissolved in water, and diluted with ammonium buffer pH 10	DPV, using clay-coated screen-printed electrode
Desiderio *et al.* (2000)	HQ, HQDM, HQMB, HQMM, HQMP, HQMPh	Creams	Sample is mixed with MeOH:water mixture and vortexed by heating. Finally, filtered	CEC-DAD, C_{18} capillary column at 25 C and MeCN:20 mM ammonium acetate buffer pH 6 as running buffer
Vieira and Fatibello-Filho (2000)	HQ	Creams	Sample is dissolved in MeOH. An aliquot is transferred to measurement cell	CV, sweet-potato tissue modified paraffin/graphite electrode in MeOH:phosphate buffer pH 7 containing 0.1 M TBAB and 1 mM H_2O_2

Fatibello-Filho and Vieira (2000)	HQ	Creams	Sample is dissolved in MeOH. An aliquot is transferred to measurement cell	CV, sweet potato tissue modified stearic acid/graphite electrode in MeOH containing H_2O_2
Masse *et al.* (2001)	ARB, KA	Creams	Sample is dispersed in MeCN, sonicated and centrifuged (or filtered)	LC-UV/VIS, Diol column with MeCN:0.05 M phosphate buffer pH 2.5 as mobile phase (TLC used for identification purposes)
Shih (2001)	KA, MAP	Creams and lotions	Sample is extracted with water and filtered	LC-DAD, C_{18} microbore column with 0.5 mM TBAB and 50 mM phosphate buffer pH 5 containing 5% MeOH as eluent
Zhang *et al.* (2002)	HQ		Samples are mixed with EtOH and sonicated	GC-MS
Chang and Chang (2003)	AA, ARB, MAP	Creams, gels and lotions	Sample is mixed with water and sonicated at 25 C. Then it is filtered and deoxygenated by N_2	LC-UV/VIS, C_{18} column with 5 mM phosphate buffer pH 2.5 containing 10 mM TBAH and 10% MeOH as mobile phase
Rueda *et al.* (2003)	HQ	Creams		FI-AMP, graphite electrode
Huang *et al.* (2004)	AG, ARB, HQ, KA, MAP		Sample is extracted with 0.05 M phosphate buffer pH 2.5	LC-UV/VIS, C_{18} column with MeOH:0.05 M phosphate buffer pH 2.5 as mobile phase
Liu (2004)	ARB, KA, MAP			LC
Xie *et al.* (2005)	KA		Sample is extracted with 40% aq. MeOH by sonication	LC-UV/VIS, C_{18} column with MeOH:0.01 M phosphate buffer containing 1 mM TBAB as mobile phase
Lin *et al.* (2005)	ARB	Creams and lotions	Sample is diluted with water. After mixing, it is submitted to dialysis vial and injected on-line	MD-LC-UV/VIS, PFP column with MeOH:0.02 M phosphate buffer pH 5.5 as mobile phase

(*Continued*)

Table 3.3.1 (Cont.)

Authors	Target whitening agents[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
L pez-Garc a *et al.* (2005)	HQ	Creams and gels	Method 1: Sample is dissolved in mobile phase Method 2: Sample is dissolved in 0.05 M H_2SO_4	Method 1: LC-UV/VIS, C_{18} column at 25 C with MeOH:H_2O as mobile phase Method 2: DUVS using 1st derivative spectra

[a]Key abbreviation: AA, ascorbic acid; ADP, ascorbyl dipalmitate; AG, ascorbyl glucoside; AP, ascorbyl palmitate; ARB, arbutin; AZA, azelaic acid; HQ, hydroquinone; HQDM, hydroquinone dimethyl ether; HQMB, hydroquinone monobenzyl ether; HQME, hydroquinone monoethyl ether; HQMM, hydroquinone monomethyl ether; HQMP, hydroquinone monopropyl ether; HQMPh, hydroquinone monophenyl ether; KA, kojic acid; MAP, magnesium ascorbyl phosphate.

[b]Symbol − means coupling between techniques, and symbol + means sequentially applied techniques. Key abbreviation: AMP, amperometry; C_{18}, octadecylsilica; CEC, capillary electrochromatography; CN, cyanopropyl silica; CV, cyclic voltammetry; DAD, diode-array detector; DPV, differential-pulse voltammetry; DSC, differential scanning calorimetry; DUVS, derivative ultraviolet spectrometry; FI, flow injection; FL, fluorimetry; GC, gas chromatography; LC, liquid chromatography; MD, microdialysis; MeCN, acetonitrile; MEKC,: micellar electrokinetic chromatography; MeOH, methanol; MS, mass spectrometry; NH_2, amipropyl silica; PFP, perfluorinated phenyl phase; SAX, strong anion exchanger; SPE, solid phase extraction; TBAB, tetrabutyl ammonium bromide; TBAH, tetrabutyl ammonium hydroxide; THF, tetrahydrofuran; TLC, thin layer chromatography; UV/VIS, ultraviolet/visible spectrometry.

published methods. It should be emphasized that the non-English publications have been reviewed on the basis of their respective abstracts, and thus, some data could be incomplete as shown by some blank cells in the aforementioned table.

On having a quick look at Table 3.3.1, the reader will realize that some of the abovementioned whitening agents have not been determined in any of the published methods. Also, one notes the absence of a general methodology focused on determining all the currently used whitening agents worldwide.

As shown in Table 3.3.1, whitening agents have been determined by means of different analytical techniques.

The most frequently used analytical technique for their determination has been LC with an UV/VIS spectrometry detector, either as a single wavelength or as a diode array detector (DAD). Nevertheless, a paper where the determination of AZA is carried out by means of a fluorimetric (FL) detector has also been published (Gatti *et al.*, 1995). With regard to the columns employed, octadecylsilica (C_{18}) phase is the favourite option, although papers where other stationary phases are used have also been reported, as is the case of Semenzato *et al.* (1995) who employed an aminopropyl (NH_2) bonded silica column to determine MAP, and Sottofattori *et al.* (1998) who employed a cyanopropyl (CN)-bonded silica column to determine AP and MAP. Both papers justify the use of these columns by the fact that MAP, which is a highly polar compound, is not properly retained in other less-polar columns like C_{18}. The same justification is given by Masse *et al.* (2001), who employed a diol-based column to determine ARB and KA, and presumably the same reason made Lin *et al.* (2005) use a perfluorinated phenyl (PFP) phase to determine ARB.

Other chromatography-related techniques such as GC (Zhang *et al.*, 2002), micellar electrokinetic chromatography (MECK) (Sakodinskaya *et al.*, 1992) and capillary electrochromatography (CEC) (Desiderio *et al.*, 2000) have been much less used.

Other techniques, like electroanalytical ones, have also focused on whitening agent determination. Likewise, differential pulse voltammetry (DPV) by using carbon paste (Wang, 1995), screen-printed carbon (Shih and Zen, 1999) or clay-coated screen-printed electrodes (Shih and Zen, 2000) as working electrodes have been proposed for the determination of different whitening agents (see Table 3.3.1). Cyclic voltammetry (CV) based on sweet-potato tissue modified paraffin/graphite (Vieira and Fatibello-Filho, 2000) or stearic acid/graphite (Fatibello-Filho and Vieira, 2000) electrodes (where peroxidase present in this tissue catalyses an oxidation process) has been applied exclusively to HQ. In addition, Rueda *et al.* (2003) optimized a flow injection (FI) system with amperometric detection for HQ determination.

The use of derivative ultraviolet spectrometry (DUVS) was proposed to determine HQ in creams and gels, avoiding interferences from the matrix that direct ultraviolet spectrometry caused (L pez-Garc a et al., 2005).

Finally, a thermoanalytical technique like differential scanning calorimetry (DSC) has been applied to ADP determination (Luckewicz and Saccaro, 1990).

With regard to sample preparation, no complex operations are necessary. The sample is usually dissolved in a suitable solvent, or analytes are leached from the matrix, by means of gently mixing or sonication. Centrifugation or filtration can be employed to obtain clear solutions. Only Scalia *et al.* (1997) proposed a methodology based on solid-phase extraction (SPE) procedure by using strong anion exchange (SAX) phases.

Only in the case of Gatti *et al.* (1995), an additional derivatization step was necessary to transform AZA in a fluorescence derivative to be detected by FL detector.

SUMMARY

There are no positive lists for tanning nor whitening agents in any of the three main markets dealing with cosmetic products, that is, EU, US and Japan (in US, whitening agents are considered as OTC drugs, whereas they are considered as quasi-drugs in Japan); however, the use of some of them is restricted depending on the legislation.

On the other hand, certain undesirable effects have been found to be caused by some of these agents, especially hydroquinone.

However, although the analytical control of these products seems necessary, no official analytical methods have been published to determine most of these chemicals. Only the determination of hydroquinone and some of its ethers is covered by a method published by the European Commission.

Nevertheless, an overview of the background shows that there are several published papers focusing on their determination, although most of them do not deal with all the chemicals involved.

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– 4 –

Colouring Agents in Decorative and other Cosmetics. Analytical Methods

4.1. Colouring Agents in Cosmetic Products (*Excluding Hair Dyes*): Types of Decorative Cosmetic Products

B. Valet, M. Mayor, F. Fitoussi, R. Capellier, M. Dormoy and J. Ginestar*

Laboratoires Clarins, Pontoise, France

INTRODUCTION

Decorative cosmetics are principally used to beautify or cover minor, visible imperfections. Shiny, oily, inhomogeneous colouring, as well as slight imperfections on skin surface are corrected by these kinds of cosmetics. These products play an important role, creating the effect of youthfulness and wholesomeness which are becoming more and more important in our society today.

The different types of decorative cosmetics include foundations, lipsticks, glosses, mascaras, nail lacquers and powders.

Dealing with this subject demands a wide understanding of colouring agents. The aim of this section is to introduce the reader to the topic of decorative cosmetics, discussing the different colouring agents and types of decorative cosmetic products.

*Corresponding author. E-mail: jose.ginestar@puig.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

COLOURING AGENTS

Colouring agents are added to cosmetics in order to colour the cosmetic itself and/or to impart colour to the skin (or its appendages) of users of decorative cosmetic products. The colouring agents intended for colouring hair are considered as other types of cosmetic agents under the name of hair dyes, and although there are some colouring agents that are also used to colour hair, they will not be considered here as such, since all hair dyes are reviewed in Section 4.3.

Colouring agents are classified in two main groups: colorants (or dyes) and pigments. Colorants are soluble (in water or in oil) synthetic organic colouring agents. They are used to colour cosmetic products such as skin care or toiletries, among others. Meanwhile, pigments are insoluble colouring agents, which when used, remain in the form of crystals or particles. They are divided into mineral and organic pigments, and are used in make-up, for example, and also for the making cosmetics, like toothpastes, soaps and beauty masks. A detailed classification of these groups is described further on.

On the other hand, colouring agents can be of natural origin or obtained through a synthesis procedure. In the event of natural origin, they can be obtained from minerals, plants or animals. Figure 4.1.1 shows a schematic classification according to both solubility and origin.

As mentioned in Section 1.2, unlike other cosmetic ingredients to which an international common nomenclature is followed, i.e. INCI (International Nomenclature for Cosmetic Ingredients) names established by the CTFA (Cosmetic, Toiletries and Fragrance Association), there are certain discrepancies between the three main legislations regarding cosmetic products (i.e. European Union (EU) Cosmetics Directive, Food and Drug Administration (FDA) from United States (U.S.) and Japanese regulations) in the event of colouring agents.

In EU, as mentioned in Section 1.2, the substances allowed to be used as colouring agents in cosmetics are listed in Annex IV of the EU Cosmetics Directive (Council Directive 76/768/EEC). Except for a few cases, they are usually listed by their Colour Index (CI) numbers, which is a code number assigned by the Society of Dyers and Colourists (SDC) in conjunction with the American Association of Textile Chemists and Colorists (AATCC). For example, the colouring agent tartrazine is named as CI 19140. This nomenclature is

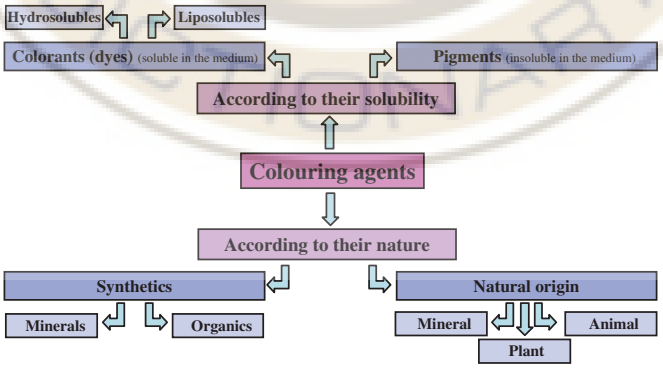

Figure 4.1.1 Schematic classification of colouring agents.

considered as INCI in the EU framework, and it is used for labelling cosmetic products. Lakes or salts of the allowed colouring agents, using substances that are not prohibited by the Cosmetics Directive, are equally allowed and they are named by using the same CI number.

On the contrary, in U.S., as mentioned in Section 1.2 and described in greater depth in Section 4.2, there are two lists of allowed colouring agents to be used depending on whether they are exempt or subject to the batch mandatory certification to be performed by the FDA. The former are listed in Part 73 of Title 21 of Code of Federal Regulation (21 CFR Part 73), and they have simple or chemical names (e.g. bismuth citrate, caramel, dihydroxyacetone, lead acetate and titanium dioxide among others), while the latter have different names depending on whether they have been certified or not. If they are not certified, they adopt the CI names assigned by SDC and AATCC (e.g. tartrazine is named as Acid Yellow 23), and in the event they are certified they are listed in 21 CFR Parts 74 and 82 and they adopt a code assigned by the FDA consisting of a prefix like FD&C, D&C, Ext. D&C (where it states that the colouring agent is authorized for food (F), drugs (D) or cosmetics (C), and for external (Ext.) use only, i.e. avoiding the lips or any body surface covered by mucous membrane), followed by a colour, and finally a number after the abbreviation "No." (e.g. FD&C Yellow No. 5, for tartrazine). In the event of lakes (which will be described further on), the type of lake must appear (e.g. Aluminium) followed by the word "Lake" at the end of the name (i.e. FD&C Yellow No. 5 Aluminium Lake). Nevertheless, an alternative proposed by industry and accepted by FDA, permits the use of abbreviated names in cosmetic labelling. These abbreviated names avoid including the aforementioned terms "FD&C", "D&C" and "No." or the type of lake (i.e. Yellow 5 for tartrazine and Yellow 5 Lake for tartrazine lake). Both abbreviated and non-abbreviated names are currently allowed by FDA to label cosmetic products to be marketed in the U.S. (FDA, 1999).

With regard to Japan (MHW, 1966), cosmetics are labelled according to their Japanese names (i.e. characters), that CTFA has transliterated into English language, followed by a number (e.g. Ki4 for tartrazine). Other colouring agents are not regulated as such, if not as general cosmetic ingredients and thus they follow the usual INCI names.

Some efforts have been made by CTFA to harmonize the nomenclature for colouring agents, by proposing a dual declaration with both U.S. and EU names on cosmetic labelling (i.e. Yellow 5 (CI 19140) in the case of tartrazine), but there is no official rule regarding this issue (Gottschalck and McEwen, 2004). In any event, in a response letter to CTFA, the FDA stated a willingness not to object to this approach (FDA, 1995).

COLORANTS

Hydrosoluble colorants

These colorants are used to colour mixtures in lotions, perfumes, emulsions, soaps and bath products, where a covering effect is not necessary. These are molecules which contain one or more water-soluble groups such as sulfonic ($-SO_3^-$ Na^+) or carboxylic ($-COO^-Na^+$) moieties.

These colorants are very sensitive to pH, UV rays, as well as to oxidative or reductor chemicals.

Examples of water-soluble colorants are: carminic acid, caramel, FD&C Yellow No. 5, FD&C Blue No. 1, D&C Orange No. 4, D&C Red No. 33, FD&C Red No. 40.

Liposoluble colorants

These colorants are used to colour anhydrous mixtures where concealing is not necessary (e.g. tanning oils, bath oils, sticks, etc.). These molecules do not contain water-soluble groups and their stability in oil rarely goes beyond a few grams per litre. They are also sensitive to UV radiation.

Examples of liposoluble colorants are β-carotene, D&C Red No. 17, D&C Yellow No. 11, D&C Green No. 6 and D&C Orange No. 5 among others.

PIGMENTS

Mineral pigments

Mineral pigments are more resistant to light than organic colorants and they are also more opaque but less shiny.

Different compounds can be found within this group. Some examples are described below. Among others, we can find iron oxides, which offer excellent stability and are the most widely mineral pigments in make-up. There are three basic shades of iron oxides: yellow (which corresponds to hydrated ferrous oxide, i.e. $FeO \cdot nH_2O$), red (which is attributed to ferric oxide, i.e. Fe_2O_3) and black (which is a mixture of both iron oxides).

Chromium oxides are also employed in decorative cosmetics. Chromium oxide greens (Cr_2O_3) and chromium hydroxide greens ($Cr_2O_3 \cdot 2H_2O$) are typical mineral pigments with excellent light fastness, heat stability and bleed resistance. They also offer a high covering capacity, but their colour-giving power is weak.

Another class of pigments is the so-called ultramarines, which have a range of shades including green, pink and blue. They are synthetic pigments composed of complex sodium aluminium sulfosilicates having a typical formula $Na_v(Al_wSi_xO_y)S_z$ with proportions of each ingredient depending of the desired colour. They are very stable to heat and pH alkaline, but not very stable in acid surroundings.

Another inorganic pigment is Manganese Violet, which is a very bright colour. Chemically it is $MnNH_4P_2O_7$. It is very stable to light and organic solvents.

Titanium dioxide (TiO_2) is a white pigment, with high coverage that offers excellent stability to heat and light. It is probably the most frequently used white pigment.

Organic pigments

There are three types of organic pigments: lakes, toners and true pigments.

Lakes are water-soluble dyes that are absorbed into insoluble substrates by means of Van der Waals forces. They give dazzling shades although they have moderate covering

capacity. Their stability is weak in the face of light and chemical agents; however, they offer good heat stability. These pigments are widely used in lipsticks and nail lacquers. When used, the preparation procedure is attained by absorption of a hydrosoluble colorant on an insoluble substrate. The most commonly used substrates are hydrated aluminium, titanium dioxide and aluminium benzoate, among others.

In the event of toners, they are water-soluble dyes that are precipitated as metal salts. They differ from lakes, where there is absorption on a substrate. The most used metals are calcium and barium.

Finally, true pigments are pigments that based on their chemistry, precipitate back to they are made from. They are insoluble compounds which contain no metal ions. Examples of which are D&C Red No. 36 and D&C Red No. 30.

A pearl effect can also be obtained by using appropriate pigments. They are the so-called pearlescents. Pearlescent pigments reflect and transmit light by their transparency. The pearlescent effect of a pigment is obtained by superpositioning plates of transparent material and different refractive indexes. This structure allows the reflection of part of the incident light and transmits the remainder to the plates below.

Various types of pearlescent materials are used in decorative cosmetic products, such as bismuth oxychloride (BiOCl), which is a synthetic pearlescent in the form of octagonal platelets giving a very shiny metallic pearlescent effect. It offers strong covering effects and good adhesion to skin surface. It is soft and silky to the touch. However, due to its poor stability to light, it has a tendency to darken after prolonged exposure.

Mica also has pearlescent properties. It gives a pearly aspect to skin, because it has a refractive index which is different to that of air. A new generation of pearlescent pigments appeared when refined (micronized) mica dust was coated with highly refringent substances such as titanium dioxide and iron oxides. These are called interferential pigments.

The first interferential pigments were created on the basis of natural muscovite mica as a substrate and coated with titanium or iron oxide. Other substrates have subsequently been used in this type of pigment, notably the silica flake and the alumina flake. The substrate provides the necessary platelet shape, while the interference colour is determined by the thickness of the metal oxide layer and its refractive index.

DECORATIVE COSMETIC PRODUCTS

Face colouring

Foundations

Foundation is a make-up product designed to be applied to the face. Its purpose is to even the complexion and cover slight imperfections. The shade should be the closest possible to ones natural carnation. It is obtained by ground iron oxides and dispersed in oil or glycols. Moreover, different white powder agents (talc, modified starch, kaolin, silica, polymethylmethacrylate, etc.) are added according to the desired matt effect and also to obtain the desired silky and velvety feel.

Foundations can be found either in the form of liquid or oil-in-water (O/W), water-in-oil (W/O) or water-in-silicone (W/Si) cream emulsions, anhydrous forms (creams, sticks, compacts) or aqueous gels, and they may be applied either with fingers or sponge. Anti-ageing, matt appearance, correcting effects and bright complexion are the main claims of these products.

This type of formulation has to fulfil different cosmetic qualities, such as easy application (glides on and smoothes out easily, homogeneity, quick drying); pleasant application (soft and comfortable); final homogeneous and unifying film, more or less matt and covering; final comfortable non-occlusive film; long-lasting day wear (resistance to sebum and perspiration); and tendency towards being make-up and skin care in one. Also it must fulfil different technical qualities, such as long-lasting stability; dermatological safety and be non-comedogenic; and respect legislation in force in the country where it is sold.

All the aforementioned properties can be achieved by formulating the cosmetic appropriately. This means, for example, by choosing the correct emulsifiers according the type of emulsion desired. Moreover, oils with good pigment dispersing properties and other fatty substances, such as waxes that give consistency to the formulation are needed to be appropriately chosen. Rheology modifiers, humectants, different active ingredients (moisturizers, regenerators, anti-age chemicals, UV filters (in case of anti-UV claim), etc.) and electrolytes helping to stabilize the foundation and preservatives are also few examples of ingredients added to these formulations.

Table 4.1.1 shows the cosmetic qualities that foundations are expected to fulfil depending on the type of formulation.

Table 4.1.1

Properties of different types of foundation

Type of foundation	Composition	Cosmetic qualities
O/W emulsion	Important aqueous phase gel-formed. Fatty phase: esters and different oils	A light slip and spread texture, less rich, rather mat
W/O emulsion	Important fatty phase gel-formed. Fatty phase: ester, silicone oils, small quantity of wax. Contains electrolytes	Good slip and spread, rich in texture, comfortable, some what thick film
W/Si emulsion	Fatty phase: silicone. Contains electrolytes	Good slip and spread, fine film, softness and silkiness
Anhydrous cream	Gel-formed fatty phase. Volatile silicones and hydrocarbons, waxes, important quantity of powders	Special silky feel and evens out nicely
Stick/compact	Gel-formed fatty phase. Volatile important quantity of waxes and powders	Important covering effect. Evens out nicely
Aqueous gel	Little or no fatty phase: small quantity of pigments	Transparency, wholesome effect, non-rich texture

Tinted day creams are different from foundations in that they have less colouring power, and they are less corrective. However, they also give a wholesome, outdoorsy effect.

In the same category as foundations we can classify concealers, such as anti-dark circles and tinted correctors. For these products the correcting and covering effects are important; the shades can be different from those of the carnation to better and enhance it.

Powders

Powders are used since antiquity to enhance the complexion and are still an up-to-date product. They are constituted by a white base which is essentially made of talc, mica and or serecite. Other powders allow improving the mat aspect, the softness, slip and holding and modulating transparency (silica, polymethylmetacrylate, polymide, modified starch, kaolin, lauroyl lysine, boron nitride powder). The powder shade and cover is achieved by means of mineral pigments (titanium dioxide, iron oxides, blue and red ultramarine ferric ferrocianide, chromonium oxide green) and organic pigments (FD&C Blue No. 1 Aluminium Lake, D&C Red No. 30, D&C Red No. 28 Aluminium Lake, D&C Red Calcium Lake). Pearls can be added to achieve a more or less satiny effect.

Powders can be found in loose form or pressed form for different uses. Loose powder is applied after moisturizer or foundation; it fixes the make-up base, evens out and mattifies skin tone. Compact powder evens out skin colour and tones down imperfections. Some are used dry for a powdered finish and others wet for a silky long-lasting finish, while bronzing powder ensures a natural, sun-kissed glow. Finally, powder blush is for a glowing effect as well as contouring.

They must fulfil different cosmetic qualities, such as evens out skin tone, acts as a complexion corrector and conceals pores; covering power; long-lasting power; pleasant to the touch and pleasant to apply; and homogeneous finish between colour and make-up. On the other hand, they must fulfil other qualities from the technical standpoint, such as long-lasting and stable to light; good compromise between mechanical resistance and wax (compact powders); dermatologically safe, non-occlusive; and they must obviously respect the legislation in force in the country where they are sold.

The use of binders is very important in this type of formulation, since these components allow powder to be compressed, make it applicable and adhere to skin; they determine the final touch and increase the intensity of the shade. Binders are of lipid nature, such as esters (e.g. octyl dodecyl stearoyl stearate, isopropyl isostearate), silicones (e.g. dimethicone, phenyltrimethicone, bisphenylhexamethicone), polymers (e.g. polybutene, trimethylsiloxisilate), fatty alcohols (e.g. octyl dodecanol, lanolin alcohol), mineral oils (e.g. paraffin oil).

Other ingredients such as UV filters (in the event of sun protection claim), preservatives, fragrance, etc. can be added.

Certain raw materials can be coated (talc, mica, serecite, pigments, pearls, etc.) or micronized (talc, mica, serecite, pigments) to modify or amplify an aspect. Globally speaking, they may facilitate pigment dispersion, compacting, skin adhesion, softness and “wet and dry” application.

Table 4.1.2 shows the composition of different powders according to their nature.

Table 4.1.2

Composition of loose powder, compact powder and powder blush

Raw materials	Loose powder	Compact powder	Powder blush
Talc (processed)	qsp	qsp	qsp
Mica and/or serecite (processed) (%)	10	10	10
Texture agents (%)	15–35	15–25	10–20
Pigments (%)	<2	<10	<10
Pearls (%)	<5	<5	<5
Binders (%)	—	3–8	1–7

qsp: Quantity enough to reach 100%.

Eye make-up

Nowadays, eye make-up includes a wide range of products: eye shadow, pencils, mascaras and eye liners, each for a specific use.

As these products become more sophisticated, they must henceforth ally seduction, practicality and safety. Only very pure products with non-irritating, non-toxic and non-allergenic properties which have been tested and proven are accepted on areas of the face where skin is particularly fragile and sensitive.

Eye shadows

Eye shadows are preparations designed to enhance the depth of eyes thanks to a coloured background that gives contour to the eyelid. The choice of shade depends upon the eye colour we wish to compliment.

These products exist in different forms, such as compact powders, which are the most commonly used. They are conditioned in cases with one shade only or a selection of several. The coloured product is applied with a flexible brush or foam applicator. The raw materials are principally pearlescent agents dispersed in a powder base composed of talc (which give softness and easy glide), kaolin, titanium dioxide or calcium carbonate. Lipophilic binders are necessary to ensure compacting and the dispersion of pigments. They help to avoid flaking or the formation of dust and give a soft texture. Likewise, volatile silicones help to avoid the chalky effect owed to powders.

Cream shadows are another form of eye shadow. These are conditioned emulsions in small-sized tubes or jars. Their consistency is more or less fluid but they must spread easily without leaving chalky deposits in the crease of the lid. They are rich in coloured pigments and pearlescent agents. Some are made resistant to water thanks to silicones.

Finally, pencils, in a category of their own, are made up of a wax base whose composition is close to that of lipstick, and in which pigments are dispersed. An appropriate mixture of waxes and oils help to obtain an extruded or moulded, coloured pencil in a cylindrical or plastic casing. The pencil should be neither too hard nor too soft, nor easily broken and allow the application of an even line along the edge of the eyelid.

Eye mascaras

Mascaras are used by 90% of women to enhance and accentuate the natural beauty of their eyes by means of colouring, lengthening, thickening or filling out their eyelashes.

There are many different colours, but the most common are black, brown, green or blue. Usually, mineral pigments are used for shades.

This type of product has to fulfil different cosmetic qualities, such as easy application; even and covering film; lash separation; quick drying; lash flexibility and no caking; long-lasting day wear (mascara should not flake or run); and easy removal. Moreover from a technical standpoint, they have to have long-term stability; to be dermatologically and ophthalmologically safe; and finally fulfil the legislation in force in the country where sold.

In its formulation, we can find synthetic or natural waxes (which give consistency to the formula), emulsifiers, polymers (as viscosity controlling agents, pigment dispersers, binders, etc.), powders (which ease sliding upon application and give volume to lashes), and other diverse additives.

There are different types of mascara, like waterproof, water resistant and basic mascaras. Some of their characteristics are summarized in Table 4.1.3.

In the past, they were sold in the form of cakes and applied with a wet brush. These types no longer exist for reasons of hygiene. Today, there are coloured emulsions contained in tubes that are applied with a brush type applicator. It is important to mention that the latter plays an essential role in the quality of the application (its shape; bristle shape, number, nature and size; the dimensions of wand and wiper).

There are also products on the market offering volume on one hand and colour on the other.

Eye liners

They have been designed to underline and over line the lower and upper lids; they intensify make-up, modify and correct the appearance of the shape of the eye, and are applied with a small brush. There are many different colours but the most common are black and brown.

We can observe two types of eye liners: liquid eye liners which are made with a non-greasy emulsion, colorants and film-forming agents, and compact (or cake) eye liners which are made with pressed powders and colorants (applied with a wet brush).

Table 4.1.3

Types of eye mascaras

Type of mascara	Composition	Cleansing	Hold and resistance to water
Waterproof	W/O or W/Si emulsions or anhydrous gel with a high level of volatile hydrocarbon solvents	Difficult	Excellent
Water resistant	O/W emulsion containing waterproof polymers	Easy	Partial
Basic mascara	O/W basic emulsion	Very easy	Weak

Liquid eye liners are made of a suspension of pigments in a base containing a film-forming material which helps to fix the product and prevents smudging. There is a large variety of formulae. Cake eye liners resemble pressed eye shadow but generally contain more binding material to form a cream or a paste.

Eye liners contain the same types of raw materials as mascaras, but they are more liquid and therefore have a smaller percentage of waxes.

Among their cosmetic qualities, can be found the ease of application; covering and even film power; quick-drying; long-lasting day wear; and ease of removal. As technical qualities, they must have good long-term stability, be dermatologically and ophthalmologically safe, and fulfil the legislation in the country where sold.

Lips

A symbol of femininity, lipstick is above all the emblem product of make-up.

Whether it be solid (stick), semi-solid (jars) or liquid (tube), it should be easy to apply, giving protection and comfort.

The cosmetic qualities that a lipstick should fulfil are attractive shades; homogeneous film colour when applied to lips, glide-on texture without feathering; lasting hold; comfort and pleasant fragrance, disguising the odour and possible taste of the base. As technical qualities, they have to fulfil long-term stability; resistance to temperatures from 4 to 40 °C without hardening or running; dermatological safety and respect for legislation in the country where sold; pliable stick but not subject to breakage, creamy and gliding without any greasy sensation; and free from exudation and crystallizing.

When preparing the formula, it is necessary to verify all of these criteria by doing corresponding tests.

Lipsticks are made up of a white base (solid fraction, liquid, semi-paste) and a coloured base.

The white base is constituted by waxes of different origin and nature (beeswax, carnauba wax, etc.) and different oils (vegetable, synthetic, etc.). The former participate in the crystalline structure of the stick, enabling adjustment of the staying power properties to heat and bestowing a more or less hardened texture upon application. On the other hand, oils give the lipstick its shine, glide and easy application quality, but are unfortunately also responsible for bleeding and exudation. Other fatty materials present such as butters, hydrogenized vegetable oils and fatty esters are the link between waxes and oils, playing an important role in homogeneity, thickness, creaminess, richness and hold of the film placed on lips; and a coloured base.

The coloured base is formed by pigments and pearls. Pigments give lipstick its colour and covering power. The concentration of pure pigments can vary from 1% to 10% depending on the type of product (lip gloss to a dark lipstick). The most widely used pigments are mineral (titanium and iron oxides) and organic pigments (true pigments, toners and lakes). Being insoluble substances, they will be finely dispersed, ground, by mechanical action in an oil support (binder), which must have affinity with the pigments to ensure the wetting, dispersion and to maintain the ground particles in suspension. In the event of pearls, they are not mandatory in the composition of lipstick but can be added to create a

Table 4.1.4

Formula types of lipstick and gloss

Raw materials	Example formula lipstick (%)	Example formula gloss (%)
Waxes	12–18	<5
Oils	40–60	10–30
Polymers	0–20	>50
Fatty materials	10–15	0–10
Pigments	1–10	<2
Powders	0–5	0–2
Pearls	0–10	0–10
Fragrance	<1	<1

more or less satiny, iridescent or glittery effect. They are added towards the end of the process to avoid losing their effects.

In addition to these, we can add optional elements depending on the market brief of the product; for example, powders to improve texture and avoid sweating (talc, kaolin, silica, starch, mica, etc.), UV filters (in case of anti-UV claim), antioxidants, etc. A fragrance is often used in most lipsticks and should be pleasantly sufficient to cover fatty base odour, but not be obtrusive. It should be neither irritant nor toxic by ingestion, and its taste must be agreeable. Small quantities of ammonium glycyrrhizate may be used to improve the latter aspect.

In Table 4.1.4, different example formulas for a lipstick and a gloss are shown.

It should be emphasized that more than just a simple make-up product, lipstick has also become lip care because of its repairing (nourishing and moisturizing) and protecting (against solar radiation by incorporation UV filters) actions. We look for the best compromise between comfort and hold.

Lipstick must live up to new expectations. Shine and persistence, this is the current trend.

In the last few years, basic lipstick has shown a certain decline. It has been replaced by the "Rouge Brillant" (semi-covering) and the cream gloss that were expected to be short-lived, have now become a part of catalogued products.

The market offers duos associating long-lasting lipstick and gloss for comfort and shine.

Nails

Nail lacquers, applied with a brush, are designed to colour and give shine to nails. The lacquer shade is obtained through mineral pigments or organic lakes. According to their percentage of use we will have a more or less transparent lacquer. Additions of pearlescents give special effects to shades.

These products have to fulfil several cosmetic qualities, such as even colour, shine, easy application, resistance to use and chipping and must not become dull, as well as easy

removal with an adapted lacquer remover. Also, they must fulfil different technical qualities, such as good ageing and light stability, good tolerance and respect the legislation in the country where sold.

Different additives are added to nail lacquers in order to fulfil the aforementioned cosmetic and technical qualities. For example, film formers are added, which are ingredients of the film that determine the principal cosmetic quality of the lacquer (nitrocellulose; plasticizers such as dibutyl phthalate, camphor and citric acid esters; resins).

Solvents are other important ingredients of these products, which have to allow quick drying and give staying power to the lacquer. They must be good solvents of the ingredients of the film, have a tolerable odour and be non-toxic. Examples of solvents are toluene, ethyl acetate, isopropyl acetate, isopropylic alcohol, diacetone alcohol, methyl and ethylcetone.

The problem of sedimentation is solved by using modified synthetic clay offering thixotropic properties to the product.

On the other hand, base and transparent coats can applied under the lacquer to make it last longer. Certain bases should make the nail more resistant and thus avoid breaking or splitting. They are less hard and richer in adherent resins than the lacquer. Also transparent top coats are applied on the nail to enhance shine and hardness. These are rich in nitrocellulose.

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4.2. Colouring Agents in Cosmetic Products (*Excluding Hair Dyes*): Regulatory Aspects and Analytical Methods

A. Weisz*, S.R. Milstein and A.L. Scher

Office of Cosmetics and Colors, HFS-106, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD 20740, USA

INTRODUCTION

As mentioned in Section 4.1, application of colour is the main purpose of many cosmetic products such as lipsticks, blushers, eye shadows, eyeliners, and nail polishes. All of these products contain one or more colouring agents—dyes, pigments or other substances—for providing the desired colours. Moreover, colouring agents may be used to colour the cosmetic products.

The aim of this section is to review regulatory information concerning colouring agents in cosmetic products, as well as the methodologies involved in their analysis. Chemicals used as hair dyes are reviewed in Sections 4.3 and thus will not be considered here.

REGULATORY ASPECTS OF COLOURING AGENTS IN COSMETIC PRODUCTS

Colouring agents are subject to a wide range of regulatory restrictions across countries. As mentioned in Section 1.1, positive lists of colouring agents that may be used in cosmetic products have been published by three main regulatory authorities—U.S. Food and Drug Administration (FDA) in the United States, the European Commission in the European Union (EU), and the Ministry of Health, Labor and Welfare in Japan. Other countries permit colouring agents approved in the U.S. and/or the EU with certain variations.

The number and identity of colouring agents permitted for cosmetic use varies among countries. Table 4.2.1 shows the number of these ingredients listed for use in cosmetic products by the three aforementioned regulatory authorities.

United States regulatory requirements for colouring agents

In the U.S., colouring agents are known as colour additives, which must comply with requirements of the U.S. Food, Drug, and Cosmetic Act (FD&C Act) and its implementing

*Corresponding author. E-mail: adrian.weisz@fda.hhs.gov

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Table 4.2.1

Number of colouring agents permitted for use in cosmetics by the three main regulatory authorities

Country	Number of colouring agents permitted
U.S.	64[a]
EU	154[b]
Japan	83[c]

[a]FDA 21 CFR Parts 73 and 74.
[b]Annex IV, Part 1 of the EU Cosmetics Directive (Council Directive 76/768/EEC and its amendments).
[c]Ordinance No. 30/1966 from MHW (as amended by MHLW Nos. 55/1972 and 126/2003) (Rosholt, 2003).

regulations. The term colour additive is defined in section 201(t) of the FD&C Act as *"(…) a material which (A) is a dye, pigment, or other substance made by a process of synthesis or similar artifice, or extracted, isolated, or otherwise derived, with or without intermediate or final change of identity, from a vegetable, animal, mineral, or other source, and (B) when added or applied to a food, drug, or cosmetic, or to the human body or any part thereof, is capable (alone or through reaction with other substance) of imparting a colour thereto (…). The term 'colour' includes black, white, and intermediate grays"*.

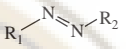
Colour additives permitted in the U.S. are classified from a regulatory standpoint as those subject to batch certification by the FDA and those exempt from certification. Certifiable colour additives (see Tables 4.2.2–4.2.9) include a variety of mainly synthetic aromatic organic chemicals (also know as coal-tar colouring agents). These colour additives are batch-certified by FDA to ensure that their composition is in compliance with the identity and specifications in Title 21 of the U.S. Code of Federal Regulations (21 CFR) in order to protect the public's health. FDA assigns a unique certification lot number to each certified batch. However, as discussed in Section 4.3, coal-tar colouring agents used in hair dyes may be exempt from this certification. The certifiable colours are listed in 21 CFR Part 74 (straights and a few lakes) and in 21 CFR Part 82 (most lakes). The definitions of straight and lake are given in 21 CFR 70.3(j) and (l), respectively, and in brief state that a lake is a straight colour extended on a substrate by adsorption, coprecipitation or chemical combination excluding any combination made by a simple mixing process. Modified definitions were proposed for these terms in the U.S. Federal Register (61 FR 8372-8417, 1996) but have not yet been officially adopted. Certification-exempt colour additives (see Table 4.2.10) include a wide variety of substances that are derived from inorganic, plant, or animal sources, and they are listed in 21 CFR Part 73.

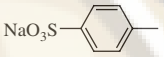
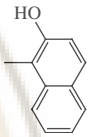
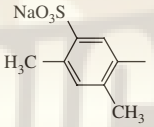
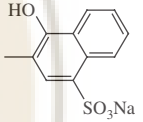
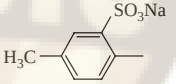
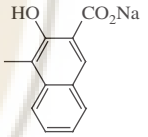
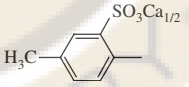
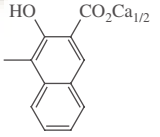
Certifiable and certification-exempt colour additives must undergo the FDA pre-market approval process in order to be listed. A proposal to list a new colour additive or new uses of a colour additive is made by petition to the FDA as described in 21 CFR Part 71. Descriptions of the approval process can be found on the FDA website (see references). The listing regulations describe the identity of each colour additive, specifications, uses and restrictions, labeling requirements, and the requirement for or exemption from batch certification. In addition, a regulation must specifically authorize use of the colour additive in the area of the eye (21 CFR Section 70.5(a)), in injections (21 CFR Section 70.5(b), currently, no colour additive is listed for use in injections), and in surgical sutures (21 CFR Section 70.5(c)).

Table 4.2.2

U.S. certifiable monoazo colour additives for cosmetic use

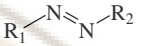
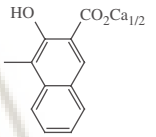
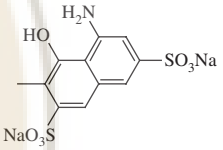
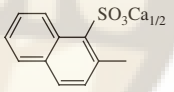
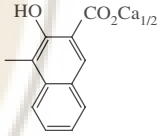
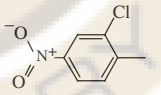
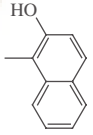
Azo-enol structure

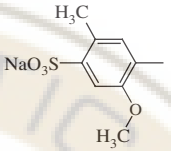
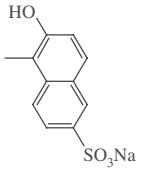
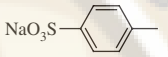
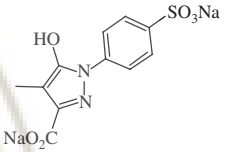
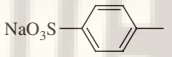
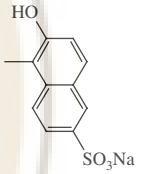


U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2
D&C Orange No. 4	CI Acid Orange 7 Orange II	15510	633-96-5		
FD&C Red No. 4	CI Food Red 1 Ponceau SX	14700	4548-53-2		
D&C Red No. 6	CI Pigment Red 57 Lithol Rubin B	15850	5858-81-1		
D&C Red No. 7	CI Pigment Red 57:1 Lithol Rubin B Ca	15850:1	5281-04-9		

(*Continued*)

Table 4.2.2 (cont.)

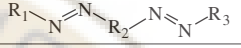
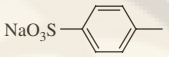
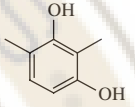
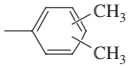
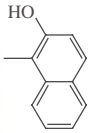
Azo-enol structure					
U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2
D&C Red No. 31	CI Pigment Red 64:1 Brilliant Lake Red R	15800:1	6371-76-2		
D&C Red No. 33	CI Acid Red 33 Acid Fuchsin D	17200	3567-66-6		
D&C Red No. 34	CI Pigment Red 63:1 Deep Maroon	15880:1	6417-83-0		
D&C Red No. 36	CI Pigment Red 4 Flaming Red	12085	2814-77-9		

FD&C Red No. 40	CI Food Red 17 Allura Red AC	16035	25956-17-6		
FD&C Yellow No. 5	CI Acid Yellow 23 CI Food Yellow 4 Tartrazine	19140	1934-21-0		
FD&C Yellow No. 6	CI Food Yellow 3 Sunset Yellow FCF	15985	2783-94-0		

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.3

U.S. certifiable disazo colour additives for cosmetic use

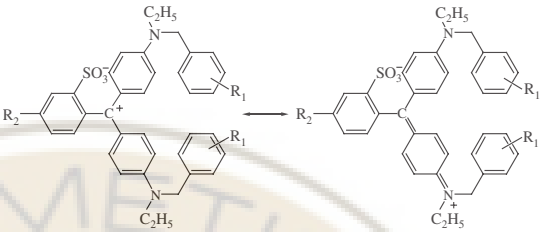
Bis azo-enol structure						
U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2	R_3
D&C Brown No. 1	CI Acid Orange 24 Resorcin Brown	20170	1320-07-6			
D&C Red No. 17	CI Solvent Red 23 Sudan III Toney Red	26100	85-86-9			

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.4

U.S. certifiable triphenylmethane colour additives for cosmetic use

Triphenylmethanium resonance structures



U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2
FD&C Blue No. 1	CI Acid Blue 9 (sodium salt) CI Food Blue 2 Brilliant Blue FCF	42090	3844-45-9	$-SO_3Na$	$-H$
D&C Blue No. 4	CI Acid Blue 9 (ammonium salt) Alphazurine FG Erioglaucine	42090	6371-85-3	$-SO_3NH_4$	$-H$
FD&C Green No. 3	CI Food Green 3 Fast Green FCF	42053	2353-45-9	$-SO_3Na$	$-OH$

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

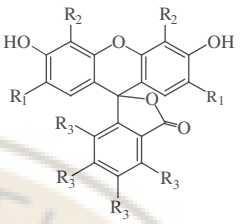
FDA considers cosmetic products to be neither adulterated nor misbranded when they are in compliance with the requirements of the FD&C Act and its implementing regulations, as well as other applicable laws and regulations. A cosmetic product (with the exception of coal-tar hair dyes, discussed in Sections 4.3) containing an unlisted colour additive or a listed colour additive that does not conform to the requirements of its listing regulation is considered adulterated under the provisions of sections 601(e) and 721(a) of the FD&C Act.

In the U.S., cosmetic products that are offered for retail sale are subject to the provisions of the Fair Packaging and Labeling Act (FPLA). Under the authority of the FPLA, 21 CFR Section 701.3 requires the label of a cosmetic product to bear a declaration of the ingredients, usually in descending order of predominance, as mentioned in Section 1.2. However, 21 CFR Section 701.3(f) states that colour additives are permitted to be declared as a group at the end of the ingredient statement, without respect to order of predominance. This requirement for colour additive labeling does not apply to professional-use-only (or salon) products unless specifically required by regulation. In addition, colour additives that are not present in shaded products or products with similar composition and that are intended for the same use may be included in the label by preceding the colour additive name with "may contain" (21 CFR Section 701.3(g)).

Table 4.2.5

U.S. certifiable fluoran colour additives for cosmetic use

Fluoran structure



U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2	R_3
D&C Orange No. 5	CI Solvent Red 72 Dibromofluorescein	45370:1	596-03-2	−H	−Br	−H
D&C Orange No. 10	CI Solvent Red 73 Diiodofluorescein	45425:1	38577-97-8	−H	−I	−H
D&C Red No. 21	CI Solvent Red 43 Tetrabromofluorescein	45380:2	15086-94-8	−Br	−Br	−H
D&C Red No. 27	CI Solvent Red 48 Tetrabromotetrachlorofluorescein	45410:1	13473-26-2	−Br	−Br	−Cl
D&C Yellow No. 7	CI Solvent Yellow 94 Fluorescein	45350:1	2321-07-5	−H	−H	−H

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

As explained in Section 4.1, colour additives may be declared on cosmetic labels either by their listed names or, for certifiable colour additives, by abbreviated names formed by omitting "FD&C" or "D&C" and "No." but including "Ext." and "Lake". FDA has stated that the agency *"(…) does not intend to object to the immediate use of abbreviated labeling for declaring the presence of certified colour additives in cosmetics (…)"* (FDA, 1999).

Both the FD&C Act and FPLA provide authority to FDA to regulate the labeling of cosmetic products. Failure to comply with the requirements for cosmetic labeling may render a cosmetic adulterated under section 601 of the FD&C Act or misbranded under section 602 of the FD&C Act.

Regulatory requirements for the marketing of cosmetics in the U.S. have been presented previously (Milstein *et al.*, 2006). Further details about colour additives permitted in the U.S. may be found on the FDA website (see references).

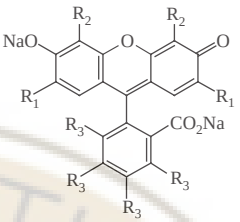
EU regulatory requirements for colouring agents in cosmetic products

Within the EU Cosmetics Directive (i.e. Council Directive 76/768/EEC), all colouring agents, except those intended to colour hair, and their field of application and other

Table 4.2.6

U.S. certifiable xanthene colour additives for cosmetic use

Xanthene structure



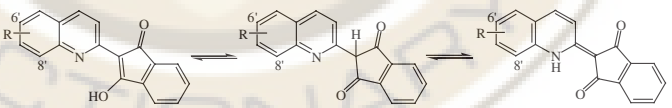
U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2	R_3
D&C Orange No. 11	CI Acid Red 95 Erythrosine Yellowish Na	45425	33239-19-9	−H	−I	−H
D&C Red No. 22	CI Acid Red 87 Eosin Y	45380	17372-87-1	−Br	−Br	−H
D&C Red No. 28	CI Acid Red 92 Phloxine B Cyanosine	45410	18472-87-2	−Br	−Br	−Cl
D&C Yellow No. 8	CI Acid Yellow 73 Uranine	45350	518-47-8	−H	−H	−H

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.7

U.S. certifiable quinoline colour additives for cosmetic use

Quinoline tautomeric structures

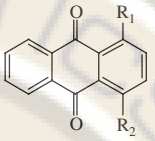
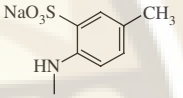
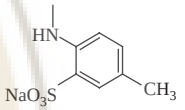
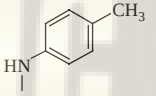
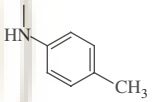
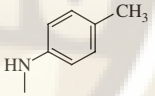
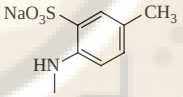


U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R
D&C Yellow No. 10	CI Acid Yellow 3 Quinoline Yellow WS	47005	8004-92-0	Mixture of 6′— and 8′−SO_3Na
D&C Yellow No. 11	CI Solvent Yellow 33 Quinoline Yellow SS	47000	8003-22-3	−H

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.8

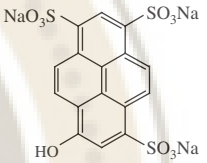
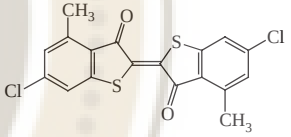
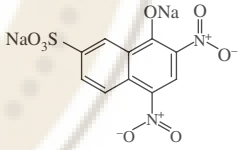
U.S. certifiable anthraquinone colour additives for cosmetic use

Anthraquinone structure					
U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	R_1	R_2
D&C Green No. 5	CI Acid Green 25 Alizarine Cyanine Green F	61570	4403-90-1		
D&C Green No. 6	CI Solvent Green 3 Quinizarin Green SS	61565	128-80-3		
D&C Violet No. 2	CI Solvent Violet 13 Alizurol Purple SS	60725	81-48-1		−OH
Ext. D&C Violet No. 2	CI Acid Violet 43 Alizarine Violet	60730	4430-18-6		−OH

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.9

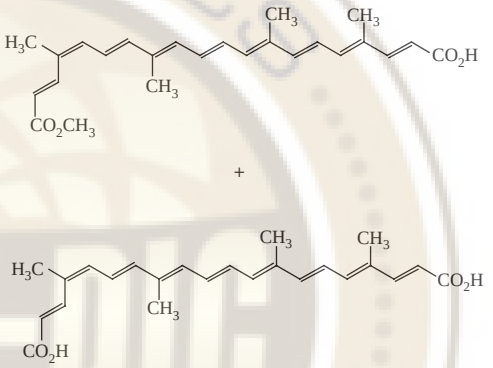
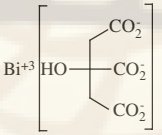
U.S. certifiable nitro, pyrene, thioindigoid, and carbon colour additives for cosmetic use

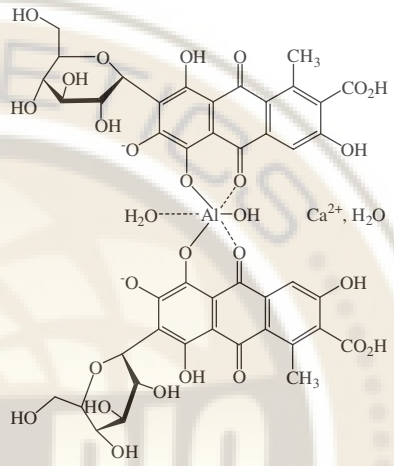
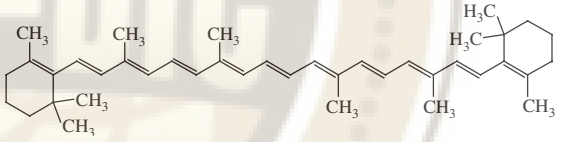
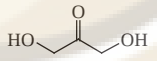
U.S. listed name[a]	Common names[b]	CI No.[c]	CAS No.[d]	Dye classification	Chemical structure
D&C Black No. 2	Carbon black (high purity furnace black)	77266		Inorganic pigment	C (Carbon)
D&C Green No. 8	CI Solvent Green 7 Pyranine	59040	6358-69-6	Pyrene	
D&C Red No. 30	CI Vat Red 1 Helidone Pink CN	73360	2379-74-0	Thioindigoid	
Ext. D&C Yellow No. 7	CI Acid Yellow 1 Naphthol Yellow S	10316	846-70-8	Nitro	

[a]Names assigned by FDA after certification, and listed in 21 CFR Part 74.
[b]Not used in the U.S. for the names of certified colour additives.
[c]Colour Index (CI) number. Not used in the U.S. for certified colour additives.
[d]Chemical Abstracts Service (CAS) number.

Table 4.2.10

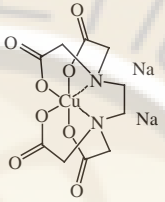
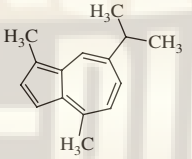
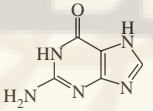
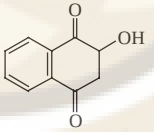
U.S. certification-exempt colour additives for cosmetic use

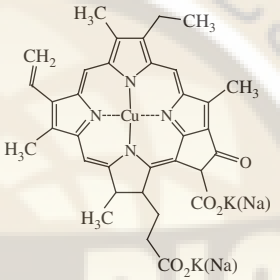
U.S. listed name[a]	CI No.[b]	CAS No.[c]	Chemical structure
Aluminum powder[d]	77000	4729-90-5	Al
Annatto (extract from *Bixa orellana* L.)[d]	75120	1393-63-1	
Bismuth citrate[e,f]	—	813-93-4	
Bismuth oxychloride[d]	77163	7787-59-9	BiOCl
Bronze powder[d]	77400	158113-12-3	CuSn alloy
Caramel[d]	—	8028-89-5	(Heat treated glucose or sucrose saturated solutions)

Carmine (Al or Ca-Al lake on $Al(OH)_3$ of aqueous extract of cochineal)[d]	75470	1390-65-4	
β-Carotene (synthetic and natural)[d]	40800 75130	7235-40-7	
Chromium hydroxide green[d]	77289	12001-99-9	$Cr_2O_3 \cdot xH_2O$
Chromium oxide greens[d]	77288	1308-38-9	Cr_2O_3
Copper powder[d]	77400	7440-50-8	Cu
Dihydroxyacetone[d,g]	—	62147-49-3	

(*Continued*)

Table 4.2.10 (Cont.)

U.S. listed name[a]	CI No.[b]	CAS No.[c]	Chemical structure
Disodium EDTA-copper[d,g]	—	14025-15-1	
Ferric ammonium ferrocyanide[d]	77510	25869-00-5	$NH_4Fe[Fe(CN)_6] \cdot xH_2O$
Ferric ferrocyanide[d]		14038-43-8	$Fe_4[Fe(CN)_6]_3 \cdot xH_2O$
Guaiazulene[d,g]	—	489-84-9	
Guanine[d] (from fish scales)	75170	73-40-5	
Henna[d,e]	75480	83-72-7	
Iron oxides[d] (synthetic)	77489[f] 77491 77492 77499	see Rosholt, 2003	$Fe_xO_y \cdot zH_2O$

Lead acetate[f,g]	—	6080-56-4	$Pb(O_2CCH_3)_2 \cdot 3H_2O$
Luminescent zinc sulphide	—	1314-98-3	ZnS + 100±5 mg/kg copper
Manganese violet[d]	77742	10101-66-3	$MnNH_4P_2O_7$
Mica[d,g] (from muscovite mica)	77019	12001-26-2	$K_2Al_4(Al_2Si_6O_{20})(OH)_4$ or $H_2KAl_3(SiO_4)_3$
Potassium sodium copper chlorophyllin[d] (chlorophyllin-copper complex)	75810	11006-34-1	
Pyrophyllite[d,g] (mineral)	—	12269-78-2	$Al_2O_3 \cdot 4SiO_2 \cdot H_2O$
Silver[d] (crystalline powder)	77820	7440-22-4	Ag
Titanium dioxide[d] (synthetic)	77891	13463-67-7	TiO_2
Ultramarines[d]	77007		$Na_v(Al_wSi_xO_y)S_z$
Ultramarines[d,e]	77013		
Zinc oxide[d] (French process)	77947	1314-13-2	ZnO

[a]21 CFR Part 73.
[b]Colour Index (CI) number. Not used in the U.S. for certification-exempt color additives.
[c]Chemical Abstracts Service (CAS) number.
[d]Treated as a cosmetic ingredient, not as a colour additive in Japan.
[e]Not permitted in the EU.
[f]Not permitted in Japan.
[g]Treated as a cosmetic ingredient, not as a colour additive in the EU.

requirements are listed under Annex IV. It should be emphasized that in the EU Inventory of Cosmetic Ingredients (Commission Decision 2006/257/EEC), colouring agents are divided into cosmetic colorants, which *"colour cosmetics and/or impart colour to the skin and/or its appendages"* and hair dyes, which *"colour hair"*. As mentioned previously, hair dyes are described in Sections 4.3, and are not the subject of this section.

According to EU Cosmetics Directive, as previously mentioned in Section 1.2, labels of cosmetic products marketed in the EU are required to declare their ingredients in descending order of predominance using the INCI names. However, colouring agents may be listed in any order after other components. As previously mentioned in Section 4.1, they are listed in EU Cosmetics Directive Annex IV by their Colour Index (CI) number or denomination, which are INCI names for these cosmetic ingredients. In the special case of decorative cosmetic products marketed in several colour shades, all colouring agents used in the collection may be listed, provided that the words "may contain" or the symbol "+/−" are added.

As mentioned in Section 1.2, Annex IV is divided into two parts: Part 1 lists colouring agents that are currently allowed for use in cosmetics; whereas Part 2, for provisionally allowed colouring agents, is empty. Footnote 1 to Annex IV permits the lakes or salts of the permanently listed straight colouring agents also to be used as cosmetic ingredients provided they are prepared from substances not prohibited under Annex II or excluded under Annex V of the EU Cosmetics Directive. The lakes or salts have the same CI numbers as the corresponding straight colouring agents.

Japan regulatory requirements for colouring agents in cosmetic products

In Japan, colouring agents are named as colorants. As mentioned in Section 1.2, a positive list for synthetic organic colorants was created for the first time in 1966 by the Ministry of Health and Welfare (MHW, 1966), and amended by the Ministry of Health, Labor, and Welfare (MHLW) in 1972 and 2003. As mentioned in Section 4.1, Japan uses alternate INCI names for colouring agents in the cosmetics marketed in Japan. These Japanese names differ from the U.S. and EU names.

It should be emphasized that only synthetic organic (or coal-tar) compounds are listed as colorants by MHLW. These colorants do not need to be certified, but they must conform to specifications. Inorganic, plant, and animal substances are regulated as cosmetic ingredients, but may be used as colorants.

Other international regulatory requirements for colouring agents

Many countries have enacted legislation and issued regulations for approving and listing colouring agents and declaring colouring agents on the labels of cosmetics. Rosholt (2003) presents detailed discussions of specific requirements, by country. Other countries have chosen approaches to control the use of colouring agents in cosmetics that reflect, to a greater or lesser extent, either the U.S. or EU regulatory models. Otterstätter (1999) notes that this is done in some cases by incorporating into national regulations reference to lists

of approved colouring agents that are virtually identical to the lists of the U.S. or the EU. Alternatively, some countries adopted parallel regulatory approaches for the approval of such colouring agents, whereby equivalence can be established.

Some countries require colouring agents to be declared on the labels of cosmetic products in their primary national language(s). Cosmetics also may have ingredient labeling in several languages if they are marketed in more than one country.

U.S. and EU international harmonization efforts for cosmetic labeling

The use of CI numbers has been an approach for harmonizing the declaration of colour additives in cosmetic products marketed in the Member States of the EU. As was also mentioned in Section 4.1, the Cosmetic, Toiletry, and Fragrance Association (CTFA) requested in 1995, in the interest of international harmonization, that FDA permit the use of dual declaration of colouring agents on the labels of cosmetic products marketed in the U.S. Dual declaration of a colouring agent would consist of the U.S. listed name followed by the CI number in parentheses. CTFA requested that FDA permit such dual labeling in the interim while the agency considered a citizen petition requesting that the colour additive regulations be amended to permit such labeling. In response, in a June 1, 1995 letter to CTFA, FDA stated that the agency "would be unlikely to object" to such interim use of dual declarations of colour additives on the labels of cosmetic products (FDA, 1995).

FDA also stated that manufacturers of finished cosmetic products (other than hair dyes) intended for sale in the U.S. should be alerted that, although a dual declaration for the colour additive name might be used for cosmetic labeling purposes, U.S. law requires the use of only colour additives in their products that are in full compliance with applicable regulations. The use of an uncertified, and therefore unapproved, colour additive subject to batch certification in a cosmetic renders such product adulterated within the meaning of the FD&C Act.

DETERMINATION OF COLOURING AGENTS

The importance of determining colouring agents in cosmetic products

This section describes several reasons for determining colouring agents in cosmetic products. The determinations have regulatory, forensic, or manufacturing significance as presented below.

(a) To ensure that only permitted colouring agents are added to the cosmetic product

As was shown earlier, colouring agents permitted in one country are sometimes not approved in others. For example, erythrosine is permitted in cosmetics as a colouring agent in the EU (as CI 45430) and as a colorant in Japan (as Aka3), but it is not permitted for use in cosmetics in the U.S. (21 CFR 81.30(u)).

A different case is the colouring agent Quinoline Yellow. This quinoline-type dye consists of a mixture of mono-, di-, and trisulfonated positional isomers, the relative proportions of

which depend on the degree of sulfonation obtained during its manufacture. A mixture of the monosodium salts of the 6′- and 8′-monosulfonic acids with up to 15% of the disodium salts of the disulfonated isomers is certifiable in the U.S. as the colour additive D&C Yellow No. 10 (Table 4.2.7) (21 CFR 74.1710). A mixture that contains mostly di- and trisulfonated components is permitted as a cosmetic colouring agent in the EU (as CI 47005) and as a colorant in Japan (as Ki203) (see Rosholt, 2003). Even though both of these variant forms are indexed as CI 47005, the latter mixture (i.e. consisting mainly in di- and trisulfonated components) is not certifiable in the U.S. and is therefore not permitted in cosmetics that are imported and sold in the U.S.

(b) To ensure that the information on the label is complete and correct

In U.S., for example, the colouring agents that are part of a cosmetic product must be declared by name on the product's label (21 CFR Section 701.3). Analysis of the cosmetic may find undeclared certified or certification-exempt colour additives. If the information is not complete or not correct, the product is misbranded and may also be adulterated depending on what is found.

(c) To determine the cause of allergic and dermatologic reactions

Contact of the human body with certain colouring agents, their impurities, or their decomposition products (that may occur during processing or storage of the cosmetic product) can produce allergic reactions, sensitization, or photosensitization in susceptible people (Rosenthal *et al.*, 1988; Wei *et al.*, 1994, 1995; Mselle, 2004; Antonovich and Callen, 2005; Klontz *et al.*, 2005). Determination of the colouring agent(s) present in the cosmetic product used may provide a clue to the source of the unexpected reaction.

(d) To help in forensic investigations

Lipstick smears left on drinking glasses, cups, and cigarette butts can link a suspect to a crime scene. When found on a suspect's clothing, they can prove a link between the suspect and victim. Results obtained from the analysis of lipstick smears in a forensic science laboratory are often found to be important evidence in criminal cases (Barker and Clarke, 1972; Andrasco, 1981; Russel and Welch, 1984; Gennaro *et al.*, 1994; Griffin *et al.*, 1994; Ehara and Marumo, 1998; Rodger *et al.*, 1998).

(e) To determine the stability of a colouring agent added to various matrices

The stability of a colouring agent can be affected by many factors during storage of the cosmetic product. Such factors are light, heat, pH, nature of the packaging, nature of the product base, etc. (Rush, 1989; Otterstatter, 1999).

(f) Quality control

Cosmetic manufacturers must determine colouring agents present in their products in order to ensure that quality standards are consistently maintained (Rodger, 1998). This quality

control may be conducted at various stages in the production and pre-marketing process. The sample tested is compared with a standard using various analytical techniques such as colorimetry and spectroscopy (DRAGOCOLOR, 2004).

Composition of colouring agents

In general terms, the manufacturing process of most colouring agents involves several steps: condensation of the starting materials, partial purification of the resulting condensation product, and introduction of functional groups to increase the colour additive's solubility in water (e.g. sulfonation, carboxylation) or its solubility in organic solvents (e.g. halogenation, nitration, alkylation). The mono- and disazo-colouring agents (Tables 4.2.2 and 4.2.3) are prepared by diazo coupling reactions. As an example, Figure 4.2.1 schematically shows the preparation of the chemically related colour additives D&C Red No. 27 (CI 45410:1, Table 4.2.5), D&C Red No. 28 (CI 45410, Table 4.2.6), and Rose Bengal (CI 45440, approved for cosmetic use in Japan, South Korea, and Taiwan).

During manufacture, in addition to the main component of a colouring agent, various impurities may be produced, depending on the purity of the starting materials used and the conditions under which the technological process was performed. These impurities can consist of intermediates (compounds from which a colouring agent is directly or indirectly synthesized), side-reaction products, and subsidiary colours (21 CFR Parts 73, 74, and 82; Leatherman *et al.*, 1977; Abrahart, 1968; Marmion, 1991). A subsidiary colour is a structural variant of the main colour component that varies in the position, number, or the length of the substituent groups. As an example, D&C Red Nos. 27 and 28 (CI 45410:1 and CI 45410, respectively, see Figure 4.2.1) may contain a limited amount of lower-halogenated

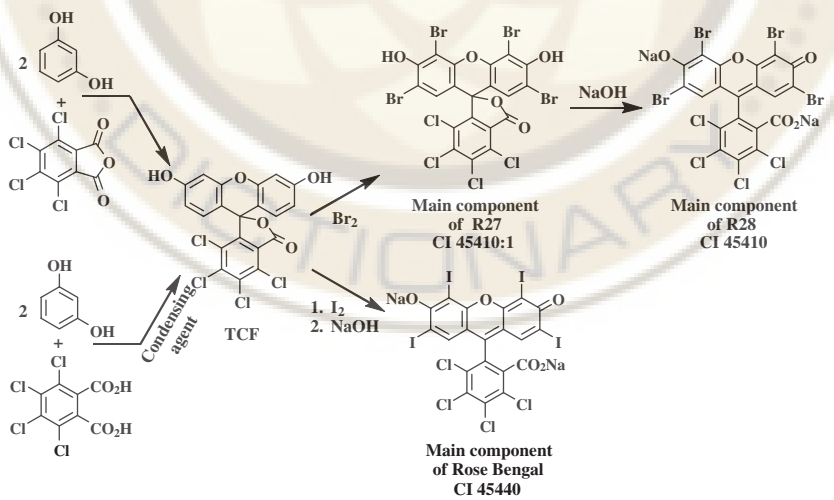


Figure 4.2.1 Preparation of D&C Red No. 27 (CI 45410:1), D&C Red No. 28 (CI 45410), and Rose Bengal (CI 45440) (adapted from Weisz *et al.*, 1995).

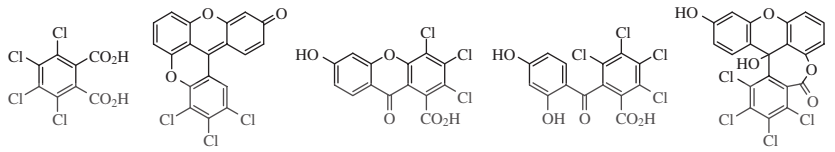


Figure 4.2.2 Impurities isolated from commercial 4,5,6,7-tetrachlorofluorescein (TCF) (adapted from Weisz *et al.*, 1995).

subsidiary colours (21 CFR Parts 74 and 82; Weisz *et al.*, 1992, 1994a, 1994b, 1996). Impurities present in the starting materials can be carried over into the final product. Figure 4.2.2 shows impurities separated from a commercial sample of 4,5,6,7-tetrachlorofluorescein (TCF) (Weisz *et al.*, 1995, 1998), which is an intermediate for the manufacture of the more-highly-halogenated dyes D&C Red No. 27 (CI 45410:1), D&C Red No. 28 (CI 45410), and Rose Bengal (CI 45440) (see Figure 4.2.1). These impurities of TCF can be halogenated during the manufacturing process and can be incorporated into the more-highly-halogenated colour additives. Often analyses reveal the presence of contaminants that vary in nature across batches of the same dye obtained from different suppliers (Van Liedekerke and De Leenheer, 1990; Gagliardi *et al*., 1995; Weisz *et al.*, 1995). Figure 4.2.3 shows the chromatograms obtained by liquid chromatography (LC) of commercial batches of D&C Red No. 28 obtained from three different sources.

As discussed previously, colouring agents are batch-certified by FDA to ensure compliance with the limiting specifications for subsidiary colours, intermediates, and some side-reaction impurities listed in 21 CFR Parts 74 and 82. Excessive levels of specified impurities may result in the failure of a batch of colour additive to meet certification criteria established by the FDA. The application of new technologies has enabled identification and quantification of colouring agents impurities that are not specified in the CFR (Yamada *et al.*, 1996; Ishimitsu *et al.*, 1997; Weisz, 1997; Andrzejewski and Weisz, 1999; Ngang *et al*., 2001; Matsufuji *et al.*, 2002; Weisz and Andrzejewski, 2003; Weisz *et al.*, 2004, 2006). In such cases, the toxicity of those impurities may be assessed by the FDA and specifications limiting their presence in the colouring agents may be added to the CFR.

Preparation of colour components as reference materials

Purified dye components as well as purified dye contaminants are needed for use as reference materials in the development of analytical methods. Such compounds are typically not available commercially. Lyon (2002) in a review on dye purity for biological staining summarized the situation: “Pure dyes are still difficult or impossible to purchase”. With regard to the alternative of purifying dyes in the laboratory, Zollinger (2003) presented a literature review and stated that “Amazingly, the purification and analysis of dyes and pigments is not well documented in the scientific literature”. Nevertheless, he referred to several studies that showed that some subsidiary colours can be obtained by separating them from a dye mixture using various chromatographic methods.

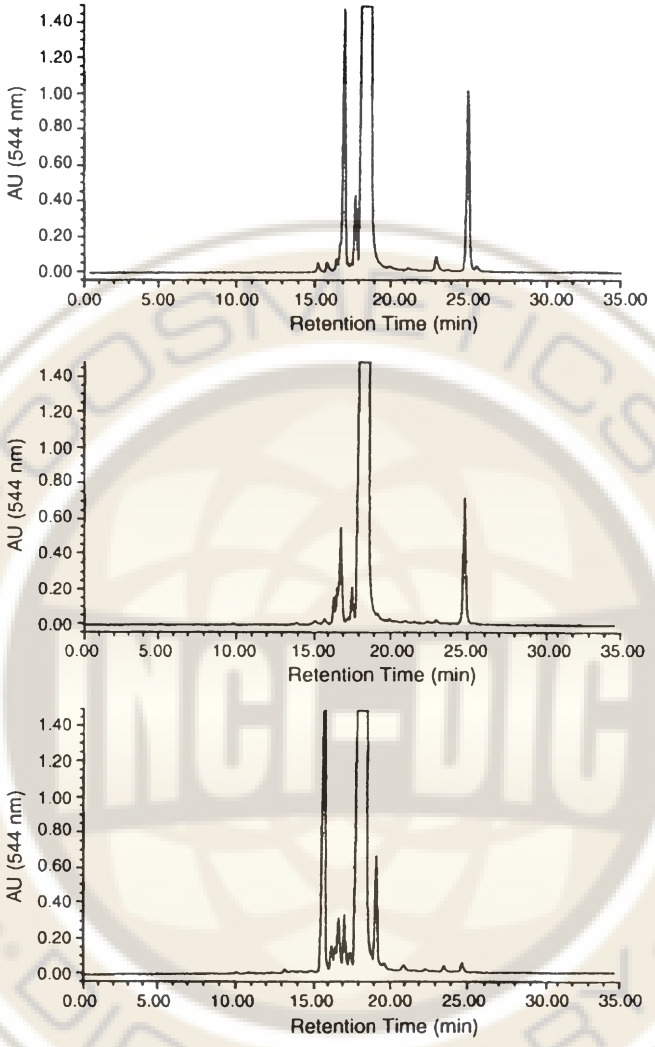


Figure 4.2.3 Chromatograms from reversed-phase LC of commercial batches of D&C Red No. 28 (CI 45410) obtained from three different sources (adapted from Weisz *et al.*, 2006).

One of the most effective methods for the separation and purification of preparative amounts of dyes and of their subsidiary colours is high-speed countercurrent chromatography (HSCCC) (Conway, 1990; Ito, 1996), both in its conventional and in its modified modes. A general approach to the separation of dyes by HSCCC is presented in Figure 4.2.4. The experimental conditions for this approach were given earlier (Weisz and Ito, 2000).

In its conventional mode, HSCCC was applied to the separation of a triphenylmethane dye (Fales *et al.*, 1985), and several azo-acid, direct, and disperse dyes (Freeman and Williard,

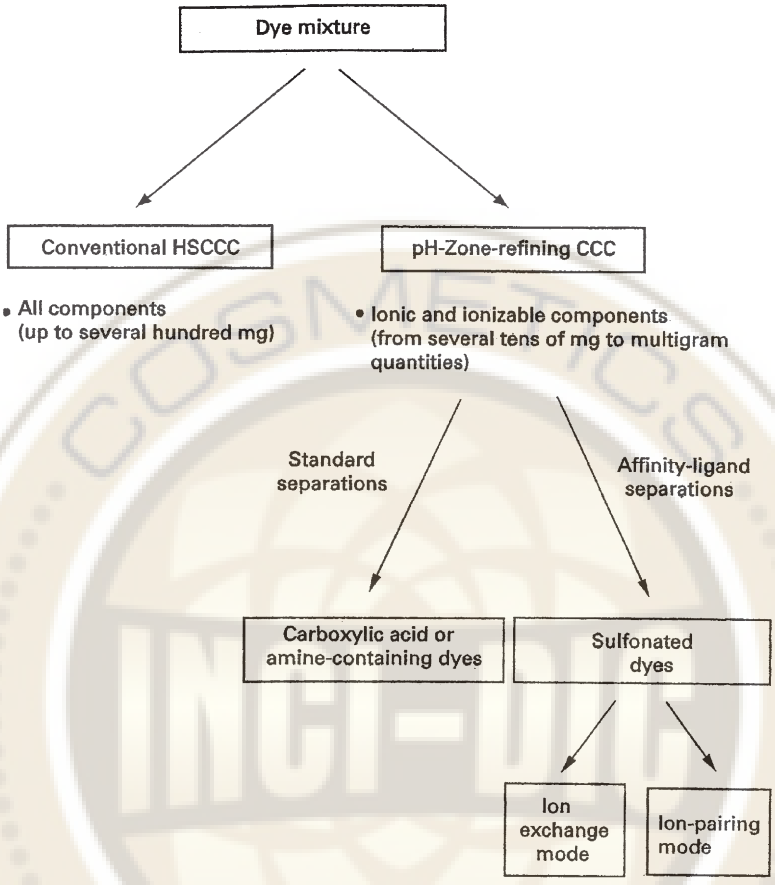


Figure 4.2.4 General approach to the preparative separation of dyes by high-speed countercurrent chromatography. (adapted from Weisz and Ito, 2000).

1986; Freeman *et al.*, 1988) in the 1980s. Zollinger (2003) included in his literature review the above-mentioned use of HSCCC (Freeman and Williard, 1986). His view that there is a scarcity of work on dye purification is apparently based on his overlooking the literature published since 1990. Specifically, HSCCC was later used for the separation and purification of components from other colouring agents, such as Sulforhodamine B (CI 45100) (Oka *et al.*, 1991), D&C Red No. 28 (CI 45410) (Weisz *et al.*, 1991), and Gardenia Yellow (Oka *et al.*, 1995). It was also used as a complement to preparative LC for the separation of a complex synthetic mixture of brominated tetrachlorofluorescein dyes (Weisz *et al.*, 1992). Conventional HSCCC was applied to the separation of quantities of dyes up to several hundred milligrams.

By contrast, a modified form of conventional HSCCC, pH-zone-refining CCC, developed in 1993, was applied from the outset to the separation of multi-gram quantities of dye mixtures (Weisz *et al.*, 1994c; Ito *et al.*, 1995) such as fluoran and xanthene dyes (Tables 4.2.5

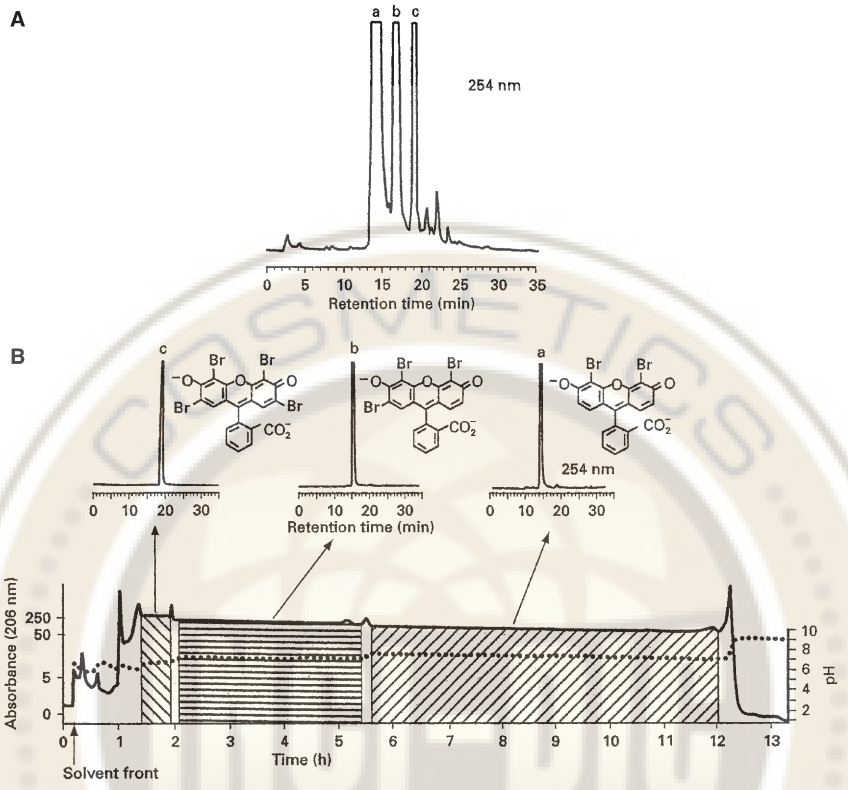


Figure 4.2.5 Separation of a 5-g test portion of D&C Orange No. 5 (CI 45370:1) using pH-zone refining CCC. (A) Reversed-phase LC analysis of the test portion, (B) pH-Zone-refining CCC chromatogram of the separation and reversed-phase LC chromatograms of the dyes isolated from the combined fractions from each hatched region (adapted from Weisz *et al.*, 1994c).

and 4.2.6) (Weisz, 1996). Figure 4.2.5 shows the separation of 5 g of D&C Orange No. 5 (CI 45370:1) using standard pH-zone-refining CCC and the LC analysis of the separated components. This separation resulted in pure (>99.5%) 4′,5′-dibromofluorescein (2.83 g), 2′,4′,5′-tribromofluorescein (1.52 g), and 2′,4′,5′,7′-tetrabromofluorescein (0.26 g).

A modified pH-zone-refining CCC procedure has been applied to the separation of gram quantities of the highly polar monosulfonated components of D&C Yellow No. 10 (CI 47005) (Table 4.2.7) and of some di- and trisulfonated components of Ki203 (also indexed as CI 47005) (Weisz and Ito, 2000). Gram quantities of other sulfonated dyes such as FD&C Yellow No. 6 (CI 15985) (Table 4.2.2) and D&C Green No. 8 (CI 59040) (Table 4.2.9) were also subjected to pH-zone-refining CCC purification (Ito and Ma, 1996; Weisz and Ito, 1996). Figure 4.2.6 shows the separation of 1.8 g of D&C Yellow No. 10 using affinity-ligand pH-zone-refining CCC in the ion-exchange mode (Figure 4.2.4). This separation resulted in 0.6 g of the 6′-monosulfonated isomer and 0.18 g of the 8′-monosulfonated isomer, both >99% pure.

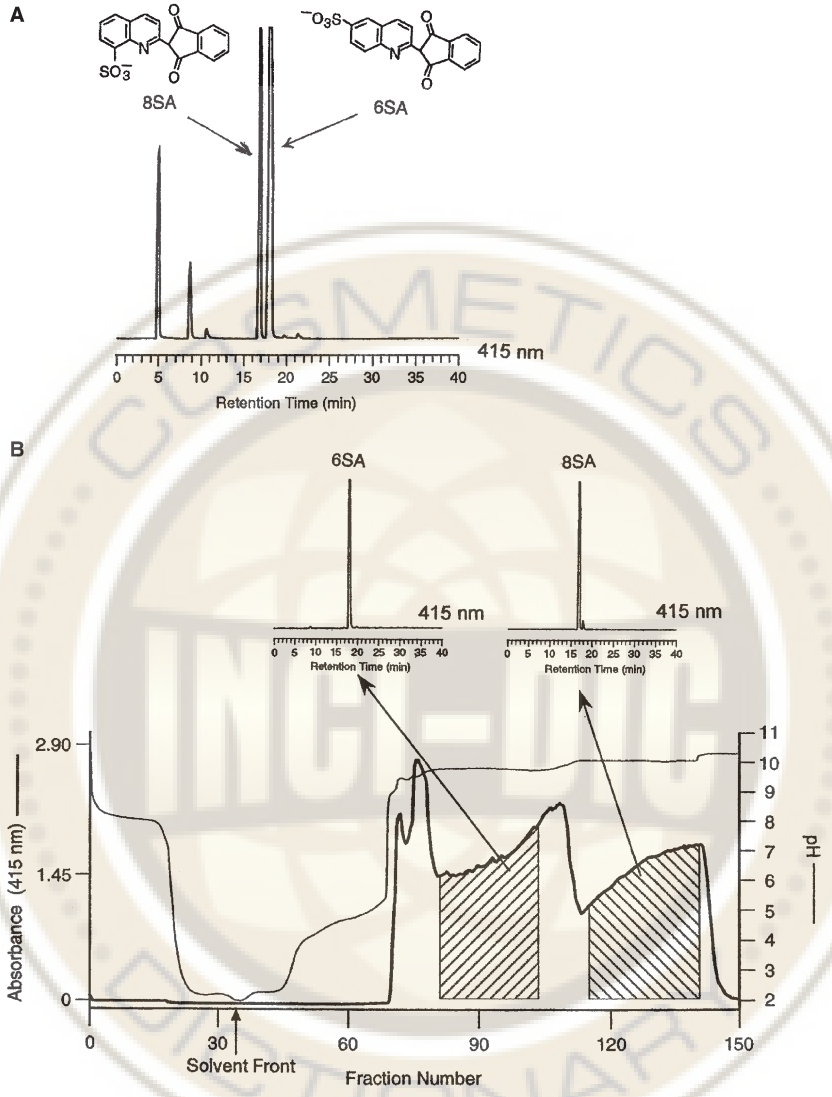


Figure 4.2.6 Separation of the main components of D&C Yellow No. 10 (CI 47005) using pH-zone-refining CCC. (A) Reversed-phase LC analysis of the certified colour additive, (B) pH-zone-refining CCC of the separation of a 1.8-g portion of colour additive and LC analyses of the separated components (adapted from Weisz *et al.*, 2001).

For use as reference material, dye contaminants can also be isolated from the dyes by HSCCC (Weisz *et al.*, 1998) or by other chromatographic methods. Alternatively, some can be produced synthetically (Weisz and Andrzejewski, 2003; Weisz, 1997), and others can be obtained by purifying a purchased material of technical grade (Andrzejewski and Weisz, 1999; Weisz *et al.*, 2004).

Analytical methods for determining colouring agent components

Analytical methods are continuously developed in order to implement FDA's colour additive batch certification program. These methods are used to enforce the limiting specifications for subsidiary colours, intermediates and side-reaction impurities listed in 21 CFR Parts 74 and 82. Some of the methods have been presented in detail by Leatherman *et al.* (1977) and Marmion (1991). Since those publications appeared, new technologies have been developed, analytical instrumentation has been improved, and, as a result, some of the described methods have been replaced. Some modern analytical techniques applicable to synthetic colour additives also have been described (Peters and Freeman, 1995). This part will focus on reviewing the methods for analyzing colour additives themselves and their components that have been published since the appearance of Marmion's (1991) book. The determination of colouring agents in cosmetic products is described further on.

Inorganic components

Triphenylmethane dyes (Table 4.2.4) are generally prepared in two steps: a condensation reaction that results in a colourless intermediate, a leuco base; and an oxidation reaction of the leuco base, resulting in the coloured material (Fierz-David and Blangey, 1949). The oxidizing agents used for the second step are typically manganese dioxide or a dichromate salt. Because traces of manganese and chromium may remain in the final product, specifications that limit the amount of these metals in the triphenylmethane colour additives are listed in the CFR. Two new methods based on X-ray fluorescence were developed for the determination of chromium (Hepp, 1996) and manganese (Hepp, 1998) in FD&C Blue No. 1 (CI 42090). The analyses are completely automated, require about 5 min per element, and can be performed in conjunction with lead and arsenic determinations in the same sample portion.

Mercury (calculated as elemental mercury) is limited to *"not more than 1 part per million"* in most certifiable colour additives listed in the CFR. A new method was developed that uses microwave digestion of the sample prior to the determination of mercury in colour additives by cold-vapor atomic absorption spectrometry (Hepp *et al.*, 2001). That method was later modified and extended to the determination of mercury in the recently approved colour additive D&C Black No. 2 (CI 77266) (Hepp, 2006), listed in 21 CFR Part 74 in 2005. It should be noted that this method of mercury determination cannot be applied to colour additives that contain iodine, such as FD&C Red No. 3 (CI 45430), D&C Orange No. 10 (CI 45425:1) and D&C Orange No. 11 (CI 45425), because digestion produces iodine, which penetrates Teflon tubing and subsequently binds mercury (Hepp *et al.*, 2001).

CFR specifications for most certifiable colour additives limit arsenic (calculated as elemental arsenic) to *"not more than 3 parts per million"*. A new method was developed that uses dry ashing followed by hydride-generation atomic absorption for the determination of arsenic at levels well below the specified limit (Hepp, 1999). That method has become the preferred one when quantification of arsenic is needed in certifiable colour additives.

Organic components

A capillary-electrophoresis (CE) method was developed for the determination of the main component and two subsidiary colours in FD&C Red No. 3 (CI 45430) (Evans III,

2003). The reference materials used for that method, 2′,4′,5′-triiodofluorescein, 2′,4′,7′-triiodofluorescein, and 2′,4′,5′,7′-tetraiodofluorescein, were obtained by pH-zone-refining CCC (Weisz *et al.*, 1994b).

An LC method was developed for the determination of the intermediates (2-chloro-4-nitroaniline and 2-naphthol) and an impurity (2,4-dinitroaniline) in the monoazo colouring agent D&C Red No. 36 (CI 12085) (Table 4.2.2) (Scher and Adamo, 1993).

An impurity found by LC in the monoazo colouring agent FD&C Red No. 40 (CI 16035) (Table 4.2.2) was identified by gas chromatography (GC) coupled with a mass spectrometry (MS) detector as 4-nitro-*p*-cresidine (2-methoxy-5-methyl-4-nitrobenzenamine) (Richfield-Fratz *et al.*, 1989). This impurity was found in all 28 certified batches of dye analyzed. This newly found impurity and other aromatic amines (*p*-cresidine and aniline) were quantified at parts per billion (μg/kg) levels in the colouring agent using an LC method.

Analytical methods were developed to determine, at μg/kg levels, the total quantity of benzidine (free aromatic amine and combined forms) in the colouring agents FD&C Yellow No. 5 (CI 19140) (Davis and Bailey, 1993; Prival *et al.*, 1993) and FD&C Yellow No. 6 (CI 15985) (Table 4.2.2) (Peiperl *et al.*, 1995). These methods have several components in common: the reduction (with sodium dithionite) of any combined benzidine present in the colour additive as azo and/or disazo dyes, to free benzidine; an extraction step and diazotization and coupling with pyrazolone-T (for FD&C Yellow No. 5) or with 2-naphthol-3,6-disulfonate (for FD&C Yellow No. 6), followed by LC analysis of the coupling product.

Various techniques were used for the determination of impurities (not specified in the CFR) in the colour additives D&C Red Nos. 21 and 27 (CI 45380:2 and CI 45410:1, respectively) (Table 4.2.5) and D&C Red Nos. 22 and 28 (CI 45380 and CI 45410, respectively) (Table 4.2.6). Thus, solid-phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS) was used to determine 2,4,6-tribromoaniline (TBA) in D&C Red Nos. 21 and 22 (Weisz *et al.*, 2004), and hexachlorobenzene (HCB) and 2-bromo-3,4,5,6-tetrachloroaniline (2BTCA) in D&C Red Nos. 27 and 28 (Andrzejewski and Weisz, 1999 and Weisz and Andrzejewski, 2003, respectively). LC methods were used to quantify 1-carboxy-5,7-dibromo-6-hydroxy-2,3,4-trichloroxanthone (HXCA) (Weisz, 1997) and the decarboxylated analog of tetrabromotetrachlorofluorescein (BCPX) (Weisz *et al.*, 2006) in D&C Red Nos. 27 and 28.

A method was developed that uses thin-layer chromatography (TLC) to separate colour components (specified in 21 CFR 74.1327 and 74.1328) in D&C Red Nos. 27 and 28 and then uses videodensitometry to quantify them (Wright *et al.*, 1997). Thus, the TLC-videodensitometry method has been developed for the *in situ* quantification of lower-halogenated subsidiary colours (such as the 2′,4′,5′-tribromo derivative) or of the ethyl ester of the main component (the 2′,4′,5′,7′-tetrabromo derivative) in multiple dye samples on the same analytical TLC plate. The total time for the analysis of five standards and four samples applied to each plate is at most 45 min. This technique replaced the classic method of quantifying the amount of dye in a spot/band by scraping the spot/band from the plate, dissolving the dye in a solvent, and analyzing the solution using ultraviolet/visible spectrophotometry (UV/VIS).

An LC method has been developed for the quantification of 2,4,6-triiodoresorcinol (I3R) and other specified intermediates and side-reaction products in the colouring agent FD&C Red No. 3 (CI 45430) (Mai *et al.*, 2006).

LC methods have also been developed for the identification and quantification of subsidiary colours in triphenylmethane colouring agents. Specifically, Matsufuji *et al.* (1998) determined five subsidiary colours in Brilliant Blue FCF (CI 42090), certifiable as FD&C Blue No. 1, and Tsuji *et al.* (2006) determined subsidiary colours in Fast Green FCF (CI 42053), certifiable as FD&C Green No. 3. The latter study compared TLC-UV/VIS and LC methods and recommended the LC method for the quantification of the subsidiary colours in Fast Green FCF.

MS was shown to be a useful technique in structural assignment of isomeric mono- and disulfonic acid components of the colouring agent Quinoline Yellow (CI 47005) (Table 4.2.6) (Weisz *et al.*, 2001, 2002). Quinoline Yellow may or may not be certifiable in the U.S. (as D&C Yellow No 10) depending on the proportion of these components.

Determination of colouring agents in cosmetic products

The previous part described the analysis of the colouring agents themselves. This section describes the isolation/separation of the colouring agents from cosmetic products (sample preparation) and the analysis of the isolated dyes.

Cosmetic products vary widely in their colouring agent contents. Decorative cosmetics contain the highest percentages of colouring agents, frequently present as mixtures of multiple colouring agents; therefore, such products—lipsticks, blushers, face powders, mascara, eye shadows, eyeliners, and nail polishes—are the subjects of most analytical studies. Their colouring agent content ranges between 1% and 25% (Gagliardi *et al.*, 1995; Schlossman, 2000; Wilkinson and Moore, 1982). By contrast, other types of cosmetics such as shampoos, bubble bath, creams, and oil-based lotions generally contain between 0.01% and 0.3% colour additives (DRAGOCOLOR, 2004) to colour the cosmetic itself.

In the majority of cases, the colouring agents present in a cosmetic product must be isolated from their matrices prior to their identification and quantification. The colouring agents in clear liquid cosmetics or in products that can be dissolved generally do not require such isolation as long as the UV/VIS spectrophotometric analysis of each additive can be achieved without interference from that of the others. Pawliszyn (2002) offers a comprehensive treatment of modern sample-preparation techniques. Although Pawliszyn's book pertains mainly to biological, food and environmental matrices, it is possible that the described techniques could be adapted to the analysis of dyes in cosmetic-product matrices.

Generally, the isolation of a colouring agent depends on the matrix that the cosmetic product is made of and on the solubility and other physical and chemical properties of the colouring agent. The solubility of cosmetic dyes in various solvents has been tabulated (Holtzman, 1962; Zuckerman, 1974; Marmion, 1993). Table 4.2.11 shows the types of colouring agents that may be used in selected cosmetic products.

The methods used most often for the separation of dye components from a product are liquid–liquid extraction (with two-phase solvent systems) and various adsorption techniques such as solid-phase extraction (SPE). Lakes and pigments cannot be separated by adsorption from the matrix; rather, fat and other components, such as soluble dyes, must be eliminated so the pigment can be collected as a residue. Details and examples of how to use these techniques for sample preparation have been previously presented (Bell, 1977;

Table 4.2.11

Types of colouring agents that might be used in selected cosmetic products

Cosmetic product	Colouring agent				
	Dye		Pigment		
	Water soluble	Oil soluble	Lake	Organic	Inorganic
Shampoo	✓				
Bubble bath	✓				
Makeup powder			✓	✓	✓
Lipstick		✓	✓	✓	✓
Nail polish and enamel			✓		✓
Cream	✓	✓			✓
Blusher		✓			✓
Eye shadow			✓		✓
Fragrance preparation	✓				

Leatherman *et al.*, 1977; Lehmann, 1986; Marmion, 1991; Otterstatter, 1999). Figure 4.2.7 (adapted from Etournaud and Aubort, 1983) shows a general method for the extraction of various colouring agents present in lipsticks, and it is also applicable to the extraction of colouring agents from fatty and non-fatty-based make-up and mascaras.

A limited number of official methods for the extraction of some colour additives from cosmetic preparations are available in several countries (e.g. Pharmaceutical Society of Japan, 2000; European Commission, 1999; Horwitz and Latimer Jr., 2005). Ashkenazi *et al.* (1991) reported a novel self-contained system, called dynamic column solid-phase extraction (DC-SPE), for extraction and concentration of five colouring agents used in food and cosmetics. Its efficacy was demonstrated by extraction and recovery of dyes spiked into commercial products.

Once the colouring agents are extracted from the cosmetic product, they can be further separated from each other, identified and quantified using methods developed for those purposes. Some such methods of analysis have been described above as applied to analyzing dyes *per se*.

There is a wealth of published work on the analysis of colouring agents in cosmetics, especially, lipsticks. Marmion (1991) reviewed the literature that was published from the mid-1960s through the 1980s. This part will highlight selected early studies that are now considered classics and will also review the methods published since Marmion's (1991) work, grouped by the analytical technique employed.

Thin-layer chromatography

TLC is one of the most common techniques of separating the multiple dyes within a mixture from each other (Touchstone, 1992; Gupta, 2003). Silica gel is the most widely used adsorbent, followed by alumina and microcrystalline cellulose. After the dye solution is spotted or streaked, the TLC plate is developed with a suitable solvent system and then it

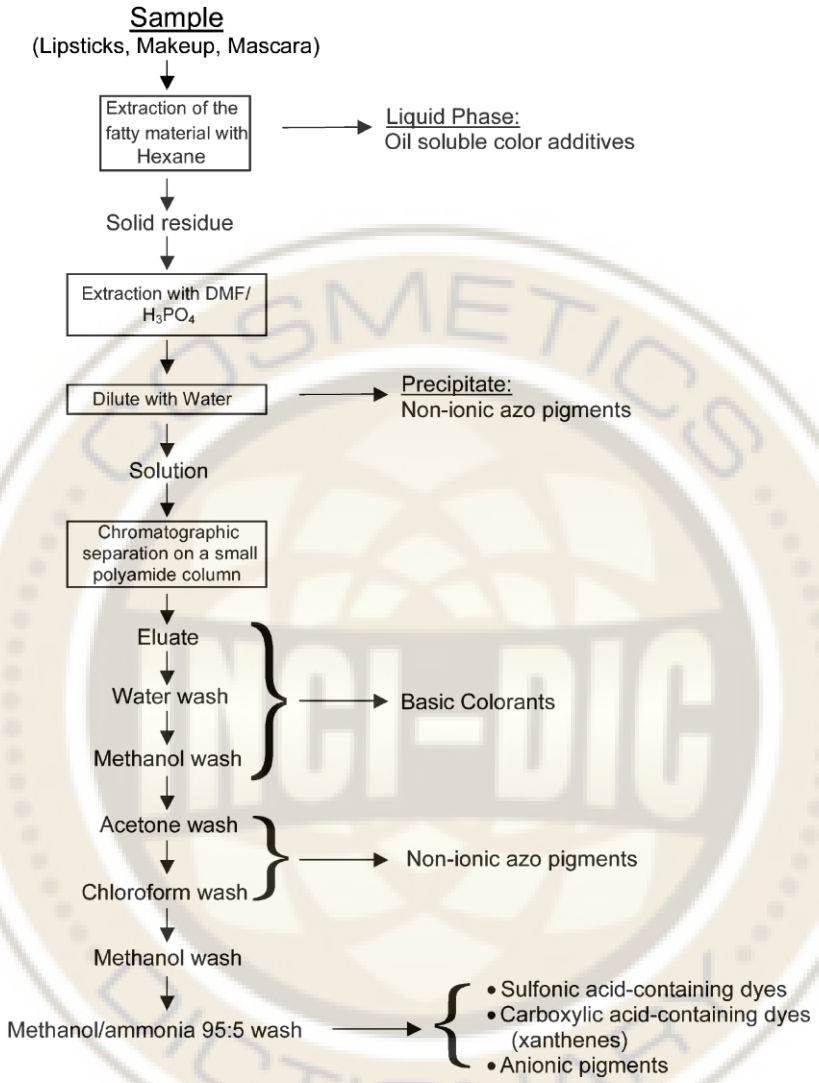


Figure 4.2.7 General method for extraction of colour additives present in lipsticks, fatty and non-fatty-based make-up and mascaras (adapted from Etournaud and Aubort, 1983).

is dried. Next, the separated dye bands are individually removed by scraping and the dyes are extracted from the adsorbent in a solvent. Finally, they are identified and quantified spectrophotometrically. The use of TLC was described for the separation of synthetic dyes (Wall, 2000; Cserhati and Forgacs, 2001a; Gupta, 2003) and natural pigments (Pothier, 1996; Cserhati and Forgacs, 2001b; Francis and Andersen, 2003).

As applied to cosmetic products, TLC was used primarily for the separation of colouring agents present in lipsticks. Thus, Silk (1965) developed a method whereby 15 colouring

agents used in lipsticks were analyzed without the need for a preliminary cleanup because the lipstick was directly applied to a warm silica gel TLC plate. The colouring agents were separated in two steps: elution with methylene chloride brings the fats and oils to the top of the plate and enables separation of the oil-soluble dyes and leaves the water-soluble dyes and the pigments at the origin; then the water-soluble dyes were separated from each other by elution with ethyl acetate:methanol:8.7% ammonium hydroxide (15:3:3). The separated colour bands were removed by scraping, and the dyes were extracted from the silica gel in a solvent and identified/quantified by UV/VIS. This method was also applied to the analysis of dyes in nail polishes and blushers (Leatherman *et al.*, 1977). By modifying the solvent systems used to develop the TLC plate, the method was extended to the separation of other oil-soluble, fluorescein-type, sulfonated, and basic colouring agents from lipsticks, blushers, make-up, and nail polish (Bell, 1977). In the above studies, the determination of the separated dyes was by UV/VIS. Sjoberg and Olkkonen (1985) analyzed synthetic organic colouring agents in lipsticks after separating them by direct application of the sample on the TLC plate and then determining the extracted colouring agents by LC. Direct application of lipsticks on the TLC plate combined with developing the plate with a series of selective eluants of increasing polarity, or use of solvent extraction with dimethylformamide followed by TLC, enabled Perdih (1972) to separate more than 150 dyes, 37 of which were found in lipsticks.

Gagliardi *et al.* (1995) presented the ratio to front (R_f) (also called retardation factor) values obtained for the 20 colouring agents most frequently encountered in cosmetic products, when they were developed on silica gel TLC plates with eight different solvent systems. Those authors also reported the volume of solvent needed for the elution of the colouring agents with three LC solvent systems. The described methods were applied to the analysis of the colouring agents present in 25 cosmetic products (lipsticks, mouthwash, toothpaste, eye shadows, and blushers). The authors considered the information obtained by the TLC analysis as preliminary, as screening tests, and complementary to the LC analyses.

Ohno *et al.* (1996) developed a reversed-phase TLC on octadecylsilica (C_{18}) gel method that complementarily employs four solvent systems to separate 45 water-soluble dyes, most of which are used for colouring cosmetics or food in Japan. They applied that method in combination with scanning densitometry to separate and identify dyes in a cosmetic lotion, a bath preparation, and imported candies. Another reversed-phase TLC-scanning densitometry method, which involves two developing solvent systems, has been used by Ohno *et al.* (2003) to separate and identify 11 oil-soluble cosmetic dyes. That method was applied to the separation and identification of colouring agents present in two kinds of nail polishes and other cosmetic products.

Liquid chromatography

Currently, LC combined with UV/VIS detection is the most used analytical technique for the determination of dyes and pigments. Ion-exchange LC uses strong anion-exchange columns (or weak anion-exchange columns for separation of azo dyes) and gradient elution with buffered eluants. Reversed-phase LC uses columns packed with short-chain alkyl-bonded silica (e.g. octyl (C_8), octadecyl (C_{18})), amino-bonded, and cyano-bonded

phases, or cross-linked polystyrene-divinylbenzene copolymer packing materials. Depending on the composition of the eluant (usually buffered to obtain an appropriate pH level and modified with an organic solvent such as methanol or acetonitrile), one can influence the affinity of the analyte for the column packing material by ion suppression or an ion-pairing mechanism, using either isocratic or gradient elution. The preferred method of detection is with a UV/VIS diode-array detector (DAD), which has the capability of simultaneously recording absorbance data from 190 to 800 nm. Another advantage of DAD is that matching with spectral libraries of previously analyzed standard compounds may identify eluted peaks.

Wegener *et al.* (1987) characterized 126 colouring agents through their retention time and UV/VIS spectra obtained with an ion-pair reversed phase LC system. A C_{18} bonded silica-packed column and gradient elution were used with an eluant made of distilled water and a dilute solution of tetrabutylammonium hydroxide (ion-pairing reagent) in aqueous methanol (pH 7.0 adjusted with phosphoric acid). The UV/VIS spectra were recorded with a DAD. The method was applied to the determination of the colouring agents used in 45 cosmetic products including lipsticks, nail polish, shampoos, foam bath, face powder, makeup, eye shadow, after-sun cream, and bar soap. Sample preparation included heating the sample with dimethylformamide (DMF) that contained 5% phosphoric acid, followed by filtration. The filtrate was diluted with aqueous 0.1 M tetrabutylammonium hydroxide and extracted twice with chloroform. The combined extracts were concentrated and analyzed by LC. This general extraction procedure was slightly modified, according to the type of cosmetic product processed (e.g. lipsticks had to be defatted by extraction of the acidic DMF solution with *n*-hexane, prior to filtration).

Rastogi *et al.* (1997) built a spectral library consisting of retention times and UV/VIS spectra of 130 organic cosmetic colouring agents using an ion-pair reversed-phase LC method. An analytical column packed with a polymeric material and gradient elution was used with a mobile phase that consisted of three solvents: citrate buffer containing tetrabutylammonium hydroxide as the ion-pairing reagent (pH 9.0 adjusted with concentrated ammonia), acetonitrile and tetrahydrofuran. The UV/VIS spectra were recorded with a DAD. The method was applied to the analysis of colouring agents present in 139 cosmetic products. Those products were collected from 52 manufacturers representing 12 European countries and the U.S. Among the products analyzed were lipsticks, nail polishes, mascara, eyeliner, eye pencil, eye shadow, shampoos, bath gel, body lotion, roll-on deodorant, skin tonic, aftershave, and beauty toner. Detailed sample preparation procedures were presented for the various cosmetic products, including an SPE method for the extraction of the colouring agents from cosmetics with complex matrices.

Several LC methods have been developed to identify xanthene dyes in lipsticks. Thus, Gagliardi *et al.* (1988) analyzed 99 lipsticks for the presence of xanthene dyes by a reversed-phase LC method. A C_{18} bonded silica-packed column and gradient elution were used with an eluant made of water (pH 3 adjusted with glacial acetic acid) and acetonitrile. Detection was performed with a variable-wavelength UV/VIS detector. The dyes were extracted from the lipsticks following the sample-preparation methods described by Etournaud and Aubort (1983) and Lehmann (1986).

Gagliardi *et al.* (1996) developed a method for the extraction, separation, identification, and quantification of the aminoxanthene dye, Rhodamine B (CI 45170), in cosmetic products

(prohibited as a cosmetic colouring agent in both the U.S. and the EU). Extraction methods are given according to the type of cosmetic (i.e. anhydrous or aqueous formulations). A reversed-phase LC method was developed that uses a C_{18} column and gradient elution with a mobile phase composed of acetonitrile and 0.1 M aqueous sodium perchlorate (pH 3 adjusted with perchloric acid). The UV/VIS spectra were recorded with a DAD. The method was successfully applied to the analysis of Rhodamine B in cosmetic products (e.g. shampoos, lipsticks, foam bath) which were spiked with the dye.

Scalia and Simeoni (2001) developed an assay of six xanthene dyes in lipsticks using an inverse supercritical fluid-extraction (SFE) method for sample preparation. The SFE extraction produced recoveries that were comparable to those with a conventional liquid–liquid extraction method. The separation of the extracted dyes was performed by LC with a cyanopropyl packed column, and eluted isocratically with aqueous sodium acetate (0.02 M, pH 4.5):acetonitrile:methanol (55:35:10). The spectra were recorded with a variable-wavelength UV/VIS detector.

Spectrophotometry

Simultaneous determination of up to four colouring agents in cosmetic products was demonstrated by applying various spectrophotometric techniques. In all cases, the colouring agents were isolated from the cosmetic product by liquid–liquid extraction with an ethanol/water/methylene chloride two-phase solvent system. The aqueous phase contained the dyes of interest and the interfering compounds remained in the organic phase. The absorbance of the colouring agents was measured directly in the aqueous phase or after isolation by SPE in Sephadex DEAE A-25 gel. Thus, solid-phase spectrophotometry was applied to the simultaneous determination of Quinoline Yellow (CI 47005) and Brilliant Blue FCF (CI 42090, certifiable as FD&C Blue No. 1) in perfumes, aftershave lotion and a shampoo gel (Capitan-Vallvey *et al.*, 1996). First-derivative spectrophotometry methods were used for the simultaneous determination of tartrazine (CI 19140), certifiable as FD&C Yellow No. 5, and Brilliant Blue FCF in cologne and *Eau de Cologne* (Capitan-Vallvey *et al.*, 1995) and of tartrazine and Sunset Yellow FCF (CI 15985), certifiable as FD&C Yellow No. 6, in shampoos, bath gel, and cologne (Capitan-Vallvey *et al.*, 1997a). A method that was based on partial least-squares multivariate-calibration UV/VIS spectrophotometry was applied to the simultaneous determination of Sunset Yellow (CI 15958), tartrazine (CI 19140), Brilliant Blue FCF (CI 42090), and Quinoline Yellow (CI 47005) in cologne, bath salts, aftershaves, deodorants, facial tonics, bath gels, and shampoos (Capitan-Vallvey *et al.*, 1997b).

Other methods

Desiderio *et al.* (1998) reported a quantitative method of analyzing dyes in lipstick using micellar electrokinetic capillary chromatography (MEKC) with diode array UV detection. This electrophoretic method was optimized for the separation of seven cosmetic dyes: Eosin Y (CI 45380), certifiable as D&C Red No. 22; erythrosine (CI 45430); cyanosine (CI 45410), certifiable as D&C Red No. 28; Rhodamine B (CI 45170); Orange II (CI 15510), certifiable as D&C Orange No. 4; Chromotrope FB (CI 14720); and tartrazine (CI 19140), certifiable as FD&C Yellow No. 5. The process was fast (3 min per separation).

The colouring agents in the lipstick samples were extracted using a modified shortened version of Etournaud and Aubort's (1983) general sample-preparation method. The method was successfully applied to the analysis of a lipstick sample in which Eosin Y and cyanosine were present.

Rodger *et al.* (1998) demonstrated the use of surface-enhanced resonance Raman scattering (SERRS) spectroscopy, without any separation procedure, to analyze dyes and pigments in lipsticks. Lipsticks smeared on glass and cotton surfaces required treatment with a surfactant, for example, poly(L-lysine), and silver colloid prior to the analysis. This *in situ* SERRS method was applied to six commercial lipstick samples. Discrimination between the samples and identification of some of the pigments present were achieved. The method is qualitative in nature and was suggested to have potential for forensic and quality-control applications.

SUMMARY

Colouring agents are central components of some cosmetic products. The number, nature, and official name of colouring agents permitted in cosmetic products vary across countries, though efforts toward international harmonization are in progress. This chapter provides an overview of the regulatory policies currently in place within the international community, and then it focuses on reviewing the analytical work that has been conducted on colouring agents over the past 15 years. Included are discussions of the rationale for analysis, the methods for preparation of reference materials, and the most recent technological developments applied to this field.

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4.3. Hair Dyes in Cosmetics. Regulatory Aspects and Analytical Methods

A. Chisvert[1*], A. Cháfer[2] and A. Salvador[3]

[1]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Ctra. San Vincente del Raspeig s/n, 03690 San Vincente del Raspeig, Alicante, Spain
[2]Deparment of Chemical Engineering, School of Engineering, University of Valencia, Valencia, Spain
[3]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain

INTRODUCTION

The white hair usually gives an old age appearance and many people (especially women) have dyed them for many years ago. More and more, not only women, but also men, change their hair colour to make themselves more attractive, and not only to hide white hair but also for changing their image, lightening the hair, removing the yellow look from grey hair, or enhancing the colour of the natural grey, and so on (Zviak and Milléquant, 2005a).

The aim of this section is to introduce the reader to the field of hair-dye products, describing the different types of products and the chemicals involved, and moreover to review the recent legislation data and the analytical chemistry procedures for quality control.

TYPES OF HAIR-DYE PRODUCTS

There are two main groups of hair-dye products according to the mechanism involved in producing the colour. One is based on a non-oxidation mechanism, whereas the other involves an oxidation mechanism.

According to how long-lasting they are, hair-dye products may be classified into three groups: temporary, semi-permanent, and permanent hair colours. The two formers are based into a non-oxidation mechanism, whereas the last is based mainly on oxidation reactions although other chemicals which impart progressive permanent hair colour do not follow this mechanism. They are discussed below.

*Corresponding author. E-mail: alberto.chisvert@ua.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

Temporary hair-dye products

These products are intended to effect a change, rapidly and simply, in natural or modified hair colour. The change must be temporary, so the colour can be easily removed at the first shampooing.

We can find a wide variety of these products like shampoos, hair sprays, lotions, foaming preparations, etc., which can be easily applied to the hair.

The chemicals responsible for the colour are not able to penetrate the cortex and are deposited on the surface of hair to give the colouring effect. Different chemicals are used in this type of products, such as azo compounds, triphenylmethane-based dyes, indoamines, and indophenols.

Semi-permanent dyes (see further on) are sometimes used at concentrations weak enough to avoid excessive duration and overintense shades.

Semi-permanent hair-dye products

These products are capable of effecting to some extent a change in the natural hair colour that fades progressively with cumulative shampoos.

The semi-permanent hair-dye products, available to professional hairdressers or directly to the consumer for home use, are often products to be applied to the wet hair after shampooing and rinsed out carefully after waiting for 10–30 min. They are available in all kinds of presentations: lotions, gels, creams, foaming preparations, etc.

The chemicals responsible for the colour are able to penetrate the cortex, and during shampooing they gradually diffuse out of the hair, thus disappearing after several shampooings.

These chemicals are the so-called nitro dyes, acid dyes and basic dyes. They are described below.

- *Nitro dyes:* This group of semi-permanent dyes are aromatic amines (to be exact, derivatives of *p*-phenylendiamine and *o*-phenylendiamine), aminophenols and aminophenyl ethers, which contain nitro groups. Some examples are shown in Figure 4.3.1.

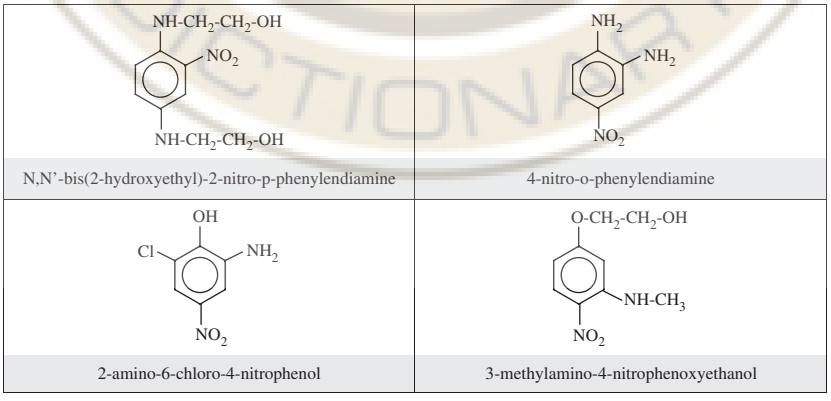


Figure 4.3.1 Example of chemical structures for four nitro dyes.

The different moieties that are attached to the ring play a crucial role on the shade of the dye, as can be seen in Table 4.3.1.

- *Acid dyes:* They contain acid groups like $–SO_3H$ or –COOH in their molecular structure. Examples of these semi-permanent dyes are azo acid dyes (e.g. Acid Orange 7) and anthraquinone acid dyes (e.g. Acid Violet 43). Figure 4.3.2 shows the chemical structure of two of these acid dyes.
- *Basic* (*or cationic*) *dyes*: They contain quaternary amine groups in their molecular structure. Examples of basic dyes are azo basic dyes (e.g. Basic Red 22) and cationic anthraquinone dyes (e.g. Basic Blue 47). Figure 4.3.3 shows the chemical structure of two of these semi-permanent dyes.

Permanent hair-dye products

Nowadays, these hair-dye products are by far the most frequently used hair colouring products and hold the dominant share of the market. They have sufficient durability so that the user only requires one application a month.

The formulation of almost all permanent hair-dye products uses the so-called oxidative hair dyes. These chemicals are often referred as intermediates, because most of them are uncoloured and produce coloured compounds through a process of oxidative condensation when mixed with oxidizing products just before use. In fact, the hair colour is formed when a dye precursor (usually referred to as base or primary intermediate) is oxidized by the oxidizing agent (also known as the developer) to produce an imine, which reacts rapidly with the so-called modifier (also known as coupler).

So, the oxidative hair-dye products consist of two bottles, one containing the oxidative hair dye (both base and coupler) and the other one containing the oxidizing agent which are mixed shortly before application to the hair.

Hydrogen peroxide is the most commonly used developer.

In general, bases and couplers are aromatic derivatives belonging to three major chemical families: aromatic diamines, aminophenols, and phenols (or also naphthols).

The primary intermediates are aromatic diamines and aminophenols where an amino or hydroxy group is positioned in *ortho* or *para* with respect to the amino group. Some of them are summarized in Table 4.3.2.

The modifiers are aromatic *m*-diamines, *m*-aminophenols, and *m*-polyphenols. Taken separately, all these modifiers yield only feeble colouring through oxidation; cooxidation of modifier mixes, too, yield only slight colouring (yellow, blond-beige). But when they are combined with primary intermediates they contribute developing highlights. Some of them are summarized in Table 4.3.3.

The addition of different moieties to the benzene ring or in the amino or in the hydroxy moieties plays a crucial role on the nature and intensity of the developed colour.

Besides primary intermediates having a benzene ring, pyrimidine and pyrazole derivatives have also been used as bases.

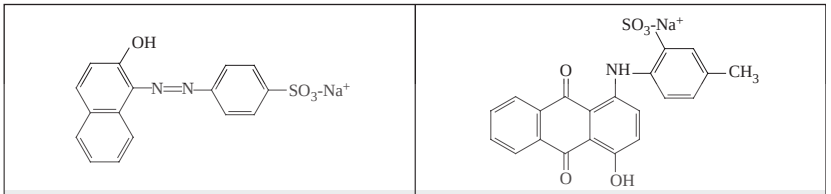
Sometimes, semi-permanent dyes are added to the oxidation dyes to provide highlight. They do not participate either in the oxidation itself or in the oxidative condensations.

Table 4.3.1

Some examples of nitro dyes (adapted from Zviak and Milléquant, 2005a)

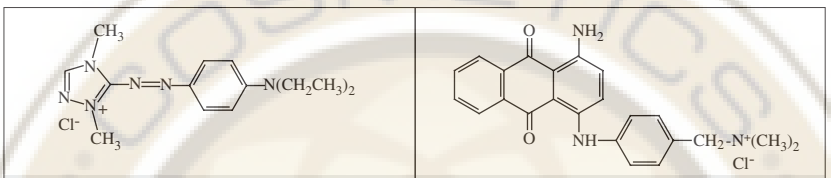
INCI name	Other names	Shade
p-Phenylendiamine nitro derivatives		
2-Nitro-*p*-phenylenediamine	1,4-Diamino-2-nitrobenzene	Orange-red
HC Red No. 7	1-Amino-2-nitro-4-*β*-hydroxyethylaminobenzene	Purple-red
HC Red No. 13	1-Amino-2-nitro-4-bis-(*β*-hydroxyethyl)aminobenzene	Red-violet
N,N′-bis(2-hydroxyethyl)-2-nitro-*p*-phenylenediamine	1,4-Bis-(*β*-hydroxyethyl)amino-2-nitrobenzene	Violet
HC Blue No. 2	1-*β*-Hydroxyethylamino-2-nitro-4-bis-(*β*-hydroxyethyl)aminobenzene	Violet-blue
HC Red No. 3	1-*β*-Hydroxyethylamino-2-nitro-4-aminobenzene	Purple-red
HC Violet No. 1	1-Amino-3-methyl-4-*β*-hydroxyethylamino-6-nitrobenzene	Purple-red
2-Chloro-5-nitro-*N*-hydroethyl-*p*-phenylenediamine	1-Amino-2-nitro-4-*β*-hydroxyethylamino-5-chlorobenzene	Purple-red
HC Blue No. 12	1-*β*-Hydroxyethylamino-2-nitro-4-(ethyl-*β*-hydroxyethyl)aminobenzene	Blue-violet
HC Violet No. 2	1-*γ*-Hydroxypropylamino-2-nitro-4-bis-(*β*-hydroxyethyl)aminobenzene	Violet-blue
o-Phenylendiamine nitro derivatives		
4-Nitro-*o*-phenylenediamine	1,2-Diamino-4-nitrobenzene	Yellow-orange
HC Yellow No. 5	1-Amino 2-*β*-hydroxyethylamino-5-nitrobenzene	Orange-yellow
Tetrahydro-6-quinoxaline	1,2,3,4-Tetrahydro-6-nitroquinoxaline	Orange-yellow
Aminophenol nitro derivatives		
4-Amino-3-nitrophenol	1-Hydroxy-3-nitro-4-aminobenzene	Orange
3-Nitro-*p*-hydroethylaminophenol	1-Hydroxy-3-nitro-4-*β*-hydroxyethylaminobenzene	Red
2-Amino-3-nitrophenol	1-Hydroxy-2-amino-3-nitrobenzene	Yellow-orange
HC Yellow No. 11	1-Hydroxy-2-*β*-hydroxyethylamino-5-nitrobenzene	Yellow
2-Amino-6-chloro-4-aminophenol	1-Hydroxy-2-amino-4-nitro-6-chlorobenzene	Red-orange
Aminophenyl ether nitro derivatives		
HC Yellow No. 4	1-*β*-Hydroxyethyloxy-2-*β*-Hydroxyethylamino-5-nitrobenzene	Yellow-green
2-Hydroxyethylamino-5-nitroanisole	1-Methoxy-2-*β*-hydroxyethylamino-5-nitrobenzene	Yellow-green
3-Methylamino-4-nitrophenoxyethanol	1-*β*-Hydroxyethyloxy-3-methylamino-4-nitrobenzene	Yellow-green

INCI: International Nomenclature of Cosmetic Ingredients



Acid Orange 7 | Acid Violet 43

Figure 4.3.2 Example of chemical structures of two acid dyes.



Basic Red 22 | Basic Blue 47

Figure 4.3.3 Example of chemical structures of two basic dyes.

Table 4.3.2

Some chemicals used as bases (or primary intermediates) in hair-dye oxidative products (adapted from Zviak and Milléquant, 2005b)

INCI name	Other names
Aromatic diamines	
p-Phenylenediamine	1,4-Diaminobenzene
Toluene-2,5-diamine	2-Methyl-1,4-diaminobenzene
2-Chloro-*p*-phenylenediamine	2-Chloro-1,4-diaminobenzene
N-phenyl-*p*-phenylenediamine	4-Aminodiphenylamine
Hydroxyethyl-*p*-phenylenediamine	1-β-Hydroxyethyl-2,5-diaminobenzene
N,*N*-bis-(2-hydroxyethyl)-*p*-phenylenediamine	1-Amino-4-bis-(β-hydroxyethyl)aminobenzene
Aminophenols	
o-Aminophenol	1-Hydroxy-2-aminobenzene
6-Amino-*m*-cresol	1-Hydroxy-5-methyl-2-aminobenzene
p-Aminophenol	1-Hydroxy-4-aminobenzene
4-Amino-*m*-cresol	1-Hydroxy-3-methyl-4-aminobenzene
p-Methylaminophenol	1-Hydroxy-4-methylaminobenzene

INCI: International Nomenclature of Cosmetic Ingredients

It should be pointed out that there are other permanent hair-dye products which produce progressive hair colouration (by reacting with the sulfur of hair keratin) which are not formulated with oxidative hair dyes. The so-called progressive hair-dye products produce gradually a darkening of the hair. Lead acetate and bismuth citrate act as active ingredients in this type of products.

Table 4.3.3

Some chemicals used as modifiers (or couplers) in hair-dye oxidative products (adapted from Zviak and Milléquant, 2005b)

INCI name	Other names
m-Diamines	
m-Phenylenediamine	1,3-Diaminobenzene
2,4-Diaminophenoxyethanol	1-β-Hydroxyethyloxy-2,4-diaminobenzene
2-Amino-4-hydroxyethylaminoanisole	1-Methoxy-2-amino-4-(β-hydroxyethylamino)benzene
2,6-Diaminopyridine	2,6-Pyridinediamino
2,6-Dimethoxy-3,5-pyridinediamine	2,6-Dimethoxy-3,5-diaminopyridine
2,6-Dihydroxyethylaminotoluene	1-Methyl-2,6-di-(β-hydroxyethylamino)benzene
m-Aminophenols	
m-Aminophenol	1-Hydroxy-3-aminobenzene
4-Amino-2-hydroxytoluene	2-Hydroxy-1-methyl-4-aminobenzene
2-Methyl-5-hydroxyethylaminophenol	2-Hydroxy-1-methyl-4-(β-hydroxyethylamino)benzene
3-Amino-2,4-dichlorophenol	1-Hydroxy-2,4-dichoro-3-aminobenzene
5-Amino-6-chloro-*o*-cresol	1-Hydroxy-6-chloro-2-methyl-5-aminobenzene
m-Polyphenols	
Resorcinol	1,3-Dihydroxybenzene
2-Methylresorcinol	1,3-Dihydroxy-2-methylbenzene
4-Chlororesorcinol	1,3-Dihydroxy-4-chlorobenzene
1,2,4-Trihydroxybenzene	1,2,4-Trihydroxybenzene
Hydroquinone	1,4-Dihydroxybenzene
1,5-Naphthalenediol	1,5-Dihydroxynaphthalene
1-Naphthol	1-Hydroxynaphthalene
1-Acetoxy 2-methylnaphthalene	2-Methyl-1-naphthyl acetate

INCI: International Nomenclature of Cosmetic Ingredients

REGULATORY ASPECTS

Some dermatological and/or carcinogenic side-effects have been attributed to some chemicals used as hair dyes (Gago-Dominguez *et al.*, 2001; Huncharek and Kupelnick, 2005). However, unlike other cosmetic ingredients, like UV filters, preservatives, and general colouring agents, there are no specific positive lists for hair dyes in the different legislations in force concerning cosmetic products in the principal markets (i.e. European Union (EU), United States (US) and Japan).

With regard to the EU framework, certain hair dyes seem to enjoy certain privileges that other cosmetic ingredients do not have. For instance, the EU Cosmetics Directive (Council Directive 76/768/EEC), in its Article 4, states that Member States must prohibit the marketing of cosmetic products containing colouring agents other than those listed in Annex IV or colouring agents listed in Annex IV used outside the conditions laid down therein; however, cosmetic products containing colouring agents intended solely to colour hair are the exception. In fact, if readers have a look at the European Inventory of Cosmetic Ingredients (Commission Decision 2006/257/EC), they will realize that a same chemical compound is named in a different way if it is used as a hair dye or if it is used to colour another part of

the body, and also the restrictions of use are different. For instance, according to the aforementioned inventory, the chemical tartrazine is named as Acid Yellow 23 when its function is for dyeing hair, whereas it is named as CI 19140 if its function is as a cosmetic colorant. No restrictions are stated in the first case, whereas for the second case it has to be used under the conditions laid down in Annex IV of the EU Cosmetics Directive.

Fortunately, this problem is changing. So, on the basis of increased bladder cancer risk caused by certain hair dyes and due to the considerable number of hair dyes used whose safety has not yet been assessed by public authorities, the European Scientific Committee on Consumer Products (SCCP) (formerly known as Scientific Committee on Cosmetic Products and Non-Food products intended for consumers (SCCPNFP)) established guidelines encouraging the cosmetic industry to submit dossiers for hair dyes containing chemical specifications and toxicological studies in order to establish a positive list for hair dyes in the near future (SCCP, 2002; SCCP, 2005). In the SCCP webpage (see references), opinions concerning different hair dyes can be consulted.

So, on the basis of the toxicological studies carried out by the SCCP (or by the former SCCPNFP), the European Commission included, in the 26th adaptation (Commission Directive 2002/34/EC) to the technical progress of the EU Cosmetics Directive, 60 hair dyes in Part 2 of Annex III, where some of them are provisionally allowed until 31 August 2006 and other until 31 December 2006. Conditions of use, that basically establish the maximum authorized concentration of use, other restrictions to fulfil when combined with other ingredients, statements to be printed on the label and obviously the deadline are stated in the aforementioned annex. After deadline, these provisionally authorized ingredients may be definitively allowed (and will then be moved to the corresponding Part 1), or on the contrary, they may be definitively prohibited if considered harmful to human health (and then will be moved to Annex II of the EU Cosmetics Directive), or they may be further maintained in an updated Part 2 for a given period of time if there are insufficient data for them to be allowed/prohibited definitely. So, for example, the colouring agent named CI 44045 when used as cosmetic colorant under Annex IV of the EU Cosmetics Directive is regulated according to Annex III under the name of Basic Blue 26 when used as hair dye, and thus it has to comply with the restrictions laid down therein. Also most of the aromatic amines and aminophenols used as hair dyes are regulated under the same Annex III.

Moreover, in the aforementioned adaptation of the EU Cosmetics Directive, 17 hair dyes were banned to be used in cosmetics and they are listed in Annex II of the EU Cosmetics Directive.

More recently, just before closing this book, the European Commission has banned by means of Commission Directive 2006/65/EC of 19 July 2006, the use of 22 hair dyes in cosmetics for which industry has not submitted any safety files at all. These substances are listed in Table 4.3.4. Some of the banned substances were in the aforementioned Part 2 of Annex III. According to the same directive, the deadline for hair dyes remaining in this Part 2 has been extended until 31 December 2007.

Moreover, lead acetate, which has been extensively used as active ingredient in progressive hair-dye products (i.e. those that produce gradually a darkening of the hair), was also banned when Commission Directive 2004/93/EC came into effect.

On the other hand, in the US the regulations are also different for certain hair dyes and other colouring agents. So, US regulations prohibit any cosmetic product to be marketed

Table 4.3.4

Hair dyes that are just banned in the EU framework according to Commission Directive 2006/65/EC

INCI[a] name
6-Methoxy-2,3-pyridinediamine (and its HCl salt)
2,3-Naphthalenediol
2,4-Diaminodiphenylamine
2,6-Bis(2-hydroxyethoxy)-3,5-pyridinediamine
2-Methoxymethyl-*p*-aminophenol
4,5-Diamino-1-methylpyrazole (and its HCl salt)
4,5-Diamino-1-((4-chlorophenyl)methyl)-1H-pyrazole sulphate
4-Chloro-2-aminophenol
4-Hydroxyindole
4-Methoxytoluene-2,5-diamine (and its HCl salt)
5-Amino-4-fluoro-2-methylphenol sulphate
N,*N*-diethyl-*m*-aminophenol
N,*N*-dimethyl-2,6-pyridinediamine (and its HCl salt)
N-cyclopentyl-*m*-aminophenol
N-methoxyethyl-*p*-phenylenediamine (and its HCl salt)
2,4-Diamino-5-methylphenetole (and its HCl salt)
1,7-Naphthalenediol
3,4-Diaminobenzoic acid
2-Aminomethyl-*p*-aminophenol (and its HCl salt)
Solvent Red 1
Acid Orange 24
Acid Red 73

[a]INCI, International Nomenclature of Cosmetic Ingredients.

in its framework if it contains a colouring agent that has not been previously approved by the Food and Drug Administration (FDA) (see Section 4.2). An exception to this prohibition is stated for synthetic organic (commonly referred to coal-tar) hair dyes provided the products display the following statement: *"Caution: This product contains ingredients which may cause skin irritation on certain individuals and a preliminary test according to accompanying directions should first be made. This product must not be used for dyeing eyelashes or eyebrows; to do so may cause blindness"* (FD&C Act, Section 601(a)), as well as adequate directions for conducting the aforementioned "preliminary test". As previously mentioned, this exception is only for coal-tar hair dyes, thus, hair dyes from other sources do not fall under this rule (21 CFR Section 70.3 (u)), and then they need to be approved by the FDA before use as hair dyes. An example of this would be lead acetate, bismuth citrate and henna (21 CFR Part 73).

Moreover, in US, there is a group of colouring agents to be used in cosmetics that need to be batch certified by the FDA prior addition to cosmetics (see Section 4.2); however, in case of hair-dye products they can contain a non-certified batch of a certifiable colouring agent if the cosmetic conforms to the aforementioned conditions of FD&C Act Section 601(a) (see FDA website concerning Colour Additives in references).

THE DETERMINATION OF HAIR DYES IN COSMETICS

Thus, bearing in mind the above-mentioned observations, it is obvious that the analytical control of hair-dye products is necessary in order to assure that the manufacturing process of these products is carried out correctly by the cosmetic industry in order to safeguard consumer safety.

If readers take a quick look at Section 2.1, which deals with the official analytical methods focused on cosmetics, they will quickly realize that there are not many official analytical methods dealing with hair dyes. According to the methods of analysis published by the EU Cosmetics Directive (European Commission, 1999), there is only an official method for the qualitative and semi-quantitative determination of 17 oxidative hair dyes, which are described in Table 4.3.5. The method proposes the extraction of the target analytes at pH 10 with ethanol by means of centrifugation, and afterwards the supernatant is run either in one- or two-dimensional thin-layer chromatography (TLC) plate. The identification and subsequent semi-quantitative determination is carried out by comparing the position and intensity of the obtained spots with those spots obtained with an appropriate range of concentration of reference substances.

Also, the international Association of Analytical Communities (AOAC) published three different methods to determine three hair dyes, namely toluene-2,5-diamine, *p*-phenylendiamine and pyrogallol. The two former ones were determined gravimetrically, whereas the latter was determined colorimetrically (Horwitz, 2005).

To our knowledge, no other official methods regarding this type of ingredients have been published.

Table 4.3.5

Substances determined qualitatively and semi-quantitatively by the official method of analysis proposed by the European Commission for the determination of hair dyes

INCI[a] name
o-Phenylenediamine
m-Phenylenediamine
p-Phenylenediamine
4-Nitro-*o*-phenylendiamine
2-Nitro-*p*-phenylendiamine
Toluene-3,4-diamine
Toluene-2,4-diamine
Toluene-2,5-diamine
o-Aminophenol
m-Aminophenol
p-Aminophenol
2,4-Diaminophenol
Hydroquinone
1-Naphthol
2-Naphthol
Pyrogallol
Resorcinol

[a]INCI, International Nomenclature of Cosmetic Ingredients.

Thus, one can deduce that there are no official methods that cover the determination of all the chemicals currently used as hair dyes in cosmetics, and moreover, those that already exist must be improved in order to perform a feasible quantitative determination, by using instrumental analytical techniques. Thus, the development of analytical methods focusing on the determination of hair dyes is necessary in order to safeguard consumer safety.

An up-to-date bibliographic search from 1980 to July 2006 using analytical chemistry databases revealed more than 30 publications focusing on hair-dye determination in hair-dye products. Table 4.3.6 gives a chronological summary of the experimental details of published papers on hair-dye determination. Those papers that do not deal with cosmetic samples are not included. It should be emphasized that the non-English publications have been reviewed on the basis of their respective abstracts, and thus, several data could be incomplete as shown by some blank cells in the aforementioned table.

Published papers on hair-dye analysis before 1980 are very scarce; nevertheless, there is evidence that a few papers were published before this date. Most of these papers are non-English publications, and although abstracts are written in English they do not contribute enough information. For this reason, because of the difficulty in reviewing these papers, they have been excluded from Table 4.3.6.

Different review articles in which bibliography concerning the analytical methods used for hair-dye determination in cosmetic products by liquid chromatography (LC) (Goetz *et al.*, 1985) and/or other chromatographic techniques (Kijima, 1991) were published a few years ago. Another review which covers the analysis of different cosmetics including hair dyes was also published by König (1985).

However, it should be emphasized that a detailed study of these published papers indicates that improvement is required, since although most of the published methods present good characteristics from an analytical point of view, most of them do not deal with the high number of hair dyes and mixtures currently used.

It is worth mentioning the interesting work carried out by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre of the European Commission aimed at proposing an LC method for oxidative hair-dye determination as official. The IRMM Analytical Chemistry Unit headed by Dr. Rodríguez performed a separation of the most commonly used hair-dye chemicals by means of LC coupled with a diode-array detector (DAD) and studied the influence of light, temperature and antioxidants on standard solutions of hair dyes (Pel *et al.*, 1998). In a second paper (Vincent *et al.*, 2002a), this unit studied the influence that common matrix compounds could cause on the qualitatively and quantitatively determination of different hair dyes, and established an extraction procedure based on a three step liquid–liquid extraction by using *n*-heptane as extractant. Under the chosen conditions, the matrix components are removed whereas hair dyes are not extracted. In a subsequent paper, the method was applied to synthetic and spiked samples, and was validated for four representative hair dyes taken as model, which represent the three major classes of oxidative hair dyes (i.e. aromatic amines, aminophenols and phenols); moreover, they appear regularly in the composition of commercial formulations. In a subsequent paper (Vincent *et al.*, 2002b), the method was further optimized and validated by quantitatively determining nine representative oxidative hair dyes. After in-house validation, a peer review exercise was carried out during September 2002 on the determination of 16 oxidative hair dyes.

Table 4.3.6

Published papers until June 2006 on hair-dye determination in cosmetic products (chronological order)

Authors	Target hair dyes[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
Choudhary (1980)	4MmPD, pPD, T25D	Not specified	Sample is mixed with ethy acetate, and NaCl is added.After stirring, supernatant is injected	GC-FID
Bernabei *et al.* (1981)	2A4NP, 2NpPD, 4NoPD, oPD			LC
Ohshima *et al.* (1982)	2NpPD, 4NoPD, mAP, oAP, pAP, RES			TLC-UAD, benzene:ethyl acetate as eluent
Ehlers (1983)	1NP, RES, T25D, T26D	Creams and lotions	Sample is mixed with ascorbic acid, dissolved in MeCN and shaken. Next it is diluted with borate buffer pH 9 and filtered	LC-UV/VIS, Ph column at 40°C with gradient MeCN:5 mM methanesulphonic acid:acetate buffer pH 4.2
Bhuee *et al.* (1984)	pPD		Sample is diazotized with *N*-1-naphthylethylenediamine and extracted with $CHCl_3$ in presence of BaO	UV/VIS
Sardas *et al.* (1985)	4A2NP, 4MmPD, mPD, pPD, T24D, T25D		Sample is dissolved in MeOH	LC-UV/VIS, C_{18} column MeCN:water
Tokuda *et al.* (1986)			Sample is dissolved in MeOH containing ammonium thioglycolate	GC-NPD
Zarapkar *et al.* (1988)	pPD		Sample is treated with catechol, resorcinol or benzaldehyde in order to form the corresponding Schiff's base	UV/VIS

Fanali (1989)	mAP, mPD, oAP, oPD, pAP, pPD	Creams	Sample is dissolved in water:EtOH:THF containing ascorbic acid	ITP-CD, PTFE capillary tube and KOH/picolinic acid pH 5.4 (containing α and β-cyclodextrin) and 5 mM acetic acid as leading and terminating electrolytes, respectively
Andrisano *et al.* (1994a)	4EmPD, pMAP, pPD	Creams	Sample is mixed with aqueous 0.85% H_3PO_4 containing 0.5% Na_2SO_3. Then, it is stirred, diluted with same solvent and filtered. On-line post-column photochemical derivatization is made	LC-DAD, C_{18} column with MeCN:sodium heptasulphonate buffer pH 4.5 containing 5 mM 1,8-diaminooctane as mobile phase
Andrisano *et al.* (1994b)	4AoC, 2EpPD, mAP	Creams	Sample is mixed with aqueous 0.85% H_3PO_4 containing 0.5% Na_2SO_3. Then, it is passed through a SCX SPE cartridge	SPE+LC-DAD, C_{18} column with MeCN:sodium heptasulphonate buffer pH 1,8-diaminooctane as mobile phase
Wu *et al.* (1997)	mAP, mPD, oAP, oPD, pAP, pPD, PYC, PYG, RES	Creams and lotions	Sample is dissolved in EtOH	GC-FID
Maffei-Facino *et al.* (1997)	2A5NP, HCR3, HCY2		Sample is dispersed in water, adjusted to pH 1 with HCl and extracted with hexane. The aqueous residue is extracted with $CHCl_3$	GC-MS, 5% phenyl/95% dimethyl polysiloxane capillary column. Carrier gas: He
Wang and Chen (1998)	2NpPD, 4MmPD; mPD, NPpPD, pPD, T25D			DPV, carbon paste electrode in acetate buffer 4.74
Scarpi *et al.* (1998)	BBl26, BBl99, BBr17, BR76, DBl1, DBl3, DV1, HCBl2, MAB139	Not specified	Sample is diluted with EtOH:water	LC-DAD, C_{18} column with gradient 5 mM heptanesulphonic acid sodium salt: MeCN as mobile phase

(*Continued*)

Table 4.3.6 (Cont.)

Authors	Target hair dyes[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
Wang and Kuo (1999)	1NP, RES	Lotions, creams	Sample is diluted with EtOH:water mixture and extracted with ethyl ether. The extract is dried and dissolved in EtOH:water	LC-UV/VIS, C_{18} column with octylammonium orthophosphate in EtOH:water as mobile phase
Lin *et al.* (1999)	4A2HT, 4MmPD, HQ, mAP, mPD, oAP, oPD, pAP, pMAP, pPD, PYC, RES, T25D	Not specified	Sample is diluted in the running buffer	MECK-UV/VIS, 13 mM HTAB in 50 mM phosphate pH 5 as running buffer
Peng *et al.* (2000)	mPD, oPD, pPD		Sample is agitated with ethyl acetate, and NaCl is added. Next it is centrifuged, and the organic layer diluted with ethyl acetate	GC-FID, PEG column with N_2 as carrier gas
Penner and Nesterenko (2000)	HQ, mAP, oAP, pAP, pPD PYC, RES	Creams	Sample is mixed with MeOH:water solution, stirred, diluted and passed through C_{18} cartridge	LC-UV/VIS, hypercross-linked polystyrene column with MeCN:0.3 M ammonium phosphate pH 5.15
Shao *et al.* (2001)	26DP, mAP, pAM, pPD, RES			LC-UV/VIS, C_{18} column with MeOH:0.1% triethylamine containing 20 mM acetate buffer pH 5.2 as mobile phase
Rastogi (2001)	1NP, HQ, mAP, mPD, oAP, oPD, pPD, RES, T24D, T25D, T26D, T34D		Sample is mixed with MeCN and 25 mM phosphate buffer pH 6 containing 0.1% sodium heptanesulphonate and 0.0% sodium ascorbate, and heated. Then, is sonicated and filtered	LC-DAD, amide-bonded silica column at 25°C and gradient MeCN:25 mM phosphate buffer containing 0.1% sodium heptanesulphonate

Lawrence *et al.* (2001)	pPD	Not specified	Sample is diluted with water, and added to measurement cell containing phosphate buffer pH 7	SWV, glassy carbon electrode as working electrode
Vincent *et al.* (2002a)	mAP, mPD, pPD, RES	Creams and shampoos	Sample is dissolved in MeOH:tetraborate buffer pH 8 containing sodium ascorbate. Then, it is extracted three times with *n*-heptane	LC-DAD, C_{18} column kept at 48°C and gradient MeOH:0.05 M ammonium acetate buffer pH 5.9 as mobile phase
Vincent *et al.* (2002b)	1NP, 24DPE, HBNHpPD, HQ, mAP, pPD, RES, T24D, T25D	Creams and shampoos	Sample is dissolved in MeOH:tetraborate buffer pH 8 containing sodium ascorbate. Then, it is extracted three times with *n*-heptane	LC-DAD, C_{18} column kept at 48°C and gradient MeOH:0.05 M ammonium acetate buffer pH 5.9 as mobile phase
Li *et al.* (2004)	1NP, 2NP, 4A2HT, mPD, NNDpPD, oAA, oPD, pPD, T24D		Sample is extracted with ethyl acetate by means of sonication	GC-MS
Zhou *et al.* (2004)	HQ, oAP, pAP, oPD, pPD, RES	Not specified	Sample is mixed with mobile phase, sodium sulphite is added and sonicated. Afterwards, it is filtered and diluted with the same solvent	LC-DAD and LC-CLI, C_8 column with MeOH:0.1% triethylamine containing 25 mM acetate buffer and 5 mM TBAB at pH 6 as mobile phase
Liu *et al.* (2004)	1NP, 2NP, 2MA, 4A2HT, 4MA, mAM, mPD, oPD, pPD, PYC, RES, T24D, T34D		Sample is solved in ethyl acetate and sonicated	GC-MS
Di Gioia *et al.* (2005)	pPD	Creams	Sample is suspended in THF and derivatized with benzaldehyde (5 h at 70°C) to yield the corresponding diimine. Then filtered and diluted with THF	GC-MS, 5% phenyl/95% dimethyl polysiloxane capillary column. Carrier gas: He
Zhu *et al.* (2005)	HQ, mAP, oPD, pAP, pMAP, pPD, RES, T25D			GC-MS

(*Continued*)

Table 4.3.6 (Cont.)

Authors	Target hair dyes[a]	Type of matrix	Sample preparation[b]	Analytical technique[b]
Wang and Huang (2005)	4AmC, mAP, mPD, oAP, oPD, pAP, pMAP, pPD, T25D, T34D	Not specified	Sample is diluted with water and filtered	LC-DAD, C_8 column with MeOH:15 mM triethylamine adjusted to pH 8 with H_3PO_4 as mobile phase MECK-UV/VIS, 55 mM CTAC in 50 mM borate buffer pH 9.2 as running buffer

[a]Key abbreviation: 1NP, 1-naphthol; 24DPE, 2,4-diaminophenoxyethanol; 26DP, 2,6-diaminopyridine; 2A4NP, 2-amino-4-nitrophenol; 2A5NP, 2-amino-5-nitrophenol; 2EpPD, 2-ethoxy-*p*-phenylenediamine; 2MA, 2-methoxyaniline; 2NA, 2-nitroanilina; 2NpPD, 2-nitro-*p*-phenylenediamine; 2NP, 2-naphthol; 4A2HT, 4-amino-2-hydroxytoluene; 4A2NP, 4-amino-2-nitrophenol; 4AmC, 4-amino-*m*-cresol; 4AoC, 4-amino-*o*-cresol; 4EmPD, 4-ethoxy-*m*-phenylenediamine; 4MA, 4-methoxyaniline; 4MmPD, 4-methoxy-*m*-phenylenediamine; 4NoPD, 4-nitro-*o*-phenylenediamine; 6HI, 6-hydroxyindole; BBl26, Basic Blue 26; BBr17, Basic Brown 17; BBl99, Basic Blue 99; BR76, Basic Red 76; DBl1, Disperse Blue 1; DBl3, Disperse Blue 3; DV1, Disperse Violet 1; HBNHpPD, hydroxypropyl-bis-(*N*-hydroxyethyl-*p*-phenylenediamine); HCBl2, HC Blue No. 2; HCR3, HC Red No. 3; HCY2, HC Yellow No.2, HQ, hydroquinone; mAP, *m*-aminophenol; MAB139, Melangi Acid Black 139; mPD, *m*-phenylenediamine; NNDpPD, *N*,*N*-diethyl-*p*-phenylenediamine; NPpPD, *N*-phenyl-*p*-phenylenediamine; oAA, *o*-aminoanisole; oAP, *o*-aminophenol; oPD, *o*-phenylenediamine; pAP, *p*-aminophenol; pMAP, *p*-methylaminophenol; pPD, *p*-phenylenediamine; PYC, pyrocathecol; PYG, pyrogallol; RES, resorcinol; T24D, toluene-2,4-diamine; T25D, toluene-2,5-diamine; T26D, toluene-2,6-diamine; T34D, toluene-3,4-diamine.

[b]Symbol "−" means coupling between techniques, and symbol "+" means sequentially applied techniques. Key abbreviation: C_{18}, octadecylsilica; CD, conductivity detector; CLI, chemiluminiscence inhibition; CTAC, cetyltrimethylammonium chloride; DAD, diode-array detector; DPV, differential-pulse voltammetry; EtOH, ethanol; FID, flame ionization detector; GC, gas chromatography; HTAB, hexadecyltrimethylammonium bromide; ITP, isotachophoresis; LC, liquid chromatography; MeCN, acetonitrile; MEKC, micellar electrokinetic chromatography; MeOH, methanol; MS, mass spectrometry; NPD, nitrogen–phophorus detector; PEG, polyethyleneglycol; Ph, phenyl-bonded silica; SCX, strong cation exchanger; SPE, solid phase extraction; SWV, square wave voltammetry; TBAB, tetrabutyl ammonium bromide; THF, tetrahydrofuran; TLC, thin layer chromatography; UAD, ultraviolet absorption densitometry; UV/VIS, ultraviolet/visible spectrometry.

Participating laboratories were: Department of Analytical Chemistry of L'Oréal, Department of Analytical Chemistry of University of Valencia and the IRMM-Analytical Chemistry Unit. The evaluation meeting of the peer review exercise took place in November 2003, and it was concluded that the method was suitable without any restriction for the identification and quantification of 11 hair dyes, whereas for the other 5 hair dyes the method was suitable with restrictions (Vincent *et al.*, 2004). The target 16 hair dyes are listed in Table 4.3.7. To our knowledge, at this moment the method is being evaluated by the Directorate-General Enterprise of the European Commission before its publication in the Official Journal of the European Communities.

Apart from the paper published by Scarpi *et al.* (1998) which deals with semi-permanent triphenylmethane, azo and anthraquinone dyes, all the others deal mainly with oxidative hair dyes. This is because oxidative hair dyes seem to be the most interesting topic at the moment. Also, as many triphenylmethane, azo and anthraquinone dyes are used as cosmetic colorants, the papers published on this matter and reviewed in Section 4.2 will be useful to determine these substances in hair-dye products.

To our knowledge there are no published methods focusing on the determination of lead acetate and bismuth citrate in progressive hair-dye products.

Analytical techniques employed for hair-dye determination

As is shown in Table 4.3.6, different analytical techniques have been employed for the determination of hair dyes. Nevertheless, chromatographic techniques, such as thin layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), and other chromatography-related techniques, have been by far the most extensively used techniques. This is due to the fact that there are more chemicals used as hair dyes, and they are usually mixed in the hair-dye products, thus it is not an easy task to determine them by

Table 4.3.7

Hair dyes determined without and with restrictions by the LC method proposed by the IRMM as reference method for analysing hair-dye products

Without restrictions	With restrictions
1-Naphthol	2,4-Diaminophenoxyethanol
2-Methylresorcinol	*p*-Aminophenol
Hydroquinone	*p*-Phenylenediamine
Hydroxypropyl-bis-(*N*-hydroxyethyl-*p*-phenylenediamine)	Resorcinol
m-Aminophenol	Toluene-2,5-diamine sulphate
o-Aminophenol	
m-Phenynelediamine	
o-Phenynelediamine	
Toluene-2,4-diamine	
6-Hydroxyindole	
2-Methyl-5-hydroxyethylaminophenol	

direct measurement without a previous separation step. Moreover, it is also necessary to take into account that matrix components might also interfere (Vincent *et al.*, 1999).

Next, the different analytical techniques focusing on hair-dye determination in hair-dye products will be discussed.

Chromatographic techniques

Among the chromatographic techniques, LC with octadecyl silica (C_{18}) columns and ultraviolet/visible (UV/VIS) spectrometry detection or by using a diode-array detector (DAD) has been widely employed. It is worth mentioning the paper published by Zhou *et al.* (2004), in which the inhibition effect on luminol-dimethylsulfoxide chemiluminiscence was exploited as a detection system.

The fact that LC can deal with low-volatile compounds makes it the technique of choice for hair-dye determination. However, substances containing amino groups (as is the case of most of the hair dyes) give broad and asymmetric chromatographic peaks on C_{18} columns that prevent their quantification, which can be avoided by using other types of columns, like hypercross-linked polystyrene (Penner and Nesterenko, 2000) or amide-bonded silica (Rastogi, 2001) columns. Also, different modifiers can be added to the mobile phase to improve the shape of the chromatographic peaks. Thus, Shao *et al.* (2001), Zhou *et al.* (2004) and Wang and Huang (2005) used triethylamine, whereas Andrisano *et al.* (1994a, 1994b) added 1,8-diaminooctane. An ion-pairing reagent, like methanesulphonic acid, was also added in order to improve resolution in some cases, as shown in Table 4.3.6.

Despite the restrictions of GC, because it is not very suitable for high hydrophilic substances due to high polarity and low volatility and/or low thermostability, GC has sometimes been used for hair-dye determination. Different detectors like flame ionization (FID), nitrogen-phosphorous (NPD) and mass spectrometry (MS) have been used. MS has the advantage of enabling accurate on-line identification. Derivatization reactions have been proposed in some cases (Di Gioia *et al.*, 2005).

Concerning TLC, this chromatographic technique has traditionally been employed for identification purposes, by scraping the spots from the plate and off-line measuring other characteristics like, for example, their UV spectra. Quantification methods employing a densitometric detector on the plate have also been published (Ohshima *et al.*, 1982).

Finally, as shown in Table 4.3.6, other chromatography-related techniques, as is the case of electrophoretic techniques like isotacophoresis (ITP) (Fanali, 1989) and micellar electrokinetic chromatography (MEKC) (Lin *et al.*, 1999; Wang and Huang, 2005) have also been employed to determine hair dyes but to a lesser extent.

Spectroscopic techniques

Individually, this group of techniques has not been used much. They are usually used as detectors for chromatographic techniques. As mentioned previously, the reason is that it is difficult to measure directly due to the interference produced by each hair dye on the measurement of the others, and also the interferences produced by matrix components, which make it necessary to perform a previous separation step. Nevertheless, Bhuee *et al.* (1984) proposed an UV/VIS methodology to determine *p*-phenylenediamine after diazotation with *N*-1-naphthylethylenediamine, and Zarapkar *et al.* (1988) also determined

p-phenylenediamine after the formation of the corresponding Schiff's base by using catechol, resorcinol or benzaldehyde.

Electrochemical techniques

There are only two published papers dealing with the determination of hair dyes by means of electrochemical techniques. Differential-pulse voltammetry (DPV) using carbon paste electrode (Wang and Chen, 1998) or square wave voltammetry (SWV) using glassy carbon electrode (Lawrence *et al.*, 2001), have been used to determine different hair dyes successfully.

Consideration on sample preparation

Sample preparation depends on different aspects, like type of sample, target analytes and the analytical technique to be used, which is reflected in the different sample preparation procedures described in Table 4.3.6.

Usually the sample is dissolved in an appropriate solvent. Sometimes, if total solubilization of the sample is not complete, slightly cloudy homogeneous solutions are obtained due the presence of few insoluble substances, which can be removed by means of filtration or centrifugation. Using sonication can help to leach analytes from the matrix if necessary.

On the other hand, leaching of target analytes could also be interesting in order to avoid interferences from matrix, or on the contrary, matrix compounds can be removed by means of an extraction procedure. Vincent *et al.* (1999) studied the influence of different matrix compounds on the determination of hair dyes, and an extraction procedure based on a three-step extraction with *n*-heptane was finally proposed to remove matrix constituents from sample solutions. The same authors claimed that solid-phase extraction (SPE) procedures did not give more satisfactory results. However, Andrisano *et al.* (1994b) proposed passing samples through a strong cation exchange (SCX) cartridge for clean-up purposes.

Finally it should be emphasized that in case of oxidative hair dyes, these chemicals are sensitive to air oxidation, and thus their preservation with antioxidant compounds is very important. Authors have employed different chemicals, such as ammonium thioglycolate (Tokuda *et al.*, 1986), ascorbic acid (Fanali, 1989; Rastogi, 2001; Vincent *et al.*, 2002a, 2002b) and sodium sulphite (Andrisano *et al.*, 1994a, 1994b; Zhou *et al.*, 2004) with preservation purposes.

SUMMARY

On one hand there are no positive lists for hair dyes in any of the three main legislations in force in the three principal markets regarding cosmetic products, i.e. EU, US and Japan.

However, on the other hand, different side-effects have been found to be caused by some of these compounds, which all goes to show it is recommendable to establish positive lists. Nevertheless, there are some restrictions of use for some of the hair dyes in the EU Cosmetics Directive, and also there are others that have been prohibited.

Taking into account all the above-mentioned remarks, it is evident that there is a need for the analytical control of hair-dye products; notwithstanding, there are no official analytical methods to cover all the chemicals used as hair dyes. Nevertheless, there are more than 30 published papers in which analytical methodologies to determine hair dyes in cosmetic products are proposed. However, although most of these published methods have good characteristics from an analytical point of view, most of them do not deal with the extensive number of hair dyes and mixtures currently used. The validated LC method proposed by the Institute for Reference Materials and Measurements of the Joint Research Centre of the European Commission, which aims to become a reference method for hair-dye determination deserves special notice.

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– 5 –

Preservatives in Cosmetics. Analytical Methods

5.1. Preservatives in Cosmetics. Regulatory Aspects and Analytical Methods

S. Polati, F. Gosetti and M.C. Gennaro*

Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro" Via Bellini 25 G, 15100-Alessandria (Italy)

PRESERVATIVE USE IN COSMETICS

Cosmetic composition and stability are of particular relevance in our daily life, since the average adult is estimated to use at least seven different cosmetic products a day. Therefore, antimicrobial chemicals that prevent microorganisms from growing play a crucial role. We should bear in mind that not only can a cosmetic product be damaged by microorganisms but also by other external agents, such as air (if it contains easily oxidative ingredients) and sunlight (in the event it contains light-sensitive ingredients). Thus, using compounds with antioxidant and light absorbent properties can help lengthen the life of cosmetics. However, according to the definition provided by the European Union Cosmetics Directive 76/768/EEC, preservatives are only considered as *"(…) substances which may be added to cosmetic products for the primary purpose of inhibiting the development of micro-organisms in such products"*, and therefore, antioxidant and light absorbent substances are not considered as such.

*Corresponding author. E-mail address: mariacarla.gennaro@mfn.unipmn.it

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

This chapter mainly focuses on antimicrobial and antioxidant preservatives. Cosmetics containing UV filters were previously discussed in-depth in Section 3.1. On the other hand, cosmetics containing antimicrobial preservatives with other specific functions than protect the cosmetic product, like for example, as antidandruff agents in shampoos or as deodorants, are reviewed in Section 8.8.

ANTIMICROBIAL PRESERVATIVES

The addition of antimicrobial preservatives to cosmetic and toiletry products is necessary to prevent bacterial or fungal growth and is essential in all formulations that contain water. Not only can microbial contamination cause organoleptic alterations, such as offensive odours and changes in viscosity and colour, but it can also put the consumer at risk. Therefore, the concentration of preservatives added plays a key role and two concentration levels are given: MIC (minimum inhibitory concentration), which is the lowest concentration of preservatives for which there is no evidence of organism growth and MBC (minimum biocidal concentration), which is the lowest preservative concentration ensuring no microorganism growth within the first 24 h.

However, the use of preservatives can also produce other undesirable effects, which can appear either after the first application or after years of cosmetic use. These effects range from mild skin irritation to estrogenic activity, and recently the possibility that they could potentially induce human breast tumours has been discussed (Richter and Barth, 1992; Harvey, 2003; Jensen *et al.*, 2005).

Thus, the correct choice of preservatives must guarantee the absence of undesirable side-effects while at the same time meet the requirements of guaranteeing the absence of bacterial action.

On the other hand, certain preservatives, such as 5-bromo-5-nitro-1,3-dioxane (bronidox) and 2-bromo-2-nitropropane-1,3-diol (bronopol) can decompose liberating nitrosating agents that can react with aliphatic amines like diethanolamine (DEA), triethanolamine (TEA) and monoethanolamine (MEA), which are commonly added to shampoos and other personal hygiene products, to form carcinogenic nitrosamines that are readily absorbed through the skin (Havery and Chou, 1994; Matyska *et al.*, 2000).

Moreover, recently has been shown that the preservative known as triclosan reacts with chlorine of tap water yielding chloroform gas (Rule *et al.*, 2005), which can cause depression, liver problems and, in some cases, cancer, what has special relevance in the event of toothpastes.

On the basis of such evidences or suspicions, on occasion of the first Nordic Round Table in March 2005 in Gothenburg, specialists in toxicology, microbiology, epidemiology and dermatology discussed the risks and benefits of preservatives added to cosmetic products and agreed that the challenge is to simultaneously protect the products from spoilage and the consumers from potentially pathogenic microorganisms without running the risk of adverse side-effects.

The rapid development of cosmetic products has forced the authorities of different countries to regulate cosmetic products to ensure consumer safety. As described in Section 1.1, the three main regulatory systems on cosmetic products which have influenced different

countries around the world, are the European Union (EU) Cosmetics Directive (Council Directive 76/768/EEC), the federal Food, Drug and Cosmetic Act (FD&C Act) and the Fair Packaging and Labelling Act (FPLA) drawn up by the Food and Drug Administration (FDA) in United States, and finally the Pharmaceutical Affairs Law (PAL) adopted in Japan, which regulate everything concerning cosmetic products, obviously including preservatives, in all the countries subject to their corresponding legislation.

Owing to the continuous increase in the variety of raw materials used in cosmetics and the lack of comprehensive official guidelines for cosmetic preservative use, it is frequently necessary to rely on pharmaceutical and food regulations and on pharmacopoeias. But while for food and pharmaceuticals the microbiological limits are defined, for cosmetic products the only requirement according to the EU Cosmetics Directive is that they *"(…) must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use (…)"*. Such differences are often a matter of disagreement. While health authorities would like cosmetic companies to fulfil the "golden standards" imposed by pharmaceutical regulations and recommendations through the steps of manufacturing, filling and testing, it is also true that cosmetics are not meant to be ingested or injected in the human body nor are they to be used for therapeutic purposes. The scientific information on cosmetic preservatives that provide good protection against microbial contamination is scarce, since most biocide studies deal with antibiotics for human treatment. Thus, for microbiologists to achieve effectiveness against microorganisms and avoid direct toxicity for consumers, they must work within a narrow range of preservative concentrations.

With regard to preservatives and contents authorized in the EU, Annex VI of the EU Cosmetics Directive, which appeared for the first time in the 8th adaptation of the technical progress of the original directive, rules on the use of preservatives in cosmetic products (Commission Directive 86/199/EEC). This annex indicates the list of preservatives that cosmetics can contain, makes reference to the relative admissible concentration and also indicates the substances that may be present in concentrations other than those reported in the annex when used for other different specific purposes. In particular, Part 1 of the Annex VI lists all the permissible preservatives and indicates the maximum concentration, limitations, requirements and label warnings, while Part 2 lists the preservatives that are allowed provisionally. The list does not include essential oils or alcohols, used in cosmetic formulations, which could contribute to product preservation. On the other hand, Annex II of the aforementioned directive contains substances that can no longer form part of cosmetic products, among which can be found different substances formerly used as preservatives, such as hexachlorophene, bithionol, different halogenated salicylanilides, 4-tert-butylphenol and sodium pyrithione.

As has been pointed out, according to Annex VI of the EU Cosmetics Directive, different compounds used as preservatives can be used in other conditions than those regulated in this annex when they are used for other specific purposes, and, consequently, manufacturers are free to add as much as they want when using them for purposes other than to protect cosmetics. So, for example, triclosan content must not exceed 0.3% of the finished product when used as a preservative but can freely exceed this limit when used to prevent the growth of microbes on the body, like for example in deodorants.

On the other hand, the 7th amendment to the Cosmetics Directive (Directive 2003/15/EC) incorporates a new concept based on microbiological criteria, *i.e.* the Period After Opening (PAO) that should not be confused with the expiration date. PAO expresses the time during

which the cosmetic product can be used after opening without causing any harm to the consumer. It is indicated on the label by an open-jar symbol accompanied by the recommended number of months within which the product should be used after opening, followed by the abbreviation M (months). Obviously, PAO is not relevant where there is no risk of microbial deterioration, as in the case of sealed pressurized containers or single-use products.

In the United States framework, according to FDA, for safety purposes, the total number of aerobic microbes per gram should be lower than 500 CFU (bacterial colony forming units) for eye-area products and lower than 1000 CFU/g for other products (Hitchins *et al.*, 1998). However, unlike EU Cosmetic Directive, although a microbiological limit is established, there is not a positive list of preservatives, but in Title 21 of the Code of Federal Regulations (21 CFR) published by the FDA, there is a short list of substances that are banned or restricted in cosmetics, including different compounds formerly used as preservatives, such as hexachlorophene, mercury compounds, bithionol and halogenated salicylanilides.

Finally, in Japanese legislation there is also a positive list of preservatives, similarly to the EU, but where the permissible substances and authorized contents are quite different. Moreover, there is a list showing different substances whose use is banned in cosmetics, including hexachlorophene, bithionol and halogenated salicylanilides among others, like in the aforementioned two legislations.

In general, permissible preservatives and their maximum allowed concentrations are related to the specific cosmetic product, because it is also necessary to consider their compatibility with all the ingredients of the often intricate cosmetic formulas, their antagonism or synergism. In this sense, for instance, Bianco-Prevot *et al.* (2000) deal with the issue giving the example of formulations like shampoos, which consist of a micellar surfactant solution. In such formulations the partition process taking place between the aqueous and the micellar phase could affect the preservative action, because the concentration is lowered in the aqueous fraction, in which bacteria activity is predominant. Therefore, the correlation between antimicrobial activity and micelle/water partitioning of preservatives must be considered.

All in all, an ideal preservative should be effective at low levels, tasteless, odourless, colourless, effective against both Gram-positive and Gram-negative bacteria and fungi (yeast and mould), useable in both hot and cold phases, effective and stable in the pH range from 2.5 to 10.5, acceptable for the regulatory agencies worldwide and cost-effective.

Efforts have also been made to avoid the need of preservatives, such as, for example, irradiating the cosmetic products or preserving them in special packaging. There is also an increase in the use of naturally derived preservatives, like for example, benzoic acid extracted from cranberries, which would avoid using toxic impurities that may remain as residues coming from the raw materials used in synthesis.

CLASSES OF ANTIMICROBIAL PRESERVATIVES

The wide range of preservatives in use can be grouped into chemical classes, according to the predominant functional group present in their molecular structure.

Organic acids and their salts and esters

Examples of organic acids used as preservatives are: dehydroacetic acid, sorbic acid, salicylic acid, propionic acid and their salts; also benzoic acid and its salts and alkyl esters.

In addition, 4-hydroxybenzoic acid is widely used together with its alkyl esters (generally known as parabens) and salts. The most common are methyl-, ethyl-, propyl- and butyl- esters and are generally known as methylparaben, ethylparaben, etc. The antimicrobial activity of these chemicals increases as the carbon number of the alkyl chain increases, while, by contrast, their water solubility decreases in parallel. The antimicrobial activity of the individual esters is generally selective, so their mixtures offer powerful antimicrobial activity against an extremely broad spectrum of microorganisms. This is the reason why a lot of cosmetics and skin care products are preserved with parabens both in Europe and the United States.

We can also consider carbamate-derivatives within this class, like for example, iodopropynylbutylcarbamate.

Aldehydes and formaldehyde-releasing preservatives

The most commonly used aldehyde is formaldehyde known as oxymethylene or formalin. It is a cheap preservative, more easily soluble in water than in oil and fat, used in watery concoctions like shampoo, conditioner, shower gel, liquid hand wash and bubble bath. However, formaldehyde, which is a colourless, pungent-smelling, irritant gas, can cause watery eyes, burning sensations in the eyes and throat, nausea, difficulty in breathing, allergies and at higher concentrations may trigger headaches and asthma attacks (Agner *et al.*, 1999). Furthermore, formaldehyde is a cancer suspect and is banned from cosmetics and toiletry in Sweden and Japan. The safety of formaldehyde was reviewed in 1984 by a panel of scientific experts commissioned by the Cosmetic, Toiletry and Fragrance Association (CTFA). The conclusion, which recognized that the study suffered from insufficient data, was that cosmetics containing more than 0.2% cannot be considered as safe (Bergfeld *et al.*, 2005). According to the EU Scientific Committee on Consumer Products (SCCP), formerly known as Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) intended for consumers, formaldehyde is safe when used "at low levels". As a result, cosmetics and toiletries sold within EU may contain formaldehyde as a preservative with the following restrictions: oral hygiene products must not contain more than 0.1%, while cosmetics and toiletries to be applied externally must not contain more than 0.2%, except in aerosol sprays where it is forbidden. These limitations apply when formaldehyde is used as a preservative but when used in nail hardeners, it can reach concentrations of 5%.

It should also be considered here as those preservatives which in aqueous polar solvents act by releasing formaldehyde, such as imidazolidinyl urea, diazolidinyl urea, sodium hydroxymethylglycinate and benzylhemiformal. All these are regulated on the basis of their releasing formaldehyde content, expressing the maximum allowed concentrations as the concentration of formaldehyde released (0.2%). So for example, benzylhemiformal 0.15% corresponds to 0.044% formaldehyde, sodium hydroxymethylglycinate 0.5% to 0.215%,

imidazolidinyl urea 0.6% to 0.186% (SCCNFP, 2002). Also 2-bromo-2-nitropropane-1,3-diol (also known as bronopol) and DMDM hydantoin act by releasing formaldehyde and are the principal constituents of largely diffused commercial formulations of preservatives in toiletry products. Also, Quaternium-15 (also known as azonium-adamantane chloride or methenamine-3-chloroallylochloride), used in many cosmetics and pharmaceutical preparations, is a formaldehyde releaser preservative.

Moreover, glutaral (glutaraldehyde), the action of which is activated on adding a slightly alkaline buffer (Danielson and Thompson, 1996) is efficacious against all types of microorganisms and is widely used for cold sterilisation of clinical, surgical and dental instruments.

Amines, amides, pyridines and benzalkonium salts

Triclocarban, hexamidine, dibromohexamidine, dibromopropamidine, chlorhexidine, benzalkonium chloride, methenamine, Quaternium-15, sodium pyritione, cetylpiridinium chloride, 3-chloro-*N*-methyl propionamide and dichloro-*N*-methyl acetamide are just a few examples of this numerous group of preservatives.

Phenols and derivatives

Phenols are also often used as preservatives. The most commonly used are: phenol, *o*-phenylphenol, *p*-chloro-*m*-cresol, *o*-cymen-5-ol, chlorophene and triclosan, among others.

Alcohols and derivatives

In this class we find phenoxyethanol, phenoxyisopropanol, dichlorobenzyl alcohol, benzyl alcohol, chlorobuthanol, 2-bromo-2-nitropropane-1,3-diol.

Imidazole derivatives

This category comprises compounds such as climbazole, DMDM hydantoin, imidazolidinylurea and diazolidinyl urea, among others.

Other preservatives

Other compounds commonly used as preservatives in cosmetics, which escape from the above mentioned categories are, for example, bromo-5-nitro-1,3 dioxane (bronidox), methyldibromo glutaronitrile, methylchloroisothiazolinone and methylisothiazolinone.

ANTIOXIDANT PRESERVATIVES

This type of compounds are able to inhibit reactions promoted by oxygen, thus avoiding the oxidation and rancidity of commonly used fats, oils, waxes, surfactants, perfumes, etc. in cosmetics. They are usually reducing agents and free radical scavengers.

Different compounds have been used as antioxidants in cosmetics: citric acid, gallic acid and its esters, nordihydroguaiaretic acid, thioctic (or lipoic) acid and its derivative dihydrolipoic acid, glycolic acid are just a few examples, but without any doubt the most commonly used are BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole). However, BHA and BHT, also employed for long-term preservation of food products and pharmaceuticals, can cause secondary effects, as proven by recent studies. In particular BHT, when applied to skin, is reported to damage lung tissues (Lanigan and Yamarik, 2002) and BHA to induce underdevelopment of the reproductive system (Jeong *et al.*, 2005).

Vitamins (see Section 8.6), often added to cosmetic formulations, act as antioxidant preservatives due to their general antioxidant properties towards free radicals. Examples are retinol (vitamin A) and its precursor β-carotene, tocopherol (vitamin E) and ascorbic acid (vitamin C). Moreover, vitamin derivatives, such as retinyl acetate, retinyl palmitate, ascorbyl palmitate, magnesium ascorbyl phosphate and tocopheryl acetate among others, are also employed as antioxidant agents.

Likewise, different antioxidants of natural origin can be used. Even though their preservation ability is generally lower than that of synthetic compounds, natural products generally offer the advantage of not inducing secondary toxic effects as was mentioned above. Flavonoids make up a family of compounds reported to have health benefits through antioxidant action. Recently, there has been an increase in the use of these polyphenolic compounds in cosmetics. Antioxidant and preservative properties, able to prevent rancidity of oils and fats in creams, have been assigned to flavonoids and plant extracts, such as grapefruit, rosemary and essential oils of tea and thyme (Lupo, 2001).

ANALYTICAL METHODS USED TO DETERMINE PRESERVATIVES

As mentioned before, in order to protect consumer health and ensure compliance to existing government regulations, there is a practical demand for the development of analytical methods to identify and determine preservatives in cosmetics both accurately and sensitively.

In spite of the relatively high number of preservatives used in cosmetics, and their restrictions, there are not many official analytical methods to control all these substances in these products. As mentioned in Section 2.1, within the EU framework there are several official analytical methods proposed to determine some preservatives in cosmetic products, which are described in different EU Directives and grouped in a book edited by the European Commission (1999). Table 5.1.1 summarizes all these official methods regarding preservative substances.

Table 5.1.1

Official analytical methods for preservatives determination in cosmetic products in the EU framework

Target preservatives	Type of cosmetic sample	Sample preparation[a]	Analytical technique[a]	EU directive
- (free) formaldehyde	Whichever	(a) Sample is heated to 60 °C with pentane-2,4-dione in presence of ammonium acetate to form 3,5-diacetyl-1,4-dihydrolutidine. Finally, is extracted with 1-butanol and absorbance is measured	(a) UV/V (in absence of formaldehyde donors)	82/434/EEC
		(b) Sample is extracted with CH_2Cl_2 in hydrochloric acid medium. The extract is purified by solid phase extraction, and immediately injected. Formaldehyde is derivatized with pentane-2,4-dione in a post-column reactor system	(b) LC-UV/V (in presence of formaldehyde donors)	
- hexachlorophene	Whichever	(a) Sample is mixed with Celite AW and H_2SO_4, and dried. After cooling, is extracted with ethyl acetate, centrifuged, evaporated and re-dissolved in ethyl acetate prior running	(a) TLC (for identification)	83/514/EEC
		(b) Sample is mixed with H_2SO_4, Celite AW and acetone. After drying, is transferred to a glass column and eluted with ethyl acetate. Then, it is methylated by using diazomethane, diluted with hexane and injected into the GC system	(b) GC-ECD (for quantitation)	
- thiomersal - phenylmercuric salts	Whichever	(a) Sample is suspended in water, heated and NaCl added. After cooling is centrifuged, filtered, acidified with H_2SO_4 and extracted with dithizone in CCl_4. The extract is dried, re-dissolved in CCl_4 and run	(a) TLC (for identification)	83/514/EEC
		(b) Digest the sample with HNO_3, H_2SO_4 and $KMnO_4$ by heating. Dilute with water, and mix with tin oxide in acidic medium to reduce all ionic mercury to the metallic state	(b) AAS (for quantitation)	

- chlorobutanol	Whichever (except aerosol)	Sample is dissolved in ethanol, and injected into the GC system	GC-ECD	85/490/EEC
- sulphites - disulphites	Whichever (containing aqueous or alcoholic phase)	Sample is mixed with water and methanol, and distilled in H_3PO_4 medium, by collecting the distillate into a mixture of H_2O_2/water. Afterwards is titrated with NaOH	Titration	85/490/EEC
- sodium iodate	Rinse-off	(a) Sample is extracted with water, filtered, diluted with water and run	(a) TLC (for identification)	85/490/EEC
		(b) Sample is diluted with water, filtered and injected into LC system, by using both reversed-phase and anion exchange columns connected in series	(b) LC-UV/V (for quantitation)	
- benzyl alcohol	Whichever	(a) Sample is dissolved in $CHCl_3$, diluted with ethanol and TLC run	(a) TLC (for identification)	93/73EEC
		(b) Sample is extracted with methanol by heating, and after cooling, is centrifuged and re-extracted again with methanol. The combined extracts are injected into the LC system	(b) LC-UV/V (for quantitation)	
- hexamidine - dibromohexamidine - dibromopropamidine - chlorhexidine	Whichever	Sample is dissolved in 5 mM 1-heptanesulphonic acid in methanol at pH 3.5, sonicated, filtered and injected into the LC system	LC-UV/V	93/73/EEC
- benzoic acid - 4-hydroxybenzoic acid - sorbic acid - salicylic acid - propionic acid	Whichever	(a) Sample is extracted with acetone in acidic medium, by heating if necessary, filtered, diluted with water and adjusted to pH 10. After adding $CaCl_2$, is cleaned-up with ethyl acetate. Afterwards is acidified and extracted with ethyl acetate, evaporated, re-dissolved in ethyl acetate and run	(a) TLC (for identification)	95/32//EEC
		(b1) Sample is acidified with H_3PO_4 and diluted with ethanol. Filter through membrane filter and inject into the GC system.	(b1) GC-FID (for propionic acid quantification)	

(*Continued*)

Table 5.1.1 (Cont.)

Target preservatives	Type of cosmetic sample	Sample preparation[a]	Analytical technique[a]	EU directive
		(b2) Sample is acidified with H_2SO_4 and extracted with methanol/water mixture by heating. After cooling is run	(b2) LC-UV/V (for the rest)	
- Phenoxyethanol - Phenoxyethanol - phenoxyisopropanol - methylparaben	Whichever	(a) Sample is extracted with acetone in acidic medium, by heating if necessary, filtered, diluted with water and adjusted to pH 10. After adding $CaCl_2$, is cleaned-up with ethyl acetate. Afterwards is acidified and extracted with ethyl acetate and run	(a) TLC (for identification)	96/45/EC
- ethylparaben - propylparaben - butylparaben - benzylparaben		(b) Sample is acidified with H_2SO_4, suspended in ethanol/water mixture, by heating. Extracts are injected into the LC system within 24 h	(b) LC-UV/V (for quantitation)	

[a]Key abbreviation (in alphabetical order): AAS: atomic absorption spectrometry; ECD: electron capture detector; FID: flame ionization detector; GC: gas chromatography; LC: liquid chromatography; TLC: thin layer chromatography; UV/V: ultraviolet/visible spectrometry. Symbol "-" means coupling between techniques.

As was also mentioned in Section 2.1, there are no official methods for preservative determination published by FDA and Japanese authorities.

On the other hand, most papers on preservative analyses can be found in the analytical chemistry databases, most of which are focused on food products, given the major implications of these products have on health. However, a relatively high number of articles have reported the analysis of preservatives in cosmetic products for external use. For easy consultation, Table 5.1.2 summarizes the methods reported in literature chronologically: for each paper it shows the preservative studied, the cosmetic matrix, the employed analytical technique, a brief description of sample pretreatment and, when present, the results of the analysis in terms of limit of detection (LOD) and limit of quantification (LOQ).

In order to use or adapt a method cited in literature or develop a new one, it is necessary to know in which concentration preservatives are generally added and, if available, the threshold concentration. As mentioned before, concerning the maximum permissible concentrations, some discrepancies exist between different legislations as well as the kind of cosmetic to which the preservative is added. So for example, the cosmetic ingredient review (CIR) expert panel from CTFA concluded that iodopropynyl butylcarbamate is safe as a cosmetic ingredient in concentrations lower than or equal to 0.1% (non-aerosolized products). However, the Blue List of Germany's regulatory agency lists all this compound can give rise to unusual allergic reactions. Most cosmetic formulations require concentrations below 0.0125% of this preservative for proper preservation. In the EU the concentration approved when used as a preservative is up to 0.05% but it is not allowed in oral hygiene or lip-care products.

Another point that should also be taken into account concerns the restrictions for a mixture of preservatives. For example, according to EU Cosmetics Directive, the maximum admitted concentration for parabens, expressed as *p*-hydroxybenzoic acid content, is 0.4% if only one paraben is present and 0.8% if a mixture of them is used.

TREATMENT OF THE SAMPLE AND EXTRACTION

Preservative determination in cosmetics is often difficult due to matrix complexity, therefore great care must be devoted to developing suitable extraction procedures and reliable evaluation of the mean recovery values.

The procedure used to extract preservatives from cosmetics depends on the nature of the products (emulsion, cream, shampoos, etc.) and also the characteristics of the analytical techniques to be employed to determine the active substances. For chromatographic analyses, based on liquid, gas or thin layer chromatographic techniques, it is generally possible to dilute aqueous samples in a suitable solvent or extract target compounds with organic or hydro-organic mixtures. For example, Hashim *et al.* (2005) determined different parabens contained in different products (gel, creams, lotions, etc.) by simple vortex extraction with a water/acetonitrile mixture (under heating) and analysed by liquid chromatography (LC).

However, sometimes, the extraction process also needs a clean-up step using a suitable solid-phase extraction (SPE) sorbent, as done by Matissek (1986a) or Hild (1993), for example, who employed silica gel or octadecylsilica cartridges to clean-up thiazolone type preservatives, respectively, or Baltes and Hirsemann (1986), who used a cation exchange

Table 5.1.2

Published papers on preservatives determination in cosmetics (chronological order)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
Wisneski (1980)	Quaternium-15	Different cosmetics products		TLC	
Curea and Cernat (1980)	BHA, methyl benzoate, hexachlorophene,	Dermocosmetics		TLC	
Matissek and Dross (1983)	5-Bromo-5-nitro-1,3-dioxane	Shampoos and bath preparations, non-emulsion cosmetics	Sample is shaken with ethyl ether, filtered, and partially evaporated Then TLC is performed	TLC, Si plates and toluene:acetone, toluene:ether or hexane:$CHCl_3$ as mobile phase	LOD $<$10 mg/kg
Stack and Davis (1984)	Quaternium-15	Different cosmetics products	Sample is dissolved in water and injected into LC system. The eluate is derivatized on-line with acetylacetone by means of a post-column reactor	LC-FD, C_{18} column, EtOH:MeCN containing 30 mM acetate buffer pH 6.5 as mobile phase	Recoveries: 94 and 106% for 0.1 and 0.2% preservative content
Gagliardi *et al.* (1984a)	Dichlorobenzyl alcohol phenoxyethanol, phenoxyisopropanol	Creams, lotions, bath foam and shampoos	Extraction with 2 M H_2SO_4 and MeOH, ultrasonication and dilution with MeOH prior injection	LC-UV/V, C_{18} column, with gradient MeCN:H_2O as mobile phase	
Cortesi *et al.* (1984)	Formaldehyde, imidazolidinylurea	Standard solutions	Derivatization with 2,4-dinitrophenylhydrazine and extraction in CH_2Cl_2	LC-UV/V, C_{18} column and MeCN:H_2O 70:30 as mobile phase	
Gagliardi *et al.* (1984b)	Benzoic acid, benzyl benzoate, ethyl benzoate, ethylparaben, 4-hydroxybenzoic acid, methyl benzoate, methylparaben, sorbic acid, propylparaben	Skin cosmetics, shampoo and bath foam	1 g of sample is mixed with 2 M H_2SO_4 and MeOH, ultrasonicated, centrifuged and diluted in MeOH	LC-UV/V, C_{18} column, with gradient MeCN:5 mM sodium acetate buffer pH 4.4 as mobile phase	

Wyhowsky-de-Bukanski and Masse (1984)	Chlorhexidine, dibromohexamidine, dibromopropamidine, hexamidine	Creams		LC-UV/V, C_{18} column and gradient H_2O:MeOH containing 5 mM heptane sulphonate pH 3.5 as mobile phase	LOD: 50 ng/L
Gagliardi *et al.* (1985a)	Butylparaben, chlorophene, dichloro-*m*-xylenol, ethylparaben, isopropylcresol, isopropyl sorbate, methylparaben, *o*-phenylphenol, propylparaben, tetrabromo-*o*-cresol	Cosmetics formulations		LC-UV/V, C_{18} column with gradient MeOH:5 mM KH_2PO_4 as mobile phase	
Gagliardi *et al.* (1985b)	Bromochlorophene, chloroxylenol, chlorphenesin, cloflucarban, diclorophene, hexachlorophene, *p*-chloro-*m*-cresol usnic acid, triclocarban, triclosan	Cleansing lotion, bath foam, day creams	Sample is extracted with MeOH and H_2SO_4, and fractionated with ethyl ether (acid and neutral compounds)	LC-UV/V, C_{18} column with gradient MeCN:5 mM KH_2PO_4 buffer pH 2.8 as mobile phase	
Gagliardi *et al.* (1985c)	Dibromohexamidine, dibromopropamidine, chlorhexidine, hexamidine	Creams		LC-UV/V, C_{18} column at 40 °C with gradient H_2O:MeCN containing 50 mM $NaClO_4$and 5 mM tetramethyl ammonium bromide pH 3.0 as mobile phase	
Cozzoli *et al.* (1985)	Methylparaben, propylparaben, sodium dehydroacetate	Different cosmetics products		LC-UV/V, C_{18} column with MeOH:H_2O as mobile phase for hydroxybenzoates and MeCN:citrate phosphate buffer pH 3.0 for sodium dehydroacetate	

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
Matissek (1986a)	Dichloromethylisothiazolinone, methylchloroisothiazolinone, methylisothiazolinone	Different cosmetics products	Extraction from silica gel column with ethyl acetate, elution of the compounds into MeOH:acetic acid mixture and flash chromatography on C_{18} column with MeOH:acetic acid as eluent was done	LC-UV/V, C_{18} column by using MeOH:0.4% acetic acid as mobile phase	LOD: 1 mg/L Recovery was 86% for samples containing 31 mg/kg of active substance
Baltes and Hirsemann (1986)	Benzethonium chloride, cetrimonium chloride, cetylpyridinium chloride, chlorhexidine, chlorhexidine, diacetate, dodecylguanidine acetate, hexamidine, hexamidine diisethionate, hexetidine	Different cosmetics products	Sample is cleaned-up by cation exchange column by first elution with MeOH:HCl mixture and second elution with MeOH	TLC, C_{18} plates, and H_2O:MeOH containing 3.84% ammonium acetate as mobile phase LC-UV/V or LC-RI, CN column at 40 °C and with MeCN:MeOH:H_2O: methanesulphonic acid: sodium acetate at pH 5.0 as mobile phase	Recoveries >90% on commercial products
Matissek (1986b)	Chloro-*N*-methylpropionamide, dichloromethylisothiazolinone, dichloro-*N*-methylacetamide, methylchloroisothiazolinone, methylisothiazolinone	Different kinds of cosmetics	(a) Sample is extracted with ethyl acetate (b) Sample extraction with ethyl acetate from a silica gel column and flash chromatography on C_{18} column with MeOH:acetic acid as eluent	(a) GC-MS(EI), GC-MS(CI) Stationary phase: 5% phenyl/1% vinyl/94% dimethyl polysiloxane capillary column. Carrier gas: He (b) LC-UV/V, C_{18} column with MeOH: acetic acid as mobile phase	

De Kruijf *et al.* (1987)	Benzethonium chloride, benzoic acid, benzyl alcohol, benzyl benzoate, benzylhemiformal, benzylparaben, boric acid, 5-bromo-5-nitro-1,3-dioxane, 2-bromo-2-nitropropane-1, 3-diol,butyl benzoate, butylparaben, captan, climbazole, cloflucarban, chlorhexidine digluconate, chlorhexidine hydrochloride, chloroacetamide, chlorobutanol, chlorophene, chloroxylenol, chlorphenesin, dehydroacetic acid, dibromohexamidine isethionate, dichlorobenzyl alcohol, dichloro-*m*-xylenol, dichlorophene, dimethoxane, dimethyl oxazolidine, DMDM hydantoin, dodecylguanidine acetate, ethyl benzoate, ethylparaben, formaldehyde, formic acid, hexachlorophene, hexetidine, hexamidine isethionate, isopropyl sorbate, 4-hydroxybenzoic acid, MDM hydantoin, imizalodinyl urea, methenamine, methyl benzoate, methylchloroisothiazolinone, methyldibromo glutaronitrile, methylisothiazolinone,	Commercial formulations	TLC, in Si and Al plates, with ethyl acetate: isopropyl ether:EtOH:25% NH_3 as eluent	LOD: 0.1% (in sample)

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
	methylparaben, *N*-methylolchloroacetamide, *o*-cymen-5-ol, *o*-phenylphenol, oxyquinoline, *p*-chloro-*m*-cresol, piroctone olamine, phenoxyethanol, potassium metabisulfite, propionic acid, propyl benzoate, propylparaben, Quaternium-15, salicylic acid, sodium dehydroacetate, sodium iodate, sodium o-phenylphenate, sodium pyrithione, sorbic acid, tetrabromo-o-cresol, triclocarban, triclosan, undecylenic acid, undecylenamide MEA, usnic acid, zinc pyrithione				
Krull and Matissek (1988)	Benzoic acid, sorbic acid	Cosmetics formulations	Sample is extracted with acidified acetone, filtered, diluted with water and adjustment of pH to 10 with KOH. Then, $CaCl_2$ was added. After filtration, the solution is washed and extracted with ether, and run.	TLC, Si plates, toluene:acetone as mobile phase.	
Matissek and Lehnguth (1988)	Methylchloroisothiazolinone, methylisothiazolinone, octylisothiazolinone	Standard solutions		LC-UV/V, C_8 column, and MeOH: acetic acid	
Langner *et al.* (1988)	Methyldibromo glutaronitrile	Standard solutions	Sample is dissolved in 0.1 M tetrapropylammonium perchlorate and acetonitrile	DPP, dropping-Hg and Ag-AgCl as working and reference electrodes respectively	

Benassi *et al.* (1989)	Formaldehyde	Different cosmetics formulations	1 g of sample is diluted in 10 mL of THF:H_2O mixture, derivatized with 2,4-dinitrophenylhydrazine in acidic medium, and then is buffered to pH 6.8	LC-UV/V, C_{18} column and H_2O:MeCN as mobile phase	LOD: 0.2 μg/mL Recoveries >90% for spiked sample at 0.04% concentration level
De Kruijf *et al.* (1987)	Benzethonium chloride, benzoic acid, benzyl alcohol, benzyl benzoate, benzylparaben, 5-bromo-5-nitro-1,3-dioxane, 2-bromo-nitropropane-1,3-diol, bromophene, butyl benzoate, butylparaben, chlorhexidine digluconate, chlorhexidine dihydrochloride, chlorophene, chloroxylenol, chlorphenesin, climbazole, cloflucarban, dehydroacetic acid, dibromohexamidine isethionate dichlorobenzyl alcohol, dichloro-*m*-xylenol, dichlorophene, dipyrithione, ethyl benzoate, ethylparaben, hexachlorophene, hexamidine diisethionate, 4-hydroxybenzoic acid, isopropyl sorbate, methyl benzoate, methylchloroisothiazolinone, methylisothiazolinone, methylparaben, *o*-cymen-5-ol, *o*-phenylphenol, oxyquinoline, *p*-chloro-*m*-cresol, phenoxyethanol, phenxyisopropanol, propyl benzoate, propylparaben, salicylic acid, sodium pyrithione, sorbic acid,		Sample is mixed with 4 M formic acid and MeOH, and extracts are injected into the LC system.	LC-UV/V, different C_8 and C_{18} columns, with different mobiles phases	LOD: 0.1% w/w (in sample)

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
	tetrabromo-*o*-cresol, triclocarban, triclosan				
Gagliardi *et al.* (1990a)	Benzylparaben, bromochlorophene, butylparaben, chlorophene, chloroxylenol, chlorphenesin, cloflucarban, dichlorobenzyl alcohol, dichlorophene, ethylparaben, hexachlorophene, isopropyl sorbate methylparaben, *o*-cymen-5-ol, *o*-phenylphenol, *p*-chloro-*m*-cresol, phenoxyethanol, tetrabromo-*o*-cresol, triclocarban, triclosan	Cosmetics formulations		LC-UV/V, C_{18} column, with gradient MeOH:1 % acetic acid as mobile phase	LOD: 5–60 ng Recoveries: 92–101% RSD <3.2%
Bettero *et al.* (1990)	Benzalkonium chloride (a), formaldehyde (b), sodium dehydroacetate (c)	Cosmetic emulsions	1 g of sample is diluted to 10 mL with H_2O:THF and homogenization by stirring	LC-UV/V, different systems: (a) C_{18} column and MeCN:0.02 M phosphate buffer pH 3.5 as mobile phase (b) C_8 column with MeCN:H_2O as mobile phase and pre-column derivatization with 2,4-dinitrophenylhydrazine (c) C_8 column with MeCN:0.01 M phosphate buffer pH 4.7 as mobile phase	
Gagliardi *et al.* (1990b)	Climbazole	Shampoos	Sample is mixed with H_2O:MeOH and sonicated. Then is cleaned-up in a cation exchange column by elution with MeOH:HCl	LC-UV/V, C_{18} column with MeCN:50 mM $NaClO_4$ pH 3	LOD: 10 ng

			and MeOH. Eluate was neutralized with conc. NH_3, dried and diluted with MeOH		
Sarlin and Cellerino (1990)	BHA, BHT, ethylparaben, methylparaben, propyl gallate, propylparaben, 2′,4′,5′-trihydroxybutyrophenone	Hand lotions	Sample is shaken in MeOH, centrifuged and diluted in mobile phase	LC-ED	LOD: of the order of μg/g Recoveries: 87 to 102%
Maffei-Facino *et al.* (1990)	Benzoic acid, benzylparaben, bromochlorophene, 2-bromo-2-nitropropane-1, 3-diol, butylparaben, captan, chlorhexidine, chloroxylenol, chlorphenesin, dehydroacetic acid, dichlorobenzyl alcohol, dichloro-*m*-xylenol, dichlorophene, ethylparaben, methylparaben, *o*-phenylphenol, *p*-chloro-*m*-cresol, phenoxyethanol, propylparaben, salicylic acid, sorbic acid, tribromosalicylanilide, triclocarban, triclosan, undecyldeneamide DEA, undecyleneamide MEA, undecylenic acid, usnic acid	Deodorants, shampoos, syndets, foot cosmetics, face powder and mascara		MIKES(CAD)	LOD: 0.5 pg for triclosan
Ianniello (1992)	Sodium hydroxymethylglycinate	Shampoos, conditioners, styling gel and skin moisturizers	Sample is diluted with H_2O, cleaned-up by solid-phase extraction with Maxi-Clean 300 IC-Ba2+ cartridge by heating of the eluted solution and cooled prior injection	IC-PAD, anion exchange column with 0.15 M NaOH as mobile phase	LOD: 50 mg/L

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
Hild (1993)	Thiazolone type preservatives	Shampoos and creams	Sample is extracted with MeOH:0.4% acetic acid mixture, filtered and cleaned-u by solid-phase extraction C_{18} cartridge eluting with H_2O:MeOH mixture	LC-UV/V, C_{18} column and MeOH:0.4% acetic acid or MeOH:MeCN:0.4% acetic acid as mobile phases	
Weyland *et al.* (1994)	5-Bromo-5-nitro-1,3-dioxane, 2-bromo-2-nitropropane-1, 3-diol, methyldibromogluta-ronitrile	Different cosmetics, emulsions	Sample is treated with 30 mL of H_2O:MeOH, heated, homogenized and after cooling is diluted with H_2O:MeOH and filtered	LC-ED, C_8 column set at 40 °C, and H_2O:acetone containing 2.84 g Na_2SO_4 and 0.12 g NaCl. Au and Ag as working and reference electrodes, respectively	Recoveries: 97–100% for emulsion samples spiked at 0.03% concentration level RSD <2%
Qi *et al.* (1994)	Butylparaben, ethylparaben, methylparaben, propylparaben	Cosmetics formulations	Sample is diluted with MeOH, sonicated, filtered and further diluted with mobile phase	LC-UV/V, C_{18} column set at 45 °C. MeOH: 20 mM ammonium acetate as mobile phase	LOD: 6.25–20.83 ng Recoveries: 87.7–99.5%
Haruyama and Okaya (1995)	Benzalkonium chloride, benzethonium chloride, cethylpiridinium chloride, chlorhexidine gluconate	Cosmetics formulations	Sample is dissolved in THF or MeOH, and passed through a cation exchange cartridge by eluting with 0.2 M KH_2PO_4:MeCN	LC-UV/V, C_{18} column set at 50 °C, and MeCN:H_2OTHF:acetic acid containing 0.2% sodium lauryl sulphate as mobile phase	LOD: 10, 2, 0.5 and 0.3 μg/g, respectively
Rastogi and Johansen (1995)	Methyldibromo glutaronitrile	Body shampoos, creams and lotions	(a) sample is suspended in 10 mL of MeOH, ultrasonicated and filtered	Different methods are compared: (a) LC-UV/V, MeCN: THF:H2O	LOD: (a) 50, (b) 50 and (c) 0.5 mg/kg

			(b) sample is suspended in 10 mL of isopropanol, heated at 60 °C and filtered (c) sample is suspended in H_2O:MeOH, heated at 60 °C and centrifuged	(b) LC-UV/V, hexane :i-PrOH (c) LC-ED, H_2O: acetone containing 20 mM Na_2SO_4 and 2 mM NaCl. Au and Ag/AgCl as respectively working and reference electrodes
Imrag and Junker-Buchheit (1996)	Benzoic acid, benzyl alcohol, BHA, BHT, bromochlorophene, 5-bromo-5-nitro-1,3-dioxane, 2-bromo-2-nitropropane-1,3-diol, butyl benzoate, butylparaben, cetrimonium bromide, cetylpiridinium chloride, dehydroacetic acid, DMDM hydantoin, ethyl benzoate, ethylparaben, imidazolidinylurea, lithium benzoate, methyl benzoate, methylchloroisothiazolinone, methylisothiazolinone, methylparaben, phenoxyethanol, propyl benzoate, propylparaben, Quaternium-15, sodium benzoate, sodium dehydroacetate, sorbic acid, triclosan	Cosmetics formulations	Samples are suspended in 50% EtOH, sonicated and centrifuged. Supernatant is analysed by TLC	TLC, Si plates developed in toluene:acetone, CN plates in EtOH:H_2O: acetone containing 0.1 M tetraethylammonium chloride, C_{18} plates in H_2O:acetone and Diol plates in hexane:acetone 9:1 are used
Danielson and Thompson (1996)	Glutaral, phenol	Germicidal formulations		GC-FID, 20% phenyl/ 80% dimethyl polysiloxane capillary column. Carrier gas: He

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
Schlegel *et al.* (1997)	Basic and quaternary ammonium preservatives	Creams and gel samples	Homogenization with MeOH or 0.1 M HCl acidified MeOH, centrifuged and submitted to a cation exchange cartridge. Eluate is evaporated and re-dissolved in MeOH for running the TLC	TLC-UV/V	
Gagliardi *et al.* (1997)	Ascorbyl palmitate, benzylparaben, BHA, BHT, butylparaben, di-t-butyl hydroquinone, dodecyl gallate, ethylparaben, methylparaben, octyl gallate, propyl gallate, propylparaben, sorbic acid, t-butyl hydroquinone, tocopheryl acetate, triclosan	Cosmetics and pharmaceutical formulations (oil-rich products)	Emulsions are dissolved in MeOH:MeCN by sonication. Lipsticks are refluxed with DMF. After filtration are injected	LC-UV/V C_8 column, with gradient MeCN: MeOH:H_2O containing $HClO_4$	LOD: 5–30 ng
Facino *et al.* (1997)	Preservatives and antioxidants	Mascara	Mascaras are vortex-extracted with hexaneand $CHCl_3$, evaporated to dryness and dissolved in hexane prior GC-MS analysis	GC-MS(EI), 5% phenyl/1% vinyl/94% dimethyl polysiloxane capillary column. Carrier gas: He	
Wang and Chang (1998)	Butylparaben, ethylparaben, methylparaben, propylparaben	Home-made creams	Sample is rolled in a piece of paper and placed in an extraction cell. Extracts are collected in MeCN and diluted with borate buffer	SFE+CZE CO_2 with 0.05% MeCN as extractant	Recoveries > 99.8%
Hu and Wang (1999)	Benzoic acid, benzyl alcohol, 2-bromo-	Cosmetics formulations	1 g of sample is stirred with 10 mL of MeOH,	LC-UV/V, C_8 column, with 50 mM phosphate	LOD: 5–500 ng Recoveries:

	2-nitropropane-1,3-diol, butylparaben ethylparaben, isobutylparaben, isopropylparaben, methylchloroisothiazolinone, methylisothiazolinone, methylparaben, phenoxyethanol, propylparaben		sonicated and centrifuged. Supernatant is injected into the LC system	buffer pH 3.5:MeOH: MeCN containing 2 M hexadecyltrimethyl ammonium chloride as mobile phase	85–107% RSD: 2.2–7.5%
Gámiz-Gracia and Luque de Casto (1999)	Formaldehyde	Different cosmetics	Sample is diluted in water and placed into a thermostated pervaporation unit. The analyte is evaporated and collected into al pararosaniline acidified stream, which was merged with a Na_2SO_3 stream, and finally the coloured derivative is measured.	FI-UV/V	
Sottofattori and Anzoldi (2000)	Ascorbyl palmitate, butylparaben, ethylparaben, imidazolidinylurea, kojic acid, magnesium ascorbyl phosphate, methylparaben, propylparaben, triclosan	Creams	Cream sample is mixed with THF:25 mM M phosphate buffer pH 3.5	LC-UV/V, CN column, with gradient H_2O: MeOH as mobile phase	
Bianco-Prevot *et al.* (2000)	Benzyl alcohol, butylparaben, chloroxylenol, ethylparaben, methylparaben, *o*-phenylphenol, p-chloro-m-cresol, phenoxyethanol, propylparaben,	Shampoos, emulsions	Dilution in SDS solution	MEKC-UV/V phosphate buffer:sodium tetraborate containing 60 mM SDS at pH 6.90	
Boyce and Spickett (2000)	Benzoic acid, BHA, BHT, dodecyl gallate,	Cosmetics, shampoos		MEKC, Electrolyte solution: buffer 15 mM	

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
	butylparaben, ethylhexyl gallate, ethylparaben, methylparaben, propyl gallate, propylparaben, salicylic acid, sorbic acid			SDS, 35 mM sodium cholate, 10 mM boric acid and 10 M sodium tetraborate, pH 9.5	
De Villiers and Bergh (2000)	Sorbic acid	Non-ionic creams		LC-UV/V, C_8 column with H_2SO_4:KH_2PO_4: MeOH mixture as mobile phase UV/V	LOD: 0.0082 ng/mL for LC-UV/V and 0.15 ng/mL for UV/V analysis.Quantitative recoveries
Cruces-Blanco *et al.* (2001)	Butylparaben, ethylparaben, methylparaben, propylparaben	Standard solutions		CZE-UV/V, Stationary phase: fused-silica capillary tubing, and 35 mM tetraborate buffer pH 10.0	LOD: 0.65–0.81 mg/L
Frauen *et al.* (2001)	Iodopropynyl butylcarbamate	Cosmetics	Sample is mixed with THF: MeOH by ultrasonication. Then, is diluted with H_2O sonicated again and centrifuged. Supernatant is filtered and injected	LC-MS(ESI)	LOD: 50–100(ng/g
Bodor *et al.* (2001)	Benzoic acid, glutamic acid, parabens, sorbic acid	Food products and cosmetics	Minimal sample preparation: dilution and filtration	ITP and ITP-ZE with column-coupling conductivity detection	LOD: of the order of μg/L.
Marengo *et al.* (2001)	Benzoic acid, benzyl benzoate, benzylparaben, butyl benzoate, butylparaben, dehydroacetic acid, ethyl benzoate, ethylparaben, 4-hydroxybenzoic acid, methyl benzoate,	Cosmetic lotions		LC-UV/V, C_{18} column, with MeCN:H_2O containing 3.25 mM hexylammonium orthophosphate as mobile phase	LOD: 10 to 820 μg/L

	methylparaben, propyl benzoate, propylparaben, salicylic acid, sorbic acid, triclocarban				
Mahuzier *et al.* (2001)	Butylparaben, ethylparaben, methylparaben, propylparaben	Standard solutions		MEEKC, SDS:octane: BuOH in buffer sodium tetraborate	LOD: 0.1%
Piccoli *et al.* (2002)	Triclosan	Deodorant stick, dentifrice gel, mouth rinse, tooth paste and hand wash		LC-UV/V, C_{18} column with MeCN:70 mM TEA phosphate pH 3.5 LC-MS(ESI), capillary voltage: 30 V, capillary temperature: 180 °C, positive ions	
Mikami *et al.* (2002)	Benzoic acid, dehydroacetic acid, salycilic acid, sorbic acid	Milk and cream lotions		LC-UV/VIS, C_{18} column. Mobile phase: H_2O:MeOH containing 2.5 mM tetrabutylammonium hydroxide at pH 7.0	LOD: 2.5–5.5ng Recoveries >94.8% for spiking between 0.5 and 2.0 mg/g
De Rossi and Desiderio (2002)	Benzylparaben, butylparaben, ethylparaben, 4-hydroxybenzoic acid methylparaben, propylparaben	Cosmetics and drugs	Sample is extracted with MeCN by sonication and heating. Filtered and diluted with H_2O prior its injection	CEC-UV/V, C_{18} capillary. Mobile phase: MeCN:H_2O containing 5 mM ammonium formate pH 3.0	LOQ: 1.25–2.50 μg/mL
Baalbaki et al. (2002)	Imidazolidinylurea, methylparaben, propylparaben	Pharmaceutical ointment		MEKC-UV/V, electrolyte solution: 40 mM SDS in 10 mM NaH_2PO_4 solution	Recoveries >97% for 0.03–0.50% spiked samples
Li *et al.* (2003a)	BHA, BHT, butylparaben, ethylparaben,	Cosmetics formulations	Sample is mixed with 20 mL of ethyl acetate,	GC-MS(EI) 5% phenyl/95% dimethyl	LOD: 0.5 μg/mL Recoveries: 90–102%

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Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
	methylparaben, propylparaben		ultrasonicated and diluted with ethyl acetate	polysiloxane capillary column. Carrier gas: He.	
Li *et al.* (2003b)	Benzyl alcohol, butylparaben, chlorophene, chloroxylenol, dichloro-m-xylenol, ethyl benzoate, ethylparaben, isopropylparaben, methyl benzoate, methylparaben, *o*-cymen-5-ol, *o*-phenylphenol, *p*-chloro-*m*-cresol, phenoxyethanol phenyl benzoate, propylparaben	Cosmetics	Sample is sonicated with acetone prior its injection	GC-MS	LOD: 0.05–0.5 mg/L Recoveries: 92–101%
Huang *et al.* (2003)	Benzoic acid, butylparaben, dehydroacetic acid, ethylparaben, imidazolydinylurea, methylparaben, propylparaben, sorbic acid, triclosan	Ointment, liquid formulation, water-based lotions		MEKC SDS in tetraborate buffer pH 9.0 MEEKC microemulsion buffer with octane, BuOH and running buffer (2.1–9.5) sodium tetraborate/NaOH	
Marengo *et al.* (2004)	Benzoic acid, benzyl benzoate, benzylparaben, 2-bromo-2-nitropropane-1, 3-diol, butyl benzoate, butylparaben, chlorhexidine, dehydroacetic acid, 4-hydroxybenzoic acid, ethyl benzoate, ethylparaben,	Shaving foam	Sample is extracted with water by sonication, then diluted with mobile phase and filtered	LC-UV/V, C_{18} column. Mobile phase: H_2O:MeCN, containing butyl, hexyl, and octylamine in H_3PO_4 medium	LOD: $<$0.35 mg/L

	methyl benzoate, methylparaben, o-phenylphenol, p-chloro-m-cresol, propyl benzoate, propylparaben, salicylic acid, sodium pyrithione, triclocarbam				
Sirichai *et al* (2004)	Butylparaben, ethylparaben, methylisothializolinone, methylparaben, propylparaben,	Lotions		MEKC-UV/V, 30 mM NaH_2PO_4 pH 7.2 solution containing 30 mM SDS.	LOD: 0.3–1.0 mg/L
Rivero and Topiwala (2004)	Formaldehyde	Nail polish, shower gel, mascara, body creams and surfactant	Sample is derivatized with pentafluorophenyl hydrazine, and the hydrazone derivative is extracted by SPME and injected	SPME+GC-FID SPME+GC-MS	LOD: 0.39 μg/L Recovery >89% in 20 mg/L spiked samples
Rastogi *et al.* (2004)	Methyldibromo glutaronitrile	Creams, shampoos, mascara	Sample is mixed with 80 % aqueous MeOH and heated. After cooling is diluted with MeOH and injected	LC-ED, C_8 column at 40 °C, with acetone: 0.5 M Na_2SO_4:0.2 M NaCl:H_2O Au and Ag/AgCl as working and reference electrodes, respectively	LOD: 0.5 mg/L Recoveries >95% for 0.005–0.04 5% spiked samples
Borremans *et al.* (2004)	Benzylparaben, butylparaben, ethylparaben, methylparaben, phenoxyethanol, phenoxyisopropanol, propylparaben	Shampoos, body milk		LC-UV/V, C_{18} column with H_2O:MeCN:MeOH: THF as mobile phase	
Capitan-Vallvey *et al.* (2004)	BHA, BHT, propyl gallate	Fat foods and cosmetic samples	Sample is mixed with hexane and extracted with	FI-UV/V, Flow cell filled up with C_{18}	Recoveries: 101–105% for real samples

(*Continued*)

Table 5.1.2 (Cont.)

Authors	Target preservatives[a]	Type of sample	Sample preparation[b]	Analytical technique[b]	Analytical features[c]
			MeOH:H_2O, filtered, diluted and injected	adsorbent. MeOH:H_2O as carrier.	
Hashim *et al.* (2005)	Butylparaben, ethylparaben, methylparaben, propylparaben	Shampoo, facial cleanser, hand and body lotion, shower gel, moisturizer	Sample extraction with H_2O:MeCN by vortex mixer and heating	LC-UV/V, C_{18} column	Recoveries >86.0% for 0.1, 0.2 and 0.4% spiked samples
Guan *et al.* (2005)	BHA, BHT, propyl gallate, *t*-butyl hydroquinone	Cosmetics formulations	Sample is extracted with EtOH by sonication, and the extract is diluted with running buffer and filtered prior injection	MEKC-ED, 20 mM borate running buffer (pH 7.4) containing 25 mM SDS. Detection: amperometric detector in combination with a carbon disc, saturated calomel and Pt as working, reference and auxiliary electrodes, respectively	LOD: $0.3–5\times10^{-6}$M
He *et al.* (2006)	Butylparaben, ethylparaben, methylparaben, propylparaben	Cosmetics formulations	Sample is dissolved in MeOH:H_2O and injected	MEKC, 50 mM phosphoric acid buffer (pH 2.28) containing 100 mM SDS and 1% MeOH	LOD: 3×10^{-7} M

[a]Ordered in alphabetical arrangement.
[b]Symbol "-" means coupling between techniques, and symbol "+" means sequentially applied techniques. Key abbreviation (alphabetical order): Al: alumina gel; BuOH: butanol; C_8: octylsilica gel; C_{18}: octadecylsilica gel; CAD: collisionally activated decomposition ionization; CI: chemical ionization; CN: cyanopropylsilica gel; CZE: capillary zone electrophoresis; DPV: differential pulse polarography; ED: electrochemical detection; EI: electron ionization; ESI: electrospray ionization; EtOH: ethanol; FD: fluorimetric detection; FI: flow injection system; FID: flame ionization detection; GC: gas chromatography; IC: ion chromatography; ITP: isotacophoresis; LC: liquid chromatography; MeCN: acetonitrile; MeOH: methanol; MS: mass spectrometry detection; MECK: micellar electrokinetic chromatography; MEECK: microemulsion electrochromatography; MIKES: mass-analysed ion kinetic energy spectrometry; PrOH: isopropanol; PAD: pulse amperometric detection; RI: refractive index detection; SDS: sodium dodecyl sulphate; SFE: supercritical fluid extraction; Si: silica gel; SPME: solid-phase microextraction; TEA: triethanolamine; THF: tetrahydrofuran; TLC: thin layer chromatography; UV/V: ultraviolet/visible spectrometry; ZE: zone electrophoresis.
[c]LOD: limit of detection; LOQ: limit of quantification.

sorbent to determine basic preservatives. By choosing the correct volume of eluting solvent, this step can also represent a pre-concentration process. The clean-up is necessary to determine preservatives in complex matrices or heterogeneous samples, in which interference problems are likely. To a lesser extent, supercritical fluid extraction (SFE) with carbon dioxide as extractant (e.g. Wang and Chang, 1998) or solid-phase micro-extraction (SPME) (e.g. Rivero and Topiwala, 2004), which do not require great amounts of solvent, have been also used for preservative determination.

The organic solvents most commonly used in the extraction–preconcentration step and sample dilution are hexane, methanol, ethyl ether, ethyl acetate and acetone. When determination involves the separation of compounds with different acid–base properties, i.e. acidic, basic and neutral analytes, separation occurs as a function of pH: acidic and basic molecules are extracted making use of hydro-organic mixture also containing acids or bases while neutral species are dissolved in organic phases.

The first pre-treatment procedure for non-aqueous formulations is represented by the homogenisation of the sample that is obtained by stirring, ultrasonication or centrifugation and filtration. Sometimes, heating or cooling processes are employed, to favour substance transport between phases or to induce precipitation.

ANALYTICAL TECHNIQUES

The most commonly used methods to determine congeners or classes of preservatives were shown in Table 5.1.2. However, one must bear in mind that cosmetic formulations very often contain mixtures of preservatives belonging to different chemical classes and characterized by different functional groups. Therefore, multicomponent analysis methods are required. In this sense, chromatographic techniques are those most commonly used to determine preservatives in cosmetic products. Within this group of techniques, LC is the most commonly used technique to separate and determine preservatives, in particular both ion-pair and reversed-phase LC with UV/Vis detection. Thin layer chromatography (TLC), as well as capillary electrophoresis (CE) and capillary zone electrophoresis (CZE) have been widely used. Recently, CE has become a popular separation technique in preservative analysis, used to determine both charged and hydrophobic compounds after addition to the running buffer of a surfactant or a modifier. Also, papers have been published related to gas chromatography (GC) with flame ionization detector (FID), electron capture detector (ECD) or mass spectrometry (MS) detector used for preservative determination.

In summary, according to the analysis to be done, the kind and concentration level of the preservatives of interest, the matrix in which they are contained, the degree of accuracy required and the instrumentation available, readers can adapt the most suitable methodology to their individual needs, including the extraction step and the analysis itself.

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– 6 –

Perfumes in Cosmetics. Analytical Methods

6.1. Perfumes in Cosmetics. Regulatory Aspects and Analytical Methods for Fragrance Ingredients and other Related Chemicals in Cosmetics

A. Chisvert[1*] and A. Salvador[2]

[1]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Ctra. San Vincente del Raspeig s/n, 03690 San Vincente del Raspeig, Alicante, Spain
[2]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain*

INTRODUCTION

Odours play a crucial role in human behaviour. While a pleasant scent can have a calming effect or make one feel better, unpleasant odours can alter our mood negatively and produce anxiety and discomfort.

Perfumes are the substances responsible for providing us with pleasant redolence that we smell everyday. Each perfume is made up of hundreds of aromatic chemicals (also

*Corresponding author. E-mail: alberto.chisvert@ua.es

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referred to as fragrance chemicals), each of which contributes with its characteristic odour, together giving the characteristic aroma of the perfume.

The first evidence of humans using perfumes goes back thousands of years, when Egyptians used plant, gums, and resins in religious rites (Pybus and Sell, 1999). Nowadays, the use of perfumes is well established in our daily lives. They are used in different cosmetics and household products.

The aim of this section is to give the reader a general vision of the use of perfumes in cosmetic products, dealing with the types of perfume, as well as legislation and analytical methods focused on fragrance ingredients and other related chemicals in cosmetics.

TYPES OF PERFUMES

Perfumes can be classified according to their nature, since they can be obtained from different sources. So, there are "natural" perfumes, which are obtained from natural products, either from plants or animals, or they may be "synthetic" if they are made of synthetic chemicals.

Natural perfumes

Natural perfumes, also referred to as essential oils, are obtained from different parts of plants like flowers (e.g. jasmine, rose, gardenia), fruits (e.g. lemon, orange, vanilla), roots (e.g. vetiver, cistus, angelica), leaves (e.g. violet, patchouli, peppermint), wood (e.g. vetiver, sandalwood, cedarwood), bark (e.g. cinnamon, nutmeg), resin (e.g. benjui, tolu, galbanum) and seeds (e.g. angelica, celery, anis), or from whole plants (e.g. lavender, geranium). Also they may be obtained from animal glands and organs, like for example: musk, which is obtained from the testicles of the musk deer; civet, which is a secretion from glands of the civet cat; ambergris, which is obtained from a secretion from the intestine of the sperm whale; and finally castoreum, which is obtained from glands near the reproductive organs of the beaver.

All natural perfumes are obtained from their corresponding source by extraction procedures, like those described below. The selected process depends on the natural product and also on the chemicals responsible for giving the odour. The particular method applied greatly affects the quality of the perfume produced.

Hydrodistillation

The natural product is immersed in water and heated until boiling. The essential oil is dragged out by water vapour. When the distillate is condensed back into liquid, the essential oil is easily separated from the water since it floats to the top. Nevertheless, some fragrance chemicals are soluble in the water distillate, and thus they need to be recovered from the distillate by an additional distillation step or extracted by other means, and returned to the separated oil. This process is known as cohobation.

One advantage of hydrodistillation is that the oil temperature never rises above 100°C, and thus thermal decomposition is minimized, although the process can be carried out at a lower temperature if reduced pressure is applied.

Steam distillation

Pressurized-flowing steam is passed through plant material, and the aromatic components are extracted. They are condensed back to liquids, and the essential oil is easily separated from the water since it floats to the top, nevertheless, cohobation (mentioned above) may be necessary.

This method is the most commonly used at present, and it is especially useful to obtain heat-resistant essential oils. Nevertheless, as steam is under pressure, the temperature can be adjusted to achieve a maximum rate of extraction with low thermal decomposition.

Solvent extraction

Hydrocarbon solvents, like hexane, petroleum ether, methanol or ethanol, are added to the plant or animal material in order to extract the delicate fragrance substances. The extraction by means of Soxhlet manifolds could, in some cases, facilitate the extraction procedure. After distillation to remove the solvent, the remaining extract is known as concrete. This extract can be used as such, or cold ethanol may be added to re-extract oils or fats in order to purify the extract. After ethanol evaporation, the resulting oils are more purified, and the extract is known as absolute, which is the purest and most concentrated form of essential oil. This method is useful for fragrance chemicals which are thermolabile and/or have too high a boiling point to be extracted by the steam distillation method.

A variant of this method is using animal fats as extractive solvents of plant materials. So, the method called enfleurage involves plant material being steeped in pig or cow fat for several days, often being repeating with fresh plant material. The resulting substance is known as pomade, which can be used as such or is also preferably re-extracted with ethanol in order to obtain the absolute extract. It is worth mentioning that this extraction method is becoming obsolete because it is costly and time-consuming.

A very similar method to the one mentioned above is that called maceration. In this process the natural materials are steeped in vats of oil until fragrance chemicals are dissolved. The oil may be heated to accelerate the process, although this process is very time-consuming. Once more, the pomade can be extracted by means of ethanol in order to purify the extract.

Percolation is a characteristic method based on solvent extraction, where the material to be extracted is packed into a column with a tap at the lower end. The tap is opened and the extraction solvent is poured in at the top and allowed to flow through the material.

Expression

This extraction method is mainly used to obtain essential oils from fresh fruit rinds, which are cold pressed by means of rollers or sponges. This method is very suitable for thermally labile components.

Supercritical fluid extraction

This method makes use of carbon dioxide in supercritical state to extract the essential oils. This method can considerably reduce the extraction time by adding modifiers to the carbon dioxide. Moreover, carbon dioxide has the advantage of “disappearing” when it is depressurized, since it changes to the vapour state, which escapes and leaves the extract alone, and

since it is not a contaminant, the method could be considered as a green extraction procedure. However, it is an expensive method, which requires special instrumentation.

It should be noted that besides the extraction procedures described above, there are other operations like rectification (i.e. an additional distillation), fractional distillation (i.e. collecting the distillate in different batches), terpene removal (because some terpene and sesquiterpene obtained from certain plants are difficult-to-solve in ethanol and are easily oxidized and polymerized), decolourization, etc., that are needed in order to obtain good-quality extracts.

Also, a pure fragrance chemical can be obtained from these natural perfumes by means of isolation procedures applied to essential oil.

Synthetic perfumes

They are made of chemicals obtained by synthesis processes that try to imitate the natural fragrance ingredients found in the natural extracts.

These synthetic ingredients appeared as a consequence of the high demand for perfumes in the 20th century, which made their cost increase while the availability of some of them decreased. For example, musk and ambergris are very difficult to obtain as a result of the Washington Treaty that bans international trade in protected species of wild animals and plants (Mitsui, 1998).

The main advantage of using synthetic ingredients is that it decreases perfume costs compared to natural perfumes. Also, they can always be obtained without problems related to poor crop quality or lack of supply (as mentioned above), so, it is expected that they are not subject to market variations affecting quality.

Another advantage is the fact that new chemicals can be synthesized, and thus new scents are developed that are not found naturally.

Unfortunately, there are also some drawbacks that are worth mentioning. First we should mention that a natural perfume could be made of hundreds (sometimes thousands) of fragrance chemicals, so it is difficult to reproduce the desired perfume exactly. Nevertheless, one could think that the synthetic perfume could be created by just mixing the main ingredients (i.e. those in higher concentration), but one should bear in mind that all the components, including those present at trace level, have a synergistic effect that is difficult to imitate synthetically, and thus the resulting perfume will have a slightly different smell (Scott, 2005).

Moreover, another disadvantage is that the fragrance chemical responsible for a characteristic odour is one of the two possible isomeric forms of this compound, or in the worst case, the other isomeric form is the one responsible for a different, and sometimes unpleasant, odour. For example, D-linalool has a floral scent with a woody note, while L-linalool has a sweet floral scent. Therefore, it is of interest to develop chiral synthesis methods using either optically active catalysts or optical separation strategies.

On the other hand, perfumes, and also the pure fragrance chemicals that compose the perfume, can also be classified according to the note they provide, i.e. according to the fragrance type. So, one can find different types, like floral, which reminds one of scents similar to jasmine, rose, heliotrope, etc; citrus, which are aromas reminiscent of lemon, orange, lime, grapefruit, etc.; fruity, based on non-citrus fruity odours like peach,

apple, banana, etc.; green, which create the sensation of smelling recently cut grass and leaves; woody, which reminds one of dry wood and trees; oriental refers to those sweet strong fragrances reminiscent of vanilla, ambergris, etc.; spice, giving off a redolence coming from clove, cinnamon, thyme, pepper, etc.; animal refers to scents provided by musk, civet and castoreum; and leather, which try to reproduce the characteristic smell of leather, tobacco, smoke, etc.

TYPES OF FRAGRANCE INGREDIENTS

Among the complex mixtures that comprise a perfume, the fragrance chemicals they contain can be classified in different families according to their chemical structure, where it is usual to find the five-carbon isoprene unit in most of them, giving them the names of terpenes. So, one can find: monoterpene hydrocarbons (e.g. limonene), sesquiterpene hydrocarbons (e.g. α-farnesene), alcohols (e.g. *cis*–3–hexenol), monoterpene alcohols (e.g. linalool), sesquiterpene alcohols (e.g. farnesol), phenols (e.g. eugenol), aldehydes (e.g. 2,6-nonadienal), terpene aldehydes (e.g. citral), ketones (e.g. cyclohexanone), terpene ketones (e.g. β-ionone), lactones (e.g. γ-undecalactone), esters (e.g. methyl salicylate), terpene esters (e.g. linalyl acetate), and oxides (e.g. eucalyptol), etc. Some examples are depicted in Figure 6.1.1.

Perfumes in cosmetic products

Perfumes are closely related with cosmetic products, since it is very common to add perfumes to cosmetics in order to make cosmetic users feel good, like feeling clean, or simply to make the user feel more comfortable and attractive because they smell nice. On the other hand, perfumes are sometimes added to cosmetics to mask undesirable odours caused by other cosmetic ingredients.

The perfumery raw materials used to perfume cosmetics are not usually pure fragrance chemicals or crude extracts obtained via the methods described above, but these are mixed by perfumers in such a way as to create a "perfume" (usually referred to as fragrance compound) that fulfils the established fragrance requisites, which usually fall in line with a market study, considering the type of cosmetic to which it has to be added, cosmetic image, target consumer (age, sex, etc.), originality, fashion, etc.

Perfume type and its content in the cosmetic product obviously depend on the type of cosmetic, but also on other factors related to the potential customers. So, we can divide cosmetics into two broad groups depending on the perfume content. Fine fragrances, which are hydroethanolic solutions of perfumes, usually have higher contents than other cosmetic formulations, since their main function is to transmit a pleasant redolence to the user. Fine fragrances can also be subdivided according to their perfume content. Table 6.1.1 shows the contents usually found in cosmetic products.

With regard to the type of cosmetics, for example, for fine fragrances the perfume employed should be pungent, beautiful and elegant or on the contrary may be sweet and refreshing, depending on the specific case; for skin and hair care sweet or tenuous notes are usually used whereas for toothpastes, high refreshing power notes are preferred. On the

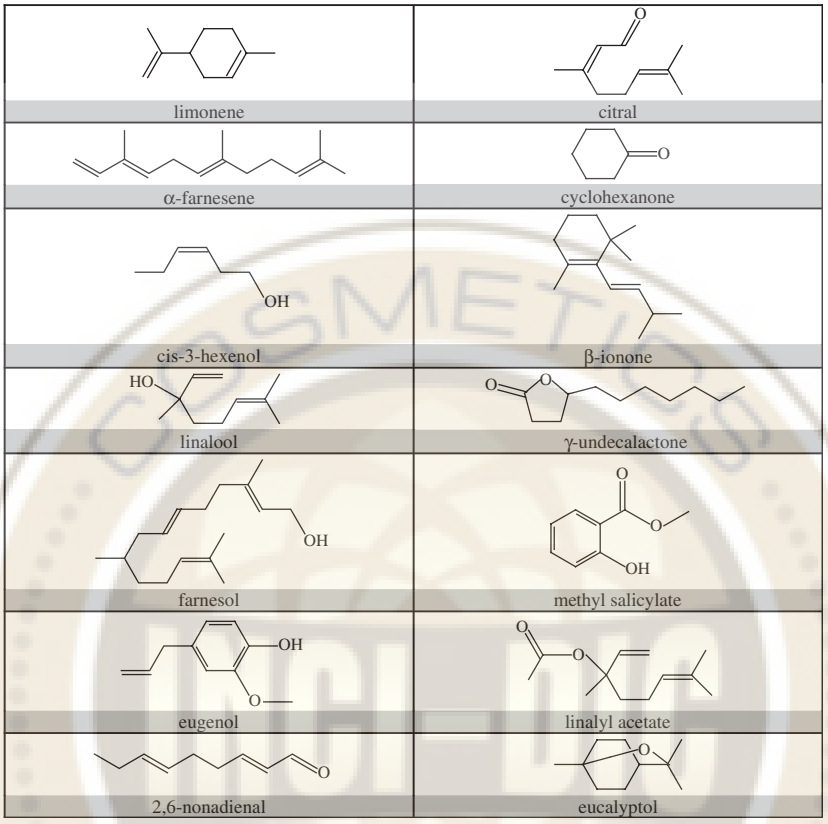

Figure 6.1.1 Examples of fragrance chemicals*.
*Reader should know that different *cis*/*trans* and L/D-isomers could exist for some compounds.

Table 6.1.1

Perfume contents usually found in cosmetic products

Cosmetic product	Content (%)
Fine fragrances	
– Baby cologne	1–2
– Cologne	2–3
– Eau de Cologne	3–4
– Eau Fraiche	4–5
– Eau de Toilet	5–8
– Eau de Parfum	8–12
– Pafum	12–18
Skin care products	0.01–0.5
Hair care products	0.01–1
Bath preparations	0.1–3
Toothpastes	0.5–1

other hand, depending on the formulation, there are perfumes that could be incompatible with the cosmetic itself, due to solubilization problems (which affect the manufacturing process), chemical and/or physical interactions with other ingredients, etc.

Another thing to bear in mind is how long the scent persists on the body, especially necessary for fine fragrances. There are three notes that can be distinguished in a perfume, which are responsible for the major or minor persistence. The so-called top note is responsible for imparting the first impression, which lasts for around 1–2 h, and is created by high-volatility fragrance chemicals. The middle note, composed by medium-volatility fragrance chemicals, plays the most important role in a perfume, since it is responsible for the identity of the perfume, and it should usually remain up to six hours after application. Finally, the lasting note lasts for more than six hours, and is given off by low-volatility chemicals. The balance between all these notes makes it possible for perfumes to be more or less persistent. Adding fragrance modifiers and fixatives helps to increase perfume persistence.

Regulatory aspects for fragrance ingredients in cosmetic products

Legislations in force in the three principal markets regarding cosmetic products, i.e., in the European Union (EU), the United States (US) and Japan, establishes that all the ingredients for cosmetics should be indicated on the label (see Section 1.2). This could be an extremely difficult task, since, as mentioned previously, a perfume could be composed of hundreds of chemicals, which should be mentioned on the label together with the other cosmetic ingredients.

However, according to the EU Cosmetics Directive (Council Directive 76/768/EEC), in the event of perfume and aromatic compositions and their raw materials, all together they can be referred to under the word "*perfume*" or "*aroma*". However, the presence of any of the 26 aromatic substances declared as potentially allergenic fragrance substances under Annex III of the EU Cosmetics Directive must be declared on the cosmetic labelling list (see Section 6.2).

Also, according to the US Food and Drug Administration (FDA) regulations established in its Title 21 of the Code of Federal Regulations (21 CFR) Part 701.3 about cosmetic labelling, mentions that fragrances or flavours may be listed under the word "*fragrance*" or "*flavour*".

Since fragrance ingredients do not have to be declared on the label, except those 26 required by the EU Cosmetic Directive for the products to be marketed in its framework, it is impossible to know exactly which fragrance substances are in the cosmetic products, which is not particularly useful to consumers since some of the fragrance chemicals have been shown to cause various side-effects, like skin sensitivity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. (Anderson and Anderson, 1997; De Groot and Frosch, 1997; Bickers *et al.*, 2003).

The Research Institute for Fragrance Materials (RIFM), created in 1966, is an independent entity which evaluates and distributes scientific data on the safety assessment of fragrance substances found in cosmetics and other products. RIFM maintains the largest database on toxicology data for flavour and fragrance materials available worldwide, classifying more than 4500 materials. The database can be accessed online (see RIFM's website http://www.rifm.org).

On the other hand, the International Fragrance Association (IFRA), created in 1973, establishes usage guidelines for fragrance ingredients based on RIFM evaluation results. In

its Code of Practice, available online (see IFRA's website http://www.ifraorg.org) one can find recommendations to avoid many ingredients, although the last decision on restricting or avoiding a fragrance ingredient is taken by the corresponding regulation in force.

In the EU framework, besides the 26 potentially allergenic substances (PASs) mentioned above (regulated under Annex III), the EU Cosmetics Directive prohibits the use of more than 50 fragrance substances (including some extracts) in cosmetic products, under its Annex II. Special attention is paid to synthetic musks, especially to musk ambrette, which are shown to be linked with different types of dermatitis, carcinogenic effects and endocrine dysfunction (Parker *et al.*, 1986; Lovell and Sanders, 1988; Dietrich, 1999; Eisenhardt *et al.*, 2001). So musk ambrette and musk tibetene are prohibited in cosmetics under Annex II, whereas musk xylene and musk ketone are permitted with the restrictions laid down in Annex III, on the basis of the Scientific Committee on Cosmetic Products and Non-food products intended for consumers (SCCNFP) recommendations (SCCNFP/0817/04). Moreover, in Part 2 of the European Inventory of Cosmetic Ingredients (see Section 1.2), the substances usually employed as fragrance ingredients in cosmetics are listed, independently of the other functions they may have, and which are listed in Part 1 of the aforementioned inventory.

The US FDA's list of prohibited ingredients has only a few compounds (see Section 1.2), but none of them are used as fragrance ingredients.

Regulations on other fragrance-related ingredients in cosmetic products

There are other substances related to fragrances that are also worth mentioning, since different restrictions have been set as a consequence of their undesirable side-effects.

For example, phthalate esters (or commonly known as phthalates) have been used as solvents and vehicles for fragrance ingredients, as perfume fixatives and as alcohol denaturants.

Some phthalates, like dibutyl phthalate, diethylhexyl phthalate, dimethoxyethyl phthalate, pentyl and dipentyl phthalates (mixed isomers) and benzyl butyl phthalate, are prohibited under Annex II of the EU Cosmetics Directive, on the basis of being classified as toxic for reproduction under Council Directive 76/769/EEC.

However, other phthalates like dimethyl phthalate and diethyl phthalate are allowed in cosmetic products without any restriction, despite some evidence that links exposure to phthalates with DNA damage in human sperm (Duty *et al.*, 2003). Moreover, a recent investigation shows a relationship between phthalates and respiratory problems in adults (Hoppin *et al.*, 2004).

DETERMINATION OF FRAGRANCE INGREDIENTS AND OTHER INTERESTING FRAGRANCE-RELATED INGREDIENTS IN COSMETICS

Analysis of perfumes and perfumery raw materials

Analytical aspects related to perfumes involve, overall, characterizing the extracts obtained by perfume manufacturers to check whether they fulfil the desired quality

requirements (ratio of fragrance ingredients, presence/absence of undesired compounds or contaminants, etc.), i.e., for quality control, and also to characterize new extracts obtained from different sources or obtained by different methods. Also, quality control should be required when the different extracts and/or pure fragrance ingredients (synthetic or natural) are blended in order to create the perfumery raw material (fragrance compound), which will later be sold to cosmetics manufacturers.

In this last case, additional quality control of perfumery raw materials by cosmetic manufacturers is not needed, since perfume manufacturers issue a certificate that guarantees the quality of the perfumery raw material. Nevertheless, cosmetic manufacturers can obviously perform a quality control of the raw material that they are buying to manufacture their cosmetics, in order to avoid quality variations in their final product.

Different assays can be carried out. Measurement of physical properties like refractive index, optical rotation, density, colour and/or solubility in different solvents, are commonly applied to perfumes and perfumery raw materials.

Also, the "noses" of trained personal are also useful for checking the notes of the perfume.

From a chemistry standpoint, the determination of the acidity and the measurement of the saponification and carbonyl indexes, provide general information on the global quality control of the perfumes. The use of spectroscopic analytical techniques, like ultraviolet/visible spectrometry (UV/VIS), infrared spectrometry (IR) and nuclear magnetic resonance (NMR), also provide valuable information about quality. However, these measures do not provide feasible qualitative and quantitative information of the fragrance chemicals they contain. They are limited to providing general qualitative information of the perfume as a whole. So, more sophisticated analytical techniques are necessary, especially taking into account that any one perfume could be made up of hundreds (sometimes thousands) of fragrance ingredients.

Separation techniques like chromatography are the most suitable analytical techniques for these purposes. Bearing in mind that the fragrance chemicals usually have a low-boiling point, gas chromatography (GC), both by injection or in headspace (HS) mode, is the most widely used technique in the perfume industry. After appropriate sample preparation, and optimized experimental conditions, using flame ionization (FID) or thermal conductivity (TCD) detectors can establish the individual Kovats Index (KI) for each compound, which represents a relative measurement of the retention time with respect to a group of known hydrocarbons. The identification is carried out by comparing the experimentally determined KI with values kept in databases. However, sometimes it is a little bit more difficult, since there could be several peaks with very close KI. In addition, although databases are frequently updated, new ingredients might be present, and thus the database will not be able to identify these new compounds. Nevertheless, sometimes, a mass spectrometry (MS) detector coupled with GC can help to solve this problem, since the chemical structure can be elucidated by studying the mass spectra of the compound, as well as being databases that also identify predefined compounds. Moreover, the MS detector provides greater sensitivity and higher selectivity than the other aforementioned detectors. The use of GC coupled with electronic noses (see Section 6.3) can also be useful in some cases.

Obviously, GC is also used for quantitative purposes. Chiral columns can be used to separate and quantify the different optical isomers from the same chemical, which is very

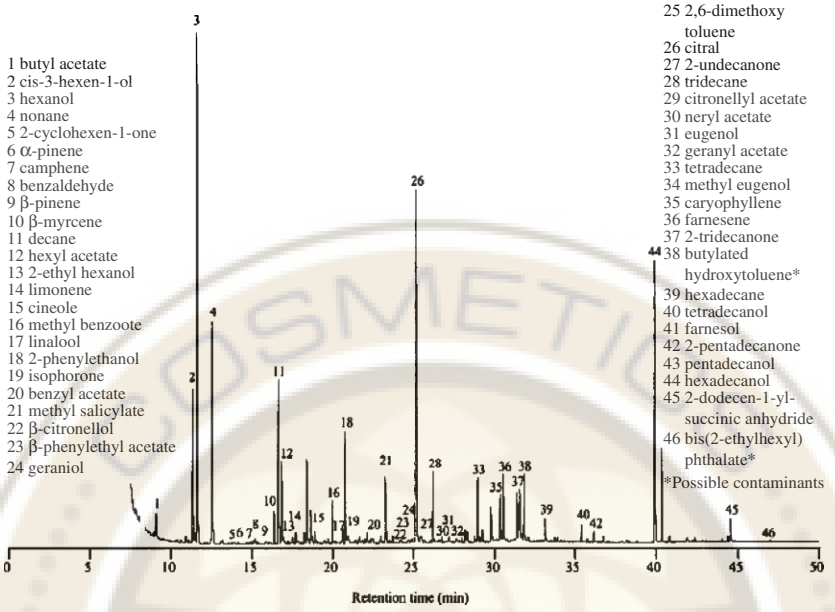

Figure 6.1.2 GC-MS chromatogram of fragrance chemicals from *Rosa hybrida* (adapted from Kim *et al.*, 2000).

important, since, as mentioned previously, there are compounds which exhibit different properties depending on their isomeric form.

On the other hand, liquid chromatography (LC) and thin-layer chromatography (TLC) have also been applied for quantitative and/or qualitative purposes in perfume analysis, more specifically to determine both low volatile or thermolabile fragrance chemicals.

It should be emphasized that the characterization of essential oils, covers a huge area of analytical chemistry, upon which many papers are published. Different review papers covering this topic can be found in scientific literature (Marriot *et al.*, 2001; Van Asten, 2002; Schulz and Baranska, 2005). So, papers focusing on perfume characterization have not been reviewed here, since the aim is to review those papers focusing in the determination of fragrance chemicals in cosmetic products.

To give an example of the many fragrance chemicals that can be present in an extract, Figure 6.1.2 shows a GC–MS chromatogram obtained in the analysis of *Rosa hybrida*.

Determination of fragrance chemicals in cosmetic products

Different fragrance chemicals have been determined in cosmetic products for one reason or another. As mentioned previously, these types of chemicals usually have low boiling points, and consequently GC is the technique of choice. However, other techniques are also used, making it feasible to determine fragrance chemicals that are difficult to determine by GC, due to their low volatility and/or their thermostability.

First of all, it should be pointed out that the analysis of cosmetic products focusing on fragrance chemicals by means of electronic noses is discussed in Section 6.3, so articles dealing with this technique will not be covered in this section.

Numerous papers have been published that deal with the determination of different fragrance chemicals currently banned by the EU Cosmetics Directive. Thus, Wisneski (1976) proposed a method based on TLC with fluorimetric (FL) detection to determine bergapten (also known as 5-methoxypsoralen) and other lactones in different fine fragrances. Later, Bettero and Benassi (1983a) also determined bergapten (and citropten) but by LC with FL detection. Wisneski *et al.* (1983) also used LC-FL to determine the carcinogen safrole, which is currently banned under Annex II of the EU Cosmetics Directive.

Other methods to determine substances currently cataloged by the EU Cosmetics Directive as PASs and restricted under its Annex III, were published in the years before these restrictions came into effect (Directive 2003/15/EC). Thus, coumarin (and also 6-methylcoumarin, which is also restricted by Annex III) was determined by LC with ultraviolet/visible (UV/VIS) detection (Bettero and Benassi, 1983b), and more recently by GC with electron capture detection (ECD) (Wisneski, 2001a); cinnamal, previously derivatized, by LC-FL (Wisneski *et al.*, 1984); isoeugenol (both *cis* and *trans* isomers) by cation exchange LC with FL detector (Wisneski *et al.*, 1988a); cinnamyl alcohol by LC-FL (Wisneski *et al.*, 1988b) and by TLC with ultraviolet absorptive densitometry (UAD) (Sherma and Brubaker, 1989); limonene (jointly with other four non-PASs fragrance chemicals called menthone, menthol, carvone and anethole) was determined in toothpastes by Tavss *et al.* (1988) by means of GC-FID using HS injection mode; linalool and five other non-PASs substances (benzyl acetate, allyl heptanoate, undecavertol, 3-phenylethyl isobutyrate, methyldihydrojasmonate) were determined in different shampoos by Chen *et al.* (2006) by GC-MS and previous extraction of the target analytes by solid-phase microextraction (SPME). Nevertheless, since Directive 2003/15/EC came into effect, efforts have been focused on the determination of all PASs together. This topic is reviewed in great depth in Section 6.2.

The determination of musks arouses special interest since, as mentioned previously, several side-effects have been described. Consequently, Wisneski *et al.* (1982) proposed a GC-MS method to determine the synthetic musk called acetyl ethyl tetramethyl tetralin; musk ambrette, musk ketone, moskene, musk tibetene and musk xylene were determined by TLC and by LC-UV/VIS in fine fragrances (Bruze *et al.*, 1985) and later by GC in different cosmetics (Sommer, 1993). A method for only qualitative determination was proposed by Goh and Kwok (1986) based on TLC or GC-FID or also GC-MS for identification of musk ambrette, musk ketone and musk xylene in cologne; and two years later, Porcu and Spanneda (1988) and more recently Wisneski (2001b) also determined the same musks by GC but by means of an electron-capture detector (ECD); finally, Sommer and Juhl (2004) proposed a GC-MS method to determine different macrocyclic musks in cosmetics.

Other aroma chemicals have been determined in different cosmetic products. So, in fine fragrances we can find papers focusing on cinnamyl anthranilate determination by LC-FL (Demers *et al.*, 1987) or by TLC with fluorescence densitometry (FD) (Sherma and Pilgrim, 1988), and benzylidene acetone determination by LC-FL (Yates and Wenninger, 1988) or

by TLC-UAD (Anderton and Sherma, 1996); α-ionone by LC-UV/VIS (Trivedi, 1988) and gluconolactone by UV/V (Zhang *et al.*, 1996) have been determined in toothpastes; 4-terpineol have been determined in shampoos, creams, shower gels, etc., by GC-FID. It is worth mentioning the paper published by Coleman *et al.* (1998), who separated and quantified D-menthol and L-menthol in different cosmetic products by using GC-MS with chiral capillary columns.

It should, however, be pointed out that there are no official analytical methods for the determination of fragrance ingredients in cosmetic products, except the method proposed by the IFRA to determine 24 PASs (IFRA, 2003) (see Section 6.2).

Determination of other interesting fragrance-related ingredients in cosmetic products

As mentioned previously, phthalates represent a group of analytes that are interesting to identify in perfumed cosmetics, since various undesirable effects have been associated with exposure to this family of chemicals. However, to our knowledge, no official analytical methods focus on their determination in cosmetics.

Nevertheless, different methods have been found in the scientific literature. To our knowledge, the first published paper focusing on this topic is that published by Hancock *et al.* (1966), who determined DEP by three different methods, namely GC-FID, LC-UV/V and gravimetry. The gravimetry method, which requires the conversion of DEP to phthalic acid by alkaline hydrolysis and the subsequent reaction with aniline to convert in phthalanil, is the most accurate.

Godly and Mortlock (1973) proposed a GC methodology for the determination of DEP, DMP, dipropyl phthalate (DPP) and dibutyl phthalate (DBP) in hair lotions and after-shave lotions.

Later, Koo and Lee (2004) determined DBP, DEP, diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) by LC in different cosmetic products, in order to predict human exposure to phthalates.

Moreover, Chen *et al.* (2004, 2005) proposed both LC with diode-array detector (DAD) and GC-FID methods to determine six phthalates, namely DMP, DEP, DBP, BBP, DEHP and dioctyl phthalate (DOP). Lower detection limits were obtained with the GC-FID method.

Recently, Hubinger and Havery (2006) have also published a LC-UV/V method for the quantification of DMP, DEP, BBP, DBP and DEHP in different cosmetics.

SUMMARY

Perfumes are extensively used in cosmetics. Different fragrance chemicals and other chemicals present in perfume composition have been shown to cause undesirable side-effects. The EU Cosmetics Directive prohibits some of these in cosmetics to be marketed within its framework. Other international organizations, like IFRA and RIFM, work on the safety of fragrance chemicals, and release reports on the convenience or not of using certain fragrance chemicals in cosmetics, depending on their toxicity.

However, there are no official analytical methods focusing on the determination of these substances in cosmetics, which could be used to monitor them, except the method published by the IFRA focusing in the determination of the 24 potentially allergenic chemicals declared by the EU Cosmetics Directive. Nevertheless, different methods can be found in scientific literature focusing on this topic, but they obviously do not cover all the fragrance chemicals involved in cosmetic manufacture, which are in thousands. However, researchers should be encouraged to develop methods to control almost all those fragrance chemicals that have been shown to pose a safety hazard.

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6.2. Analytical Methods to Determine Potentially Allergenic Fragrance-Related Substances in Cosmetics

A. Chaintreau*

Firmenich S.A., Corporate R&D Division

INTRODUCTION

The European Union Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers (SCCPNFP), currently known as the Scientific Committee on Consumer Products (SCCP), published a review on potentially allergenic substances (PASs) related to fragrances (SCCNFP, 1999). In this respect, the European Union (EU) published an amendment to the Annex III of the EU Cosmetics Directive (Directive 2003/15/EC) in 2003, which clearly sets out the conditions of use in cosmetic products for 26 fragrance-related substances classified as likely to cause allergenic reactions (Directive 2003/15/EC). Of these 26 substances, 24 are chemically defined volatile compounds whereas the other two are natural moss extracts and do not correspond to defined chemicals (see Table 6.2.1). Some of the constituents contained in these natural extracts, such as atranol and chloroatranol, have proven to be skin sensitizers (Bernard, 2003; SCCP, 2004). Nevertheless, they are not present in mosses themselves but originate from the degradation of atranorin and chloroatranorin during the extraction process. However, these compounds are not regulated as such.

The above-mentioned amendment, within the EU framework, stipulates it as mandatory to declare the presence of any of these aforementioned 26 PASs on the product label when present at concentrations exceeding 0.001% in cosmetics intended to remain on the skin or 0.01% in those rinsed off the skin. This is in contrast with other fragrance chemicals that are not needed to be declared individually, but can be grouped and labelled under the word "*perfume*" or "*aroma*", as mentioned in Section 1.2.

Two years before this amendment came into effect, no valid analytical method existed to monitor PASs either in cosmetics or in fragrance concentrates used to perfume cosmetics. On one hand, this has posed a great challenge to the fragrance industry to establish methods capable of quantifying traces of these 26 chemicals from among the huge number of perfume constituents of any given formula, which are often present in much greater concentrations than PASs. On the other hand, this challenge has promoted new developments currently improving analytical techniques in the field of fragrances and cosmetics.

*Corresponding author. E-mail: alain.chaintreau@firmenich.com

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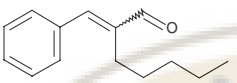
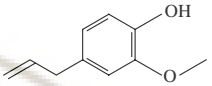
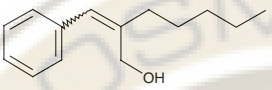
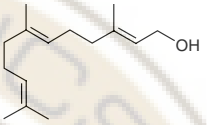
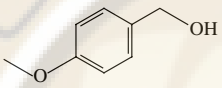
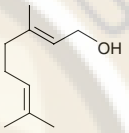
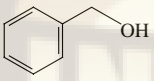
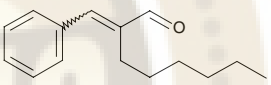
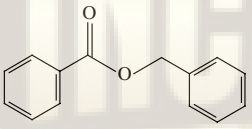
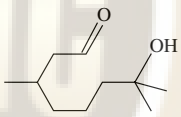
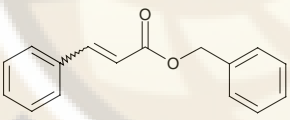
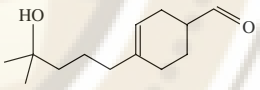
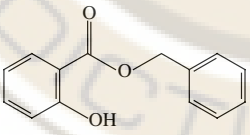
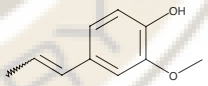
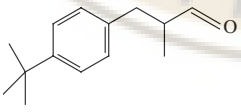
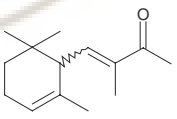
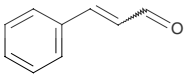
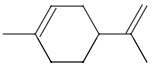
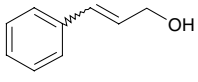
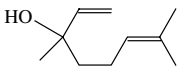
Amparo Salvador and Alberto Chisvert

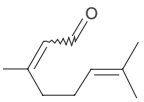
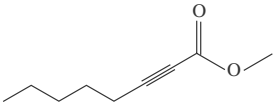
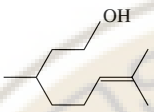
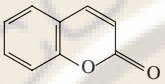
Table 6.2.1

List of the 26 potentially allergenic fragrance-related substances according to the European Union Cosmetics Directive

INCI name [CAS number]	Structure	INCI name [CAS number]	Structure
Amyl cinnamal [122-40-7]		Eugenol [97-53-0]	
Amylcinnamyl alcohol [101-85-9]		Farnesol [106-28-5]	
Anise alcohol [105-13-5]		Geraniol [106-24-1]	
Benzyl alcohol [100-51-6]		Hexyl cinnamal [101-86-0]	
Benzyl benzoate [120-51-4]		Hydroxycitronellal [107-75-5]	
Benzyl cinnamate [103-41-3]		Hydroxyisohexyl 3-cyclohexene carboxaldehyde [31906-04-4]	
Benzyl salicylate [118-58-1]		Isoeugenol [97-54-1]	
Butylphenyl methylpropional [80-54-6]		Alpha-isomethyl ionone [127-51-5]	
Cinnamal [104-55-2]		Limonene [5989-27-5]	
Cinnamyl alcohol [104-54-1]		Linalool [78-70-6]	

(*Continued*)

Table 6.2.1 (Cont).

INCI name [CAS number]	Structure	INCI name [CAS number]	Structure
Citral [5392-40-5]		Methyl 2-octynoate [111-12-6]	
Citronellol [106-22-9]		Evernia prunastri extract [90028-68-5]	(Oakmoss extract)
Coumarin [91-64-5]		Evernia furfuracea extract [90028-67-4]	(Treemoss extract)

This section is divided into two sections: the former reviews the different analytical techniques used to determine PASs in cosmetics and/or fragrance-related products, while the latter focuses on sample pretreatment.

ANALYTICAL TECHNIQUES FOR THE DETERMINATION OF PASs

To determine the aforementioned 24 chemically defined PASs at once, GC is a good candidate as this technique is used to analyse volatile compounds and offers high separation capabilities of complex mixtures. The determination of chemicals contained in the two potentially allergenic natural moss extracts will be discussed later.

Gas chromatography

The variety of PAS functional classes requires a universal detection system (i.e. not restricted to a given functional class). Moreover, fragrance concentrates used in cosmetics are sometimes made up of over a hundred constituents occurring at concentrations ranging from µg/kg up to 20% or more. Therefore, quantifying PASs in cosmetics cannot be achieved with a simple flame-ionization detector (FID) coupled to the GC instrument, as the targeted analytes are very frequently eluted together with the fragrance constituents or other volatile ingredients coming from the cosmetic matrix. However, an early paper, dealing with just 11 PASs, proposed quantifying target compounds in different cosmetic products by GC-FID, but it was necessary to identify the target analytes previously by means of gas chromatography-mass spectrometry (GC-MS) under electron impact (EI) ionization mode (Rastogi, 1995). This approach doubles the total analysis time when any PAS is present, and moreover, fails to overcome biases due to co-elutions of target compounds with other ingredients. Later, the same author also proposed using MS detector selectivity in the quantification step

(Rastogi, 2002). However, this latter work used ions extracted from an analysis in scan mode, which is less sensitive and accurate than selected ion monitoring (SIM) mode (Gilbert, 1987; Gross, 2004), which only monitors a few previously selected ions. We can find the reason for this in the fact that the detector measures a given ion for much less time in scan mode than in SIM mode, since scan mode performs a screening of the whole spectrum (usually more than 100 ions) in a given period of time, whereas SIM mode only measures a few ions (i.e. those needed, that are around three ions) in the same period of time. This makes the signal for an ion obtained by SIM mode higher than that obtained by scan mode, resulting in a greater area, and thus in a greater accuracy of the peak integration. Also, as higher sensitivity is obtained, the sample amount to be injected can be reduced and so overloading the column is avoided allowing better peak resolution. Using a SIM approach based on monitoring two ions, Ellendt *et al.* (2001) analysed different deodorants, and reported recoveries in the 98–106% range, with a detection limit (LOD) of 2 mg/L. However, this paper does not mention whether abundance ratios of these ions were used to check the identity of compounds in the SIM experiment. Under these conditions, analysing a complex fragrance concentrate containing 168 constituents spiked with 5 PASs at level of 20 mg/L led to false positives and negatives mainly due to unsatisfactory analyte identification (Chaintreau *et al.*, 2003), which clearly shows that this method was not optimum for complex fragrances.

The SIM approach was fully reconsidered by the International Fragrance Association (IFRA), which proposed a method that can be used as a reference in the laboratories of the fragrance industry to determine PASs in fragrance concentrates (IFRA, 2003; Chaintreau *et al.*, 2003). The GC separation was optimized and three ions per analyte were used to help identify the analyte: one of them for quantification, and the other two as qualifiers. For a compound eluting in the expected, pre-defined retention window, its identity is checked by comparing its ion abundance ratios with those of a reference compound using the value Q (see equation 6.2.1). When its value is at least equal to 90, the analyte is considered as being positively identified.

$$Q = 100 - \frac{\sum_{i=1}^{i=n} 100^{*} \left| r_i - r_i' \right| \left(\ln\left[100 r_i + 1\right] \right)^2}{21.3^{*} \sum_{i=1}^{i=n} r_i} \qquad (6.2.1)$$

where: n: Number of ions per compound,
r_i: Reference peak area ratio, and
r_i': Observed peak area ratio.

Three PAS-free fragrance concentrates of increasing complexity (32, 57 and 168 constituents) were spiked with five compounds randomly selected from the list of 24 PASs (see Table 6.2.2). The mean recovery, calculated from the results in Table 6.2.2, was 100.5%, with a coefficient of variation of 16%. Some peaks with Q values below 90 and eluting in the expected PASs time windows required confirmation in scan mode to confirm their presence. Moreover, linalool and benzyl benzoate were over-evaluated in the most complex sample, due to co-elution of isobaric ions. Thus, although in general terms the method provides good results, the aforementioned problem of co-elution may still occur.

Fragrances may contain low- or non-volatile constituents that remain in the injector and cause analyte retention and/or artefacts (Chaintreau *et al.*, 2003). Figure 6.2.1 (top) shows

Table 6.2.2

Quantification of three PAS-free spiked fragrance concentrates by using a GC-MS(EI) SIM approach with three ions per compound (adapted from Chaintreau *et al.*, 2003)

Analyte	Sample 1[a] (containing 32 constituents)		Sample 2[a] (containing 57 constituents)		Sample 3[b] (containing 168 constituents)	
	Amount (mg/L)	*Q*	Amount (mg/L)	*Q*	Amount (mg/L)	*Q*
Phenylacetaldehyde[d]	4.7	75	**50.1**	**95**	20.2	54
Limonene			296.9	16		
Linalool	2.9	26			**127.6**	**97**
Estragole[d]	**46.5**	**99**				
Citronellol	329.5	24	**49.5**	**96**	3700	25
Geraniol	**45.1**	**99**			30.0	1
Anise alcohol					**98.0**	**97**
Hydroxycitronellal	**44.5**	**99**				
Methyl 2-nonynoate[d]	**42.7**	**98**	4.2	50	1548	25
Cinnamyl alcohol			18.9	27		
Eugenol			**45.2**	**99**	**96.6**	**92**
Methyl eugenol[d]	**47.7**	**98**				
Alpha-isomethyl ionone			364.2	33	**98.9**	**98**
Amyl cinnamal	14.7	26	**49.5**	**97**	112.3	28
Hydroxyisohexyl 3-Cyclohexene carboxaldehyde	11.8	1	8.4	1	72.1	1
Amylcinnamyl alcohol	442.15	80 [c]	16.2	1	5460	62
Farnesol	28.7	1	**49.8**	**80**[c]	204.2	1
Hexyl cinnamal	6.7	66[c]			32.8	78
Benzyl benzoate	3.7	69[c]			**145.6**	**99**
Benzyl salicylate	47.1	77[c]	14.1	11	459.3	86

[a]Spiked with 50 mg/L of each 5 compounds reported in bold.
[b]Spiked with 100 mg/L of each 5 compounds reported in bold.
[c]Presence/absence checked in scan mode.
[d]Other compounds, not listed in the EU Cosmetics Directive list of the 24 potentially allergenic fragrance-related substances.

that re-injecting one of the calibration standards after the calibration and using a dirty injector gives a lower determination than the expected concentration, whereas repeating the same experiment with a clean injector (Figure 6.2.1 (bottom)) gives the correct determination. The fact that injector cleanliness appears to be a prerequisite to PASs quantification implies that crude cosmetic extracts should not be injected directly into the GC-MS instrument without previously removing non- and low-volatile constituents with an appropriate clean-up step. The same work also shows that a GC-MS calibration may only be used for a few days (Figure 6.2.1 (bottom)), due to the drift of the MS instrument over time.

To overcome possible false positives (mainly due to co-elutions of perfume constituents exhibiting isobaric ions in common with PASs) and false negatives (due to the shift that a large non-related eluting peak in front of the SIM window can promote on the PAS retention time), a recent paper proposed successively injecting the sample into two columns

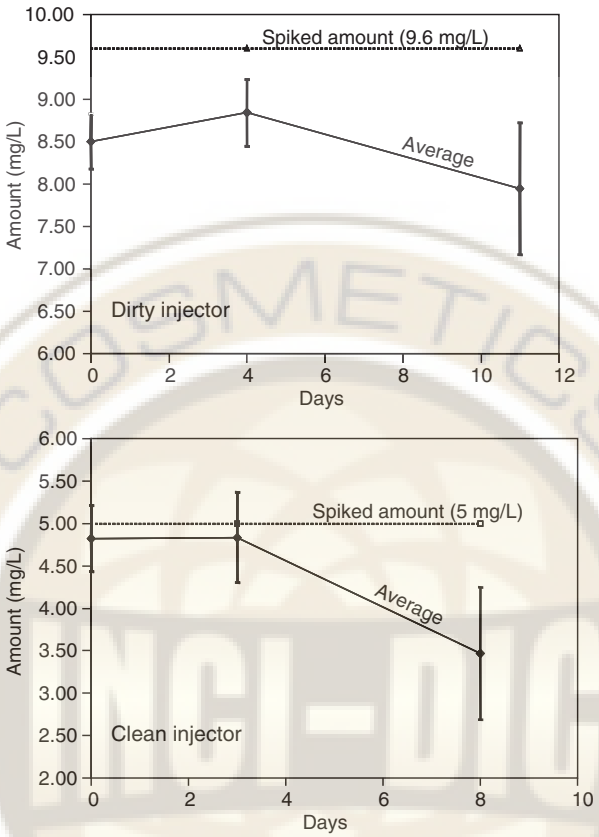


Figure 6.2.1 Calibration alterations due to injector pollution (top) and to the drift of the MS detector (bottom) (adapted from Chaintreau *et al.*, 2003).

with a different phase polarity, installed in a single GC-MS, and quantifying by using ions extracted from full scan monitoring mode (Leijs *et al.*, 2005). This approach minimizes the risk of analyte over-evaluation due to co-elutions, as the latter are unlikely to occur in both columns for the same compound. If results obtained with both columns for a given PAS do not match, the right one (absence of co-elution) can be chosen by examining the whole spectrum. On the other hand, when a target compound is shifted because it is preceded by an abundant peak, it can easily be relocated, as, unlike the SIM mode, there is no retention time window. In addition, if a given quantification ion is co-eluted with an isobaric one, another interference-free ion can be chosen from the whole mass spectrum. On the one hand, injecting the sample twice increases the run time, but on the other, since data interpretation is the time-consuming step, this procedure does not significantly alter the laboratory's throughput whereas it simplifies the analyst's task and improves result reliability.

In Table 6.2.3, five cases of co-elution can be observed in a spiked perfume, using the non-polar column, whereas they are separated and determined using the polar column. The reverse situation (co-elutions in the polar column) may also occur.

Table 6.2.3

Quantification of a perfume spiked with 10 PASs at a level of 50 mg/kg by GC-MS(EI), by running on both non-polar polydimethylsiloxane-based (PDMS) and polar polyethyleneglycol-based (PEG) columns (adapted from Leijs *et al.*, 2005)

Analyte	Contents (mg/kg)		
	PDMS column	PEG column	Nominal
Benzyl alcohol	50	54	54
Benzyl cinnamate	52	53	53
Benzyl salicylate	Co-eluted	55	56
Cinnamal	Co-eluted	51	53
Citronellol	Co-eluted	53	52
Eugenol	Co-eluted	50	53
Butylphenyl methylpropional	49	54	53
Limonene	57	57	50
Linalool	53	50	51
Hydroxyisohexyl 3-Cyclohexene carboxaldehyde	Co-eluted	52	52

However, quantifying by using ions extracted from scan mode is known to jeopardize determination accuracy, as well as sensitivity (Gilbert, 1987; Gross, 2004), as we discussed earlier. For a certain amount of a given analyte, the scan mode allocates less time to measure a given ion during peak elution than in SIM mode. This limits the signal-to-noise (S/N) ratio, i.e. the sensitivity, compared to that resulting from a SIM analysis. By contrast, this S/N can be further increased in SIM by enhancing the detector voltage, without significantly modifying the background level. As a consequence, not only is sensitivity improved, but the greater peak area gives greater accuracy of the peak integration than in scan, especially for trace analysis. This should be taken into account when determining PASs, since this method could not be suitable for this purpose. As reported by David *et al.* (2004), detection limits ranging from 5 to 60 mg/kg have been observed in scan mode, versus 0.1 to 2 mg/kg in SIM mode. The limit of quantification (LOQ) is hence higher (at least 3.3 times higher, i.e. 17–200 mg/kg and 0.33–6.6 mg/kg in scan and SIM mode, respectively). Such a scan LOQ does not fulfil the limit of 0.001% (i.e. 10 mg/kg) in leave-on cosmetic samples required by the EU Cosmetics Directive. Due possibly to this difference of sensitivity, the injected amounts in Leijs' paper were five times higher than in the IFRA procedure, which increases the risk of column overloading, hence the risk of peak overlapping and retention time shift. Therefore, this method is only reserved to determine PASs in mixtures where they occur in a significant concentration.

Concurrently to Leijs' paper, a technical note also reported GC-MS determination in scan mode of PASs, using a single, fast-GC column (Baier, 2005). However, given the above-mentioned drawbacks, the use of a single fast column is unlikely to be applicable to the complexity of fragrances; moreover, no attempt to validate the quantitative results was reported.

Another alternative to overcome interferences due to co-elutions, is to choose more selective ions in the SIM mode. Under EI conditions, as those used in the above-described methods, compounds are usually very fragmented, and so some compounds may only exhibit very common low-mass fragments that may be confused with other

Table 6.2.4

Results obtained by GC-MS in EI and CI ionization modes, and GC×GC-FID and GC×GC-MS(EI) strategies, in the analysis of a PAS-free fragrance concentrate containing 168 ingredients spiked with five PASs at individual levels of 50 mg/kg (adapted from Debonneville *et al.*, 2004)

Analyte	GC-MS(EI)[a]	GC-MS(CI)[a]	GC×GC-FID	GC×GC-MS[a]
	PDMS[b]	PDMS[b]	PDMS×PEG[b]	PDMS×PCAS[b]
Linalool	6296[c]	57	510[c]	41
Anise alcohol	782[c]	67	n.q.[d]	52
Eugenol	43	39	50	48
Alpha-isomethyl ionone	64	35	52	53
Benzyl benzoate	54	62	54	51
Mean	n.a.[d]	54	n.a.[d]	49
RSD	n.a.[d]	21	n.a.[d]	10

[a]MS determination were performed in SIM mode.
[b]PDMS: Polydimethylsiloxane-based, PEG: polyethyleneglycol-based and PCAS: polycyanoalkylsiloxane-based columns.
[c]Co-elutions.
[d]n.q.: not quantifiable due to poor precision of automated peak detection; n.a.: not applicable.

perfume constituents. In contrast, the softer chemical ionization (CI) gives rise to less fragmentation and yields more abundant ions at higher masses, which increases selectivity. Cadby *et al.* (2003) employed this alternative by using ammonia as the reagent gas, and the observed ions mainly corresponded to the following reactions:

$$\text{Ammonium adducts: } NH_4^+ + PAS \rightarrow [PAS + NH_4]^+$$
$$\text{Proton transfers: } NH_4^+ + PAS \rightarrow [PAS + H]^+ + NH_3$$

Ammonium adducts and proton transfer ions are preferentially observed with polar and basic compounds, respectively. Increased specificity is exemplified with the ammonia CI of farnesol, which exhibits abundant fragments at high masses (205, 207, 224 and 240 uma), whereas all ions exhibiting a significant abundance obtained by EI are very common and below 65 uma.

A complex PAS-free fragrance concentrate (containing 168 ingredients) spiked with five PASs was analysed by using GC-MS (CI). Results are shown in Table 6.2.4, and compared with those obtained by using the routine IFRA method (i.e. GC-MS (EI)). As can be seen, linalool and anise alcohol, which could not be determined accurately using the EI mode due to co-elutions, were satisfactorily evaluated by the CI mode. This shows that this technique provides an alternative to the EI method, when the latter fails to determine a co-eluted analyte in complex samples. However, its routine application is limited by the stability of the CI source pressure that requires frequent calibrations.

Multidimensional gas chromatography

Multidimensional gas chromatography (MDGC) is known to allow quantitative determinations of fractions that have been heart-cut from the first column and further separated in

the second column (e.g. White *et al.*, 1990). In the case of PASs, heart-cuts corresponding to their respective elution zone from the first column were selectively transferred in the second dimension. To my knowledge, the quantitative performance of the MDGC technique applied to PASs is still being developed (David *et al.*, 2004). An example of the peak resolution improvement is shown in Figure 6.2.2.

Comprehensive two-dimensional gas chromatography

Comprehensive two-dimensional gas chromatography (GC×GC) is a recent technique, proposed in the 90s by Philips (Liu and Phillips, 1991). Analytes eluting from a first and

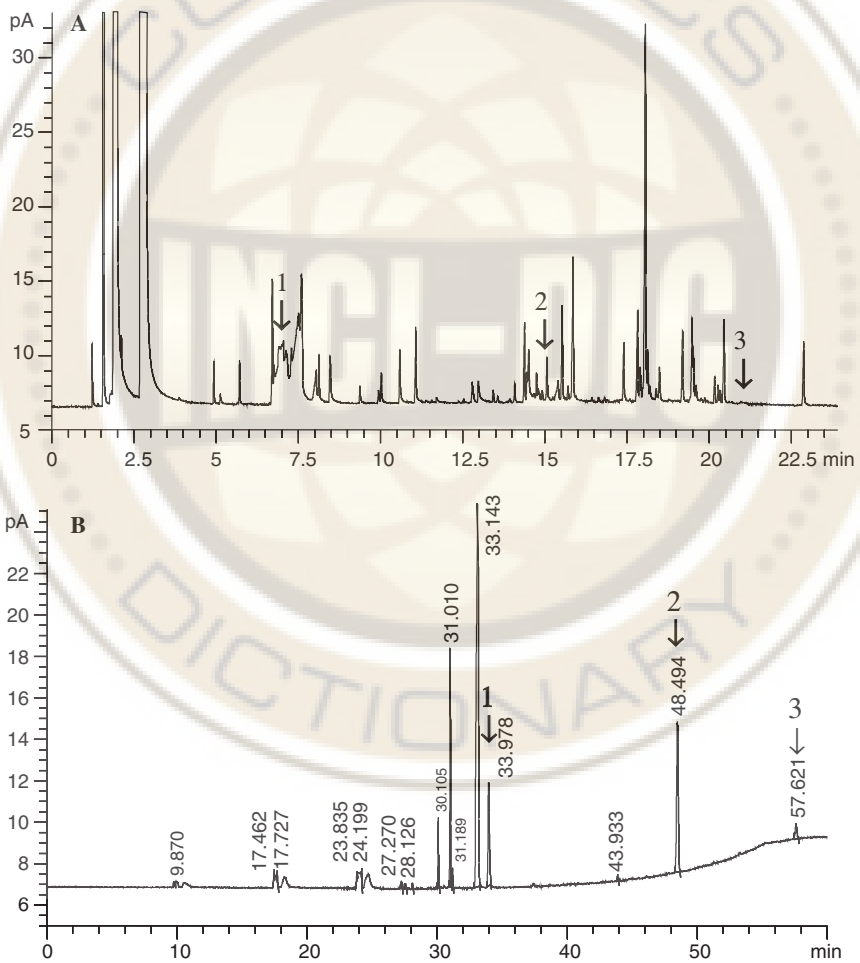


Figure 6.2.2 MDGC analysis of a perfume. A: first dimension, B: second dimension after three heart-cuts. (1) benzyl alcohol, (2) coumarin, and (3) benzyl salicylate (adapted from David *et al.*, 2004).

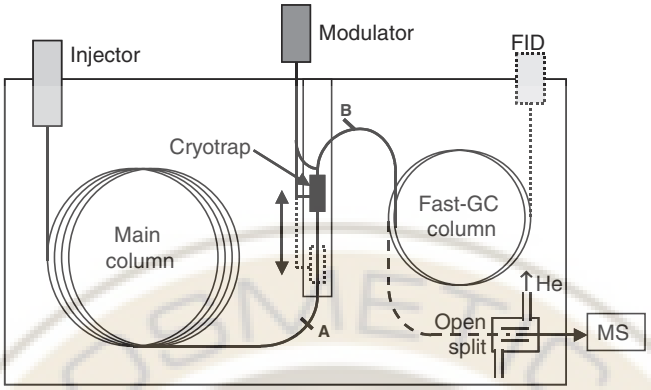


Figure 6.2.3 Comprehensive gas chromatography with either MS (long dotted line) or FID (short dotted line) detection and using a longitudinally modulated cryogenic system (from Debonneville *et al.*, 2004).

classical capillary column are re-focused (e.g. in a cryo trap as shown in Figure 6.2.3) and periodically transferred into a second, fast-GC column to be further separated, which requires a high sampling rate detector. In practice, this technique is like a MDGC, where the heart-cut and re-injection in the second column would be constantly repeated (every 2–5 s) along the whole chromatogram. In contrast to MDGC, there is no need to target the analytes in question or to adjust the time windows accordingly, as the first chromatogram is permanently "sliced".

A priori, the overall peak capacity of GC×GC is the product of peak capacities of both columns. But, in fact, the peak capacity of the first column can only be multiplied by up to 10 (Blumberg, 2003), which, in any event, greatly exceeds the abilities of any other GC technique. A detailed description of the technique can be found in various reviews (Ong and Marriott, 2002; Shellie and Marriott, 2003).

Chromatograms resulting from a GC×GC analysis can be represented as three-dimensional figures (Figure 6.2.4 (left)). The virtual chromatogram along the first axis (^{1}D) (Figure 6.2.4 (left), in red) indicates what would be observed with a monodimensional GC. As fractions of this virtual chromatogram are periodically re-injected in the second column after each modulation of the cold trap, a series of brief chromatograms is obtained (Figure 6.2.4 (left), blue lines). If the peak width in the first dimension exceeds the modulation period, the corresponding peak may appear in several modulations. Therefore, peaks belonging to a same compound are grouped (green cycles) and chromatograms are usually represented in two-dimensions as "contour plots" (Figure 6.2.4 (right)) where peak intensity is coded by appropriate colors within a spot and then integrated using a dedicated software.

Two configurations have been tested for PASs determination. First an FID was used by Shellie *et al.* (2004) but, as shown in Table 6.2.4, it did not perform better than a GC-MS for a complex sample. This was presumably due to the fact that GC×GC-FID and GC-MS are both two-dimensional techniques, which seems to be insufficient for the resolution of

most complex co-elutions. Therefore, coupling GC×GC to MS, would become a powerful tool. Thus, in another paper by the same group (Debonneville and Chaintreau, 2004) a quadrupole MS in EI ionization mode was used as a detector (see Figure 6.2.3). However, as mentioned above, the short and narrow column of the second dimension is operated in fast mode and requires a high sampling rate, which does not make quadrupole an ideal candidate as a GC×GC detector. In spite of its low sampling rate, good quantitative performances were observed when only one ion was monitored separately, as can be seen in Table 6.2.4. The resulting 2-D chromatogram (Figure 6.2.5) shows that the target analytes were clearly separated from any other peak. However, these experiments were done using a prototype hyphenation between a quadrupole MS and a GC×GC chromatograph, which is not yet commercially available.

The use of GC×GC hyphenated to a time-of-flight MS (TOF-MS) detector, which provides a higher sampling rate than quadrupole, has also been reported (LECO, 2004). It offers the unrivalled advantage of allowing quantification and identification of peaks with full scan unlike the aforementioned approach. However, despite the great increase in peak resolution obtained, coelutions of target peaks with matrix peaks can still be present, which makes it necessary to apply mathematical approaches, such as peak deconvolution.

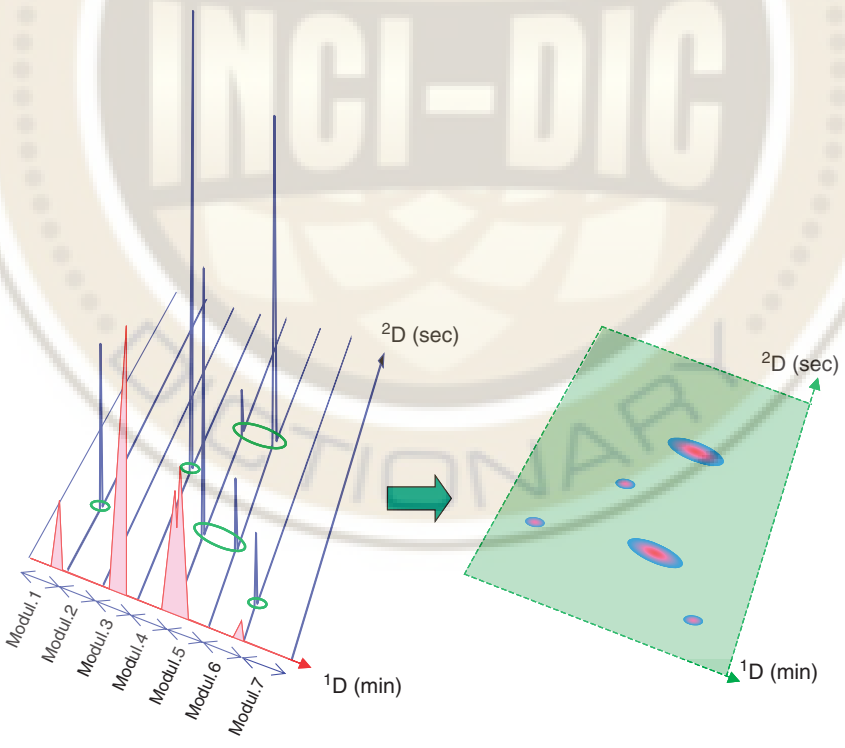


Figure 6.2.4 Principle of three- and two-dimensional representations of GC×GC chromatograms.

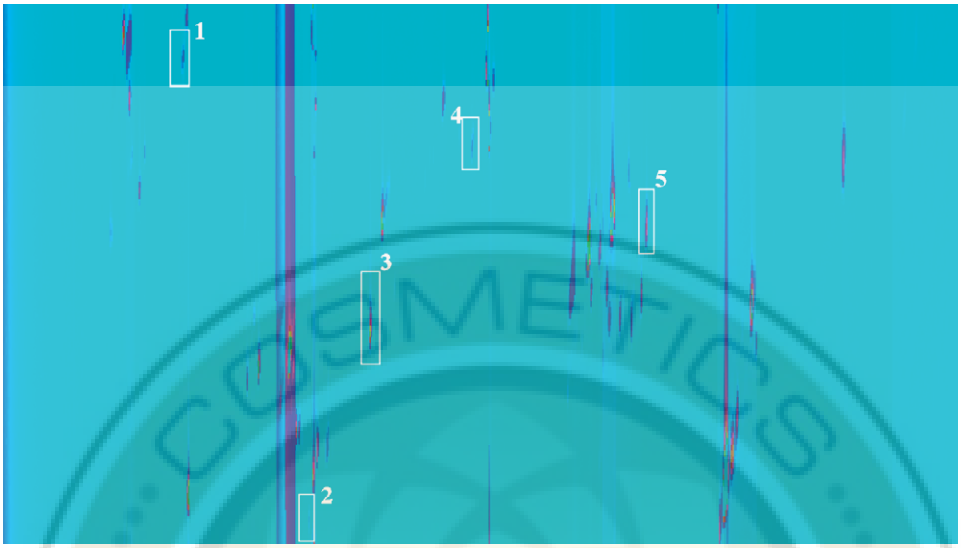


Figure 6.2.5 Contour-plot resulting from the GC×GC-MS(EI) analysis of a PAS-free fragrance concentrate containing 168 constituents spiked with five PASs at a level of 50 mg/L. (1) Linalool, (2) anise alcohol, (3) eugenol, (4) alpha-isomethyl ionone, and (5) benzyl benzoate (from Debonneville et al., 2004).

However, some problems of quantification accuracy persist, such as a lack of linearity of calibration lines (LECO, 2004) and last, but not least, the cost and complexity of such instruments are not suitable for quality control.

Gas chromatography with tandem mass spectrometry

A recent paper compares the performances of GC-MS in SIM mode to those of GC coupled to tandem mass spectrometry (GC-MS/MS), either using an ion-trap or a triple quadrupole (Kinani *et al*., 2006). The ion trap was not suitable for the PASs determination due to a high LOQ (up to 40 mg/kg), lack of linearity and high variability (up to 26% of standard deviation when re-injecting a standard at a concentration of 10 mg/kg). The statistical comparison of the GC-MS method (with a single column) with the triple quadrupole results shows, in the latter case, a lower risk of false positives and negatives. However, the authors conclude that none of the tested methods is fully satisfactory from this point of view.

Liquid chromatography with tandem mass spectrometry

Although GC is the ideal technique for volatile substances such as PASs, the presence of chromophore groups in moss constituents led Schulz and Albroscheit (1989) to analyse them by liquid chromatography (LC) with ultraviolet detection. Then Hiserodt *et al.* (2000) proposed the use of tandem mass spectrometry (LC-MS/MS) to identify atranorin

derivatives. A quantitative determination based on LC-MS/MS was finally proposed by Bossi *et al.* (2004), with a LOQ of 5.0 and 2.4 mg/L for atranol and chloratranol, respectively. Recoveries from perfumes ranged from 41 to 96%. The method was applied to investigate the presence of both chemicals in perfumes and similar products on the European market (Rastogi *et al.*, 2004).

SAMPLE PRE-TREATMENT

As seen in the previous section, GC is the technique of choice to determine PASs. Therefore, on determining PASs in cosmetic samples, they should ideally be quantitatively isolated from the matrix, without any trace of non-volatile compounds.

Although no sample pre-treatment is required for volatile samples such as fragrance concentrates and perfumes, some problems of inaccuracy might occur after several injections as previously mentioned, due to the accumulation of low-volatile compounds in the injector (Chaintreau *et al.*, 2003). Obviously, other cosmetic products with lower volatility will promote this problem. All this implies that the direct injection of a solvent extract from cosmetics is not viable, because traces of the matrix are co-extracted and then rapidly pollute the GC injectors.

Alternatively, methods used to recover PASs at a known and reproducible yield would be suitable. In this respect, many sample preparation techniques exist in the area of flavour analysis, such as direct solvent extraction followed by a solid-phase extraction (SPE) clean-up step (Rastogi, 1995, 2002), simultaneous distillation-extraction (Chaintreau, 2001), static or static-and-trapped headspace, multiple headspace extraction, etc. (Chaintreau, 2000). However, these techniques are generally applicable only to a restricted number of samples due to the time factor. They are unsuitable for answering the recent regulatory challenges mentioned previously, as they require monitoring a great number of samples. To ensure a sufficient throughput, online sample preparation techniques are required (Goosens *et al.*, 1998).

Due to these different challenges, the analysis of PASs in cosmetics remains a hot research topic and, at the moment of writing this section, most of these research works are still unpublished or in press.

Different recent approaches based on sorbtive methodologies, which allow fully automated sample preparation, are presented next.

Sorbtive extraction methods

Sorbtive extraction methods involve the extraction of compounds into a solid absorbent (fibre) and—for volatile compounds—a subsequent thermal desorption in a GC injector. The two main techniques are solid phase micro-extraction (SPME) (Pawliszyn, 1997) and stir-bar sorbtive extraction (SBSE) (Hanaoka *et al.*, 2000). Both methods are good candidates for the automation required in the context of quality control, as commercially available robots are capable of controlling the sampling and injecting the extracted analytes into the GC.

SPME and SBSE can be used either by allowing a direct contact of the extraction phase with the sample, or by exposing the fiber to the headspace of the sample (Yang and Peppard, 1994; Bicchi *et al.*, 2000). As the latter (SPME in headspace) only recovers volatile compounds, this avoids polluting GC injectors with low-boiling ingredients of cosmetics. Quantification based on sorbtive extraction is mostly achieved under equilibrium conditions. The recoveries from direct extraction and from the headspace are given by equations (6.2.2) and (6.2.3), respectively:

$$\frac{n_f}{n_0} = \frac{K_{fs} V_f}{K_{fs} V_f + V_s} \tag{6.2.2}$$

$$\frac{n_f}{n_0} = \frac{K_{fs} V_f}{K_{fs} V_f + K_{hs} V_h + V_s} \tag{6.2.3}$$

where: n_f: amount of analyte in the fibre;
n_0: total amount of analyte in the sampling system;
K_{fs}: fibre coating-to-sample partition coefficient;
K_{hs}: air-to-sample partition coefficient;
V_f: volume of fibre coating;
V_s: volume of sample matrix; and
V_h: volume of headspace.

Recoveries of different perfume ingredients from soaps using sorbtive extraction, either via direct contact with the sample, or via the headspace, were compared to those of simultaneous distillation-extraction, but without real quantification (Fujiwara, 2004). Also, an attempt to quantify several perfume ingredients in cosmetics and hygienic products by SPME under equilibrium conditions was inconclusive (Offant *et al.*, 2002). Recently, a SBSE methodology was proposed to quantify PASs in diluted samples (David *et al.*, 2006b). This approach requires a calibration for each of the PASs in a solvent mimicking the target matrix. Recoveries from a spiked lotion ranged from 70% to 120%, the limit of detection was below 1 mg/kg and the relative standard deviations (RSD) ranged from 5% to 10% for most analytes.

However, the SBSE approach does not fulfil all criteria for routine application. The partition of analytes between the bar coating and the liquid phase (K_{fs}) depends on the composition of the latter, and so recoveries (n_f/n_0) vary from one cosmetic formula to another. Therefore, a new matrix requires a new calibration, which is time consuming. To overcome this tedious task, the matrix can be standardized by dilution of an aliquot in a large amount of another homogeneous matrix (e.g. water), as investigated in a recent work (Chen *et al.*, 2006a). After dilution in water at a level of 0.01%, the calibration curves of the fragrance ingredients in three different shampoo matrices were almost identical, which shows that the quantification is independent of the matrix. Using this strategy, Chen *et al.* (2006a) quantified six perfume ingredients (including the PAS linalool) in four spiked different shampoos. As is shown in Table 6.2.5, satisfactory determinations were obtained with a single calibration for all shampoos.

The everyday use of SPME and SBSE is based on a partition between the sample and the fibre, which only recovers a portion of analyte from the sample due to the small

Table 6.2.5

Quantification of six perfume ingredients in four different shampoos (adapted from Chen *et al.*, 2006a)

Compound	Target (mg/L)	Shampoo 1		Shampoo 2		Shampoo 3		Shampoo 4	
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Linalool	125	122	102	113	103	75	73	111	96
Benzyl acetate	313	565	410	521	409	465	399	469	377
Allyl heptanoate	83	92	80	85	77	44	47	70	66
(E)-4-methyl-3-decen-5-ol	8	18	19	17	14	14	17	15	17
2-Phenylethyl isobutyrate	42	50	42	46	42	40	41	39	38
Methyldihydrojasmonate	42	62	33	45	38	42	33	44	36

Note: (1) SBSE after dilution at 0.01% in water.
(2) Quasi-exhaustive extraction method (50 µl of 1% diluted sample in water in a 20 ml vial).

extraction volume of the fibre coating. An alternative way to overcome the matrix effect lies in optimizing the extraction conditions to obtain a quasi-exhaustive extraction of the volatile compounds from the non-volatile fraction. To maximize the recovery using a given fibre, equations (6.2.2) and (6.2.3) shows that the coating volume (V_f) should be increased, or the sample volume (V_s) and the headspace volume (V_h) should be decreased. As the former parameter is limited in size, only the last two may be significantly modified using a tiny sample amount in a small vial.

Applying this approach to the previously mentioned four shampoo formulas spiked with a same perfume enabled quantification of the aforementioned perfume ingredients in all four cases, as shown in Table 6.2.5. The resulting determinations are very satisfactory in that sample preparation time of traditional methods is greatly shortened. In contrast to the previous method (standardization of the matrix), calibration can be achieved in pure water.

If recoveries are not quantitative, they can still be improved by using a cold fibre, while heating the sample (Figure 6.2.6). Increasing the matrix temperature increases the air-to-matrix partition (K_{hs}), whereas the headspace-to-fibre coefficient (K_{fs}) is maintained due to fibre cooling. In addition, the temperature gap creates convection, which increases mass transfer rate. Under such conditions, good recoveries have been observed from a micro-drop of shampoo diluted in water, together with an excellent linear range and limit of detection.

Here again, the extraction of analytes can be fully automated owing to the miniaturization of the cold-fibre device and its compatibility with an injection robot (Chen *et al.*, 2006b).

On-line sample fractionation

The on-line thermal fractionation of a sample can be achieved either with a thermo desorber of headspace cartridges (Esteban *et al.*, 1996; Valero *et al.*, 2001) or in the injector itself by using specially designed liners (Zehringer, 2001), or with programmable thermal vaporizers (PTV) (Villen *et al.*, 1996). However, the use of empty liners or cartridges does

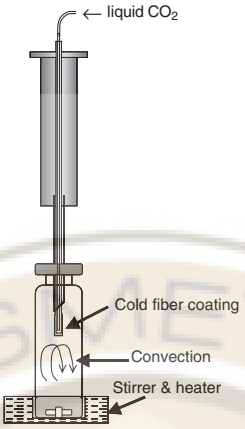


Figure 6.2.6 Headspace micro-extraction with a cold fiber.

Table 6.2.6

Determination of five PASs by GC-MS in SIM mode in a non-spiked and in a spiked mimosa absolute oil pretreated by using ALEX technique (adapted from David *et al.*, 2006a)

Analyte	Non-spiked sample		Spiked sample		Recoveries
	mg/L	RSD (%)	mg/L	RSD (%)	
Benzyl alcohol	122	6.5	195[a]	2.9	72
Anise alcohol	87	3.9	87	6.1	—
Cinnamyl alcohol	—	—	106[a]	8.5	103
Benzyl benzoate	46	2.4	44	1.1	—
Benzyl cinnamate	21	7.1	118[a]	6.2	97

[a]Spiked amount: 100 mg/L.

not enable quantitative transfer of analytes while retaining low-volatile compounds (Zehringer, 2001; David *et al.*, 2006a). Solid packing in the injector insert (e.g. quartz wool) does not solve this problem. Conversely, if the liner or the cartridge is filled with polydimethylsiloxane (PDMS), an inert absorbent with a high sample capacity, the thermal fractionation of the sample becomes easy. Liners can be packed with PDMS particles or with PDMS foam as the latter offers more reproducible permeability. The packing material then behaves as a pre-column that retains low-volatile ingredients under pre-defined injection conditions. The injection can be fully automated owing to a thermal desorber for headspace cartridges (Debonneville *et al.*, 2006), or to an injection robot performing automated liner exchange (ALEX) (David *et al.*, 2006a).

The ALEX technique has been tested using mimosa absolute oil, a natural raw material used in the fragrance industry containing many high-molecular-weight compounds (David *et al.*, 2006a). The sample was spiked with three PASs (Figure 6.2.7), and recoveries ranged from 72% to 103%, as can be seen in Table 6.2.6.

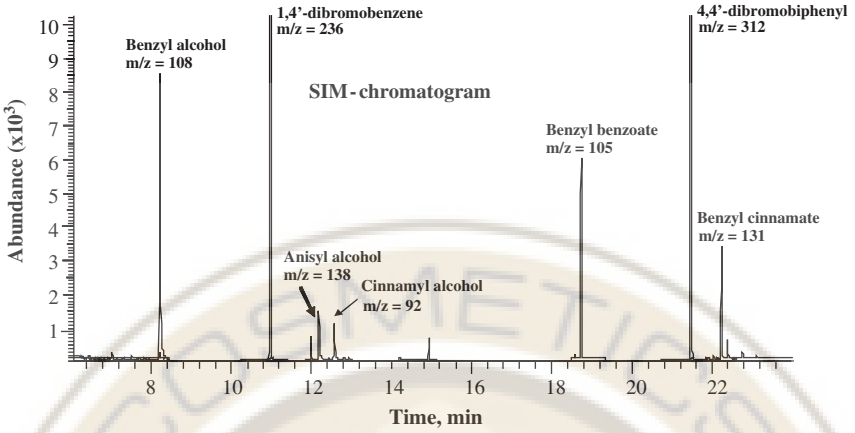


Figure 6.2.7 Chromatograms from a GC-MS analysis in SIM mode of mimosa extract pretreated by using ALEX technique (from David *et al.*, 2006b).

With this technique, calibrations are achieved by injecting the standards directly into the PDMS and subsequent desorption. The nature of the cosmetic matrix does not interfere with quantification, as volatile analytes are isolated from the rest of the sample. To show the versatility of this approach, three shampoos differing in their surfactant composition that were perfumed with the same fragrance were spiked with all volatile PASs at a level of 100 mg/kg, the limit above which PASs content must be labelled in rinsed-off products according to the EU Cosmetics Directive. The three products were analysed using PDMS-filled cartridges and a thermal desorber (Debonneville *et al.*, 2006). The mean standard deviations from the mean and from the spiked amount were less than 19% and 24%, respectively, without taking farnesol and benzyl salicylate into account. The former is known to display high determination variability for all techniques, while the latter was co-eluted with a constituent of the perfume, which does not question the on-line fractionated distillation technique.

SUMMARY

From the state of the art reported in this section, it is clear that the analysis of PASs in cosmetics remains a hot research topic. However, a brief summary of current knowledge follows:

(1) When there is no concern about the matrix (fragrance concentrates and extracts, alcoholic perfumery) the complexity lies in the huge number of perfume constituents, which may interfere with PASs. GC with MS detection in SIM mode gives the most satisfactory results for routine determination. Among more sophisticated multidimensional approaches, GC-MS/MS lowers possible false positives and negatives, whereas

the on-going advances of comprehensive GC×GC-MS might overcome the risk of co-elution in the near future.

(2) Most cosmetics are based on a non-volatile matrix from which PASs must be recovered. This implies additional difficulty concerning the previously mentioned complexity of the volatile fraction. On-line fractionation is presumably the most convenient way to solve this critical step, as the same calibration may be used for a variety of different matrices.

It is obvious that there is a need for simultaneous progress in both areas, i.e., to increase selectivity of PAS determination among other volatile ingredients and recover them from non-volatile cosmetics, to enable laboratories involved in the analysis of fragrances and cosmetics to monitor PASs with high throughput and minimum data interpretation. Nevertheless, the recent developments reported here represent a considerable improvement given the lack of suitable analytical techniques to be found in scientific literature.

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6.3. Electronic Noses in Perfume Analysis

R.M. Negri*

Institute of Chemical Physics of Materials, Environment and Energy (INQUIMAE), Department of Inorganic, Analytical and Chemical Physics, School of Sciences, University of Buenos Aires, Argentina

THE OBJECTIVES OF ELECTRONIC NOSE METHODOLOGY

An electronic nose (e-nose) could be defined as a device composed of an array of non-selective gas sensors that, after exposure to a vapour, should be able to provide a characteristic signal pattern of that vapour. However, the term "electronic nose" refers not only to a particular device, but also to a particular methodology to analyse complex multicomponent vapours and gases. Although the term e-nose is known by cosmetic chemists and flavour industries, in many cases there is still confusion about the methodology involved, which differs from that most commonly used in analytical chemistry. Therefore, to begin with we must describe the methodology which is behind the device, clarifying the differences with respect to traditional methodologies, particularly compared to GC, which is perhaps the most popular technique employed in perfume analysis (see Section 6.1).

The main objective of electronic noses, and also electronic tongues (its analogues for the liquid phase), is to discriminate or differentiate samples of complex and unknown composition without needing to identify their individual chemical components. Hence, its objective contrasts completely with that of GC, where at least the most relevant components must be identified. As the objectives are different, so the results and information obtained will also be different and the evaluation of the methodologies is dependent on what practical information is being looked for. The more common situation in which e-noses are applied is to discriminate between products in the field of food, beverages, pharmacy, and perfumery and for safety and environmental control (Gardner and Bartlett, 1993, 1999; Dickinson *et al.*, 1998).

The methodology and currently developed devices are inspired by the natural olfaction system, where odours are sampled, detected and analysed. In the natural olfaction system of mammalians, odours are sampled by aspiration and conduced into the nasal cavity where they are detected by a series of olfaction cells that provide input signals to the brain. The brain stores the signals in its memory and provides a comparison when a new odour is detected.

In electronic devices these steps are more clearly identified, in concordance with its relative simplicity compared to the natural system. For example, sampling is generally performed by an aspiration pump without previously separating the individual components; detection is

*Corresponding author. E-mail: rmn@qi.fcen.uba.ar

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

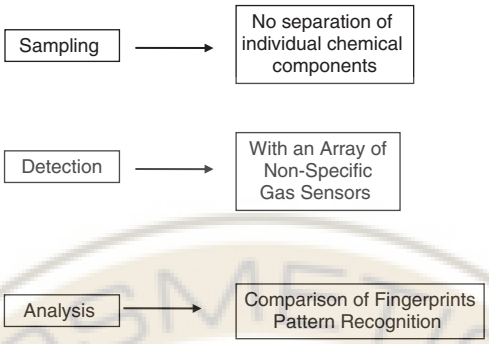


Figure 6.3.1 Simplified scheme of the steps involved when using electronic noses.

carried out by an array of gas sensors and analysis by using pattern recognition methods in a computer. Figure 6.3.1 describes schematically the three main steps involved in e-nose methodology.

The central point in electronic noses is that the individual gas sensors are completely unspecific, as are the olfactory cells in the natural olfaction system. This means, not only that an individual gas sensor may provide exactly the same electrical signal in the presence of two different pure gases, but also for two very different odours, hence one individual gas sensor is not able to discriminate between two complex odours (except in very simple cases which are not of interest). For example, an individual sensor may provide the same signal for someone smoking very close to the sensor and for something burning nearby; hence, the sensor cannot discriminate between both situations.

E-noses try to solve this problem by using an array of non-specific gas sensors, where the sensors could have different sensitivities for a certain vapour. First of all, the output of the array is not just one signal, but a group of signals (S_1, … S_N), where N is the number of sensors and S_i is the signal of the ith sensor. This group constitutes a pattern for the odour, or more precisely, a pattern for the interaction between the odour and the particular sensor array that is used. Therefore, it is important to see that each odour gives rise to a different pattern and that the e-nose recognizes each different odour by recognizing its particular pattern. Secondly, even though some of the individual sensors may provide identical signals for different odours, the probability that other sensors provide different signals for the different odours increases with the number of sensors. In other words, there is a higher probability of obtaining different patterns if there is a good selection of sensors composing the array. To give an example of this, Figure 6.3.2 shows the signal provided for each one of the five sensors constituting a fictitious array. Note that sensor number 4 provides the same signal for the two vapours A and B, but that the whole array is completely different for both vapours.

It should be pointed out that the signal provided for any sensor generally increases in line with the time the sensor is exposed to the vapour, until the signal reaches a plateau (steady-state situation), as shown in Figure 6.3.3. In the steady-state situation the signal is recorded and processed. In some cases, the same results may be obtained even though the signal has not reached the steady-state situation.

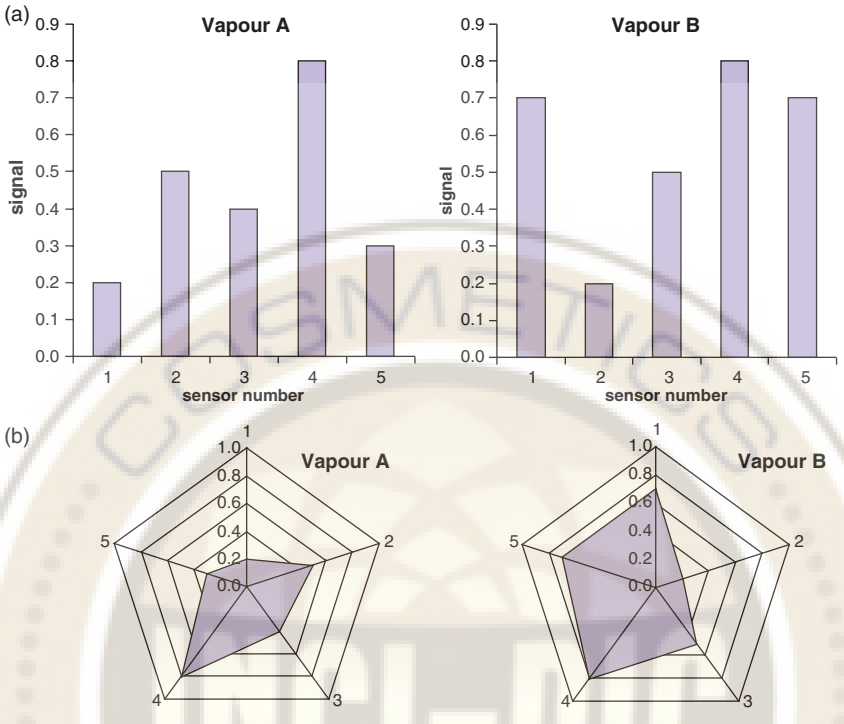


Figure 6.3.2 (a) Bar-plots and (b) radar-plots showing the signal obtained by a fictitious array for two fictitious vapours. Each vertex of the radar-plots corresponds to each one of the individual sensors of the e-nose.

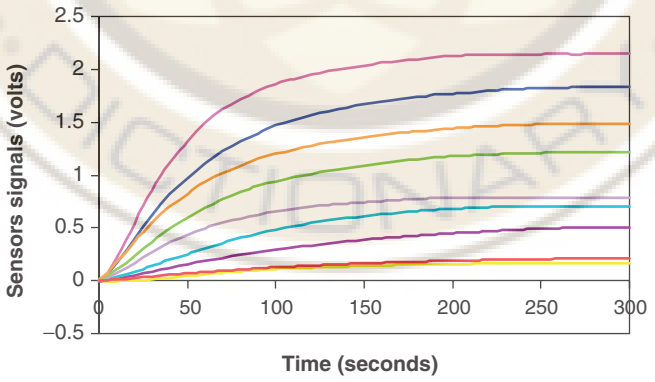


Figure 6.3.3 Example of sensor's signals recorded by an electronic nose developed at the University of Buenos Aires (in this case the array consists of eight metal oxide sensors). Each curve corresponds to one different sensor of the e-nose. In the present case the odour is collected from a headspace of limonene since time zero, up to reaching a plateau in all the sensors signals.

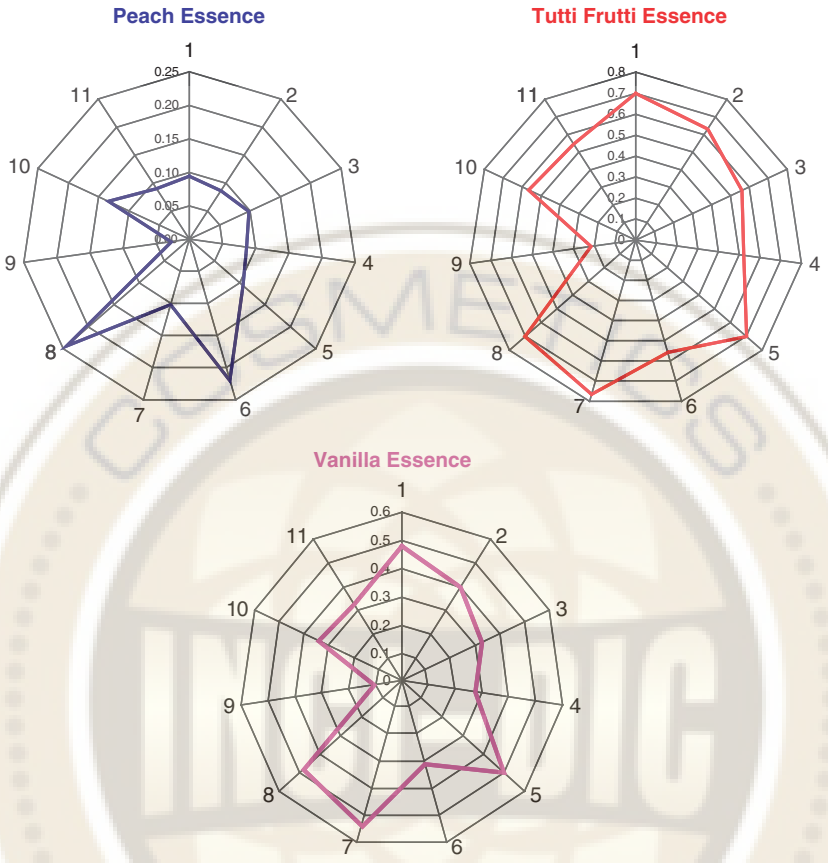


Figure 6.3.4 Radar plots of three different essences (peach, tutti-frutti and vanilla) obtained by using an array of 11 sensors.

Therefore, in principle a given pattern is obtained for each sample analysed. Figure 6.3.4 shows radar-plots obtained with one of the e-noses developed at the University of Buenos Aires for three different essences, where each vertex of the radar is associated to one defined sensor of the array. The shape of the radar, obtained by joining the signals of each sensor, constitutes the fingerprint of the essence for this sensor array. Note that not only are the shapes of the radar plots different but also the intensities of the signals are different (see scales).

The shape of the radar plot, and thus the pattern signal provided by the sensors, does not change with the concentration of the substance. As an example, Figure 6.3.5 illustrates the release of limonene, which was encapsulated in a pectin gel and the release was followed as a function of the release time. Each radar plot was recorded at different times during the whole release process, which takes several hours. It is observed that the shape of the radars does not change during the release, this being representative of limonene, while only an increase in the signal is observed as the limonene is released, becoming more concentrated in the headspace above the gel.

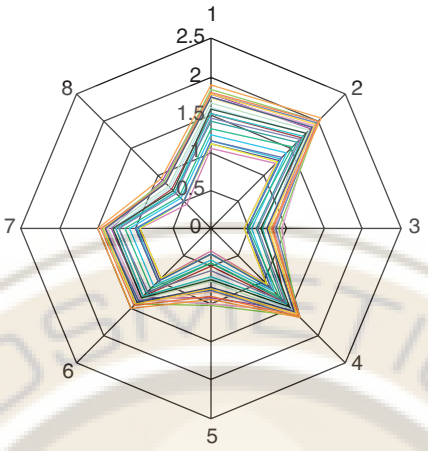


Figure 6.3.5 Release of encapsulated limonene from pectin gel. The signals are recorded at different times during the release.

Only in the very simple cases where very different compounds have very different odour patterns, the products can be discriminated by visual inspection of a radar plot (see Figure 6.3.4). But, in most practical cases the radar plots display very small differences, and so mathematical methods are necessary to demonstrate whether the differences are statistically significant. Therefore, in those cases the last step of the analysis is to try to discriminate mathematically between the groups of signals obtained.

The central idea is that the discrimination between samples is statistically similar when using human panellists. For example, if the objective is to use an e-nose to discriminate between products A and B, then the e-nose must "smell" many replicates of A and B to create a database. This intrinsically means that some mathematical criterion must be used in order to classify the samples into two groups. This "criterion" is a statistical algorithm or method based on what is called Multivariate Data Analysis, like Principal Component Analysis (PCA), Cluster Analysis, Artificial Neural Networks, etc. (Miller and Miller, 2002; Massart *et al.*, 2003). It should be pointed out that some of these methods may not be suitable, that is, the method does not identify two groups clearly. Deciding whether to use one method or another greatly depends on the sensitivity of the sensor array and, in practice, is commonly a matter of trial and error. Finally, once a method is put into use and a database is built up, the whole system must be tested using unknown samples (samples that have not been used to build up the database, but are actually known by the operator of the e-nose). After measuring many different unknown samples, the quality of the e-nose is assigned, for example, by means of the percentage of unknown samples that have been successfully assigned to the right group.

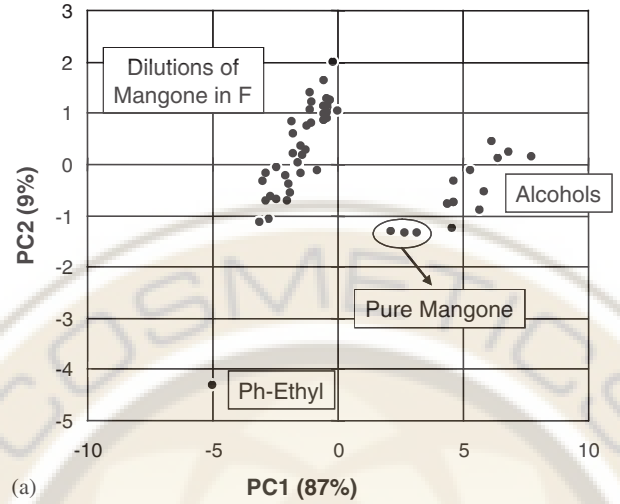
As mentioned above, there are several well-known methods of multivariate data analysis (Jurs *et al.*, 2000), but perhaps the most popular is PCA (Jollife, 1986). This feature extraction method consists in projecting the N-dimensional data set (in this case N is the number of sensors) onto a new base of the same dimension N, but now defined by the eigenvectors of the covariance or the correlation matrix of the data set. The components (projections) of the original data vectors onto this new base are the so-called Principal Components,

obtaining one set of principal components (PC_1, PC_2, … PC_N) for each data set (S_1, S_2, … S_N). The important point is that, when analysing the new data set (PC_1, PC_2, … PC_N), a large percentage of the total data variance is accumulated in a few of the principal components. For example, in most of the studies associated to e-noses, 95% of the total variance is accumulated within the three first principal components, representing a substantial reduction in the dimension and complexity of the problem. In those cases where an important percentage of the total data variance is contained in the first two or three principal components, the data points can be qualitatively discriminated by observing how they group in a 2D or 3D plot (PCAmap) of the principal components (e.g., a 2D plot of PC_2 *vs.* PC_1 or any other combination of principal components). As a realistic example, see Figure 6.3.6.

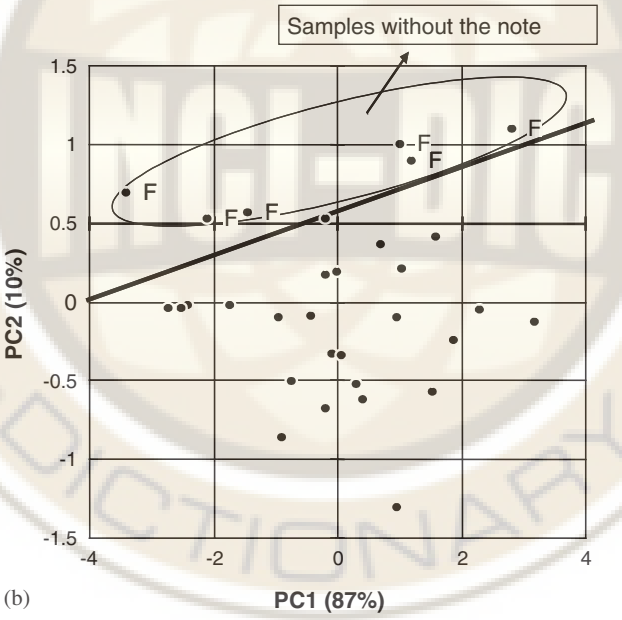
Another multivariate classification method is Cluster Analysis (CA), which groups the input data into clusters. The information that must be specified is the number of classification groups desired (i.e. the number of "clusters"). For example, suppose that you want to classify all the different samples you are interested in within exactly four groups (e.g. related to four olfactory groups, etc.). The number of groups (four) must be indicated in the programme. The number of different samples may be different to four, but the interest is to group them into exactly four. Then, the data input (for example the set of vectors (S_1, … S_N), where N is the number of sensors) are loaded into the programme, which groups these data inputs into "clusters" (four in our example). The association of the input data into a cluster is the output of the analysis. The classification is performed without the need of introducing any additional information about the samples (i.e. the method is unsupervised, as it is also in PCA). A mathematical criterion for grouping must be defined, which is generally the Euclidean distance between data points. In many cases the principal components of the PCA (PC_1, PC_2) are chosen as inputs for CA instead of (S_1, … S_N).

Artificial Neural Networks (ANN) is a more complex classification method, which usually requires a much larger amount of data than PCA (Jollife, 1986; Hertz *et al.*, 1991; Bishop, 1995; Jurs *et al.*, 2000). Perhaps the most popular ANN is the so-called Multilayer Neural Networks with Back Propagation, which are mainly used to classify samples into classes. The mathematical details are very hard to explain in the present context and are out of the scope of this section; however, they will be summarized. Let us suppose that the objective of a given work is to classify many real samples into two Groups (A and B). For example, suppose a technician would like to discriminate the samples of a complex fragrance that have a perfumery note added to the formulation (Group A) from those which do not have the note (Group B), using a neural network. The neural network must "learn" how to recognize samples A and B. This process is achieved through three consecutive steps.

The first step is to teach the device to recognize a positive input from Group A and a positive input from Group B. This is achieved by introducing N_1 measurements into the neural network as numerical data inputs with the e-nose of Group A samples; and N_1' of Group B samples, indicating to the neural network which measurement comes from each group every time. Each of these inputs are usually the vectors (S_1, … S_N), although in many cases the vectors (PC_1, PC_2) are used instead (N is the number of sensors of the e-nose). This first step is named as the learning phase. In the example of the fragrances with and without the note, every time that a vector is introduced to the network during the learning phase it must be indicated with a numerical code if the respective input corresponds to a sample with or without the note. Thus, the neural network "learns".



(a)



(b)

Figure 6.3.6 Example of a principal component map (usually referred to as a PCA-map). (a) Base alcohol-based fragrances with small amounts of a perfumery note (mangone) are well differentiated from ethanol and from mangone. (b) A PCA plot considering only the samples of the pure fragrance (F) without the note (mangone) and dilutions of the note in F. Samples F occupies a well-defined region of the PCA map, suggesting that strongest quantitative multivariate data analysis method may discriminate F samples from the others (from Branca *et al.*, 2003).

The second step is the training phase: now another set of inputs, lets say N_2 vectors of Group A measurements and N_2' vectors of Group B measurements are entered into the neural network, which must classify these measurements into outputs A or B. In this training phase, the neural network checks if its classification was right or wrong for all the inputs in this phase $\left(N_2 + N_2'\right)$ () in a feedback process that enables the network to readjust itself to improve the classification. So the neural network is trained and receives a feedback in this second step. The training phase is also part of a learning process, but from a mathematical point of view is separated from the learning phase.

Finally, the third step is to test the device, the testing or executive phase: other sets of samples are measured (e.g., N_3 from Group A and N_3' from Group B) and the numerical vectors are used as inputs to the neural network. In this third phase the device will have a percentage of success, which may be acceptable or not for the operator, and so on. For example, the operator may give the information that: "*from the total number of test samples* $N_3 + N_3'$, *90% were correctly assigned*". In the case of the fragrance with the note, the operator knows that when he/she tries to discriminate samples with or without the perfumery note then about 90% of the samples that are "shown" to the network will be correctly classified.

Many mathematical parameters related to these phases, especially to the learning phase, can be optimized and modified in order to improve the success rate. The result of the ANN is expressed in statistical terms, which are very much influenced by the quality of the data inputs (drift of the sensors, systematic errors, etc.), and the natural variability between samples.

Sampling and sensors

In most commercial equipment, the headspace of the samples is taken with injector systems, which inject the headspace vapours into a carrier gas that goes through a sensory chamber. In other cases, the perfumes are closed inside bottles (typically five cubic centimetres of the liquid sample) from which the vapours of the headspace are transported into a sensory cell by a flow of dry air (Penza *et al.*, 2001). The flow must be kept constant and bubble formation inside the bottles must be avoided. Other strategies can capture the sample by aspirating the vapour directly from the headspace towards the sensory chamber without injecting it into a carrier gas.

We refer to a gas sensor as an element (active material + transducer) that provides an electrical signal, which varies in the presence of a gas or vapour. The signal changes because the gas modifies some physical or chemical property of the sensor. There are many different types of sensors, based on which property is changed. The following are just few examples, corresponding to the most popular gas sensors, listed without aiming to describe the work principle, but to indicate the advantages and drawbacks. An excellent review about chemical sensors was published by Ellis and Walt (2000), and in the same year, Albert *et al.* (2000) published a review describing sensors used in e-noses. Another interesting review, where some aspects relating to e-noses are described, is that published by Schaller *et al.* (1998).

Metal oxide

This is the case of sensors based on metallic oxides, such as tin-dioxide (SnO_2) or zinc oxide (ZnO), commonly referred to as Metal Oxide Semiconductors (MOS). These gas sensors are based on changes in the electrical conductivity of the sensor and literature is very extensive (e.g. Yamazoe *et al.*, 1983; Capone *et al.*, 2001). They are relatively cheap, robust, the electronic component is relatively simple and easy to implement in an array. For example, an array can easily be built up by using sensors based on SnO_2 with each sensor having a different catalytic metal entered *ad-hoc* (electron donors or acceptors, like Pd, Sb, etc.). It is highly sensitive for molecules of relatively low-molecular weight (e.g. carbon monoxide, ethanol, methane, etc.). One drawback is that the responses are not linear with gas concentration. Another important point is that they require high operating temperatures (provided by a heating element which is part of the sensor) so power consumption is high. Although in general sensitivity is high, it can decrease to an important extent when the molecular weight of the gas increases and aging effects may become important.

Conducting polymers

The conductivity of polymers, most of which are based on polypirroles, polyanilines and polythiophenes, change in the presence of gas. They have an advantage over metal oxide sensors in that they work at room temperature, preparation at lab scale is much easier and size can be reduced. The drawbacks of this method are much greater sensitivity to humidity and long-term drift (Gardner and Bartlett, 1999).

Acoustic wave sensors

These sensors detect the effect of an adsorbed gas molecule on the propagation of an acoustic wave generated on a piezoelectric substrate (typically quartz), which is coated with a film (lipid monolayer or polymers). An electrical field applied to the material (radio frequencies) induces a mechanical change in the piezoelectric (electromechanical effect), which is perturbed by the presence of the adsorbed gas to be detected. The gas is adsorbed on the coating and the response of the sensor depends on the nature and composition of the sensing film that is coated onto the piezoelectric. Thus, the array is formed by changing the nature of the film. There are two types of the acoustic wave sensors: bulk acoustic wave (BAW, which is a quartz crystal microbalance) and surface acoustic wave (SAW, which is propagated through two electrodes) (Gardner and Bartlet, 1999). Traditionally, BAW are easiest to implement, but SAW are generally more sensitive.

Although these are the most common gas sensors, technology is changing daily and many different materials with different properties are currently being developed. The potential of many new sensors for detecting fragrances is normally reported in scientific articles. For example, Létant *et al.* (2000) used an array based on photoluminescence sensors to detect flavours, where the photoluminescence of the porous silicon films is quenched by the presence of gas, so a gas sensor is obtained. On the other hand, Battiston *et al.* (2001)

developed a micro fabricated array of eight silicon cantilevers (similar to those used as sensitive points in atomic force microscopy) having a polymer coating and actuated at their resonance frequency, which was used to detect perfume oils. When the gas to be detected is in contact with the polymer coating, the polymer swells inducing a change in the bending of the cantilever, which is detected.

EXAMPLES OF DIFFERENT POSSIBLE TESTS AND RELATIVE DIFFICULTIES

Based on our experience, some typical problems or situations are addressed concerning the e-noses used by cosmetic industries. Some examples are listed here below:

Problem 1: Is it possible to affirm that a Product A is different of a known Product B?

In principle it is not a difficult task for the e-nose. But there is common confusion about this point: if the used e-nose does not discriminate between A and B; then, this does not imply that the products are the same. In fact, A and B may "smell" the same as far as the device used is concerned, but different for another e-nose. In summary, if the e-nose used detects significant differences, then A and B are different. But if the e-nose used does not differentiate between them, then nothing can be affirmed.

Problem 2: Is it possible to reject in a routine quality control, the "bad" samples that are different to a known standard? It is accepted that the e-nose sometimes rejects a "good" sample, but it is inadmissible to accept a "bad" sample.

This problem poses two difficulties: (a) In every routine quality control, the e-nose must "smell" the known standard very frequently in order to avoid deviations due to shift of the sensors responses; and (b) more fundamental: a sample can be "bad", but still smell very similar to the "good" standard (this is similar to the case of Problem 1). In conclusion, Problem 2 is not an easy task for the e-noses and in some cases the e-nose may be not the suitable technique to solve it.

Problem 3: Is it possible to discriminate between the products A_1, A_2, A_3,…A_n?

This is a much simpler problem: (a) the analysis is performed in a short period of time (a few hours, depending on the number of samples) by analysing one sample after other so no sensor drift is expected; and (b) no stable external standard compounds are necessary. In connection with this is the following, more difficult problem: is it possible to detect the presence of a trace of a perfumery note in a fragrance? This problem requires the manufacturer to provide a large number of samples containing the note in order to create a good database to perform ANN (a first exploratory approach to this problem was reported by Branca *et al.*, 2003).

Problem 4: If odour patterns are built into a database, so odours O_1, … O_n are distinguished, then: is it possible to assign a new given sample *X* to one of those previously identified odours O_1, … O_n?

It is very difficult, but perhaps not as difficult as in Problem 2. If the database was built up many months before the analysis, then it is possible that it is no longer valid if an

important shift of the sensors has occurred. Therefore, it is convenient to re-build the data base. Moreover, it normally involves using more powerful classification methods, such as ANN.

Problem 5: Can quantification be carried out by using an e-nose?

In some cases it is possible to quantify the concentration of a particular compound, which is present in a multicomponent vapour using multivariate data analysis, but this is not an easy task. It usually requires one to know the composition of the vapour and to calibrate the response of each sensor of the array as a function of the concentration of the most relevant compounds and also to calibrate by varying the proportion of the target compound in the vapour (Gardner and Bartlett, 1999; Negri and Reich, 2001).

All this implies that the analysis of the data provided by an e-nose is performed in statistical terms. When searching for discrimination between different products, not only should the variability caused by instrumentation be considered, but also mainly inter-sample variability for a given product.

RESEARCH GROUPS AND ARTICLES REPORTED IN INTERNATIONAL SCIENTIFIC JOURNALS

It is not possible to give a full list of research groups that use e-noses, as the list is continuously lengthening, including groups all around the world. Many of them have developed their own devices and many others use commercial devices. At Network on Artificial Olfactory Sensing (NOSE) website (see references) it is possible to find a partial list, not exhaustive at all, of some research groups in the field of e-noses. At the same site there is a list of many companies that produce commercial devices (updating must be checked, as some companies can be absorbed by others, etc.). A detailed descriptive report has been published by Gardner and Bartlett (1999).

Hong *et al.* (1996) tested a thin-film oxide semiconductor micro array (based on 1% Pd doped SnO_2, 6% Al_2O_3-doped ZnO, WO_3 and ZnO) to discriminate between a woman's *eau de cologne* and a man's *eau de toilette* using PCA. Later, Penza *et al.* (2001) manufactured metal oxide sensors using WO_3 films incorporating different metals such as Pd, Au, Bi, and Sb (hence, each sensor of the array differs in the nature of the metal that was incorporated). They used this array to classify seven products: three fine fragrances for men (Sergio Tacchini, Caractere and Gian Marco Venturi), two fine fragrances for women (Chanel No 5 and Iceberg Twin) and two after-shaves (Axe and Patrichs Noir). They could differentiate between all the products using PCA, with a partial overlap between Chanel No 5 and Gian Marco Venturi, which was attributed to the relatively low discriminating power of the sensors. Meanwhile, the volatile composition of the headspace from *Citrus unshiu* blossom was investigated by Choi (2003). The odours are given off by the presence of *p*-cymene, γ-terpinene and beta-caryophyllene among others. By using an electronic nose consisting of six metal oxide sensors, PCA of the volatile compounds showed aroma discrimination of the fresh and dried blossom samples. Finally, Branca *et al.* (2003) also used metal oxide sensors to detect the presence of

a perfumery note in a fragrance and the results were comparable with those of human panellists that used triangle tests. The same group developed and used different electronic noses to detect the release of encapsulated flavouring essences and essential oils (Monge *et al.*, 2004a, 2004b). A similar system was used by Moy *et al.* (1994) to investigate the stability of perfumes on the body.

Yokoyama and Ebisawa (1993) compared the classification of 37 fragrances evaluated by eight women and by an array of eight piezoelectric sensors coated with different polymers. Similar discrimination between the human panel and the array was obtained for most of the studied cases. Cao *et al.* (1996) also used piezoelectric crystal sensors coated with many different adsorptive substances (PVC, lipids, etc.). Different arrays were built up with these sensors and their ability to discriminate between several commercial Chinese perfume preparations was tested, although the results are difficult to evaluate. Later, Nakamura *et al.* (1999) proposed a method based on computational chemistry to predict the extent of sorption of different fragrance chemicals (citral, β-ionone, 2-hexanone, and 2-pentanone) into the polymer films that are used as coatings in BAW sensors. They compared the computer prediction for the adsorption partition coefficient and the experimental values obtained from the response of the BAW sensors, obtaining good linear regressions. Some years before, the same group had used the BAW sensors, although on this occasion coated with lipids, to discriminate between perfumes (Ema *et al.*, 1989; Nakamoto *et al.*, 1991, 1993; Ide *et al.*, 1993, 1994). Yang *et al.* (2000) discriminated five fragrance chemicals (phenethylalcohol, ionone, vanillylalcohol, ethylisobutyrate and thymol) using a developed array of SAW sensors.

Hoffarth and Zesiger (2000) differentiate headspace vapours of perfumes using PCA and other multivariate data analysis methods by using a high-sensitivity quadrupole mass spectrometer-based electronic nose.

Létant *et al.* (2000) have compared the discrimination ability of an array composed by photoluminescence sensors based on porous silicon films. An array of these sensors was connected in series with a commercial electronic nose, and the discrimination ability was compared for samples of citrus/*cis*-3-hexenal, ethyl cinnamate and ethyl valerianate, with good performance for the silicon array.

Battiston *et al.* (2001) applied a developed cantilever array to the detection of perfume oils from different companies diluted in ethanol (Pinnrad GmbH, Germany: Rose, Camomile, Heliotropin, Corps and Leather), using ANN to identify each perfume.

Okabayashi *et al.* (2001) reported that they could discriminate between different terpenes (limonene, linalool, citral and α-pinene) at low concentrations (about 1 mg/kg) by analysing the temperature dependence of chemiluminescence emitted by the terpenes when adsorbed on an alumina substrate mixed with rare-earth. The chemiluminescence is registered as a function of substrate temperature, observing different peaks, which are used as input for PCA.

Miettinen *et al.* (2002) studied the effects of emulsion structure (oil-in-water) and composition of the matrix on the release of linalool (nonpolar) and diacetyl (polar), by using sensory evaluation, static headspace GC and an electronic nose.

Recently, Kermani *et al.* (2005) studied the appropriateness of a particular algorithm for training neural networks (the so-called Levenberg–Marquadt algorithm) and compared it with back-propagation (see also Kermani *et al.* (1999) for ANN analysis using

the so-called Genetic algorithm). They used coffees, cola beverages and fragrances. Different fine fragrances (Chloé, Liz Clairbone, Eternity and Polo) were selected for the experiment and the pattern obtained for ethanol was used as a reference. The authors show the benefits of using one algorithm over back-propagation.

The electronic nose in industry

It might be worth mentioning the importance of e-noses in wine analysis, as it shares common aspects with perfume analysis: quality analysis by oenologists and panellists, degradation assays, adulteration detection, the common presence of relatively high percentages of ethanol, etc. However, this topic falls outside the scope of this book, which deals with cosmetic products.

As far as cosmetic industries are concerned, information is not easily available. The web pages of the most relevant cosmetic chemistry companies do not usually mention the studies already carried out with e-noses. However, some information can be summarized.

Between 1998 and 2001 Givaudan–Fragrance Research (Dübendorf, Switzerland) was the beneficiary of a European Programme in collaboration with the University of Leeds, Departments of Biochemistry and Molecular Biology, to use sensor arrays developed at the University of Leeds, with the participation of Bloodhound Sensors Ltd. (see website in references).

In France, Elf has used an electronic nose to discriminate Yves Saint Laurent fine fragrances (Carrasco *et al.*, 1998). In this case, perfume samples were deposited onto a pare strip inside a vial then heated and the vapours were carried by gas flow through the gas sensors. They measured Paris *eau de toilette* and Paris *eau de toilette* with off-odours and Opium *eau de toilette*. The group of Elf-Aquitaine has also examined diesel fuels scented with perfume mixtures (Feldhoff *et al.*, 2000).

International Flavours & Fragrances (IFF) in United States (US) purchased a commercial e-nose and evaluated others as it was a test site for a particular company that sells these devices. IFF Argentina in collaboration with the University of Buenos Aires studied the detection limits for the presence of one perfumery note (mangone) in a given perfume, using an e-nose, gas chromatography coupled with a mass spectrometry detector (GC-MS) and human panellists of intermediate training (triangle test with two sets of 20 panellists). In this case the e-nose had the best detection limit, but ANN had to be used to identify the samples (Branca *et al.*, 2003).

Firmenich US has also performed studies with e-noses, but the results have not been disseminated to our knowledge. Merck used e-noses to discriminate between various flavours used in pharmaceutical formulations, obtaining a good correlation between GC, sensory panel and e-nose. Zhu *et al.* (2004) of Merck research Laboratories used the electronic-nose technique to qualitatively distinguish raspberry, red berry, strawberry, pineapple, orange, and cherry in placebo formulations. Raspberry flavour samples from different batches made by the same manufacturer, as well as freshly prepared and aged samples, were also distinguished by an electronic-nose. They used not only PCA and CA but also other methods such as Discrimination Function Analysis (DFA).

Prospective of e-noses in cosmetic chemistry

Users who are not familiar with e-noses must first bear in mind that e-noses imply a methodology that is the opposite of those that identify the individual chemical compounds of the perfume.

Secondly, it is the responsibility of the researchers and companies that develop e-noses to show clearly where e-noses can be useful to solve well-defined and different issues, problems or situations, with their respective degrees of difficulty. Otherwise, high expectations are created with the consequent deception if the e-nose cannot solve the problem. For example, at the present state-of-the-art in instrumentation technology, the main problem seems to be the presence of shifts in the sensor's responses, which prevents storing odour databases for periods of months. Another difficulty is to relate the results of the e-noses to universally accepted standards for describing and communicating specific flavours. Pearce and Gardner (1998a, 1998b) have already proposed a so-called odour mapping scheme to be applied when using e-noses, but it seems that much effort is needed for inter comparison of data collected with different instruments. The last point concerns simplifying the data manipulation or making it more user-friendly, so a technician can obtain simple results without having to know how multivariate data analysis works.

In most of the cases where the problems were well defined and sampling, measurements and data analysis were performed by academic researchers, very good performance and promising results were obtained, showing the potential of e-nose methodology.

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– 7 –

Surfactants in Cosmetics. Analytical Methods

7.1. Determination of Surfactants in Cosmetics

M.C. Prieto-Blanco[1*], P. López-Mahía[1,2], S. Muniategui-Lorenzo[1] and D. Prada Rodríguez[1,2]

[1]Departament of Analytical Chemistry, University of A Coruña, A Coruña, Spain
[2]Institute of Environment, University of A Coruña, Pazo de Lóngora, A Coruña, Spain

INTRODUCTION

Surfactants are possibly the most versatile components among all the ingredients of cosmetic products; since they afford properties that are both suitable to cosmetic functions and also make stable and homogeneous formulations possible. Surfactants are responsible for the cleansing, foaming and antimicrobial properties of cosmetics as well as conditioning pearlizing and opacity. In terms of design, the processes of emulsification and solubilization using surfactants enable different ingredients to be mixed (oils, water, solids, dyes, fragrances …) in cosmetic formulations (Rieger and Rhein, 1997; Somasundaran *et al.*, 2001).

Surfactants are a group of organic compounds obtained by chemical synthesis whose properties are the product of a special molecular composition constituted hydrophobically by an alkyl chain and hydrophilically by an ionic or polar group (Figure 7.1.1).

*Corresponding author: E-mail: mcprieto@udc.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

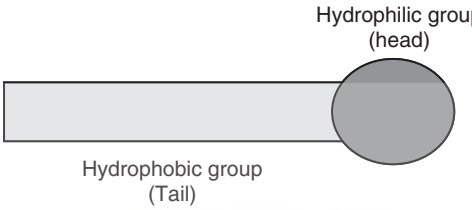


Figure 7.1.1 Structure of a surfactant.

Different surfactants used in cosmetics can be classified according to the charge of the ionic group. Four types of surfactants are attained following this criterion: anionic (negative charge), cationic (positive charge), nonionic (hydrophilic group) and amphoteric (negative and positive charge on the same molecule) (Table 7.1.1).

Besides, in the hydrophobic part, a surfactant may have homologous chains and positional isomers; for example, cocoamidopropyl betaine may contain alkyl chains with a carbon pair number from 8 to 18, the majority being those with a carbon number of 12 and 14. Aromatic groups are usually bonded in alkyl chain, like in alkylphenols (nonionic) or alkylbenzenesulfonates (anionic). Also, the hydrophilic part may contain oligomers of ethylene oxide or propylene oxide. In this article, cationic polymers have been included as a subclass of cationic surfactant based on the classification made by Richmond (1990), although many authors consider this polymer to be another ingredient of cosmetic products. Another subclass, quaternary ammonium compounds (four alkyl chains around a nitrogen atom) will be frequently mentioned components.

A great many commercially available surfactants are used in several industries: household products, paints, pharmaceuticals, textiles … but in cosmetics only those exhibiting minimum dermatological and toxicological effects at controlled concentrations are used (see Consolidated Text of the European Commission (2005) on Cosmetics). Four types of surfactants are used in different types of cosmetics depending on the function required. In cosmetics, like shower gels, shampoos and dental products with cleansing characteristics, the main ingredients are anionic surfactants such as alkyl sulfates; and amphoteric surfactants such as cocoamidopropyl betaine. In cosmetics that possess conditioning characteristics, like hair conditioner and shampoos, cationic surfactants like alkyltrimethylammonium compounds are employed. Other cationic surfactants with antimicrobial properties, such as cetylpyridinium chloride, are useful in mouthwash products. The nonionic surfactants containing ethylene oxide solubilize and emulsify cosmetic formulations, like milks, creams and lotions (Schueller and Romanowski, 1994).

Specific methodologies have been developed to analyse surfactants and their application to different matrices of formulated products (López-Mahía *et al.*, 2005). It is difficult to determine surfactants in cosmetics due to their nature, since they are compounds with low volatility and, moreover, frequently possess numerous isomers, homologues and oligomers. Their determination is also difficult due to certain factors related to cosmetic matrices, as for instance, a large number of components, some of which are in a minority (bactericides, antioxidants, fragrances) and the type of vehicle cosmetic in which they can be found, e.g. solution, emulsion, dispersion and solid (Cozzoli, 1993). The type of

surfactant, the complexity of the cosmetic matrix, the analytical information required (qualitative or quantitative analysis), the step in the cosmetic production cycle (production control, consumer product) are some of the factors that should be considered when choosing an analytical method.

In this section, we will consider the analytical techniques that have been employed to determine surfactants in cosmetics: classical techniques; electrochemical techniques; spectroscopic techniques; and chromatographic and related techniques. Both the type of surfactant and the kind of cosmetics are taken into account for each of the techniques discussed. Residual products coming from surfactants are examined in cosmetics separately due to their potential toxicity, which is the subject of a number of published papers and current legislation. Some publications devoted to determining surfactants after the application of cosmetics to the hair or body can be found in the literature; however, only those devoted to the analysis of cosmetic products have been considered here. Tables 7.1.1–7.1.5 summarize the aforementioned fields of interest and references are also included to make it easier and quicker to access the aspects considered.

ANALYTICAL METHODS FOR SURFACTANT DETERMINATION IN COSMETICS

Classical techniques

Volumetric methods are the traditional methods employed in routine control, specifically two-phase titration for anionic and cationic surfactant determination, and are characteristically highly sensitive. They are based on the reaction between an anionic surfactant and cationic surfactant (one of which is a sample surfactant and the other a titrant surfactant) in a two-phase system (chloroform–water). The endpoint is detected by a transfer-phase indicator, the most common of which is a mixture of dimidium bromide and disulfine blue although methylene blue (Epton method), which is the first indicator chronologically, is also applied. Two commonly used titrants are sodium lauryl sulfate for cationic surfactants and benzethonium chloride, currently named Hyamine 1622, for anionic surfactants.

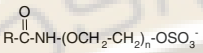
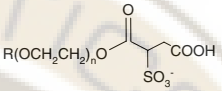
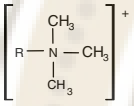
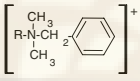
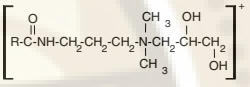
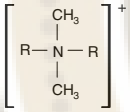
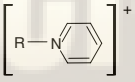
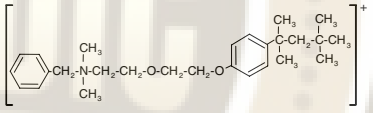
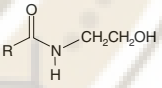
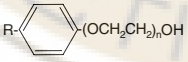
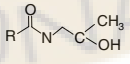
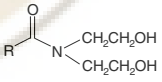
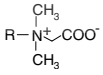
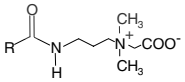
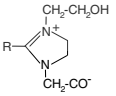
A mixture of anionic surfactants (salts of fatty acids (currently named soaps), alkyl sulfates and alkyl sulfonates may be determined by three titrations. These three surfactants are titrated using dichlorofluorescein as indicator; a dimidium bromide and disulfine blue mixture are used for titration of alkyl sulfates and alkyl sulfonates, while disulfine blue is used for alkyl sulfonates titration after acid hydrolysis (Cozzoli, 1993).

Electroanalytical techniques

There are certain drawbacks of classical two-phase titration, such as the use of chlorinated solvents (chloroform); interferences from cocoamidopropyl betaine (a common ingredient of cleansing cosmetics); and difficult detection of the visual end point when nonionic surfactants are present in formulations. However, these drawbacks can be solved

Table 7.1.1

Main surfactants used in cosmetic products classified according to class of surfactant

Anionics					
Soap		**Sulfate**		**Sulfonate**	
$R\text{-}COO^-$	Alkyl sulfate $ROSO_3^-$	Alkyl ether sulfate $R(OCH_2CH_2)_nOSO_3^-$	Alkyl sulfonate RSO_3^-	Olefin sulfonate $R_1\text{-}CH_2\text{-}SO_3^-$	Paraffin sulfonate $R\text{-}CH_2\text{-}SO_3^-$
		Alkylamide ether sulfate 		Alkyl isethionate 	
Cationics					
Alkyltrimethylammonium 		Alkylbenzyldimethylammonium 		Alkylamidopropyl -N-N-dimethyl-N-(2, 3-dihydroxypropyl) ammonium 	
Dialkyldimethylammonium 		Alkylpyridinium 		Alkylphenoxyethoxyethylbenzyl-dimethylammonium 	
Nonionics					
Alkyl alcohol		Alkylphenol	Alkyl amides	Alkyl monoethanolamide	
R-OH					
Ethoxylated alkyl alcohols		Ethoxylated alkylphenol	Alkyl isopropanolamide	Alkyl diethanolamides	
$R(OCH_2CH_2)_nOH$					
Amphoterics					
Alkylbetaine 		Alkylamidopropyl betaine 		Alkylimidazolinium 	

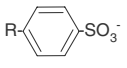
(*Continued*)

Table 7.1.1 (Cont.)

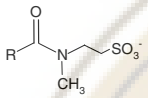
Anionics

Sulfonate

Alkylbenzenesulfonate

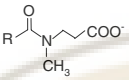


N-alkyl-N-methyl taurate

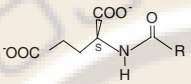


Carboxylate

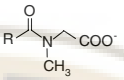
Acylalaninate



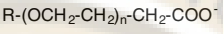
Acylglutamate



Acylsarcosinate

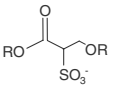


Alkyl ether acetate

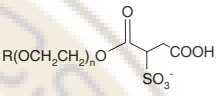


Sulfosuccinates

Dialkyl sulfosuccinate



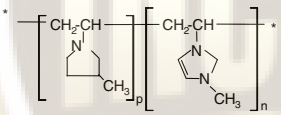
Alkyl ether sulfosuccinate



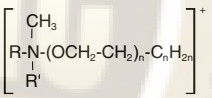
Cationics

Alkyldimethylamine oxide

Copolymer of 1 -vinyl-2-pirrolidone and 1 -vinyl-3-methylimidazolinium chloride - LUVIQUAT^{R}

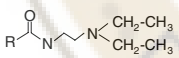


Polyethoxylated alkyl-methylammonium chloride

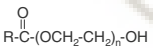


Nonionics

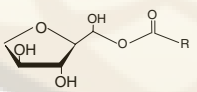
Diethylaminoethylalkylamide



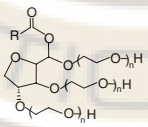
Ethoxylated esters



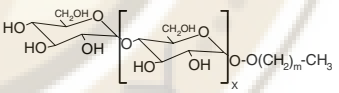
Sorbitan esters



PEG sorbitan esters

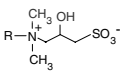


Alkylpolyglucosides



Amphoterics

Alkyl hydroxysultaine



Alkyl amidopropyl hydroxydsultaine

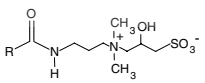


Table 7.1.2

Analytical references of anionic surfactants according to type of cosmetic product and employed analytical technique

Class	Reference	Compound	Matrix	Technique
Anionic	Walling and Dabney (1986)	Sodium lauryl sulfate; ammonium lauryl sulfate	Shampoo	NIR
	Koening and Strobel (1988)	Coco sarcosinate, *N*-coco-*N*-methyltaurate	Toothpastes	LC
	Marini *et al.* (1990)	Magnesium amidethoxysulfate	Makeup remover	Titration/ion exchange resin chromatography/LC
	Marini *et al.* (1990)	Lauryl ether sulfate; lauryl sulfate	Shampoo	Titration/ion exchange resin chromatography/LC
	Kondoh (1991)	*N*-methyl-*N*-palmitoyltaurine; ($C_{10–13}$) benzenesulfonates; (C_{12}, C_{14}, C_{16}) sulfates, polyethylene glycol (PEG) C_{14} sulfate; 2-pentadecenyl 1-sulfonate	Cosmetic product	LC
	Li and Jandik (1991)	($C_{4–14}$) benzenesulfonate	Shampoo	Solid-phase reaction (SPR-LC)
	Molever (1993)	Lauroylsarcosinate	Personal care products	GC
	Chattaraj and Das (1994)	Sodium lauryl sulfate	Toothpaste	Indirect AAS
	Heinig *et al.* (1996a)	($C_{12–14}$) ethoxy sulfate	Shampoo; face wash	CE
	Heinig *et al.* (1996a)	Sodium lauryl sulfate	Toothpaste; face wash	CE
	Heinig *et al.* (1996b)	Sodium lauryl sulfate	Toothpaste	LC; CE
	Salimi-Moosavi and Cassidy (1996)	(C_{12}, C_{14}) sulfate	Shampoo	CE
	Masukawa and Tsujimura (1997)	($C_{12–16}$) acylmethyltaurine	Cosmetic product	LC
	Maffei Facino *et al.* (1997)	Myristyl/cetyl/steraryl soaps, ($C_{8–18}$) acids	Mascara	FAB-MS; GC/MS
	Grunewald *et al.* (1998)	Sodium lauryl ether	Bubble bath lotion	NIR
	Zhang (1998)	(C_{12}, C_{14}) sulfate, (C_{12}, C_{14}) ether sulfate (OE=1–8); *N*-acyl *N*-methyl taurate; lauryl ether sulfosuccinate	Shampoo	ESI-MS

(*Continued*)

Table 7.1.2 (Cont.)

Class	Reference	Compound	Matrix	Technique
	Schulz and Bruttel (1999)	Lauryl sulfate	Mouth care	Titration sensor selective
	Goff (2000)	Lauryl ether sulfate; Lauryl sulfate	Shampoo	NMR
	Lin and Shu (2000)	Lauroylsarcosinate; Lauroylalaninate; Lauroylglutamate; *N*-lauryl-*N*-methyltaurate; PEG lauryl ether acetate	Cosmetic; toiletry products	LC; CE
	Carolei and Gutz (2005)	Lauryl ether sulfate	Shampoo; liquid soap	FTIR-ATR

with potentiometric titration using an ion-selective electrode that is suitable for surfactants. This technique is based on the same chemical reaction as the classical two-phase one and, furthermore, there are several common titrants (i.e. sodium lauryl sulfate for cationic surfactants); nevertheless, several aspects are specific to this technique. Potentiometric titration is a precipitation reaction and the compound obtained from the reaction between titrant surfactant and sample surfactant, does not have to dissociate. In this type of titration, it is important to minimize micelle formation in order to achieve a clear inflection point. Parameters, such as the control of titration pH avoid the interference of cocoamidopropyl betaine. Additionally, either micelles or adhered insoluble-component can be suppressed by adding low amounts of methanol. Potentiometric titration is often carried out using a titrator and this automated system saves time compared to the classical two-phase titration.

Schulz (1996) carried out a study on potentiometric titration of anionic and cationic surfactants in raw materials and cosmetics with a commercial surfactant-sensitive electrode in aqueous medium. Various types of anionic surfactants used in cosmetics (alkyl sulfates, alkyl ether sulfates, sulfosuccinates, isethionates, anionic surfactants contained in pearl lustre concentrates) were determined using 1,3-didecyl 2-methyl imidazolinum chloride as a titrant at pH 3. Some of them require special conditions like a strict control of pH in sulfosuccinates. Others exhibit specific characteristics like alkyl sulfates that show a derivative curve of titration with several peaks corresponding to homologues. These methods were applied to 67 commercial products that included 2-in-1 products and formulations containing sulfosuccinates and other pearl-lustre components. The relative standard deviation was in the range of 0.4–2.6%. In other formulations, such as hair conditioners, quaternary ammonium compounds were determined optimizing pH and using sodium lauryl sulfate as titrant. Also, gargle solutions based on benzalkonium chloride, mouthwash based on cetylpyridinium chloride and toothpaste and mouthwash based on amine fluoride were titrated with dioctylsulfosuccinate. In another paper, interferences caused by cocoamidopropyl betaine, sulfosuccinates, lauroyl sarcosinate, cocoyl isethionate in the determination of some ionic surfactants were minimized in two-phase titration using a solvent-resistant surfactant electrode as an indicator (Schulz, 1998). This method was also

Table 7.1.3

Analytical references of cationic surfactants according to type of cosmetic product and employed analytical technique

Class	Reference	Compound	Matrix	Technique
Cationic	Kadono *et al.* (1987)	– [a]	Shampoo; hair conditioner	LC
	Benassi *et al.* (1989)	Cetylpyridinium chloride	Mouthwash; dentifrices	LC
	Caesar *et al.* (1989)	C_{14},C_{18} amidopropyl-*N*,*N*-dimethyl-*N*-(2,3-dihydroxypropyl) ammonium	Shampoo; skin moisturizer	LC
	Bettero *et al.* (1990)	$C_{12–16}$ benzyldimethylammonium	Cosmetic product	LC
	Elfakir *et al.* (1990)	Cetylpyridinium chloride	Mouthwash	LC
	Lowy *et al.* (1991)	C_{16} trimethylammonium	Cosmetic product	Sensor selective
	Taylor and Cheng (1992)	Benzalkonium chloride	Cosmetic product	Sensor selective
	Chattaraj and Das (1992)	C_{12} trimethylammonium	Shampoo	AAS
	Okumura (1992)	Cetylpyridinium chloride, ($C_{16,18}$-trimethyl-, C_{16} benzyldimethylammonium; distearyldimethylammonium)	Shampoo; rinse	LC
	Matsuzaki *et al.* (1993)	–	Cosmetic product	LC
	Gmahl and Ruess (1993)	Copolymer (1-vinyl-2-pyrrolidone; 1-vinyl-3-methylimidazolinium)	Cosmetic products; raw materials	GC-MS
	Suzuki *et al.* (1994)	$C_{12–24}$ trimethylammonium	Cosmetic product	CG
	Haruyama and Okaya (1995)	Benzalkonium chloride, cetylpyridinium chloride; benzethonium chloride	Cosmetic product (lotions, creams, tonics, foams)	LC
	Heinig *et al.* (1996b)	Cetylpyridinium chloride	Mouthwash	LC; CE
	Schulz (1996)	Benzalkonium chloride; cetylpyridinium chloride	Gargle solutions; mouthwash	Sensor selective
	Imrag and Junker-Buchheit (1996)	C_{16} trimethylammonium; cetylpyridinium chloride	Cosmetic product	HPTLC
	Heinig *et al.* (1997)	Cetyltrimethylammonium	Face lotion	CE; LC
	Choi *et al.* (1997)	Cetylpyridinium chloride; benzalkonium chloride, C_{14},C_{18},C_{22} trimethylammonium	Hair treatment cream	LC

(*Continued*)

Table 7.1.3 (Cont.)

Class	Reference	Compound	Matrix	Technique
	Maffei Facino *et al.* (1997)	PGE oleyl, linoleyl, ricinoleyl methylammonium	Hair dye	FAB-MS
	Coran *et al.* (1998)	C_{16} trimethylammonium	Hair softeners	FAB-MS; FIA-ISI-MS
	Patel and Patel (1999)	Cetylpyridinium chloride	Shampoo; soap	FIA
	Patel and Patel (1999)	C_{12},C_{14},C_{16} trimethylammonium; cetylpyridinium chloride	Shampoo; soap	FIA
	Lin and Shu (2000)	C_{16} trimethylammonium	Cosmetic; toiletry products	LC; CE
	Kulapin and Arinushina (2000)	C_{10},C_{12},C_{18} pyridinium; C_{16} trimethylammonium	Hair rinses	Sensor selective
	Morrow *et al.* (2001)	Cetylpyridinium chloride	Oral rinse	MALDI-TOFMS
	Chen *et al.* (2001)	Benzethonium chloride; cetylpyridinium chloride	Cosmetic product	LC
	Miyamae *et al.* (2002)	Lauryldimethylamine oxide; C_{18} trimethylammonium; distearyldimethylammonium	Cosmetic lotions; creams	LC/MS
	Baptista *et al.* (2003)	Cetylpyridinium chloride	Oral disinfectant	FIA
	Tsai and Ding (2004)	$C_{12–18}$ trimethylammonium	Hair conditioner	CG-MS
	Liu and Ding (2004)	$C_{12–18}$ trimethylammonium	Hair conditioner	CE
	Oetzekin and Eim (2005)	Benzethonium chloride; cetylpyridinium chloride	Cosmetic powder	CE

[a]The compound is not provided by authors.

applied to the analysis of cationic and anionic surfactants in hair preparations (dyes and conditioners) (Schulz and Bruttel, 1999).

During recent years, the development of surfactant-sensitive electrodes has led to different proposals, such as electrodes based on membranes of cetylpyridinium chloride with laurylsulfate and tetraphenyl borate. Using such electrodes, cationic surfactants have been determined in hair rinse and anionic and nonionic surfactants in shampoos (Kulapin and Arinushkina, 2000). A cationic surfactant, benzalkonium chloride, was quantified in cosmetics in the range of 0.02–1% using a potentiometric method and obtained results were comparable to those obtained in two-phase titration using methylene blue as indicator (Taylor and Cheng, 1992). Also, different titrants to those employed in classical two-phase titrations were tested, such as heteropolyanionic for quaternary ammonium compounds (Lowy *et al.*, 1991). A review with 158 references by Kulapina *et al.* (2001) includes the characteristics of the selective-membrane electrodes and their application to the analysis of anionic, cationic, nonionic surfactants in different products including cosmetics.

Table 7.1.4

Analytical references of nonionic surfactants according to type of cosmetic product and employed analytical technique

Class	Reference	Compound	Matrix	Technique
Nonionic	Kadono *et al.* (1987)	Lauryl isopropanolamide	Shampoo; hair conditioner	LC
	Kimura *et al.* (1997)	Sorbitan laurate; PEG sorbitan laurate; PEG sorbitan palmitate, PEG C_6,C_8,C_{12} alcohol	Cosmetic products	FAB-MS
	Masukawa and Tsujimura (1997)	C_8–C_{14} diethanolamides; C_{12}–C_{14} PEG esters	Cosmetic products	LC
	Heining *et al.* (1998)	C_8,C_{10},C_{12} polyglucosides; C_{12},C_{14} alcohol ethoxylates (OE=2–5)	Shower gel	LC, CE
	Goff (2000)	Lauryl diethanolamide	Shampoo	NMR
	Kulapin and Arinushina (2000)	PEG octylphenyl alcohol, PEG nonylphenyl alcohol	Shampoo	SS
	Miyamae *et al.* (2002)	Diethylaminoethyl stearamide; Sorbitan stearate; PEG sorbitan stearate; PEG monosterarate; PEG oleate; PEG oleyl alcohol; PEG 2-hexyldecyl alcohol	Cosmetic lotions; creams	LC/MS
	Carolei and Gutz (2005)	Coco diethanolamide	Shampoo	FTIR-ATR
	Carolei and Gutz (2005)	Coco diethanolamide	Shampoo; liquid soap	FTR-ATR

Flow injection analysis (FIA) is an automated methodology in which the samples are dipped at regular intervals into a flowing stream of solvent or reagent and are subsequently measured potentiometrically or by other types of detection. The main advantage is a great many samples can be processed in a short time without handling (Válcarcel and Luque de Castro, 1988). FIA has been used by Baptista *et al.* (2003) to determine cetylpyridinium chloride in oral disinfectants. Diminution of the reagent (sodium picrate) caused by formation of ion pair with cationic surfactant was measured potentiometrically. Cetylpyridinium chloride was determined in the concentration range of 5.0×10^{-5}–7.5×10^{-5} M at a sampling rate of 60/h.

Spectroscopic techniques

Several spectroscopic techniques have been applied to determine surfactants in cosmetics with different aims: conventional infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) for qualitative analysis; near infrared spectroscopy (NIR) and attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR) for quantitative analysis; atomic absorption spectroscopy (AAS) to determine specific surfactants. Mass

Table 7.1.5

Analytical references of amphoteric surfactants according to type of cosmetic product and employed analytical technique

Class	Reference	Compound	Matrix	Technique
Amphoteric	Kondoh and Takano (1986)	– [a]	Cosmetic product	LC
	Kadono *et al.* (1987)	–	Shampoo; hair conditioner	LC
	Cozzoli *et al.* (1989)	(C_{12}–C_{18}) amidodimethyl betaine	Cosmetic product	LC
	Matsuzaki *et al.* (1993)	–	Shampoo	LC
	Wilkes *et al.* (1994)	Cocoamidopropyl betaine	Facial cleanser	LC
	Masukawa and Tsujimura (1997)	(C_8–C_{16}) amido betaines	Cosmetic product	LC
	Grunewald *et al.* (1998)	Cocobetaine	Bubble bath lotion	NIR
	Lin and Shu (2000)	Lauryl amidopropyl betaine	Cosmetic; toiletry products	LC; CE
	Goff (2000)	(3-Aminopropyldimethylamino) acetic acid betaine	Shampoo	NMR
	Carolei and Gutz (2005)	Cocoamidopropyl betaine	Shampoo	FTR-ATR

[a]The compound is not provided by authors.

spectrometry (MS) allows structural characterization of the surfactants with homologues and oligomers that are components in complex cosmetic mixtures. The potential use of this technique is dealt with separately below.

Conventional IR has been used to estimate the quantitative composition of the formulations or to identify ingredients that are determined later by chromatography or other techniques. IR spectrum absorption bands may be associated with functional groups like alcohols, amines, acids, esters, amides … and consequently indicate the type of surfactant present in the formulation.

NIR is a technique that covers the electromagnetic spectrum region from 13,000 to 4000 cm^{-1} and is widely applied in the quality assurance of cosmetics. Walling and Dabney (1986) used this technique to predict the surfactant percentage composition in two brands of shampoo. One of them contained ammonium lauryl sulfate and the other a mixture of ammonium lauryl sulfate and sodium lauryl sulfate and various coloring agents and fragrances. Authors estimated a 97% time saving as compared to classical methods. Prediction equations, obtained through setup multiple linear regression for shampoos with two anionic surfactants, can be used to predict the surfactant content in shampoo containing ammonium lauryl sulfate. Error standards of prediction are comparable with standard deviation obtained with conventional methods (potentiometric titration).

Good manufacturing practices (GMP), established in the cosmetics industry (COLIPA, 1994), involve the control of the contents of the container according to the manufacturer's specifications. NIR is recommended as an analytical technique by Grunewald *et al.* (1998) for rapid and nondestructive identification of raw materials, product control and end-product analysis. As an example, sodium lauryl ether sulfate and cocobetaine in bubble bath lotion were identified.

ATR-FTIR is a useful analytical tool for multicomponent analysis that employs a mathematical data-treatment process. Also, Carolei and Gutz (2005) have used this technique combined with chemometrics, to determine three surfactants and water simultaneously in shampoo and in liquid soap without either sample dilution or pretreatment. The surfactants analysed were an amphoteric one (cocoamidopropyl betaine), two nonionic ones (coco diethanolamide in shampoo and alkylpolyglucoside in liquid soap), (minor components) and an anionic one (sodium lauryl ether sulfate). Overlapping bands and water absorption were resolved by two multivariate quantification methods: classical least squares (CLS) and inverse least squares (ILS) (Massart *et al.*, 1997, 1998). The wave numbers chosen for the calculation process were preferably those of maximum absorption of the minor components. This method can be applied during the production process but not in final product analysis because of interference caused by the fragrance added in the last step (Figure 7.1.2).

NMR, a traditional technique for organic compound identification, when applied to surfactants provides essential structural information about the average of chain length, type of branching in the chain and type of aromatic substitution. Goff (2000) identified and quantified components of one formulation by NMR without using previous separation methods. This technique is usually used for the de-formulation of competitive products (such as shampoos) on the cosmetics market.

Atomic absorption spectrometry (AAS) has been used to determine cationic and anionic surfactants indirectly. Two methods have been put forward based on the formation of the ion pair between surfactant and hexanitrocobaltate (for cationic compounds) or bis(benzoyl)pyridine thiosemicarbazone cobalt (III) (for anionic compounds). In the former case, the complex is extracted with 1,3-dicloroethane and in the latter with an isopentylacetate and isopentyl alcohol mixture. Concentration of cobalt is determined in the organic phase using electrothermal atomic absorption spectroscopy (ETAAS), while for anionic surfactants, flame atomic absorption spectroscopy (FAAS) can also be used. Interferences like metal ions, anions and organic compounds do not have a great relevance. The two methods were applied to determine dodecyltrimethylammonium bromide in shampoos (Chattaraj and Das, 1992) and sodium lauryl sulfate (SDS) in toothpastes (Chattaraj and Das, 1994).

Patel and Patel (1999) proposed a FIA-spectrophotometric method that enables $C_{12–16}$ trimethylammonium and cetylpyridinium chloride to be determined by means of forming a complex between the cationic surfactants and Fe (III)-SCN^-. The effect of FIA variables on determining surfactant in nitric acid medium was studied; however, possible interferences (ions present in formulation) were not encountered. This method of determining cetylpyridinium chloride in soap and shampoo was compared with an established FIA method, in which the complex was formed with an anionic dye (Orange II), and the former method attained better sensitivity, selectivity and reproducibility.

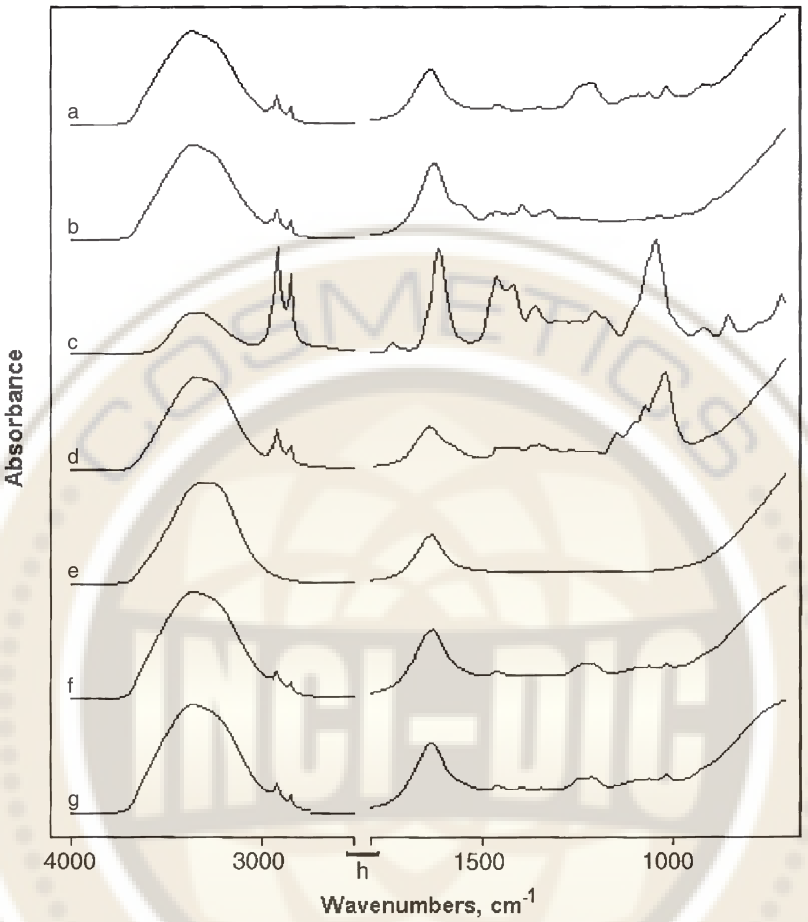


Figure 7.1.2 Infrared spectra of (a) sodium lauryl sulfate, (b) cocoamidopropyl betaine, (c) cocodiethanolamide, (d) alkylpolyglucoside, (e) water, (f) liquid soap and (g) shampoo. Adapted from Carolei and Gutz (2005) with permission.

Chromatographic and related techniques

We will go on to review different proposals to determine surfactants in cosmetics using high-performance liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). In the last decade, the main objective of these techniques was to determine surfactants present in formulations individually.

Liquid chromatography

LC methods have been more successfully employed to achieve the separation of homologues and oligomers, mixtures of different types of compounds of same type (i.e. into

cationic surfactants, alkyltrimethylammonium and dialkyldimethylammonium or even of different types (mixtures of amphoteric and cationic surfactants) in numerous cosmetics (shampoos, toothpastes, creams …). Examples of these separations under different chromatographic conditions considering the type of surfactant are described below.

Anionic surfactants. Anionic surfactants of different types of alkyl sulfates and alkyl ether sulfates were determined by Marini *et al.* (1990) using reversed-phase chromatography with ultraviolet (UV) detection. Triethanolamine lauryl sulfate and magnesium lauryl ether sulfate were quantified in shampoo and magnesium amide ether sulfate in makeup remover. Li and Jandik (1991) determined alkyl sulfonates in shampoo by reversed phase with gradient elution and simultaneous conductivity and UV detection. A solid-phase reagent was used to remove countercations of the eluent (tetrabutylammonium borate) and decrease background conductivity in a post-column reaction. Besides, the method allowed simultaneous separation of homologous series of alkyl sulfates and alkyl sulfonates and could be useful in fingerprint analysis. Information about the unknown impurities could be gained through two detection modes.

Different types of anionic surfactants (alkyl sulfates, alkyl sulfonates, alkylbenzenesulfonates, alkyl ether sulfates and **α**-olefinsulfonates) were determined in cosmetic products. The separation was carried out in an octadecylsilica column and the mobile phase made up of an acetonitrile/aqueous $NaClO_4$, using conductivity detection (Kondoh, 1991). In toothpastes, a sulfonate-type surfactant (cocomethyltaurate) and another carboxylate type (cocosarcosinate) were determined by reversed-phase LC using refractive index (RI) detection. Before chromatographic separation, a sample was dissolved in a methanol/water mixture and centrifugated to separate abrasive and insoluble components (Koening and Strobel, 1988).

Cationic surfactants. Cationic surfactants, frequently belonging to alkylpyridinum, alkylbenzyldimethylammonium and trimethylammonium types, are commonly determined in skin care cosmetics and hair and mouth formulations, using reversed-phase LC with UV, conductivity or RI detection. Thus, Benassi *et al.* (1989) developed a method to analyse cetylpyridinium chloride and alkylbenzyldimethyl chloride in cosmetic products (mouthwashes, dental products …) by reversed-phase LC. The necessary chromatographic conditions comprise a deactivated bonded-phase column and an acetonitrile/aqueous phosphate in acid pH solution as a mobile phase. LC analysis of multicomponent heterophasic matrixes, such as cosmetic products, drugs and foods, has been studied by Bettero *et al.* (1990). $C_{12–16}$ alkylbenzyldimethylammonium homologues were separated using UV detection after dilution in a tetrahydrofuran:water solution.

Using LC-UV, low concentrations of cetylpyridinium chloride (about 50 μg/ml) were quantified in mouthwash containing polyoxyethylenated castor oil (about 0.8%), but with poor repeatability (Meyer and Takahashi, 1983). Elfakir *et al.* (1990) demonstrated the influence of castor oil on repeatability using a light scattering detector (LSD) connected to a UV detector. The chromatographic conditions involved a cyano column and acetonitrile/buffer, which allowed variability levels to be reduced and a good routine method to be achieved. Tetramethylammonium hydroxide and triethylamine adjusted to acid pH were the buffers used for UV detection and LSD detection, respectively. Moreover, by comparison LSD provided

greater sensitivity than RI detection. Chen *et al.* (2001) have quantified different homologues of alkylbenzyldimethylammonium chloride in cosmetic products in a silica gel column but using aqueous mobile phases instead of the organic solvents that are usually used in the normal phase.

Alkylamidopropyl-*N*,*N*-dimethyl-*N*-(2,3-dihydroxypropyl)ammonium chlorides are conditioning and emulsifying agents which are separated into their homologues using two alkylcyano columns in series with ion-pairing reactive and a mixture of water/acetonitrile or water/acetonitrile/tetrahydrofuran depending on the formulation. This method was applied to separate the myristyl quaternary in a conditioning shampoo and the oleyl quaternary in skin moisturizer from the other formulation components, quantifying them in percentages of below 3% (Caesar *et al.*, 1989).

Other methods that enable more than two subclasses of cationic surfactants to be separated were proposed by Choi *et al.* (1997) and Okumura (1992). Six cationic surfactants (cetylpyridinium chloride, C_{16} and C_{18} trimethylammonium chloride, behenyltrimethylammonium chloride, benzalkonium chloride and benzyldimethylcetylammonium chloride) were separated by Choi *et al.* using an octadecylsilica column, with an acetonitrile/HCl solution as a mobile phase and UV detection, except in the case of cetylpyridinium chloride and C_{16} trimethylammonium. In the case of these last compounds, they were quantified using both UV and suppressed conductivity detector in series. Analytical recovery values from a hair-treatment cream and hair rinse were good. The method proposed by Okumura enabled $C_{16,18}$-trimethylammonium and benzyldimethylammoniun, cetylpyridinium chloride and distearyldimethylammonium to be determined by reversed-phase LC at acid pH using a RI detector in shampoos and rinses.

Sometimes, cationic surfactants have a preservative function in cosmetic formulations. For such formulations, Haruyama and Okaya (1995) proposed a method to separate cetylpyridinium, benzalkonium and benzethonium chloride. The three surfactants were simultaneously determined in lotions, creams, tonics and foams by reversed-phase and UV detection after pretreatment in the solid phase. Another technique, high-performance thin-layer chromatography (HPTLC) was used to analyse 30 preservatives in cosmetic products following a screening procedure. Two of them (cetylpyridinium and cetyltrimethylammonium chloride) were cationic surfactants (Imrag and Junker-Buchheit, 1996).

Amphoteric surfactants. Cocoamidopropyl betaine and other amphoteric surfactants, used less commonly in cosmetics, are usually analysed by reversed-phase and ion-exchange LC. Kondoh and Takano (1986) quantified carboxybetaine after derivatization with 4-(bromomethyl)-7-methoxycoumarin in shampoo by reversed-phase LC with gradient elution. High sensitivity and high selectivity were achieved in this way after derivatization. Cocoamidopropyl betaine was also determined in shower gel, using mixed mode reversed-phase C_8 and a cation silica column and isocratic elution by methanol/water using either UV, RI and an evaporative light scattering detector (ELSD) (Wilkes *et al.*, 1994).

Five amphoteric surfactants (cocoamidopropyl betaine, cocoamidopropyl sultaine, lauroamphoglycinate, dihydroxyethyl tallow glycinate and isostearoamphopropionate) were separated by reversed-phase LC. According to the authors (Cozzoli *et al.*, 1989), this method could be applied to cosmetic matrices. Tegeler *et al.* (1995) set out to determine amphoteric surfactants in typical cleansing formulation, which could contain anionic (e.g. alkyl ether

sulfate), nonionic (e.g. alkyl polyglucoside) surfactant. They used a cation-exchange column with isocratic elution in acetonitrile/water at strongly acidic pH and the result was that anionic and nonionic surfactants were not retained and amphoteric surfactants were more retarded. Six homologues of cocoamidopropyl betaine (C_{8-18}) and two (C_{12} and C_{14}) of alkyl betaine were separated using UV detection with an elution order from the longest alkyl chain to the shortest. The pretreatment of samples involved a dilution and filtration step. Precise results were obtained for two shampoos containing both betaines (Figure 7.1.3).

Separation of two or more types of surfactants in cosmetics. To separate two or more types of surfactants in cosmetics involves using several detectors or columns as well as studying separation variables in particular pH. Kadono *et al.* (1987) proposed a reversed-phase LC method using a combination of the UV with RI as detector. A ratio of the UV/RI area versus a retention time that was specific to each surfactant. This method was applied to hair cleanser (shampoos and hair conditioners) containing anionic, cationic, amphoteric and nonionic surfactants.

Matsuzaki *et al.* (1993) separated cationic compounds (alkyltrimethylammonium chlorides) and carboxybetaine amphoterics on a cation-exchanger column and methanol/water containing glycine-perchloric acid salt as a mobile phase. The mobile phase pH was the

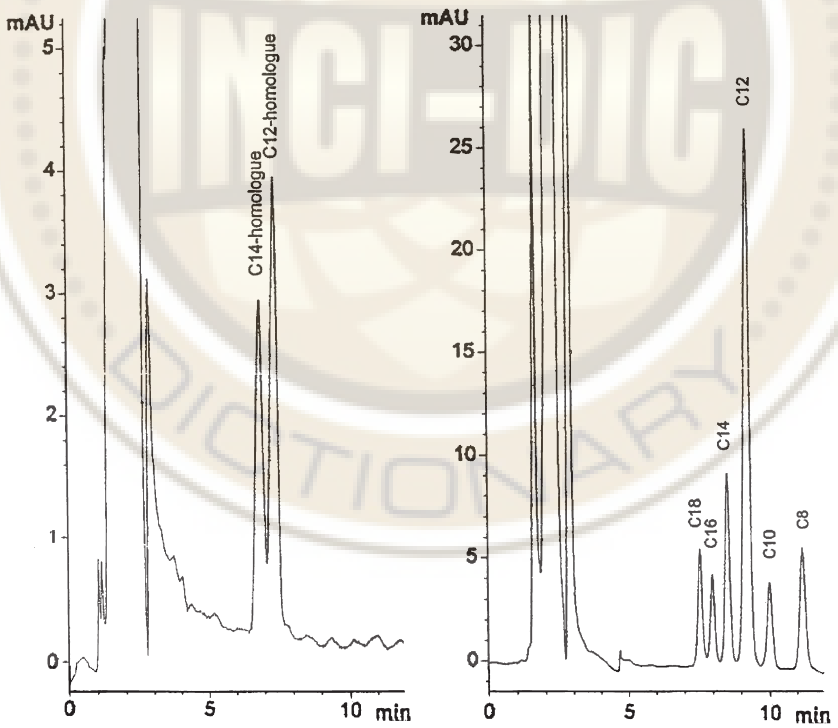


Figure 7.1.3 HPLC chromatogram of shampoo containing alkylbetaine (left) and cocamidopropylbetaine (right). Adapted from Tegeler *et al.* (1995) with permission.

variable that differentiates the two types of compounds; a value of pH below 4 was necessary for amphoteric retention, while cationic surfactants were retained throughout the pH range. In homologous series, a decrease was observed in retention with an increase in alkyl chain length. The method was proposed for quality control of cosmetics.

Two-dimensional LC to determine anionic surfactants (types of carboxylates, sulfates and sulfonates), cationic surfactants (quaternary ammonium and alkyl pyridinium salts) and amphoteric surfactants (amidobetaines) was proposed by Noguchi *et al.* (1998). First column (anion exchange column for anionics and cation exchange column for cationic and amphoteric surfactants) separated according to functional group and a second column (octadecylsilica) allowed the separation of alkyl homologues. The method was applied to shampoo.

Masukawa and Tsujimura (1997) patented a method to determine surfactants in cosmetic products by reversed-phase LC without pre-treatment. Chromatographic conditions involved a binary gradient with methanol and water, ammonium carbonate salt as buffer and LSD detection. Amphoteric surfactants (C_8–C_{16} amidobetaines), an anionic surfactant ($C_{12–16}$ acylmethyltaurine) and two types of nonionic surfactants ($C_{8–14}$ diethanolamides and $C_{12–14}$ alkyl polyoxyethylene alcohols) were identified on applying this method.

Gas chromatography

Although GC is not the most suitable technique to determine low volatile compounds like surfactants, pre-treatments for decomposition or derivatization make it possible. An anionic surfactant, lauroyl sarcosinate was analysed in personal care products by capillary GC using flame ionization detection (GC-FID) (Molever, 1993). Before being placed inside the chromatographic system, the sample was dissolved in acidified dimethylformamide and derivatizated using bis(trimethylsilyltrifluoroacetamide). Cationic surfactants, ($C_{12–24}$) trimethylammonium chloride, are often analysed in cosmetic products like hair rinse, using injection port pyrolysis GC. This type of analysis could be used for routine control and is based on thermal decomposition of quaternary chloride into alkylamines and alkyl chlorides. Compounds are identified with GC coupled to a mass spectrometry detector (GC-MS) using the chemical ionization mode (Suzuki *et al.*, 1994).

Capillary electrophoresis

Many of the analytical problems resolved by LC can also be resolved highly efficiently using CE. Because the latter is a relatively new technique, in many of the proposed methods a comparison is made with LC, normally in terms of sensitivity and reproducibility.

CE methods have been developed to separate homologues, mixtures of surfactants and different types of surfactants in the most commonly used cosmetic products, frequently using capillary zone electrophoresis (CZE); however, in some cases micellar electrokinetic chromatography (MEKC) has also been applied. Besides, methods in aqueous and non-aqueous media have been tested (Heinig and Vogt, 1999).

Anionic surfactants. The main anionic surfactants, alkyl sulfates and alkyl sulfonates as well as ethoxylate derivatives were analysed. C_{12} and C_{14} homologues of alkyl sulfates

were separated in shampoo using a nonaqueous system with methanol and indirect UV detection with *p*-toluenesulfonate, which is highly sensitive and has similar mobility to alkyl sulfates. Other anionic surfactants, such as alkyl sulfonates and linear alkyl sulfonates are separated, but they are not applied to cosmetic products (Salimi-Moosavi and Cassidy, 1996).

Heinig *et al.* (1996a) developed methods in aqueous and nonaqueous medium for three types of anionic surfactants: alkyl sulfates, alkyl ethoxy sulfates and alkyl sulfonates. Several separation parameters, such as chromophore buffer type, concentration, pH, non-absorbing buffers and organic modifiers were examined. Anionic surfactant with low electrophoretic mobility, like sulfonates and alkyl ethoxy sulfates could be separated and good peak shapes displayed using dodecylbenzene sulfonate, whereas *p*-hydroxybenzoate is more suitable for surfactants like alkyl sulfates that have higher mobility. Nonaqueous methods provide better resolution and detection limits than aqueous methods but the latter give better area reproducibility. Sodium lauryl sulfate in toothpaste and face wash was determined using an aqueous method, while alkyl ($C_{12–14}$) ethoxy sulfate were separated in shampoo and face wash using a nonaqueous method. The authors recommended using these methods for product control, considering that electropherogram may be a fingerprint of surfactant formulations.

Acylglutamates, anionic surfactants used in cosmetics due to their lack of irritation and good biodegradability, can be analysed by CE method. The effect of the pH and concentration of buffer and organic solvents on ($C_{12–18}$) *N*-acyl-L-glutamate separation was examined. Methanol was more effective than acetonitrile in minimizing the variation of mobility and zone width caused by changes in sample concentration. The method applied to cosmetic lotion enabled *N*-lauroyl-L-glutamate to be separated from preservatives like methylparaben (Kunimasa and Kameyama, 1999).

Cationic surfactants. Concerning cationic surfactants, like LC, the most common surfactants are analysed by CE using direct and indirect UV detection. Two cationic surfactants (benzethonium and cetylpyridinium chloride) were determined with direct UV detection in cosmetic powder and mouthwash, respectively. Simultaneous determination with good analytical recovery, repeatability and a detection limit at a level μg per ml was achieved using an acetonitrile/water mixture with acid pH (Oeztekin and Erim, 2005). Cetylpyridinium chloride can also be quantified in mouthwash by a mixed MEKC-CZE with UV detection. Moreover, two homologues of ($C_{12–18}$), benzalkonium and cetylpyridinium chloride, can be separated using sodium deoxycholate (a biosurfactant, surfactant derived from biological sources) in a background of electrolyte and ethanol or tetrahydrofuran at optimized concentrations (Herrero-Martínez *et al.*, 2000).

Liu and Ding (2004) developed a method to separate simultaneously four homologues of alkyltrimethylammonium and four of dialkyldimethylammonium with internal standard (octylammonium) and using indirect UV detection. Decylbenzyldimethyl ammonium chloride was used as chromophore and the influence on separation of buffer concentration, pH and organic solvent (methanol and tetrahydrofuran) was studied. Besides, methanol concentration in a sample solution was tested to avoid the formation of micelles and surfactant adsorption onto the capillary. The method was applied to determine cationic surfactants in five hair conditioners, finding only four homologous ($C_{12–18}$) trimethylammonium at a total concentration of between 0.14 and 2.15%. In one of them, analysis with

electrospray MS revealed the presence of another quaternary ammonium compound, steralkonium.

Comparison between LC and CE

Heinig *et al.* (1998) analysed two nonionic surfactants, fatty alcohol ethoxylates and alkyl polyglucosides (frequently used in combination) in cosmetic products by CE and LC with two detectors, UV and ELSD. In CE, phthalic anhydride is the derivatizing agent that transforms a nonionic surfactant with no electrophoretic mobility or UV absorption properties into an anionic surfactant with chromophore group. Using LC-UV, derivatization was carried out with phenyl isocyanate and separation in an octadelcylsilica column and using acetonitrile/water as a mobile phase; however, no derivatization was necessary using ELSD. Homologues of alkyl chain and ethylene oxide were separated in saturated and unsaturated fatty alcohol ethoxylates using the three methods. By comparing the peak patterns of these products with the peak obtained in formulations like shower gel, enabled fatty alcohol ethoxylates to be identified. In this matrix, alkyl polyglucosides were identified using LC-ELSD. The fact that CE is more sensitive than LC is not a decisive factor, given the high concentration of surfactants in samples (Figure 7.1.4).

Also, Heinig *et al.* (1997) quantified cetyltrimethylammonium bromide in face lotion by CE with indirect detection (dodecylbenzyldimethylammonium as chromophore) and without pre-treatment. The influence of an organic modifier and electrolyte chromophore was examined obtaining conditions that permit the separation of alkyl and dialkyl ammonium compounds. In reversed-phase LC, benzyltrimethylammonium was used as a chromophore. Comparison of both methods gave results with better reproducibility for LC and similar detection limits for both methods.

Anionic (sodium lauryl sulfate) and cationic (cetylpyridinium chloride) surfactants were determined using different methods in dental products (toothpaste and mouthwash, respectively) through CE with direct and indirect detection. Different LC methods using conductivity detection were also employed for these analyses. For the two types of cosmetics, better reproducibility and detection limits in surfactant analysis were achieved using LC than with CE (Heinig *et al.*, 1996b).

In addition, analyses of mixtures containing different types of surfactants were compared using these techniques. Lin *et al.* (2000) proposed simultaneous separation of anionic, cationic and amphoteric surfactants using both techniques. All proposed surfactants, five anionic carboxylate-type surfactants (acyl sarcosinates, alaninate, glutamate and ether acetate) and one amphoteric (alkylamidopropyl betaine) have a common linear alkyl chain C_{12}, with the exception of the cationic surfactant (cetyltrimethylammonium). Separation using LC was carried out in an octadecylsilica column and mobile phase of water/methanol with ammonium chloride, tetrabutylammonium hydrogen sulfate and, dibasic ammonium phosphate. This complex mixture was necessary for a number of different reasons; the use of NH_4Cl avoids micelle formation from surfactants; tetrabutylammonium hydrogen sulfate forms ion pairs with surfactants and improves their resolution, while buffer regulates the retention. The parameters studied in CE method were buffer-type at basic pH (necessary for mobility of anionic surfactants), voltage and temperature. Under optimizing conditions, the elution order is cationic, amphoteric and anionic surfactant. LC and CE methods with UV detection were used to analyse 20 cosmetic products in

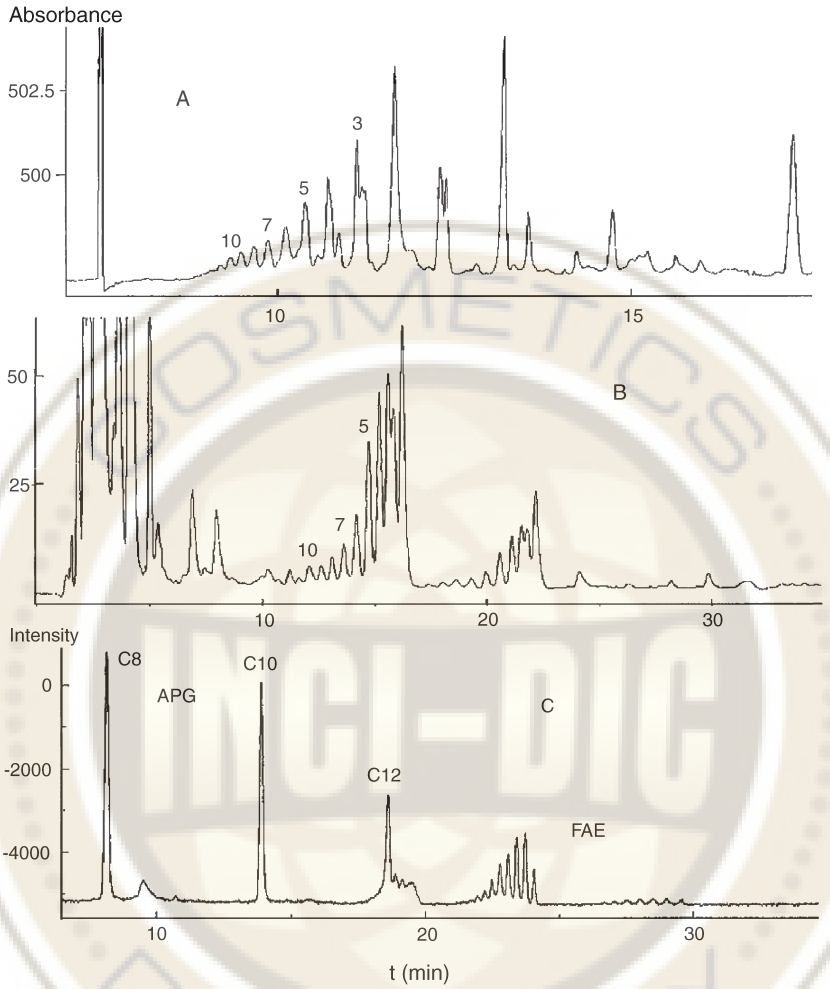


Figure 7.1.4 Analysis of shower gel containing C_{12}–C_{14} ethoxylated alcohols by CE (A), HPLC-UV (B). (numbers above peaks indicate the degree of ethoxylation). Separation of fatty alcohol ethoxylates and of C_8–C_{10} polyglucosides by HPLC-ELSD (C). Adapted from Heinig *et al.* (1998) with permission.

which there are betaine and cetyl trimethylammonium or anionic surfactant and cetyltrimethylammonium in a similar formulation. The authors concluded that the LC method leads to better separation and higher precision than the CE method.

Mass spectrometry and hyphenated techniques

Use of MS provides structural information about the surfactants employed in cosmetic formulations. Owing to the low volatility of surfactants, ionization techniques such as fast

atom bombardment (FAB) and electrospray ionization (ESI) are usually employed. For an unequivocal identification of surfactants, tandem mass spectrometry (MS/MS) might be chosen. If several types of surfactants are present, a previous chromatographic separation step could be necessary (see the hyphenated techniques section).

Zhang (1998) examined 30 brands of shampoo from 1996 to 1997 on the Asian market, using ESI-MS/MS, which is intended to identify the surfactants present. A step to separate anionic, cationic and nonionic surfactants by ion-exchange chromatography was necessary before identification could take place. The most frequently identified anionic surfactants were alkyl sulfates and alkyl ether sulfates, with C_{12} and C_{14} homologues in an alkyl chain and ethoxylation degree between 1 and 8. Other less common anionics, like paraffin sulfonate and *N*-acyl-*N*-methyl taurate, were encountered in 2 of the 30 tested brands, and laureth sulfosuccinate in 1 of the 10 brands. The most commonly found amphoteric surfactant was cocoamidopropyl betaine with a homologue series from C_{12} to C_{18}. In a small number of brands they identified cocobetaine, cocoamidopropyl hydroxylsultaine and lauryl hydroxylsultaine. The latter presented complex fragmentation pathways. Nonionic surfactants were detected by the presence of proton and ammonium adducts and the most common was ethoxylates alcohol type with a $C_{12–14}$ chain and the ethoxylation degree of between 2 and 20. Other nonionics encountered were coco diethanolamide (20 of the 30 brands) and coco monoethanolamide. A cationic surfactant like alkyltrimethylammonium was identified in only two brands, and (myristoyl-*N*-hydroxylethyl)-aminoethyl-2-hydroxypropyltrimethylammonium salt in just one brand.

FAB-MS was the technique chosen by Kimura *et al.* (1997) to identify nonionic surfactants of oxyalkylated type according to alkyl chain or oxyalkylene distribution. Previously, elution using a silica gel column enabled the anionic surfactants to be separated in the acetone and methanol fractions. This step is integrated into a systematic analytical method of cosmetic products that includes the use of NMR and GC-MS.

Coran *et al.* (1998) developed two methods to simultaneously determine $C_{12–16}$ trimethylammonium using two techniques of ionization, continuous flow FAB (CF-FAB) and ion spray ionization (ISI). One of the methods was based on FIA-ISI to avoid previous separation and quantification was carried out in selected ion monitoring (SIM) and selected reaction monitoring (SRM) mode. On comparing the two methods, FIA-ISI was more sensitive (detection limit of 10 pg/μL) than CF-FAB. Two samples of hair conditioner were quantified using the proposed methods and only C_{16} trimethylammonium was found.

The most recent advances in MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) were applied in positive ion mode to the analysis of three oral rinse and one commercial disinfectant formulation. In the latter, a complex mixture containing three dialkyldimethylammonium and alkylbenzyldimethylammonium (C_{10}, C_{14}, C_{16}) salts was detected and in three oral rinse, cetylpyridinium chloride was the cationic surfactant present. Besides, the dissociation was verified as the mode of ionization of quaternary ammonium under MALDI conditions (Morrow *et al.*, 2001).

Analytical processes of separation and identification may be carried out through chromatographic techniques combined with MS. In GC-MS, the identity of previously separated compounds is confirmed. In the case of the LC-MS combination, other problems like

the lack of UV absorption of long-alkyl-chain cationic surfactants (alkyltrimethylammonium, dialkyldimethylammonium) may be also resolved.

The content and distribution of four homologous ($C_{12–18}$) trimethylammonium have been determined in six hair conditioners by GC-MS. The total content varied between 0.4 and 6.9% and C_{16}, C_{18} were the main chains of homologues encountered. Before the chromatographic analysis, sample dilution and exchange of counter ion of quaternary (chloride or bromide) to iodide salts was carried out using liquid–liquid extraction with dichloromethane. The formation of alkylamines was produced in port-injection GC by demethylation of alkyltrimethylammonium iodide salts. The ionization modes employed were electron impact (EI) and positive-ion chemical ionization (PICI) modes. In the latter, methanol was the reagent gas used to enhance protonated molecular signals (Tsai and Ding, 2004). Cationic polymers of Luviquat types (copolymer of 1-vinyl-2-pyrrolidone and 1-vinyl-3-methylimidazolinium chloride) were also identified in cosmetic products by pyrolysis GC-MS and their monomer ratio was determined (Gmahl and Ruess, 1993).

In complex formulations, the combination of FAB-MS and GC-MS may be useful to identify all the ingredients. Two products (mascara and semi-permanent hair dye) are chosen as formulations in which the characterization in quality control is important given the possible toxicity of by-products from colorants. These formulations are complex due to the number of compounds (30 or 40) and different functionality. Mascara is a formulation in which inorganic pigments are dispersed in oil/water emulsion. The emulsifying agent is identified by FAB-MS as salts of myristic, cetylic, stearic acids and confirmed by GC-MS and GC-FTIR. In semi-permanent hair dye, polyethoxylated oleyl/lynoleyl/ricinoley-methylammonium chloride are detected in the positive-ion mode FAB (Figure 7.1.5) and $C_{8–18}$ fatty acids from a natural source in the negative-ion mode. Two extractions with hexane at pH = 1 and chloroform at different pH values were necessary using GC-MS analysis. In a hexane extract, the presence of fatty acids was confirmed and $C_{16–18}$ fatty alcohols and their ethoxylated derivatives were detected. Besides surfactants, other components such as perfumes, dye solvents and antioxidants were identified to a total of 30 ingredients. In a chloroform fraction, next to colorants, a nonionic surfactant (lauryl diethanolamide) was found (Maffei Facino *et al.*, 1997).

LC-MS was the analytical technique chosen to determine anionic and amphoteric surfactants in shampoos without pre-treatment by Miyamae *et al.* (2001). Moreover, using this combined technique, these authors determined cationic (dialkyldimethylammonium, alkyltrimethylammonium) and nonionic (amine oxide, fatty amides, polyglycerol alcohols …) surfactants in cosmetic lotions and creams (Miyamae *et al.*, 2002).

DETERMINATION OF RESIDUAL PRODUCTS FROM SURFACTANTS

In this section, *N*-nitrosamines, 1,4-dioxane and other residual products coming from surfactants (amines, ethylene oxide …) have been selected on the basis of their toxicity and common occurrence (frequency of presence in cosmetics). Analytical methods and detected concentration levels of residual products are summarized briefly for each of them (Table 7.1.6).

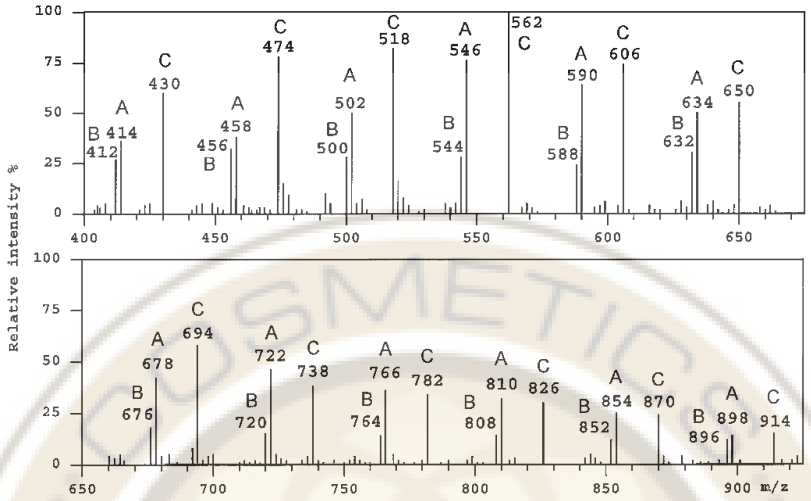


Figure 7.1.5 Positive-ion FAB mass spectrum of semipermanent hair dye containing polyethoxylated (n=3–14) oleyl (A)/linoleyl (B)/ricinoleyl (C)methylammonium chloride. Adapted from Maffei Facino *et al.* (1997) with permission.

1,4-Dioxane

1,4-Dioxane may be formed as a by-product during the synthesis of ethoxylated alcohols and ethoxylated alkylamines owing to dimerization of ethylene oxide. Ethoxylated alcohols are employed as emulsifiers in formulations and they may also be used as raw material in the production of ethoxylated sulfates. Ethoxylated alkylamines are in the synthesis route of several cationic surfactants. 1,4-Dioxane has carcinogenic effects on animals and it is possibly carcinogenic to humans (Wala-Jerykiewicz and Szymanowski, 1998). According to European legislation, 1,4-dioxane must not form part of the composition of cosmetic products (European Commission, 2005). Also, US Food and Drugs Administration (FDA) surveys into the presence of 1,4-dioxane in cosmetics since 1979 have found a reduction in 1,4-dioxane content after modifications to the manufacturing process of raw materials (possibly vacuum-stripping procedures) (Black *et al.*, 2001).

Methods designed to determine 1,4-dioxane involve using GC, GC-MS and LC and several examples are summarized below. GC-FID using isobutanol as internal standard for application in shampoos without pretreatment was proposed by Italia and Nunes (1991). The lowest content of 1,4-dioxane was detected in one shampoo containing no ethoxylated ingredients; for those that contained ethoxylated compounds, 1,4-dioxane was in the range of 6–144 μg/ml. Also, using GC-FID, Black *et al.* (2001) determined residual 1,4-dioxane in raw materials (ethoxylated alkyl sulfates) and in cosmetic products. Two pre-treatment methods, azeotropic distillation and solid-phase extraction (SPE) were compared. The values reported between 1979 and 1997 in raw materials (commonly ammonium and sodium

Table 7.1.6

Analytical references of residual product in cosmetic products from surfactants according to type of cosmetic product and employed analytical technique

Class	Reference	Compound	Matrix	Technique
Residual products	Spiegelhalder and Preussmann (1983)	Volatile nitrosamines	Shampoos products; lotions; creams and bath foams	GC-TEA
	Dahlgran and Shingleton (1987)	Ethylene oxide	Ethoxylates surfactants	HS-GC
	Chou *et al.* (1987)	*N*-nitrosodiethanolamine	Lotion; cream; gel	TEA, LC-TEA
	Dahlgran and Jameson (1988)	Formaldehyde	Ethoxylated surfactans	LC
	Helms (1988)	1,4-Dioxane	Hair rinse	GC
	Sommer (1988)	*N*-nitrosodiethanolamine	Foam bath	GC-TEA
	LaCourse *et al.* (1989)	Triethanolamine	Hand lotion	LC-PAD
	Scalia (1990)	1,4-Dioxane	Sulfated polyoxyethylene surfactants; shampoo	LC
	Italia and Nunes (1991)	1,4-Dioxane	Shampoo	GC-FID
	Scalia *et al.* (1992)	1,4-Dioxane	Cosmetic product	SPE; LC;GC-MS
	Scalia *et al.* (1992)	1,4-Dioxane	Shampoos; liquid soaps; sun creams; Bath foams; Moisturizing lotions; Cleansing milks; aftershave balms; baby lotion; day creams; hair lotion	SPE; GC-MS
	Sasaki *et al.* (1993)	Ethylene chlorohydrin	Cosmetic products	GC
	Billedeau *et al.* (1994)	*N*-nitrosodiethanolamine	Skin cream; Lotion	LC-PB-TEA; LC-MS
	Cetinkaya (1996)	Dichloroacetic acid	Shampoo; body-cleansing products; shower; bath-additives gel	GC
	Song *et al.* (1996)	1,4-Dioxane	Shampoos; lotions; creams; cleanser; conditioners	GC EI-MS
	Diallo *et al.* (1996)	*N*-nitrosodiethanolamine	Diethanolamine	LC-Fl

(*Continued*)

Table 7.1.6 (Cont.)

Class	Reference	Compound	Matrix	Technique
	Prieto-Blanco *et al.* (1997)	Dimethylaminopropylamine	Alkylamidoamide	LC
	Chou (1998)	Diethanolamine; *N*-nitrosodiethanolamine	Raw materials	GC-FID
	Wala-Jerykiewicz and Szymanowski (1998)	Ethylene glycol	Cosmetic products	GC
	Wala-Jerykiewicz and Szymanowski (1998)	1,4-Dioxane	Cosmetic products	GC
	Matyska *et al.* (2000)	*N*-nitrosodiethanolamine	Eye makeup remover; body lotion; moisturizing hand; hand cream; lipstick	OT-CEC
	Foti *et al.* (2001)	Aminoethylethanolamine	Amphoteric samples	LC-MS
	Black *et al.* (2001)	1,4-Dioxane	Shampoo	GC-FID
	Schothorst and Stephany (2001)	*N*-nitrosodiethanolamine	Shampoo; hair gel; shower gel	GC-TEA
	Koshti and Naik (2002)	Dimethylaminopropylamine	Alkylamido betaine	IC
	Wang *et al.* (2002	1,4-Dioxane	Shampoo; bath lotion	Headspace GC
	Koshti and Naik (2003)	Sodium monochloroacetate	Alkylamido betaine	LC
	Chou (2005)	Diethanolamine	Shampoo containing diethanolamides	LC-TEA
	Fuh *et al.* (2005)	1,4-Dioxane	Shampoo	SPME-GC-MS
	Schothorst and Somers (2005)	*N*-nitrosodiethanolamine	Shampoo; shower gel; cream and foam bath; hand soap; hair oil	LC-MS-MS
	Molever (2005)	Linear alkylbenzene sulfones; linear alkylbenzene	Linear alkylbenzenesulfonate	Capillary GC

laureth sulfate), were at levels of up to 1410 μg/ml; while in finished products the levels were up to 279 μg/ml; and levels of over 85 μg/ml were registered in children's shampoo during 1994–1995. Helms (1988) determined 1,4-dioxane in shampoo at a level of 1 μg/ml by GC-FID. Wala-Jerykiewicz and Szymanowski (1998) proposed a method of headspace GC using dimethylformamide as solvent. The detection limit obtained was 2 μg/g and

was applied for shampoos and body lotions. Wang *et al.* (2002) examined the contents of 1,4-dioxane in shampoo and bath lotion from the Chinese market using headspace GC. Higher contents were detected in national products than in imported and joint-venture products. The reversed-phase LC method was proposed for routine analyses of raw materials (ethoxylated sulfates). Chromatographic conditions were performed in a C_{18} column using gradient elution (water–acetonitrile), UV detection and a previous purification step with SPE (Scalia, 1990).

1,4-Dioxane in shampoos, liquid soaps, sunscreens, bath foams, moisturizing lotions, cleansing milks, aftershave balms, baby lotion, day creams and hair lotion was analysed by capillary GC-MS. Although a prior purification step using SPE was necessary, the analysis time was less than 40 min with a quantitative limit of 3 mg/kg. 1,4-Dioxane content of 56% of the analysed cosmetics were in the range of 3.4–108 mg/kg (Scalia *et al.*, 1992). Another GC-MS with the EI method determined 1,4-dioxane using deuterated dioxane as internal standard. The pretreatment involved an extraction procedure (using hexane and methylene chloride) and SPE. A quantitative limit of 0.1 μg/g was obtained and lotions, shampoos, creams, cleanser and conditioners at levels of below 5 μg/g were analysed (Song *et al.*, 1996). Solid-phase microextraction (SPME) coupled with CG and GC-MS were used to analyse residual 1,4-dioxane in three types of nonionic surfactants (polyethylene oxide, poly(ethylene/propylene) oxide, polyhydric alcohol) and cosmetic products by Fuh *et al.* (2005). Shorter pre-treatment time, better precision and detection limits are achieved with SPME as compared to SPE. The detection limit for tested nonionic surfactants was from 0.06 to 0.51 ppm. In nonionic surfactants from the USA and Europe, 1,4-dioxane was not detected but in those from Taiwan values from 12 to 72 ppm were quantified. In 22% of shampoo and liquid soap samples, values were detected ranging from 4 to 41 ppm (Figure 7.1.6).

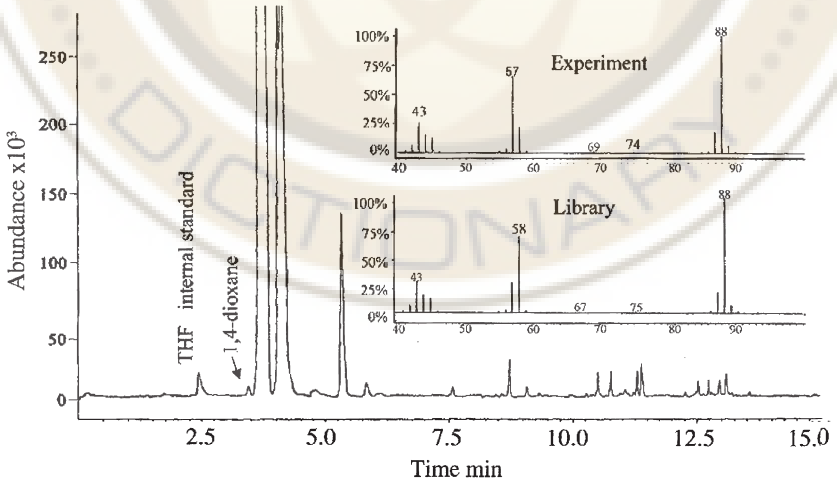


Figure 7.1.6 SPME-GC-MS chromatogram of poly(ethylene oxide) nonionic surfactants containing dioxane. Adapted from Fuh *et al.* (2005) with permission.

N-nitrosamines

N-nitrosamines, organic compounds with a functional group N-N=O, are formed by reaction between amines or amine derivatives with nitrosating agents like nitrous acid, nitrites or nitrogen oxides. These compounds have carcinogen effects on animals and can be absorbed through the skin and accumulate in organs. The presence of *N*-nitrosamines may be due to contamination from raw materials or formation in cosmetic products from precursors, with the latter being the main source. The most common raw materials that contain *N*-nitrosamines are alkanolamines, such as diethanolamine and fatty acid alkylamides like coco diethanolamide. When alkanolamines or other secondary amines and nitrite preservatives are present concurrently in cosmetics, *N*-nitrosamines can be formed. Several types of *N*-nitrosamines can be detected depending on precursor, volatiles such as *N*-nitrosodimethylamine and nonvolatiles such as *N*-nitrosodiethanolamine (the most frequent), or *N*-nitroso *N*-methyltetradecylamine (Ikeda and Migliorese, 1990). Since 1977, when *N*-nitrosodiethanolamine was detected in lotions, shampoos and skin care products, in the US the FDA carries out surveys of these contaminants in cosmetic products. In a notice published in the Federal Register on April 10, 1979, the FDA considered adulterated cosmetics to be those containing nitrosamines and subject to enforcement action (U.S. FDA, 1979). Also measures were proposed to minimize the contamination of cosmetic products by *N*-nitrosamines (U.S. FDA, 1983). In the European Union, nitrosamines, alkylamines and secondary alkanolamines and their salts, dimethylnitrosamine and nitrosodipropylamine must not form part of the composition of cosmetic products (European Commission, 2005). Contamination by dialkanolamine (i.e. diethanolamine) and *N*-nitrosodialkanolamine in fatty acid dialkanolamides (i.e. fatty diethanolamides) and monoalkanolamines should be less than 5% and 0.5 μg/kg, respectively; and in cosmetic products below 0.5% dialkanolamine (European Commission, 2005). Moreover, these raw materials and cosmetic products must be stored in nitrite-free containers, cannot be used in nitrosating systems and monoalkanolamines must have purity of at least 99%. Other compounds like dialkylamides and monoalkylamines and their salts with similar properties, are included with similar restrictions with respect to nitrosamines formation (European Commission, 2005).

A review of nitrosamines in cosmetics (Havery and Chou, 1994a) and others in sunscreens (Havery and Chou, 1994b) summarizes the most important aspects of analytical methods, occurrence, concentration levels and removal strategies.

N-nitrosamine determination is characterized by the use of a chemiluminescence detector, thermal energy analyzer (TEA) coupled habitually with GC or LC. TEA displays high selectivity for the *N*-nitroso group although several interferences have been reported. Also high sensitivity, 100 pg detection level, was achieved with GC or 10 ng and LC. Sample pretreatment is necessary, involving isolation and concentration steps. The most frequently used isolation procedures are silica gel column, C_{18} clean up or solvent fractionating. Moreover, inhibitors are added during analysis to avoid the formation of *N*-nitrosamines.

In the literature, screening methods that detect the presence of nitroso compounds are described. Chou *et al.* (1987) using TEA determined two *N*-nitrosamines *N*-nitroso *N*-methyltetradecylamine (nonpolar) and *N*-nitrosodiethanolamine (polar) in a lotion,

shampoo and cream. An aqueous extraction to determine polar *N*-nitrosamine and (methylene chloride) organic extraction to determine *N*-nitroso *N*-methyltetradecylamine were performed with good analytical recovery. Of the six analysed products, four results were confirmed by LC-TEA but two gave a false-positive *N*-nitrosodiethanolamine. Another direct screening method for *N*-nitrosodiethanolamine determination was proposed by Matyska *et al.* (2000) at the μg/ml level using open-tubular capillary electrochromatography (OT-CEC) with UV detection. In 5 and 15-year-old cosmetics, *N*-nitrosodiethanolamine was found at a concentration level of 14 μg/ml and 35 μg/ml, respectively. This fact appears to demonstrate that cosmetic products have a finite shelf life in relation to the formation of *N*-nitrosodiethanolamine.

Specific methods using GC-TEA are reported for *N*-nitrosodiethanolamine determination in several cosmetics. In foam bath, a pre-treatment involving a cleanup process using sodium ascorbate as inhibitor and formation of trimethylsilyl derivatives was performed by Sommer (1988). Different types of cosmetic products from the Dutch market were analysed using the GC-TEA method with a quantification limit of 5.3 μg/kg. In 1996, a preliminary study detected *N*-nitrosodiethanolamine above the quantification limit in 4 out of 48 samples, in a second part of the study, with a more selective survey, 7 out of 25 cosmetic samples had a content above limit quantification. Higher concentration levels, up to 1000 μg/kg, were found in hair gel, shampoo and shower gel (Schothorst and Stephany, 2001). Specific pre-treatment that included vacuum distillation enabled volatile nitrosamines to be determined in lotions, creams and bath foams by GC-TEA (Spiegelhalder and Preussmann, 1983).

Billedeau *et al.* (1994) analysed 2-ethylhexyl 4-(*N*-methyl-*N*-nitrosamino) benzoate (a *N*-nitrosodiethanolamine from Padimate O contained in sunscreens) and *N*-nitrosodiethanolamine in cosmetics for skin care (lotions, creams …) by LC-TEA using particle beam (PB) interface. *N*-nitrosamine presence was confirmed with LC-PB-MS and electron impact (EI) ionization. Reversed phase can be employed given the high efficiency of this interface for solvent removal. LC-MS enables simpler sample pre-treatment than LC-TEA, and besides, identification and confirmation can be done in only one run. *N*-nitrosodiethanolamine using the LC-MS-MS method was determined in several types of cosmetic product (shower gel, cream and foam bath, shampoo, hand soap and hair oil) from the Netherlands market in 2002. Separation was performed in reversed phase with gradient elution using electrospray as an ionization technique. In 35 of the 140 cosmetic products analysed, *N*-nitrosodiethanolamine was detected in the range from 23 to 992 μg/kg (Schothorst and Somers, 2005).

Owing to the importance of controlling raw materials, methods have been developed to determine both *N*-nitrosodiethanolamine and its precursor diethanolamine in fatty acid alkanolamides. Chou (1998) has analysed *N*-nitrosodiethanolamine and diethanolamine by LC-TEA and capillary GC-FID, respectively. In 19 samples of fatty acid alkanolamides (cocamide, lauramide, linoleamide), diethanolamine was found in the range of 1.1–14%. Although *N*-nitrosodiethanolamine can be determined at ppb levels using the LC-TEA method, it was not found in the 19 test samples. According to the author, this was due to the absence of nitrosating agents in amides. Also, a method to determine *N*-nitrosodiethanolamine in diethanolamine by normal phase LC using fluorescence detection was proposed. An extraction, alkaline denitrosation and precolumn derivatization with Coumarine 120 (7-amino-4-methylcoumarin) were necessary and parameters that influence this step were studied. A detection limit of 0.8 ng/ml was obtained (Diallo *et al.*, 1996). Moreover, Chou

(2005) quantified diethanolamine in shampoos containing fatty acid alkanolamides using LC-TEA. For diethanolamine to be detected, it was necessary to convert it to *N*-nitrosodiethanolamine with sodium nitrite in an acid medium. Out of the 20 shampoos tested, 19 of them contained diethanolamine at levels ranging from 140 to 15,200 μg/ml. LaCourse *et al.* (1989) determined other alkanolamines, like triethanolamine and aminoethylpropanol, in hand lotion and hair spray, respectively using ion pair chromatography with on-line pulsed amperometric detection.

Other toxic residual products

Apart from 1,4-dioxane, other toxic compounds may remain as residual products in ethoxylation reactions. Ethylene oxide may remain unreacted as an impurity and in European legislation its presence is forbidden as ingredient in cosmetic products (European Commission, 2005). Ethylene glycol can be produced by the hydrolysis of ethylene oxide. The method reported by Wala-Jerykiewicz and Szymanowski (1998) and described for 1,4-dioxane, also enables the two residual components to be analysed. What is more, other authors have determined ethylene oxide in surfactants (Dahlgran and Shingleton, 1987) and in shampoos and anionic surfactants (Leskovsek *et al.*, 1991) using headspace GC. Sasaki *et al.* (1993) proposed a GC method with an electron capture detector, in which ethylene oxide is converted into ethylene iodohydrin before being placed inside the chromatographic system. Ethylene oxide was detected in 5 out of 18 polyoxyethylated surfactants at levels ranging from 30 to 394 μg/g but it was not encountered in cosmetics. Aldehydes like formaldehyde may also be formed in the ethoxylation process and is considered toxic. It was analysed in ethoxylated surfactants through derivatization with 2,4-dinitrophenylhydrazine and reversed-phase LC-UV (Dahlgran and Jameson, 1988).

In the synthesis route of cocoamidopropyl betaine, the following compounds were encountered as residual products: sodium monochloracetate (unreacted product); dichloroacetic acid (by-product) from betaine formation and considered as toxic; and dimethylpropylamine a contaminant of raw material (alkylpropyldimethylamide) considered irritant. Sodium monochloracetate was analysed in cocoamidopropyl betaine by LC with UV detection; previously it was extracted through SPE and derivatized with *p*-methoxycinnamidopropyldimethylamine (Koshti and Naik, 2003). Dichloroacetic acid was determined in surfactants, shampoos and shower gels by GC after sample esterification and extraction (Cetinkaya, 1996). Dimethylpropylamine was quantified in the precursor, alkylpropyldimethylamide by reversed-phase LC-UV without pre-treatment (Prieto-Blanco *et al.*, 1997) and in cocoamidopropyl betaine using ion chromatography (IC) in a column with phosphonic acid groups and previous SPE (Koshti and Naik, 2002). In other amphoteric surfactants, amphoacetates, aminoethylethanolamine from raw material may be an allergen. Foti *et al.* (2001) detected its presence in commercial amphoacetates ranging from 4.9 to 1130 μg/ml by IC-MS.

Unsulfonated linear alkylbenzene and linear alkylbenzene sulfones are the main components of unsulfated matter in linear alkylbenzenesulfonate, an anionic surfactant. These residual products can be quantified by high-temperature capillary GC and its application to alkylbenzenesulfonate improves its synthesis process (Molever, 2005).

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– 8 –

Actives for Skin-Care Products. Actives for Personal Hygiene and Other Toiletry Products. Actives with Specific Claims. Analytical Methods

Ingredients that have a special consideration in cosmetic legislation such as: UV filters, colouring agents and preservatives were considered in Chapters 3, 4 and 5. Perfumes were dealt with in Chapter 6 and surfactants in Chapter 7. In the present chapter, actives with specific claims and actives used in general and specific skin-care, personal hygiene or other toiletry products (excluding those mentioned in previous chapters) are considered. Sections 8.1 and 8.2 try to provide a classification of the type of cosmetic products and main ingredients for face and body care and for personal hygiene. Section 8.3 describes hair products, including both hygiene and care products, except hair dye products, which were described in Chapter 4, which deals with all the colouring products. Sections 8.4 to 8.6 deal with different active ingredients used to fulfil product claims and/or specific properties, such as dental whitening products, vitamins, botanical extracts. Latest generation biotechnological actives are described in Section 8.7. Finally, a summary of the published analytical methods for actives considered in this chapter is given in Section 8.8.

8.1. General Skin-Care Products

P. Cuadrado*

RNB S.L. Cosméticos, Conde Alessandro Volta Street 7, Parque Tecnológico, 46980-Paterna, Valencia, Spain

The main aim of any cosmetic product is, as already indicated in Section 1.1, to clean, perfume, modify the appearance, and/or correct body odour, and/or protect or keep the different outer surfaces of the human body in good condition.

From this definition one can deduce that skin care is the main aim of most cosmetic products. Moreover, such products should afford care in a way that is innocuous and not aggressive but effective.

Firstly for a developed cosmetic to fulfil all the requirements, it is necessary, to have a suitable vehicle (that encompasses emulsions, emollients, moisturizers, preservatives, perfume, colour) and to include in its formulation all the active ingredients (UV filters, botanical, animal or biotechnological extracts) necessary to attain what is claimed by its advertising.

Fortunately, the cosmetic industry, including raw material suppliers, is a highly dynamic industry that makes an important effort in research, development and innovation; therefore, novel active ingredients are continuously being introduced. Thus, very often industry helps the formulator's task to achieve products on the market that are both highly effective and novel.

Currently, the cosmetic industry is strongly supporting research to obtain polyfunctional active ingredients at very low doses that are stable and easy to formulate. Work is being carried out in all areas such as the search for new active ingredients (using, for instance, new material sources like tropical plants or sources from the Arctic, minerals, etc.); with special development in the area of biotechnology (see Section 8.7); new ways to release or penetrate (liposomal multilayer, nanosomal, cyclodextrines, biospheres); new extraction modes to obtain greater purity (supercritical fluid extraction, microwave-assisted extraction, new solvents, etc.); dosage (with a trend towards minimization); modern analytical techniques to characterize and/or determine actives.

Some general skin-care products are indicated within the cosmetic classification specified in Annex I of the EU Cosmetics Directive (European Commission, Council Directive 76/768/EEC). The following list shows the EU classification of skin-care cosmetic products (excluding cosmetics for other parts of the body—such as hair or nails—perfumes, personal hygiene and decorative products):

– *General face and body products*: Creams, emulsions, lotions, gels and oils for the skin (hands, face, feet, etc.).

*Corresponding author.E-mail: idi@RNBCOSMETICOS.com

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– *Specific products*: Products intended for application to the lips, face masks (with the exception of chemical peeling products) and anti-wrinkle products.
– *Sunscreens and related products*: Sunbathing products, products for tanning without sun and skin-whitening products (they were considered in Chapter 3).

However, a more detailed classification could help one to realise the huge range of products and active ingredients incorporated in the new formulas.

The following classification has been carried out taking into account different aspects of interest:

AREA OF APPLICATION

– *Face* (including eyes and lips)
– *Body*
– *Hands and feet*

One can find all kinds of emulsions (oil-in-water O/W, water-in-oil W/O, microemulsions), gels or bi-gels, serums, powders, sticks, etc.

The active ingredients that tend to be included in the formulas are: UV filters, plant extracts, biotechnological or synthesis extracts.

SKIN TYPE

– *Normal skin*: O/W emulsions with active ingredients that fundamentally moisturize, protect and nourish the skin (plant extracts or oils, biotechnological actives).
– *Oily skin*: Oil-free emulsions and specific sebum-regulatory and pore-closing actives.
– *Mixed skin*: O/W emulsions with active ingredients to moisturize and protect the skin.
– *Dry skin*: O/W or W/O emollient-rich emulsions and lipid-replenishing-rich actives.
– *Very dry skin—atopic skin*: O/W or W/O emollient-rich emulsions and with actives rich in moisturizing substances (hyaluronic acid, tri-dimensional matrices with galactomannan), lipid replenishing, alleviators (mimosa, marigold, liquorice) and antipruritic (anti-itch) (Madecassoside, extracted from *Centellae asiaticae*).
– *Sensitive skin*: Very similar to the atopic skins. Products can include lactic proteins.

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– *Babies*: O/W emulsions with actives that basically moisturize and protect the skin (urea, allantoin, zinc oxide, bisabolol, anti-enzymatic actives, plant extracts or oils (mimosa, camomile, boswellia)).

- *Children's skin*: O/W emulsions with actives that basically moisturize and protect the skin (urea, allantoin, bisabolol, plant extracts or oils (avocado, aloe, mimosa, camomile)).
- *Young skin* (teenagers): As these skins tend towards oiliness, oil-free emulsions are used together with specific sebum-regulatory actives, antiseptics, keratolytic substances (salicylic, glycolic and lactic acids) and pore-closing products.
- *Adult skin*: Actives to moisturize, protect and nourish. O/W or W/O emollient-rich emulsions and with lipid-replenishing-rich actives (plant oils like sweet almond, borage, evening primrose oil, avocado, walnut, sheabutter, shorea, muru–muru, cupuacu, ucuuba).
- *Mature skin*: Actives to moisturize, protect and nourish. O/W or W/O emollient-rich emulsions and with actives that are increasingly specific to alleviate the effects of the menopause (soy isoflavines), calcium source, etc.

ETHNIC GROUP

- *White skin* (Caucasian): O/W or W/O emulsions for daily use that incorporate UV filters and actives to avoid oxidative stress and environmental pollution.
- *Oriental skin*: Similar emulsions to the previous one but they must also contain absorbent actives for excess oiliness to avoid shine.
- *Coloured skin*: Emulsions tend to be lighter than those used for white skin and they contain active ingredients to moisturize, protect and whiten the skin.

ACTIVE INGREDIENTS ORIGIN

- *Plants* (terrestrial and aquatic): At the moment, as well as the traditional ones, the cosmetic industry is also employing those that are obtained in specific regions like India, China, Amazon, Arctic and Antarctic and abyssal regions of the oceans.
- *Animals* (land and aquatic): Due to environmental awareness, actives of terrestrial origin are no longer used, the alternatives being fish (excluding mammalian sea creatures and sharks).
- *Minerals*: Extracted from minerals like manganese carbonate and hematite ores (for their iron content).
- *Biotechnological actives*: Hyaluronic acid, mucopolysaccharides, glycosaminoglycans.
- *Synthetic actives*: Currently the most fashionable ones are peptides, biospheres that increase in size once they have penetrated the skin and thus exert a pull-up effect on wrinkles.

ACTIVE INGREDIENTS FUNCTION

- *Traditional*: Moisturizers, nourishing, anti-wrinkle, anti-free radical and whitening products, sebum regulators, alleviators, lipid replenishers, etc.
- *Modern*: Skin redensifiers, wrinkle fillers, botox-like products, etc.

MODE OF APPLICATION

- *Traditional*
- *Oriental* (Zen)
- *Spa*: Chocolatherapy, vinotherapy, aromatherapy
- *Ayurveda*: Extracted from trees (*Aegle marmelos,* Bilva; *Cyperus rotundus,* Motha; *Emblica officinalis,* Amalki; *Morinda citrifolia,* Ashyuka; *Tinospora cordifolia,* Guduchi) or from plants (*Santalum album,* Sandalwood; *Nelumbo nucifera,* Lotus; *Cinnamomum zeylanicum,* Cinnamon*; Zingiber officinale,* Ginger; *Azadirachta indica,* Neem)

SOCIAL LEVEL

- *Mass market*: Actives habitually used and with a low economic value.
- *Prestige*: Derived from silicon, endorphin releasers (causing well-being).
- *Luxury*: Actives like gold, caviar, cashmere, champagne, pearls.

REFERENCE

European Commission, Council Directive 76/768/EEC dated 27.07.1976, *On the Approximation of the Laws of the Member States Relating to Cosmetic Products,* and its successive amendments and adaptations. <http://europa.eu.int/comm/enterprise/cosmetics/html/consolidated_dir.htm>

8.2. Personal Hygiene. Other Toiletry Products (*Excluding those Mentioned in Previous Chapters*)

M.T. Vidal Gandía[1*], Z. León González[2], M. López Nogueroles[2] and G.A. March Roselló[2]

[1]Department of Chemistry, Universidad Politécnica de Valencia, Camino de Vera s/n, E-46071, Valencia, Spain
[2]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain

As already commented for skin-care products (Section 8.1), personal hygiene and other toiletry products are also very difficult to classify due to the wide variety and application.

The EU Cosmetic Directive (European Commission, Council Directive 76/768/CEE) distinguishes between the following products (excluding those mentioned in previous chapters):

- Toilet soaps, deodorant soaps, etc.
- Bath and shower preparations (salts, foams, oils, gels, etc.).
- Deodorants and antiperspirants.
- Depilatories.
- Shaving products (creams, foams, lotions, etc.)
- After-bath powders, hygienic powders, etc.
- Products for removing make-up from the face and the eyes.
- Products for care of the teeth and the mouth.
- Products for external intimate hygiene.
- Hair cleansing products (lotions, powders, shampoos), etc.
- Products for nail care.

A short classification based on that outlined in the EU Cosmetic Directive is given in this section with the aim of helping the reader to recognize some of the analytes and samples that will be seen in Section 8.8, where analytical procedures are considered.

The most important types of cosmetic and active ingredients are indicated below. A more extensive revision of these products can be found in some interesting publications (Wilkinson and Moore 1990; Montlló, 1993; Díez-Sales, 1998; Simmons, 1995).

*Corresponding author. E-mail: mtvidal@qim.upv.es

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DEODORANTS AND ANTIPERSPIRANTS

Deodorants are substances that reduce or mask unpleasant body odours whereas antiperspirants reduce body transpiration. Deodorants are classified as over-the-counter (OTC) cosmetics by the U.S. Food and Drug Administration (FDA) whereas the EU considers them as cosmetics. They are composed of substances that inhibit the growth of microorganisms which cause bad odour. They can be formulated either with substances with anti-microbial action (such as ethanol, quaternary ammonium, triclosan) or actives which neutralize the body odour (such as Zn ricinoleate that forms complexes with the sweat). They also often contain essential oils that mask the odour of perspiration.

Deodorants may be combined with antiperspirants. They are classified as drugs by the FDA whereas they are considered as cosmetics by the EU legislation. Antiperspirants are based on actives whose function is to decrease the size of the pores (as e.g. Al or Zr salts). The most commonly used cosmetic formats are lotions, gels, water-in-oil (W/O) and oil-in-water (O/W) emulsions, aerosols, sticks or powers.

There is a restriction on the maximum authorized level of zinc 4-hydroxybenzene sulphonate in deodorants and antiperspirants established in the EU legislation (European Commission, Council Directive 76/768/CEE, 1976, Annex III), also for zinc phenosulphonate, dichlorophene and other substances used in deodorants. Triclosan and other anti-microbial treated in Chapter 5 are considered as preservatives by the EU legislation and their maximum authorized level is restricted (Annex VI); however, they have an additional anti-microbial function for deodorant users.

Aluminium and zirconium compounds used in the antiperspirants also have limited levels. The safety of using antiperspirants containing some aluminium or zirconium salts has been called into question and related to skin granulomas (European Commission, SCCNFP, 1998) and also to other unconfirmed side-effects.

PERSONAL HYGIENE PRODUCTS. BATH PREPARATIONS, SHOWER PREPARATIONS, ETC.

The most commonly used cosmetic formats are gels, oils, lotions, etc.

The most important actives are surfactants, which are used in the majority of these preparations. Chapter 7 is wholly devoted to surfactants because these are, without doubt, the main ingredients in personal hygiene products; types of surfactants and published analytical methods for their determination in cosmetic samples were also dealt with therein.

As well as surfactants that act like detergents (e.g. primary anionic tensioactives formulated in combination with secondary and amphoters), other types of surfactant and other active ingredients are also used in formulations, like for instance foam stabilizers or boosters that make the foam creamier and more stable (e.g. alkyloamides). Other actives can also be added to gain viscosity (e.g. electrolytes such as NaCl or NH_4Cl), to achieve opaque or pearly effects (e.g. magnesium and aluminium silicates), to avoid excessive de-oiling or drying of the skin (e.g. oils, esters and glycerides).

NaOH or KOH and long-chain fatty acids are used to formulate soaps.

As well as the conventional formulations, other hygiene preparations have been marketed with specific properties for moisturizing, skin protection, extra-smooth skin (products for children or for feminine hygiene) or with other properties.

HAIR CLEANSING AND CARE PRODUCTS

These types of cosmetics are discussed in detail in Section 8.3 while related decorative products, based on the use of colouring agents, are dealt with in Chapter 4.

DEPILATORIES AND SHAVING PRODUCTS

The most commonly used formats of depilatories are powders or emulsions containing keratolytic actives (alkalines or alkaline earth sulphides). Their maximum content is restricted by the EU legislation (Annex III). For physical depilation, waxes are used.

For shaving products, creams, foams, lotions, etc. are commonly used. The most frequently used ingredients in shaving products are fatty acids (e.g. stearic acid), surfactants (e.g. triethanolamine), emulsifiers (e.g. lanolin, polyoxyethylene sorbitan monostearate), solvents and emollients (e.g. glycerin and other alcohols).

After-shave products tend to contain astringent substances (e.g. hamamelis extract, ethanol), refreshing (e.g. menthol), moisturizing or soothing (e.g. aloe vera extract), anti-microbial, etc. Pre-shave products are similar but tend to contain oils (almond, peanut, safflower) to lubricate the skin and sometimes liposoluble vitamins (A, E) that soften the skin.

PRODUCTS FOR CARE OF THE TEETH AND THE MOUTH

Cosmetic formations for tooth-cleansing products are paste, gel or powders. They are formulated using abrasives that clean and/or polish (sodium metaphosphates, surfactants) and prevent caries (fluoride). Contents are restricted by EU legislation, Annex III.

Specific actives for dental whitening such as hydrogen peroxide (European Commission, SCCP, 2005) will be considered in Section 8.4.

The liquid mouthwashes and breath fresheners contain surfactants, astringents, fluoride, chlorophyll (to avoid bad breath) and different anti-septics to act against bacterial plaque (e.g. derived from quaternary ammonium).

NAIL CARE

The most common products (excluding decorative products containing colouring agents dealt with in Chapter 4) are nail-polish removers, nail builders and strengtheners, cuticle removers and moisturizers, and nail creams.

Products containing keratin, vitamins, essential oils, botanical extracts are usually employed for nail care. Ingredients which were used previously, such as formaldehyde or toluene are being replaced by other organic solvents or mixtures (e.g. ethyl acetate, butyl acetate, acetone, ethanol) that are also able to dissolve lacquers.

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8.3. Actives for Hair Products (*Excluding Hair Dyes*)

A. Salvador[1*] A. Chisvert[2] and C. del Cañizo Gómez[3]

[1]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Valencia, Spain
[2]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Alicante, Spain
[3]L'Oreal España, Madrid, Spain

INTRODUCTION

Hair products are intended to clean, care and make users' hair more attractive.

The European Union (EU) Cosmetics Directive (Council Directive 76/768/EEC) distinguishes between the following products:

- hair tints and bleaches
- products for waving, straightening and fixing
- setting products
- cleansing products
- conditioning products
- hair-dressing products

The above classification is taken here as basis to give a brief introduction to the most important hair products (excluding hair-dye products which are dealt with in Section 4.3) and the main active ingredients of these cosmetic formulations, with the aim of helping the reader to recognize some of the analytes and samples that will be seen in Section 8.8, where analytical procedures are considered. A more extensive revision of these products can be found in some interesting publications (Wilkinson and Moore, 1990; Díaz-Sales, 1998; Simmons, 1995; Bouillon and Wilkinson, 2005).

Some of the common ingredients in hair products are prohibited in EU beyond certain limits and outside the conditions laid down in Annex III of the EU Cosmetics Directive. For instance, thyoglycolic acid and its salts can be used for hair waving or straightening products but formulations must have a content level of below 8% for general use and 11% for professional use (both at pH 7–9.5). These percentages are calculated as thioglycolic

* Corresponding author. E-mail address: amparo.salvador@uv.es

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acid; the maximum authorized level of hydrogen peroxide in hair care preparations is 12% (40 volumes), present or released; selenium disulphide only can be used for anti-dandruff shampoos and the maximum authorized content is 1%, and so on. Because of this, cosmetic legislation should be carefully consulted before developing of new hair-care formulations.

HAIR CLEANSING PRODUCTS

When hair has not been washed recently, it looks dirty due to the combination of particles from the environment with the products formed from sebum lipolysis, sweat oligoelements and corneocytes, as well as those remaining from cosmetics, especially perfumes or hair-care products (Díaz-Sales, 1998).

Objective

To remove sebum, skin particles, dirt and so on without excessively degreasing the hair, as drying out the hair makes it look less healthy and difficult to comb.

Types

According to the cosmetic form

- *Conventional shampoos*. Usually hair cleaning is done by washing with the aid of the common shampoos which are based on the emulsification of sebum by surfactants.
- *Dry shampoos*. Sprayable powders, containing talc or caolin are formulated. They should only be used occasionally, if necessary, between two conventional shampoo washing.

According to the hair type to be addressed

- For normal hair, oily hair, dry hair, damaged hair, dyed hair and baby hair.

Main active ingredients and functions

Main ingredients in hair cleansing products are the following (Beauquey, 2005; Díaz-Sales, 1998; Simmons, 1995).

Surfactants
The main active ingredients are surfactants (see properties and analytical determination in Section 7.1). The major types of surfactants used in shampoos and their main functions are

- *Anionic surfactants*. They are usually the basic components of shampoos due to their high detergency and good foaming power; moreover they are effective at neutral or

slightly acid pH and easy to remove with rinsing. More common are alkyl sulphates and alkyl ether sulphates (alkaline, alkaline earth metals or ammonium salts).
- *Cationic surfactants*. These have a good affinity with keratin, are antistatic and some of them can act as bactericide. More common are ammonium tetra alkyl chlorides.
- *Non-ionic surfactants*. They enable viscosity of the formulation to be increased, they are good for refatting and foam boosting and they can also help solubilize fragrances.
- *Amphoteric surfactants*. More common are *N*-alkyl-betaines or *N*-alkyl-amido-betaines.

Ingredients for specific functions

- *Foam boosting actives*. They improve the quality of the foam produced by increasing one or more of the following properties: volume, texture and/or stability (e.g. alkanolamides).
- *Thickeners or viscosity controlling actives*. They make the product more comfortable to use (alkanolamides, methylcellulose, electrolytes such as sodium chloride, or ammonium chloride).
- *Conditioning agents*. They minimize the negative effects of excessive sebum removal (lanolin and poly ethoxyethylenated derivatives, silicones such as dimethicone, quaternary agents and ceramides).
- *Opacifiers*. They reduce transparency or translucency and improve the final appearance of the product (ethylenglycol or propylenglygol, magnesium or zinc stearates).
- *Chelating agents*. They form complexes with metal ions (like e.g. calcium present in hard waters), which could affect product stability. Most common is ethylenediaminetetraacetic (acid sodium salt).
- *pH adjusting agents*. Citric or lactic acids are usually employed in these cosmetic formulations.
- *Other*. Colouring agents, fragrance actives and preservatives (see Chapters 4, 5 and 6).

Special additives

- *Vitamins*. Vitamins E (wheat germ oil) and B (panthenol) are the most commonly used (see Section 8.6).
- *Fatty substances*. Botanical extracts are frequently used (see Section 8.5).
- *Proteins*. For instance, ribonucleic protein, collagen and placenta.
- *UV filters*. (see Section 3.1).
- *Anti-dandruff ingredients*. Specific shampoos can contain pirctone olamine, zinc pyrithione, salicylic acid and selenium disulphide.

HAIR-CONDITIONING PRODUCTS

Objective

Such products try to give hair the positive features afforded by sebum (removed by the shampoo) but at the same time avoid the oily appearance, which is associated with dirt. They are also useful for damaged hair (due to excessive brushing, waving, bleaching, sunlight exposure and others).

After using conditioning products, static electricity decreases and dry hair ends, while shine and flexibility are improved.

Types

There are different cosmetic formulations for hair-conditioning products, such as liquid, creams, gels and others. They can be used jointly with shampoo (2-in-1 products), after shampoo on wet hair or on dry hair.

Main active ingredients and functions

Different types of ingredients are necessary to achieve a series of functions:

- *Moisturizers*. They provide the necessary water content and help keep hair smooth; this is especially useful for dry hair (glycerine and propylenglycol).
- *Oils and refatting agents*. They replenish the lipids after shampooing.
- *Reconstructors*. They strengthen the hair (dimethicone).
- *Acidifiers*. They provide elasticity and make combing easier.
- *Thermal protectors*. They protect the hair from different types of drying.
- *Shiners*. They provide a healthy appearance.

A detailed classification of the chemical compounds usually employed was given by (Dubief *et al.*, 2005). The most important of these are organic acids (carboxylic acids and aromatic sulphonic acids), fatty compounds and their derivatives (fatty acids, fatty alcohols, natural triglycerides, natural waxes, fatty esters, oxyethylenated and oxypropylenated waxes, partially sulphated fatty alcohols, lanolin and its derivatives, ceramides), vitamins (A, B and E) (see Section 8.6), protein derivatives (extracts or hydrolysates of keratin, collagen and vegetable proteins), silicones (dimethicone and others), cationic surfactants, cationic polymers, amphoteric and betainic polymers.

HAIRDRESSING, SETTING AND FIXING PRODUCTS

Many different formulations have been developed as hair setting products.

Objective

To give form and style to the hair and maintain the final hairstyle.

Types

Different cosmetic forms are formulated for hair-conditioning products such as spray lotions, gels, foams, oils and creams.

Main active ingredients and functions

Way to use

- Products to use on wet hair, after shampooing (Simmons, 1995), such as fixing lotions, molding gels, molding foams and other. They must help the user to achieve the desired form or style. Hair is covered with a plastic substance or adhesive resin, hair and fixing products will be jointly dried by the user.
- Products to use on dry hair before combing, such as creams or oils (grooming oils and brilliantine).
- Products to fix the final combing, such as lacquers. They are formulated by using an ethanolic base that evaporates easily after spraying the product on the hair.

Most frequently used ingredients
Main active ingredients for hair dressing, setting and fixing (Simmons, 1995; Díaz-Sales, 1998; Beitone et al., 2005) are

- *Film-forming actives*. They produce a continuous film on hair (e.g. synthetic polymers such as polyvinylpyrrolidone, vinylpyrrolidone/vinyl acetate copolymers, natural polymers are less commonly used today).
- *Plasticisers*. They soften and make hair supple and easy to work with (lanolin derivatives, silicones, fatty acid esters, glycerine and polyethylenglycols).
- *Surfactants*. They are specially necessary for aerosol foams.
- *Other*. Colouring agents, fragrance actives, UV filters and solvents.

HAIR WAVING PRODUCTS

In wet hair the hydrogen bonding of alpha keratin can break to transform to beta keratin and then hair can be shaped by brushing or with hair-curlers. After drying, hydrogen bindings return to their initial position and the form of the hair is temporary maintained. (Diaz-Sales, 1998).

Objective

Hair waving products are formulated to achieve a lasting or permanent form of the hair. This requires breaking the disulphide binding of keratin (by reducing agents) followed by a subsequent re-structuring (with oxidizing agents) after a time of waving by hair-curlers.

Types

Several products are necessary to carry out the different steps in the waving process, and are usually presented as creams or lotions. On the other hand, nowadays the products used

to perform this process can work at room temperature or use a very slight heating by infrared radiation. The most commonly used is alkaline waving although acid waving is also been used.

The most commonly used ingredients for hair waving are summarized below (Díaz-Sales, 1998; Simmons, 1995; Zviak and Sabbagh, 2005).

Active ingredients for alkaline waving

Reducing agents

- Thioglycolic acid (or mercaptoacetic acid or 2-mercaptoethanoic acid)
- Thiolactic acid (a-mercapto-propionic acid)
- Thioglycerin (a-monothioglycerol)
- Glyceryl monothioglycolate
- Cysteine
- Cysteamine (β-mercaptoethylamine).

Oxidizing agents

- Hydrogen peroxide
- Potassium bromate
- Sodium perborate

Neutralizing agents

They are used together with oxidizing agents to ensure stability and homogeneity (alpha hydroxyacids and surfactants).

Other waving aid agents

- Alkaline bases, to neutralize the acidity of reducing agents (ammonium and monoethanolamine)
- Antioxidants, to favour the reducing effect (sodium sulphite)
- Solvents (ethanol, isopropylic alcohol)
- Surfactants to favour the homogeneous emulsification of the reducing agents
- Softeners, with keratin affinity
- Quelating (specially to avoid colouring iron effects)
- Other: colouring agents, fragrance actives.

Active ingredients for acid waving

This type of waving is based on the use of thyoglycolic acid esters and amides which can act at pH 6–7.

HAIR-STRAIGHTENING PRODUCTS

Objective

To change the structure of curly hair into a straight form.

Types

There are two ways of working, by either using reducing/oxidant treatment or by use of alkaline treatment. The process is similar to that used for waving, but hair is straightened.

Main ingredients

- *Reducing/oxidizing procedure*: Similar products and ingredients to those used for waving.
- *Alkaline treatment*: Based on the use of strong alkalis (such as sodium, potassium, lithium or guanidine hydroxide).

HAIR-BLEACHING PRODUCTS

Oxidizing action of solar UV radiation is a natural way of bleaching hair, as everyone knows. Using oxidizing agents, this natural process can be accelerated.

The different hair pigments (eumelanine, feomelanine and tricosiderine) have different bleaching potential, with the most easily bleached being eumelanine. It can be said that a depolymerization occurs, giving rise to carboxylated derivatives, which are soluble in alkalis and can be eliminated by rinsing. Details on the chemistry of bleaching can be found in the literature (Zviak and Milléquant, 2005).

Objective

To bleach hair without using dyes, only by an oxidation process.

Types

Most commonly used are emulsions, shampoos, powders and fluid gels.

Main ingredients

Oxidizing substances

Hydrogen peroxide is the main ingredient, although others, such as persulphates, sodium percarbonate, perborates, can be found in some formulations.

Alkaline substances

Hydrogen peroxide has to be mixed with an oil (shampoo with surfactants) or powder (ammonium carbonate, magnesium carbonate and surfactants) containing ammonium to achieve a pH 10–11.

At this pH, hydrogen peroxide decomposes by forming water and atomic oxygen (active oxygen) which is very reagent and can oxidise the hair pigments with the subsequent bleaching.

A bleaching stimulator can be also added, such as urea peroxide which maintains the oxidizing action of hydrogen peroxide.

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8.4. Actives for Dental Whitening

A. Torréns-Tomás* and P. Montoro-Martínez

Corporacion Dermoestética, Laboratory, 1 de Mayo Street.,
Quart de Poblet, S.A., 46930-Valencia, Spain

Dental-care products are included with the general personal hygiene products, which are dealt with in Section 8.2. In the present section, only specific dental whitening products will be commented on.

Dental whitening is a commonly requested treatment nowadays, which has led to the development of increasingly effective and user-comfortable techniques.

DENTAL WHITENING TREATMENTS

To begin with, dental whitening procedures require a diagnosis of the underlying cause of staining. Once this has been done, it makes it easier to choose the most suitable products and application modes to successfully achieve the desired effect.

There are three ways to achieve whitening:

- At home, with products that contain substances considered as cosmetic ingredients.
- In the dental clinic, using products considered as cosmetics or pharmaceuticals and special treatments based on different types of activation.
- Combining home treatment with treatment in the dental clinic.

This section will only deal with ingredients that are in cosmetic formulations and can be applied at home.

Whitening-product application at home can be carried out in different ways:

- *Using a toothbrush*: Toothpastes containing whitening active ingredients like sodium bicarbonate or urea peroxide are applied in this way.
- *Application with pen or brush-on applicator*: The commonly used whitening agent is urea peroxide, applied using an individual pot or pen applicator, between 2 and 8 hours a day for a duration of 2–6 weeks.
- *Via complementary techniques*: Commonly used ones are:
  - *Plastic strips*: These are one-use only, made of a flexible plastic, covered with a hydrogen peroxide gel. They are placed directly over the tooth for 30 minutes, twice a day for a duration of 2 weeks.

*Corresponding author. E-mail: atorrens@corpderm.com

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Amparo Salvador and Alberto Chisvert

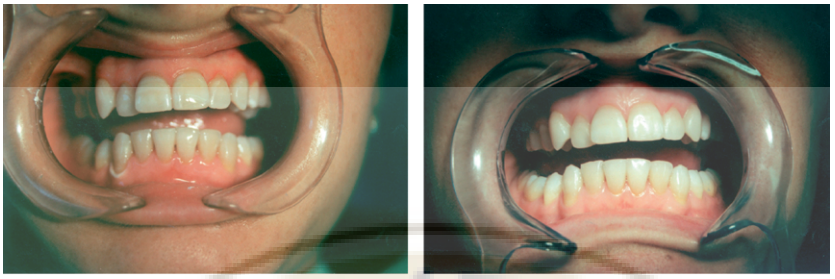

Figure 8.4.1 An example of the efficacy of cosmetic actives for dental whitening enhanced by laser irradiation. (a) before treatment and (b) after treatment.

- *Whitening mouthwashes*: These are aqueous or hydroalcoholic solutions that contain the same active ingredients as toothpastes, but at a lower concentration and without abrasive agents. They include whitening agents in their formulation at different concentrations, which help to maintain the tooth colour (Camps Miró, 2006).
- *Whitening chewing gum*: These incorporate different whitening agents in their formulation, which have the same aim as that of the mouthwashes.
- *Powders*: These are a mixture of solids, such as perborates, carbonates and polyphosphates that eliminate staining on the teeth.

The success of dental whitening depends on the ability of the whitening substance to eliminate the pigments without having secondary effects. The basis of the way in which the whitening chemical products work is generally based on redox-type chemical reactions.

This home treatment can be combined with special treatments using different activation methods (chemical, heat, visible radiation) that have to be carried out at the dental surgery.

Among such treatments we must stress laser techniques, which give excellent results. An example is shown in Figure 8.4.1.

These are highly advanced techniques with which dental colour tone can be lightened in just one session. They offer the fastest and most effective results currently known. The gums are protected with resin and afterwards a professional dentist applies a whitening gel to the teeth, which is then activated by the laser effect. The selective chemical reaction produced, lightens the colour of the teeth without affecting the enamel. A number of studies have been carried out regarding the safety of whitening gels and good results have been obtained, given that they do not produce skin sensitization and their primary irritation index (PII) is classified as having an unnoticeable irritant effect.

THE IMPORTANCE OF DISCOLOURING ETIOLOGY

The causes that bring about dental discolouring are many and diverse.

Whitening is not always recommendable. Each person, given their genetic heritage, has a specific tooth colour. This cannot be changed and it is only possible to lighten the tooth shade within the same colour range.

Whitening should be carried out when the teeth are observed to be darkened by staining or pigmentations like: staining due to ageing or diet or due to tobacco, staining caused by medicine intake, fluoride stains, an accidental blow that has led to tooth darkening, etc.

Whitening treatments are unadvisable: in the case of cracked teeth that have lost part of their enamel, in cases of sensitivity, when there is intolerance to the product, allergy or a disagreeable flavour, if there are caries or dental restructuring with some resin types or where there is extensive white staining.

To choose the suitable whitening agent, one must bear in mind that its chemical composition must allow whitening of the tooth but have low diffusion capacity in the dentine and that the pH it affords should be almost neutral.

ACTIVE INGREDIENTS USED IN DENTAL WHITENING PRODUCTS

Hydrogen peroxide

Tends to be used in aqueous or ether solution.

For a long time peroxides have been the choice whitening agents. The concentration of peroxide and its combination with other substances varied depending on the purpose of its use and the application method employed.

For dental staining the whitening process is made possible thanks to the permeability of the dental structure to hydrogen peroxide, which has the capacity to spread via the aforementioned structure, causing either oxidation or reduction of the stain molecules. Hydrogen peroxide releases oxygen, a process that is influenced by temperature, pH, light and the presence of catalysers. In contact with the tissue, the molecule of hydrogen peroxide breaks and forms free radicals that can fracture macromolecular pigments, breaking down into smaller and smaller molecules until, by diffusion, these pigments are eliminated totally.

One should be warned that an increase in environmental temperature of 10ºC doubles the speed of the reaction and the whitening process, given that the temperature catalyzes the breakdown of the whitening agent, facilitating its diffusion through the dental structure.

Urea peroxide

Urea peroxide reacts forming hydrogen peroxide. Its breakdown occurs on coming into contact with oral tissues and saliva. Hydrogen peroxide is considered to be the active agent, while the urea increases the pH of the plaque.

The hydrogen peroxide released from the urea peroxide is metabolized by the catalase, peroxydase and hydroperoxydase enzyme in the saliva and in the oral tissues. The oxygen formed softens and eliminates the interplasma waste. The low molecular weight of the peroxides, as well as of the urea could explain their free movement through the enamel and the dentine. The temperature catalyses the breakdown of the whitening agent and helps the diffusion of the whitening dissolution in the structure of the tooth. In addition to exerting an oxidative effect, hydrogen peroxide can also denaturalize protein (Saavedra, 2006).

Dental whitening using urea peroxide produces changes in the surface morphology of the enamel; these changes are related to exposure time and with the urea peroxide concentration (Pérez Vargas *et al*., 2004).

Sodium perborate

Sodium perborate is available in powdered form or in various patented commercial combinations.

This oxidant agent is a stable source of oxygen. It exists in different forms, these being anhydrous, mono, tri and tetrahydrate. The tetrahydrate is prepared through the reaction of sodium borate with hydrogen peroxide. Its characteristics in watery dissolution are very similar to those of a hydrogen peroxide solution.

The majority of these preparations are alkaline, as these can be controlled more easily and with greater safety (Saavedra, 2006).

As compared to the dissolution of hydrogen peroxide, the solid form of sodium perborate compounds provides better conditions of stability and application. By using sodium perborate mixed with water, equally satisfactory results can be obtained when compared to those achieved with the other whitening agents used in this type of treatment. Moreover, the safety margin is greater, although there is the drawback that longer treatment times are necessary.

Other whitening actives

Abrasive products

Abrasive substances are those applied to the teeth, during brushing, that eliminate the accumulated deposits.

The most commonly used abrasive substances are: micronized sodium bicarbonate, calcium carbonate, sodium benzoate, sodium phosphate, calcium phosphate (meta and piro), sodium metaphosphate, aluminium hydroxide, aluminium lactate, alumina, silicates (silice xerogel and aerogel), citroxain (a mixture of the papain enzyme, sodium citrate and alumina).

Pentasodium triphosphate

This cleans without weakening the enamel. It exerts a whitening effect on the tooth stains. Contrary to other whitening agents, which work only through exerting abrasive action that can damage the gums and dental structures, its whitening power is achieved via an enzymatic mechanism that acts directly on the stains present on the enamel surface. It works by breaking the bonds of the stain. Furthermore, this active ingredient acts on three important levels—combating dental caries, restoring natural levels of pH in the mouth and as a breath freshener.

Odontoblanxina

This active ingredient is obtained through lichen extracts; it is a substance that fights against the bacteria responsible for caries, i.e. *Streptococci mutans.* The Blanx penetrates deeply; eliminating the bacteria, the pigments and the food stains and in this way returns to the teeth their natural colour.

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8.5. Botanical Extracts

A. Benaiges[1*] and P. Guillén[2]

[1]R&D Department, Provital S.A. Polígono Industrial Can Salvatella. Gorgs Lladó 200, 08210-Barberà del Vallès, Barcelona, Spain
[2]Quality Control Department, Provital S.A., Barcelona, Spain

Extraction of active ingredients from plant material is one of the oldest procedures used in cosmetics. Extraction involves the separation of biologically active molecules (Paris and Moyse, 1976; Bruneton, 1987) from inert or inactive components usually by using suitable solvents and extraction processes. The complex systems of active substances so obtained—since they may include ballast substances of different origins—are relatively impure liquids, semi-solids or powders.

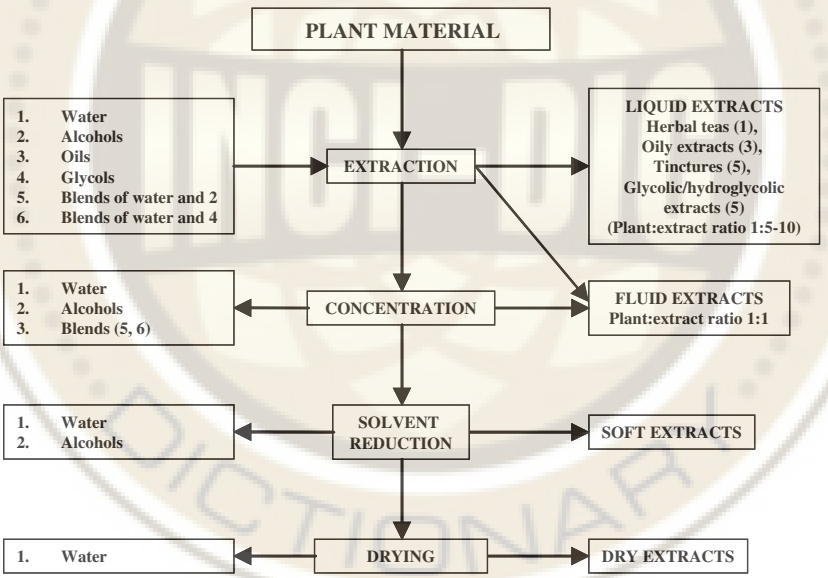

Figure 8.5.1 Diagram of obtention of different extracts from herbal material.

*Corresponding author. E-mail: tecnic@provital.org

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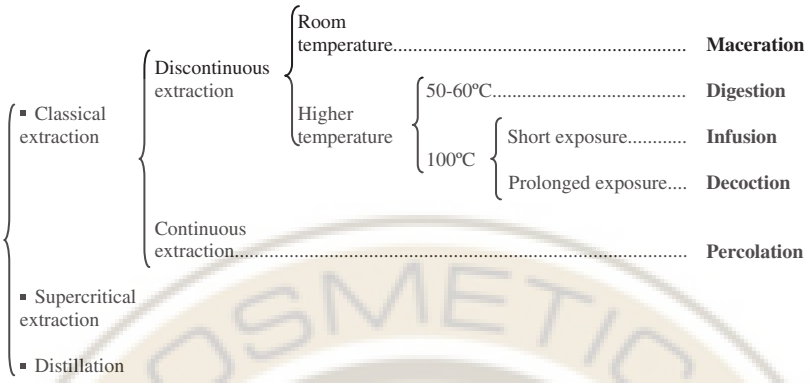

Figure 8.5.2 Main processes used in herbal extract industry.

Depending on the procedure and the concentration of the extracts the resulting preparations are known as decoctions, infusions, fluid extracts, tinctures, semisolid extracts or powdered extracts (Figure 8.5.1).

Extraction is the separation of the substances in a mixture, by dissolving each component with one or several solvents, which yields at least two components: the solution extracted in the solvent (solute) and the residue. Extraction is completed when a balance in the concentrations is reached.

Every extraction requires raw material and extraction liquid or solvent (Figure 8.5.2). The quality of the vegetable extract will depend on the quality of the starting material (Wichtl, 1994). Generally, the content of active ingredients in a drug is determined by factors prior to harvest (the harvest season, the area where the plants were grown, the type of fertilizer, the soil, the climate, etc.) as well as by processes such as ageing or degradation that might take place during drying or storage of the raw material.

Among the different extraction procedures, the most commonly used to obtain vegetable extracts is the solid–liquid extraction, which consists of the preferential separation of one or more components of a solid mixture by dissolving them in a liquid solvent. The two basic extraction procedures used in the cosmetics industry are maceration and percolation.

Maceration: This procedure consists in leaving the raw material—after reducing its particle size as finely as specified—in contact with a solvent for a long period, frequently shaking the system. The extract is separated from the residue by pressing; the residue can be re-extracted again and the two extracts are adjusted to the specified mass content.

Digestion is a maceration procedure carried out at a high temperature, using water as the extraction liquid. It has the disadvantage that precipitation generally occurs as the system cools down.

Percolation or lixiviation: It means the slow passage of a liquid through a filtering medium. Although the raw material is previously macerated, percolation requires continuously renewing the solvent, a procedure that keeps the concentration gradient as high as possible. The solvent drains from top to bottom through a layer of raw material thus extracting the components of interest without the need of pressing. Just the same as for maceration, the quality of the extract thus obtained will depend on how finely ground the

raw material is, on the diffusion rate of active substances from the raw material into the solvent and on the flow rate of the solvent.

Subsequent evaporation of the solvents plus addition of the suitable excipients produce fluid extracts, soft extracts or dry extracts, depending on the final percent of humidity (Figure 8.5.2).

In the next sections, the different plant active ingredients are described according to their chemical origin.

CARBOHYDRATES

Carbohydrates are the most widely distributed compounds in the plant kingdom. They form part of cell structures, or act as receptors, energy storage and transportation of elements or precursors of other metabolites. They are broadly classified as monosaccharides, oligosaccharides and polysaccharides. Compared with other active substances of plant origin, the cosmetic applications of carbohydrates are generally scarce.

Monosaccharides

Monosaccharides, the simplest carbohydrates existing, are polyhydroxy-carbonyls. The carbonyl group may be an aldehyde group (these are aldoses, like glucose, galactose, ribose) or a ketone group (these are ketoses, like fructose, ribulose). Monosaccharides have between three and nine carbon atoms, although the most abundant ones are those with five carbon atoms, called pentoses (D-ribose; D-2-deoxyribose; L-arabinose; D-xylose), and six carbon atoms, called hexoses (glucose, fructose, galactose and mannose).

The cosmetic relevance of monosaccharides is based on their thickening, emollient and filmogenic properties, which are due to their structure, arrangement and high content of hydrophilic groups.

Oligosaccharides

Oligosaccharides result from the condensation of two to ten monosaccharide molecules through glycosidic bonds. Acid hydrolysis easily breaks these bonds; enzymatic hydrolysis breaks them with high specificity. Saccharose (glucose + fructose) is the most abundant disaccharide. At present, most of the warm waxes for hair removal are prepared from saccharose (Tannir and Leshin, 2001). Maltose and cellobiose are degradation products of starch and cellulose polymers, respectively.

Another large group of sugars participate in heteroside combinations.

β-Glucans are oligosaccharides with at least seven glucose molecules. These oligosaccharides activate the production of macrophages and stimulate the non-specific immune system (Bland *et al.*, 2004). β-Glucan is currently being used in anti-ageing cosmetic formulas and treatments for greasy skin because they remove dead cells and stimulate cell regeneration (Olafsdottir *et al.*, 2003).

At present, oligosaccharides are used in a number of moisturizing formulas due to their hydrating and filmogenic actions, in products to normalize the desquamation of the *stratum corneum* and as anti-ageing agents (Schaeffer and Brooks, 1992).

Polysaccharides

Polysaccharides are defined as high molecular weight polymers of monosaccharides. Certain microorganisms may synthesise polysaccharides with cosmetic applications. Among them, it is worth mentioning dextran (produced by *Leuconostoc mesenteroides*) and xanthan gum (produced by *Xanthomonas campestris*). The latter one may be dissolved in hot or cold temperature conditions. The viscosity of the resulting solution does not vary with temperature or pH changes, in other words it displays a pseudoplastic and thixotropic behaviour. Xanthan gum shows a minimum of incompatibilities and may be mixed with other vegetable hydrocolloids, alcohol (up to 50%) and surfactants (15–20%). It is used as a thickening agent for toothpaste and as a stabilizer for suspensions and emulsions (Williams *et al.*, 2003).

On the other hand, different algae have characteristic glucosidic-type walls, whose composition depends on the family they belong to (Briand, 2003).

Alginic acid is a lineal polysaccharide made up of guluronic- and mannuronic-acid units present in Phaeophycea algae (*Laminaria, Fucus*). Sodium alginate is used as a thickening and dissolvent agent (Council of Europe, 1989). Different alginates are also used as emulsion stabilizers. Products of this type may be added to soap, toothpaste, shaving cream and hair gel.

Carrageenates are strongly sulphated galactose polymers typical of Rhodophyceae algae (*Gelidium*, *Gracilaria*). Carrageenates are anionic polyelectrolytes due to their abundant $(SO_4)^{2-}$ groups. They are employed as thickening and emollient agents in creams, suspensions, toothpastes, etc.

Agar–agar or gelose is another complex polysaccharide obtained from the Rhodophyceae algae (*Gelidium, Gracilaria*). It consists mainly of galactose residues. It is used as a jellifying agent and as a coadjuvant to make emulsions.

The polysaccharides obtained from higher plants may be classified as homopolysaccharides (starch and cellulose) made up of a large number of monosaccharides of the same type, and heteropolysaccharides (mucilage, gum and pectin) made up of a large number of monosaccharides of different types.

Starch is made up of two polymers of glucose units, amylose (linear chain) and amylopectin (branched polymer). It is used as the basis to manufacture talcum powder and powder make-up products due to its soothing and emollient properties. It is possible to modify its original structure to obtain products that produce gels with a variety of properties, which widens its practical applications (dextrin, pre-gelatinized starch, sorbitol, introduction of functional groups).

Cellulose is a biopolymer composed almost exclusively of glucose molecules. Some cellulose derivatives, like ethylcellulose or hydroxyethylcellulose, are used in cosmetics as thickening and filmogenic agents.

Mucilages can be classified into neutral mucilages, made of complex polyuronides or acidic mucilages made of galactomannans. Among the neutral mucilages it is worth mentioning guar gum, which is extracted from carob, *Cyamopsis tetragonolobus (L.)* and tamarind. Guar gum is used to produce viscose gels, which are not affected by pH or electrolytes. Tamarind gum in water gives dispersions with high viscosity.

Gums are substances exuded by plant organs, which may be concentrated by drying them up and are not soluble in organic solvents. Gums are complex structures characterized by a

branched polymer and the presence of at least one hexauronic acid. From this group it is worth mentioning two in particular, which are arabic gum, used for its emollient and filmogenic properties, as well as some emulsion and viscosity-increasing properties and tragacanth gum, which is used because of its good emulsion-stabilizer and filmogenic properties.

Finally, pectins are macromolecules with a rather complex structure. The majority of their structure consists of D-galacturonic acid residues but there are molecules of rhamnose and other monosaccharides such as galactose, arabinose and xylose inserted in the structure and the terminal acid groups are esterified with methanol. On the basis of their methylation degree, pectins can be classified into three categories: pectic acids, low- and high-methylated pectins, which may be used as thickening ingredients for cosmetics. These substances are added to a number of body hygiene and moisturizing products because of their hydrating and soothing properties (Thakur *et al.*, 1997).

FATTY ACIDS AND LIPIDS

The fatty acids are terminal carboxylic compounds with open aliphatic C_8–C_{24} chains that may be saturated (i.e. palmitic acid, stearic acid) or unsaturated (oleic acid, linoleic acid). They can be classified according to the length of the chain and the number, position and configuration of the double bonds.

The lipids of vegetable origin are mainly obtained from the seeds, fruits and leaves of different plant species. They are insoluble in water and soluble in organic non-polar solvents. If they are liquids they are named oils, whereas if they are solids they are known as fats. They can be classified into:

–Simple lipids: they are esters of fatty acids (described above) with alcohols. If the involved alcohol is glycerol, the resulting lipid is a mono, di or triglyceride depending on the number of fatty acids esterified with glycerol, and if the alcohol is a high-molecular-weight aliphatic alcohol, the resulting lipid is a wax. Triglycerides are very important in the field of cosmetics depending on the type of fatty acids they contain.
–Complex lipids: phospholipids and glycolipids. For example, soybean phospholipids are used as hair conditioning agents and occlusives.

Some examples of fatty acid-rich products of plant origin used in cosmetics are discussed below.

Olive oil, *Olea europaea L*, has been used as an emollient agent with moisturizing and soothing properties and protective effects (Council of Europe, 2001; Visioli and Galli, 2002). It is, above all, a source of oleic acid, linoleic acid and vitamin E. It is employed in skin and hair products, soaps and lip balms. It has excellent regenerative properties and effectively regulates skin moisture, restructures the skin and stimulates hair growth. Its high oleic acid content makes it resistant to oxidation and an excellent moisturizing agent.

Sweet almonds oil is perhaps one of the most extensively used oils in cosmetic and dermatological products due to its properties as an emollient and a treatment for sensitive skin.

Other vegetable oils like avocado, borage, maize germ, etc., rich in polyunsaturated fatty acids, are extensively used for treatment products because the organism is unable to synthesize them, though it needs them to maintain the barrier function healthy. It has been observed that patients with dermatitis have low tissular levels of all the polyunsaturated derivatives of linoleic acid and that local applications of this acid soothes the skin and reduces the transepidermal water loss.

The oil extracted from rosehip (*Rosa aff. rubiginosa L.*), a plant native to South America, has long been used with good results for anti-ageing treatments and skin-whitening products (Moreno Gimenez *et al.*, 1990; Council of Europe, 2001).

The oils extracted from coconut, onagra, quinua, soya and macadamia, among others are also extensively used in cosmetics (Marchini *et al.*, 1988).

Waxes are natural mixtures of C_{25}–C_{35} alkanes, esters, long-chain alcohols and aldehydes. They are mainly found on the surfaces of leaves and fruits, where they prevent water loss from the plant. We would like to mention jojoba, beeswax, carnauba wax and candelilla wax. The latter two are employed as excipients in the manufacture of lipsticks and wax to remove hair because they give shine and solidity to the final cosmetic formulation. Jojoba wax (*Simmondsia sinensis [Link.] C. Schneider*) is employed in facial anti-ageing treatments as a good non-greasy lubricant agent and as an emollient that conveys the cosmetic formulations smoothness and a pleasant touch (Cummings *et al.*, 2000). Additionally, its special composition makes it highly stable against lipooxidation.

Not to be forgotten in this section, is the unsaponifiable fraction, which corresponds to the non-glyceride constituents of oils. In general, oils contain between 0.2 and 1.5% unsaponifiable compounds. The relevance of unsaponifiables is based on their chemical composition. They are employed in cosmetics as antioxidant and vitamin actives because of their tocopherol and carotene content and as anti-ageing agents—their sterol content. We would like to emphasize the cosmetic properties of the unsaponifiable fractions of soya, olive and avocado oils.

ORGANIC ACIDS

These compounds—with the characteristic presence of carboxylic residues—may be found free or forming part of esters. In this group it is possible to distinguish between monoacids, diacids and triacids.

Some of the most important monoacids are the so-called fatty acids described previously, which form part of the lipids. On the other hand, it is worth mentioning the aromatic acids, such as benzoic and cinnamic acids, which may be found free or in combination, as in the case of balsams. Although less abundant, cyclic acids like hydnocarpic or gorlic acids may also be found. Among the diacids, we find the oxalic, malonic and furanic acids and among the triacids, we find aconitic acid. They are used mainly as pH-adjusters.

This group also includes the α-hydroxyacids (AHAs), which have alcohol moieties besides the acid groups (Okano *et al.*, 2003). We should highlight glycolic, malic, citric and tartaric acids, which have aliphatic structures; and quinic, shikimic and mandelic acids, which have aromatic structures. AHAs are active compounds used extensively in the field of cosmetics because they have several applications for skin and hair products

(Bergfeld *et al.*, 1997; Wang, 1999). It has been demonstrated that these acids act on the *stratum corneum* by weakening the intercorneocyte cohesion and reducing the thickness of the *stratum corneum*. AHAs promote cell proliferation, thus giving the skin a younger appearance; they reduce epidermal calcium concentration and produce decohesion, which results in skin desquamation (Jeong *et al.*, 2005). Such desquamation is increased by the action of the proteolytic enzymes present in the epidermis. More recently, β-hydroxyacids (BHAs) such as salicylic acid, have been incorporated to moisturizing and anti-ageing cosmetics with the same applications as AHAs. These active compounds are also used in hair products to treat dandruff and seborrhoea.

NITROGEN COMPOUNDS

There are a number of nitrogen-containing compounds in plants, mainly synthesized from amino acids.

Amino acids and protein hydrolysates

For a long time, the proteins used in cosmetics have only been of animal origin (mainly collagen, elastin, keratin) because they are easy to obtain and rather similar to human proteins (Gámez-Garcia , 1993). The isolation and purification of vegetable proteins posed several technical problems—more difficult than those related to animal-protein isolation—because in nature, they are usually associated to considerable amounts of carbohydrates that must be discarded. The use of vegetable proteins in cosmetics begun relatively few years ago, once the technical problems had been resolved their use has been boosted by the growing ecological awareness of society, which increasingly demands vegetable rather than animal proteins (remember bovine spongiform encephalopathy (BSE)).

Vegetable as well as animal proteins have certain characteristics, which are important for their interaction with the skin, hair and other components in the formulations and define their applications in the field of cosmetics.

Proteins are long chains made up of amino acids. Every protein has a characteristic sequence of amino acids that influences its possible interactions with the skin and hair through covalent or hydrogen bonds (Jones and Chahal, 1997). Peptides and hydrolysates are short chains of amino acids obtained after hydrolysis of proteins. The hydrolysis may be enzymatic in acid medium or in alkaline medium. Each type of hydrolysis yields characteristic types of peptides. The molecular weights of these hydrolysates may vary from 150 daltons (average molecular weight of an amino acid) to several millions daltons. It is necessary to refer to average molecular weight because the hydrolysis process never yields homogeneous peptides but mixtures of peptides of different molecular weights (Teglia *et al.*, 1993).

The molecular weight influences the degree of skin and hair adsorption, the penetration power and the interactions with other components. In general, the higher the molecular weight, the larger the filmogenic effect and the weaker the penetration power (Swift *et al.*, 2000).

The skin and hair surfaces are mostly negatively charged and weak interactions (ionic bonds, hydrogen bonds, Van der Waals forces) or covalent bonds (like disulphide bonds) may be established. Strong reductive treatments (perm, colouring) break the bonds existing in the hair-shafts, damaging them. After such treatments, the application of proteins with –SH groups during the final steps of these treatments improves the general state of hair.

The polar nature of proteins provides them with a number of points to attach water molecules through hydrogen bonds. The molecular weight has almost no influence on superficial moisturizing. If penetration and moisturizing of deeper *stratum corneum* layers is the goal, short-chain peptides will produce the best results.

It has been demonstrated that high-molecular-weight hydrolysates are best in reducing the irritant action of surfactants. Highly hydrophobic chains also produce good results.

These cosmetic properties lead to good absorption at skin and hair levels. The molecules can penetrate through the hair cortex and reach the cuticle. The molecular weight determines more superficial or deeper moisturizing actions. Vegetable proteins are added to hair products because they convey volume and shine, improve combing and elasticity, retain moisture and provide reparation properties. Used in body and facial cosmetics, they improve skin elasticity due to their moisturizing, nutritive and filmogenic properties.

Alkaloids

Alkaloids are organic alkaline nitrogenous substances with complex, highly diversified structures. They are classified on the basis of their chemical structure. This is a highly controversial group of substances. Although these compounds are not forbidden as such, some plants containing them, e.g. *Atropa belladona L, Claviceps purpurea Tul, Hyosciamus níger L* and preparations made out of them, are included in the list of substances forbidden for the composition of cosmetic products, according to the Annex II of the European Directive of Cosmetics 76/768/CEE.

Alkaloids are classified into two main groups: heterocyclic and non-heterocyclic. In this work, authors will only deal with the heterocyclic alkaloids that have a quinoline nucleus (tryptophan derivatives), which include the alkaloids of quina, and the group of diverse alkaloids, specifically the xanthine bases or purine alkaloids.

Quina, *Cinchona sp.*, is a tree native to South America, where people use the bark from trunks and branches because they contain quinoline alkaloids (quinine) and bitter principles. They are used in cosmetics because of their tonic, astringent, anti-dandruff and hair growth-stimulating properties (Council of Europe, 1989). The use of quinine and quinine salts is restricted to shampoos and hair lotions (Annexe III, Part 1).

Xanthine bases or purine alkaloids are secondary metabolites, derivatives of xanthine; caffeine, theophylline and theobromine are three very well-known examples. These compounds occur naturally in the seeds of coffee, cola, guarana and cacao, as well as in the leaves of tea and mate (Council of Europe, 1989; Zheng *et al.*, 2004). These compounds improve and stimulate blood circulation, an action that justifies the use of these vegetable compounds in anti-cellulite cosmetic formulations (Bertin *et al.*, 2001). Such an effect is the consequence of their adenosine-antagonist action, which influences the β-adrenergic system by stimulating the vasodilator response. Additionally, xanthine bases specifically

inhibit the phosphodiesterase enzyme, which mediates cyclic adenosine monophosphate (cAMP) inactivation into the adipocytes. The result is an increased intracellular level of this nucleotide, which acts as a potent stimulator of lipolysis (Tofovic *et al.*, 1991).

PHENOLIC COMPOUNDS

This is a very wide group of substances characterized by the presence of at least one benzene nucleus, which contains at least one hydroxyl group free or as a part of a chemical group.

Phenols and phenolic acids

Simple phenols are scarce and, in general, they are found as heterosides in plants. Phenolic acids are grouped into: benzoic acid derivatives and cinnamic acid derivatives. The most relevant extracted phenols used in cosmetics are: arbutin, vanillin and salicylic alcohol or saligenin.

Bearberry *Arctostaphylos uva-ursi (L.) Spreng* is a small shrub, whose leaves contain arbutin, a hydroquinone heteroside, that yields hydroquinone after degradation by a *β*-glucosidase enzyme. Hydroquinone acts by inhibiting the tyrosinase enzyme, which initiates the biochemical synthesis of melanin. At present, the use of this hydroquinone glucoside is becoming less and less frequent because of the restrictions on the use of synthetic hydroquinones for cosmetic applications and because of its well-known irritant effects on the skin (Maeda and Fukuda, 1991, 1996).

Willow is a perennial tree belonging to the Salicaceae family. Extracts of the leaves and bark of different species of *Salix sp.* are used in cosmetics (Council of Europe, 2001). Their main components are salicylic derivatives (salicin and the saligenin glucoside or salicoside), phenolic acids (salicylic, vanillic, syringic, caffeic, *p*-hydroxybenzoic, coumaric and ferulic) and flavonoids. Willow extracts and derivatives are extensively used in cosmetics and dermatology due to their moisturizing, keratolytic and purifying properties. Additionally, these products can be used as astringent, analgesic, anti-inflammatory and anti-microbial agents (Meier, 2002).

Rosemary *Rosmarinus officinalis L.* is a woody, aromatic shrub that belongs to the Labiatae family. The flowery caps contain essential oils, flavonoids and phenolic acids, especially caffeic derivatives: caffeic, chlorogenic and rosmarinic acids. Rosmarinic acid is an ester of caffeic acid and 2-hydroxy-dihydrocaffeic alcohol with antioxidant and anti-inflammatory properties (Council of Europe, 1989). The glycolic extract is recommended in concentrations higher than 5% for hair lotions with stimulating and purifying properties, for shower and bath gels, shampoos, toothpaste and products for greasy skin and skin with decreased muscle tone (Al-Sereiti *et al.*, 1999).

Phenylpropanoids

Extensions of phenylpropane yield styrylpyrones, xanthones, stilbenoids and flavonoids. This group also includes diarylheptanoids and aryl-alkanones, which are obtained from

one or two molecules of phenylpropanoic acid. In this group, there are two plants used extensively in cosmetics, *Curcuma* and ginger.

The genus *Curcuma* includes some 30 species, native to India and the southeast of Asia. An extract is produced from the rhizomes, whose main components are curcumin and its derivatives. This extract has well-known anti-inflammatory, antioxidant, wound healing, anti-microbial and tyrosinase inhibitory properties. Its versatility makes it suitable for a number of cosmetic formulations on the basis of its anti-ageing, photo-protective, sensitive skin treatment, skin whitening, antiseptic and insect-repellent properties (Srimal, 1997).

Zingiber officinale Roscoe is one of the best-known species since ancient times, extensively used in China and India. The pungency of ginger and part of its numerous beneficial actions are due to gingerols. The activity of ginger is mainly based on its action on the arachidonic acid pathway, which results in a number of applications: anti-inflammatory, analgesic, anti-pyretic and anti-platelet (Rosella *et al.*, 1996). It also acts as an antioxidant and anti-bacterial agent and has an effect on the immune system (Mustafa *et al.*, 1993)

Flavonoids

Flavonoids are widespread in the plant kingdom, making up part of different plant organs like fruits, leaves, flowers and bark. Most of them are responsible for the colour of these plant structures (Zaragoza *et al.*, 2002). They have a number of beneficial properties, the most remarkable ones being antioxidant, anti-microbial, anti-inflammatory, vitamin P and photo-protection. All of them are polyphenolic compounds with a common chemical structure, a benzopyrane skeleton. Various subgroups of flavonoids are classified according to their substitution pattern. Examples of the six major subgroups are chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavonoids. Among the more than 5000 identified flavonoids, we should point out citroflavonoids (Manthey *et al.*, 2001), coming from the genus Citrus (which includes a number of fruits) and among them, the active ingredients quercetin, hesperidin and rutin (Council of Europe, 2001). We should also mention the flavonoids from soya, or isoflavones, genistein and daidzein being the best-known ones (Toda and Shirataki, 1999).

The main sources of these compounds are fruits like citrus, apples, peaches and plant extracts like milk thistle, sophora, chamomile, roman chamomile, yarrow, ginkgo, Saint John's wort licorice, etc. (Council of Europe, 1989). This group of active compounds is extensively used in cosmetics because of their many possible beneficial applications. Below is a list of their main biological properties.

Antioxidant action

The antioxidant properties of flavonoids have been recognized since the middle of the last century. Quercetin is probably the active compound that gathers all the properties necessary for a powerful antioxidant function, and it is used in moisturizing and anti-aging products.

Anti-free radical action

Flavonoids scavenge the free radicals produced at the cell membranes, including the superoxide anion and the hydroxyl radical and, at the same time, they inhibit some enzymatic systems (xanthine oxidase, catalase, superoxide dismutase) and maintain the intracellular concentrations of glutathione. Milk thistle, *Silybum marianum Gaertn,* contains compounds called flavanolignans (silymarin) that inhibit the production of the superoxide radicals (Martelli *et al.*, 2000; Council of Europe, 2001). According to Morazzoni andBombardelli (1995), silymarin protects the membranes by preventing them from interacting with toxic agents. It is used as anti-aging, skin protectant, purifying and anti-dandruff.

Enzyme inhibition

It has been demonstrated *in vitro* that flavonoids inhibit a number of enzymes (elastase, hyaluronidase, cAMP phosphodiesterase, 5-lipooxygenase, cyclooxygenase, etc.). Quercetin, rutin, myricetin, kaempferol and morin inhibit the 5-lipooxygenase enzyme. Quercetin also inhibits the 12-lipooxygenase and the cyclooxygenase enzymes. Flavones inhibit the phospholipase A2 enzyme and can be used as anti-inflammatory and anti-irritant agent.

Capillary resistance

Flavonoids are able to reduce capillary permeability and increase capillary resistance. *Sophora japonica Linneo.* is used in cosmetics because of the protective effects of rutin on the blood-vessel walls. This active compound protects blood vessels against fragility and improves microcirculation and micronutrients transportation. The result is an improved skin texture and appearance. Several studies have demonstrated that rutin efficiently reduces oedema. This anti-oedema action is one of the main reasons for adding it to formulations aimed at the treatment of tired legs or varices and as a coadjuvant in anti-cellulite treatments (Tang *et al.*, 2002).

Anti-inflammatory activity

Many flavonoids modify the phagocytic processes of macrophages, the release of oxidant substances from neutrophils and the activation of mast cells. Their interaction with the arachidonic acid metabolism and inhibition of pro-inflammation mediators such as prostaglandins might be responsible for their anti-inflammatory activity. Chamomile exerts a powerful anti-inflammatory action due to its flavonoid content (Ramos *et al.*, 1996). This action is mainly due to apigenin and lutein (Avallone *et al.*, 2000). Traditionally, chamomile has been used in cosmetic products as soothing, anti-irritant and for sensitive skins.

Anti-allergy activity

This property is due to the actions of flavonoids on mast cells and basophils. It has been demonstrated that quercetin, myricetin and kaempferol inhibit the release of histamine and

other mediators. Luteolin has been found to be an anti-allergy active compound that inhibits the histamine release, the interleukin (IL-6) production, the tumor necrosis factor (TNFα) mediated immunoglobulin (IgE) production and the cytokine release from mast cells. These actives are used for sensitive skins, and baby products.

Anti-microbial activity

It has been demonstrated that some flavonoids—with flavonol structure—inhibit the growth of *Proteus vulgaris* and *Staphylococcus aureus* (Mitscher *et al.*, 1980).

In summary, it can be said that flavonoid-rich vegetable extracts are very useful for anti-ageing and anti-cellulite treatments and for sun-protection and hair-protection products.

Anthocyanins

Anthocyans or anthocyanidins (aglycon) are natural pigments of plants that occur mainly as heterosides (antocyanosides) and convey the fruits and flowers red, violet and blue colouring. Anthocyanidins are generally found in the anionic form, with a structure derivative of the flavylium cation although other forms are possible, depending on the pH. The red colouring cationic form is stable in strongly acidic medium, but in weakly acidic medium—between pH 4 and 6—the cation successively loses two protons and turns blue. At higher pH values, it becomes ionized and the anthocyan structure is destroyed.

Just as flavonoids, anthocyanosides reduce the capillaries' permeability and increase their resistance. The activity of these heterosides seems to be related to the function of collagen in cell-wall permeability.

The main problems for the use of anthocyanoside-rich vegetable extracts is the final colouring of the cosmetic and the fact that they are highly unstable in water medium due to their pH-dependant colour variations, their sensitivity to heat, light and other compounds of the product.

Examples of vegetable extracts used in cosmetics are grape and hibiscus (Awang, 1994). Anthocyanidins are abundant in *Vitis vinifera L.* The antioxidant activity of wine is due to the anthocyanidins (Bombardelli and Morazzoni, 1995; Morales *et al.*, 2002), which play a protective anti-oxidation role (Council of Europe, 2001). In the case of hibiscus, an anthocyan-rich fraction of it has been observed to exert *in vitro* inhibition of enzymes like elastase and, to a lesser degree, trypsin and chemotrypsin (Jonadet *et al.*, 1990). Cosmetic properties of hibiscus are refirming and anti-aging.

Tannins

This is a large group of active compounds with polyphenolic structure and the common characteristic that they can precipitate some macromolecules such as proteins, alkaloids and jelly. Tannins are classified into two groups: hydrolyzable tannins and condensed tannins.

Hydrolyzable tannins are mainly of two types: gallotannins, in which a glucose unit is surrounded by several galloyl ester groups; and ellagitannins, in which units of hexahydroxydiphenic acid (derived by linkage of two gallic acid groups) are present.

Hydrolyzable tannins are esters of a variable number of phenolic acids, gallic acid or its dimers, bond to a sugar molecule, generally glucose.

Condensed tannins or flavolans are formed by the condensation of catechin units to form dimers and then oligomers.

Condensed tannins are flavan dimers or polymers with C–C bonds between different flavan-3-ol units. An example of this is the green tea extract, a vegetable extract frequently used in cosmetics, whose main active compounds are condensed tannins that reach between 17 and 30% of the dry weight of leaves (Katiyar *et al.*, 1999, 2000). The most important ones are flavan-3-ols (catechins) and their condensed forms like (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechingallate and epigallocatechingallate. This extract is used because of the specific actions exerted by active compounds, which include anti-free radical, anti-metalloproteinase, anti-inflammatory and photo-protective actions (Kim *et al.*, 1999; Ahmad and Mukhtar, 2001).

This group of active compounds, especially the epigallocatechingallate, are responsible for the antioxidant and anti-inflammatory effects of green tea (Council of Europe, 2001). The local application of tea extract (before exposition to sunlight) results in reduced lipid peroxidation, reduced inflammation-producing leukocyte infiltration and reduced erythema (Zheng *et al.*, 2004). The condensed tannins of green tea inhibit the lipooxygenase enzyme (which is responsible for the arachidonic acid metabolism), the peroxidation of lipids involved in inflammation and the antiradical action against hydroxyl radicals (Elmets *et al.*, 2001). Another important cosmetic action of green tea is to inhibit the activity of the collagenase enzyme. This action is mainly due to the presence of compounds that contain the gallic radical (epicatechingallate and epigallocatechingallate). This property makes green tea extract useful for anti-ageing products, since it fights the deleterious effects of skin metalloproteinases. It has been demonstrated (Elmets *et al.*, 2001) that green tea has photo-protective effects against the UV radiation.

Quinones

Quinones are aromatic compounds with two ketone groups in the 1,4 position or, less frequently, in the 1,2 position. All chemical compounds with this basic structure may be classified as: benzoquinones, naphthoquinones, anthraquinones, phenanthraquinones and anthracyclones. The structure of benzoquinones derives from benzene that of naphthoquinones derives from naphthalene, anthraquinones derives from anthracene, phenanthraquinones derive from phenanthrene and anthracyclones from naphthacene. Anthraquinones and anthracene derivatives—especially those obtained from the dry juice of aloe leaves—are the most interesting group from the cosmetic point of view (Council of Europe, 1989; Vila and Guinea, 2001). Aloe gel is a mucilaginous liquid obtained from Aloe barbadensis leaves, whose main components are polysaccharides and hydroxyanthracene derivatives (Lee *et al.*, 2000). These compounds are made up of free anthraquinones (less than 1%), aloin (20–30%), the 10-C-glucoside of aloe-emodol anthrone, a mixture of R and S isomers (A and B aloines) interchangeable via anthranol.

Since ancient times, the healing properties of aloe have been well known. Low doses act as stomachic and colagoge, medium doses have laxative properties and high doses act as a purgative due to the action of aloin. Traditional uses of aloe also involve external applications to treat wounds and burns because it was observed to speed up healing. It also helps healing skin lesions produced by excessive UV radiations. Aloe has emollient, hydrating, moisturizing, epitelizing and anti-inflammatory properties. All of these make it suitable for sun-protection and after-sun products, because it protects and hydrates the skin, thus preventing problems related to prolonged sun exposure. It is extensively used in treatments for dry, irritated skin and for skin redness. It is suitable for child-care products.

TERPENOIDS

Terpenoids include a huge number of vegetable substances. All of them have a common biosynthesic origin: the isoprene molecule (Colombo *et al.*, 1998).

Mono, sesqui, aromatic compounds and diterpenes

Monoterpenes (C_{10}) are the simplest members of the terpene series. They result from the condensation of two isoprene units and may be acyclic, monocyclic, bicyclic or tricyclic. The monoterpenes can have another functional moiety like alcohol (geraniol, linalool, menthol, borneol), aldehyde (geranial, citronellal), ketone (menthone, carvone, thujone), ester (bornyl acetate, linalyl acetate), ether (1,8-cineol) and phenol (thymol, carvacol). In the case of optically active molecules, the proportions of the enantiomers vary largely from one species to another.

The sesquiterpene (C_{15}) molecules include structures like hydrocarbons (β-bisabolene, β-caryophylene), alcohols (farnesol, carotol, patchoulol), ketones (nootkatone) and aldehydes (anisaldehyde, valerenal).

Diterpenes are a large group of C_{20} compounds. Their main structural types are acyclic compounds, cyclic compounds, bicyclic diterpenes and tri- and tetra-cyclic diterpenes. The cosmetic use of this group of compounds is restricted to the antioxidant properties of the phenolic diterpenes extracted from rosemary and sage.

Tea tree oil (TTO) is an essential oil obtained by steam distillation of the leaves and terminal branches of *Melaleuca alternifolia Chell* (Nenoff *et al.*, 1996). The chemical composition of tea tree oil mainly consists of mono and sesquiterpenes and alcohols (Council of Europe, 2001); 4-terpineol, the most abundant component, is responsible for the antiseptic property of this essential oil, although the remaining components synergically contribute to this action (Carson and Riley, 1995a, 1995b). Its wide-range anti-bacterial and anti-fungal properties make it suitable for body hygiene formulations, as well as for hygiene products of local application (Lis-Balchim *et al.*, 2000). COLIPA recommends one not to use concentrations higher than 1% of *Melaleuca alternifolia* oil in cosmetics (COLIPA Recommendation no 12, December 2002).

Rosemary leaves (*Rosmarinus officinalis*) contain a concentration of essential oil between 1.0–2.5%. This composition may vary noticeably depending on the chemotype

and the stage of plant development at the moment of harvest. The main components are α-pinene, 1,8-cineol, camphor, camphene, borneol, bornyl acetate and α-terpineol. It also contains tri-cyclic diterpenes, mainly carnosic acid and carnosol. The antioxidant action of rosemary is due to its diterpenes carnosol and carnosic acid, as well as to rosmanol, epirosmanol and isorosmanol (Hui-Hui *et al.*, 2001). Rosemary glycolic extract concentrations up to 5% are recommended for hair lotions with stimulant and purifying actions, shower and bath gels, shampoos, toothpaste and products for greasy skin and skin with decreased muscle tone. Essential oil concentrations up to 3% are recommended for hair lotions, toothpaste and products for greasy skin and skin with decreased muscle tone, up to 5% for stimulating shampoos, and up to 30% for stimulating and purifying shower and bath gels (up to 10 g essential oil/bottle (Council of Europe, 1989)).

The group of products called distilled waters and colourless fluid extracts are produced by steam distillation of aromatic plants in order to obtain only the fraction extractable by this method and discard the rest. Distilled waters are usually 10 times more diluted than the plant of origin. The colourless fluid extracts are usually equivalent in weight to the plant of origin (1:1). They contain 25–40% (V/V) ethyl alcohol in order to maintain the highest possible percentage of essence in solution. Products belonging to this group available in the cosmetics market are rose water, witch hazel water (Korting *et al.*, 1993; Ramos *et al.*, 1996) and orange blossom water, which are the most widely used due to their astringent, tonic, anti-irritant and anti-couperosis actions (Council of Europe, 2001). Up to 50% witch hazel water is recommended for face tonic lotions, after-shave lotions, products for sensitive skin and products for mucosa and inflamed skin (Council of Europe, 1989).

Triterpenes and steroids

Triterpenes are C_{30} compounds with more than 40 different basic structures, almost always hydroxylated in position 3. Dammarane is the precursor of the triterpenes while 4,4-dimethyl-sterol is the precursor of the steroids. In the case of steroids, the C_{30} skeleton becomes a C_{27} or smaller one. They are used as anti-inflammatory and for peripheral blood circulation treatment.

Saponins

Saponins are heterosides with a non-glucidic portion (aglycone) called sapogenin. Saponins may be monodesmosidic or bidesmosidic. In the first group, the sugar fraction is attached to sapogenin by only one position (position 3). In bidesmosidic saponins, the sugar molecule or molecules have two points of attachment to the aglycone. The second group is classified, according to the chemical nature of the sapogenin, into triterpene saponins and steroidal saponins.

Saponins have the ability to produce foam, when a water solution containing this type of actives is stirred. This action is due to their ability to reduce surface tension. Therefore, they are known as natural surfactants. Saponins also exert a haemolytic action, a characteristic of the triterpene saponins. A number of plants used in cosmetics can be cited in

reference to this group. Examples of the most extensively used ones are: licorice, horse chestnut, ginseng and ruscus.

Licorice *Glycyrrhiza glabra L.,* belongs to the Leguminosae family. Its roots contain triterpene saponosides. The main saponoside is glycyrrhizin or glycyrrhizic acid, composed of glycyrrhetic acid and two molecules of glucuronic acid that are released by acid hydrolysis (Statti *et al.*, 2004). Glycyrrhetic acid has proven anti-inflammatory and regenerating properties (Council of Europe, 1989; Vaya *et al.*, 1997). It is therefore employed in products aimed at sensitive and irritated skin and in re-epitelizing, anti-ageing cosmetics (Saeedi *et al.*, 2003).

Horse-chestnut is used because of its high content of triterpene saponins, which give it anti-inflammatory and anti-oedema properties (Ernst and Pittler, 2002). These properties also make it suitable for cosmetic formulations such as tonic, astringent, anti-couperosis and anti-cellulite products (Council of Europe, 1989; Wilkinson and Brown, 1999). Triterpene saponins, which are found in the seeds, are composed mainly of β-aescin, a heteroside of protoaescigenin and a penta-cyclic triterpene of the oleanic group. Likewise, horse-chestnut extracts are also used in popular medicine. Aescin is a common ingredient in preparations used to treat peripheral blood-circulation conditions, especially when capillary permeability and resistance are affected (Roddick-Lanzilotta *et al.*, 2004).

For millennia, ginseng has been one of the most highly valued medicines in Chinese popular medicine, attributing properties to it for the treatment of every kind of condition (Matsuda *et al.*, 2003). However, at present, it is mainly considered for its preventive medical properties, given its beneficial effects on blood circulation, endocrine and nervous systems and general metabolism. It contains a large variety of active principles that act synergically (Attele *et al.*, 1999). Its main components are ginsenosides, ginseng-specific triterpene saponosides, which include more than 20 known molecular structures derived from three fundamental nuclei. It is important to point out that ginseng is a plant containing adaptogenic ingredients, namely, substances that protect the organism against environmental aggressions. Consequently, ginseng makes the organism more resistant to stress. It maintains body temperature and increases resistance against physical and psychological strain (Naval *et al.*, 2002). Moreover, due to its properties, it is useful for nutritive creams, products to treat wrinkled, aged skin and every kind of anti-ageing cosmetic (Council of Europe, 1989). When added to body milk and bath products, it optimises skin metabolism in the whole body. In hair-cosmetics, it is suitable for shampoos aimed at strengthening weak hair and for anti-hair loss lotions, because it stimulates hair growth.

Finally, we should mention ruscus. Its roots contain steroidal saponins (ruscogenin). Ruscogenin is a potent vein-constrictor that exerts anti-oedema actions (Thorne Research Inc., 2001). It has been found that the action of ruscogenin is due to the direct stimulation of α-adrenergic receptors as a partial agonist to α-1 and α-2 receptors on the cells of venous smooth muscle (Vanscheidt *et al.*, 2002). This direct mechanism is reinforced by the ruscogenin-mediated release of noradrenaline stored in the adrenergic terminals of the blood-vessel wall (Bertin *et al.*, 2001). Such action brings about an improvement in the blood and lymph-vessel-wall tone and improves venous return, which leads to remarkable anti-oedema effects (Council of Europe, 1989). Such effects are relevant to the formulation of cosmetic products aimed at tired legs, varicose veins and anti-cellulite treatments, as well as for anti-couperosis, eye-contour and sensitive or irritated-skin products (Carini *et al.*, 1994).

Carotenoids

Last of all we shall briefly mention carotenoids. This group of substances is only synthesized by plants. They have a basic C_{40} structure and intense yellow, orange or red colour. Carotenoids are classified into two groups: carotenes and xanthophylls. Carotenes are a group of pure polyenic hydrocarbons; xanthophylls contain oxygen functional groups (Rosen, 2003). They are sometimes used because they give the cosmetic a natural colour, e.g. the yellow colouring oil-extract of *Bixa orellana;* or the red colouring oleoresin of paprika (Council of Europe, 1989). However, their main role is as vitamins—vitamin A precursors—and as anti-free radical agents (Stahl *et al.*, 2000; Andreassi *et al.*, 2004).

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8.6. Vitamins

C. Casas*

DSM Nutritional Products, Camí de Valls, 81-87, 43206-Reus, Tarragona, Spain

Vitamins are organic compounds that are essential in small amounts to the life and well being of humans and animals. As a rule, vitamins cannot generally be synthesized in the body, but must be taken in with the diet. They play a key role in transforming energy and regulating the metabolism of the body. There are 13 major vitamins which each have a range of functions in the body and their modes of action are also very diverse.

Vitamins differ from each other in chemical structure and function. They are classified as fat or water-soluble vitamins.

Vitamins A, D, E and K are fat soluble. This means that a certain amount of fat is needed in the diet to help the body absorb these vitamins. Unused supplies can be stored in the body.

The B group vitamins (consisting of vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B8, vitamin B9 and vitamin B12) and vitamin C are water soluble. They cannot be stored in the body so we need a daily supply of them from our diet.

In food products, vitamins are mainly added to replace losses in processing, or to fortify the product, but are also used as antioxidants or colourants. In the pharmaceutical industry, vitamins are used in supplement preparations such as tablets or capsules. Vitamins are also used in the cosmetics industry in skin care, hair care and oral hygiene products. Vitamins have been added to skin care products to boost the skin's antioxidant or anti-inflammatory response. They also function as immune system strengtheners, clarifiers or wrinkle reducers.

All these vitamins can be used as active ingredients in cosmetics according to the European Union legislation, except vitamin D3 (cholecalciferol). In USA and Japan, there are no restrictions for their cosmetic use.

Some of the vitamins that are used as active ingredients in cosmetics are shown in Figure 8.6.1.

VITAMIN A

A fat-soluble vitamin occurs in two principal forms in nature: retinol and certain carotenoids. Retinol is found only in animal sources, in foods such as fish, meat, eggs and full-fat milk. In plant foods, vitamin A can be obtained from a family of substances called carotenoids that are found in brightly coloured fruit and vegetables, and leafy green vegetables. The best-known form of carotenoid is β-carotene (pro-vitamin A). β-Carotene can be converted to

*Corresponding author. E-mail address: Carmina.Casas@dsm.com

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

Figure 8.6.1 Some of the vitamins that are used as active ingredients in cosmetics.

retinol in the body when it is needed. Although it has been known since the time of ancient Egypt that certain foods would cure night blindness, vitamin A *per se* was not identified until 1913. Professor Paul Karrer defined its chemical structure in 1931. He received a Nobel Prize for his work because this was the first time that a vitamin structure was determined.

Measurement units

The International Unit (IU) is a unit of measurement for a substance, based on measured biological activity or effect, used for vitamins and other substances.

In the case of vitamin A: 1 IU vitamin A is the biological equivalent of 0.3 μg retinol or of 0.6 μg β-carotene.

Functions

Vitamin A is essential for vision, adequate growth and tissue differentiation.

Deficiency symptoms include night blindness, hyperkeratinosis of the skin and xerophthalmia, an eye condition which if untreated can lead to permanent blindness. Vitamin A

deficiency is still widespread in many developing countries. A shortage of vitamin A also affects antibody production and resistance to disease.

Cosmetic application

Vitamin A (palmitate or acetate) is also called the "normalizing vitamin", and is widely used in the cosmetic industry. Over the last 10 years, the vitamin A group received additional publicity because of the clinical studies conducted with an analogue, retinoic acid, which has shown the ability to reverse photo ageing.

Vitamin A (palmitate) is the active agent for the treatment of skin aging in beauty care. It promotes enzyme activity in the skin, improves natural skin functions, thickens the epidermis, can regenerate skin prematurely aged by UV-radiation and efficiently reduce wrinkles and improve skin elasticity (Jarrett and Jackson, 1980).

Vitamin A is soluble in oils and fats. The vitamin is not easily destroyed by heat but is readily oxidized. Preparations must, therefore, be protected from oxidation and are prepared in an atmosphere of carbon dioxide or nitrogen. In the absence of air, vitamin A is unaltered at moderate temperatures. The creams and lotions must therefore be free of oxidases, and perfumes must be free of peroxides. The addition of antioxidants is useful to maintain stability. Pure vitamin E is effective as an antioxidant in lipid solutions of vitamin A.

VITAMIN D

The generic name for a group of steroid-like substances with anti-rachitic activity, promotes bone calcification. Vitamin D is found only in animals and there are only a few foods that contain vitamin D: oily fish, fish oils, butter and eggs. Unlike other vitamins, we can actually synthesize vitamin D in our bodies as a result of exposure to sunlight, provided that vitamin C intake (ascorbic acid) is adequate.

The two most prominent members of this vitamin group are ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3).

Measurement units

The mass equivalents of 1 IU for vitamin D is the biological equivalent of 0.025 μg cholecalciferol/ergocalciferol (1/40 μg exactly).

Functions

Vitamin D is required for calcium and phosphorus absorption from the small intestine, their re-absorption in the kidneys and the mineralization process of bones. It thus supports

healthy bone growth. It also plays an important role in the proper functioning of muscles, nerves, blood clotting, cell growth and energy utilization.

Vitamin D deficiency causes delayed closure of the fontanelles in infants and impairs tooth development. In adults a shortage of vitamin D can lead to softening of the bones and spontaneous fractures. A lack of vitamin D may also prevent adequate adsorption of calcium and contribute to osteoporosis. Vitamin D also plays a role in immunoregulation and healing skin disorders. In animal production, vitamin D deficiency can cause growth depression, leg disorders and thin egg shells.

Cosmetic application

Vitamin D has several beneficial effects on the skin: prevents photo damage, wrinkles and other morphologic alterations. Its topical application has been recommended for psoriasis (Kira *et al.*, 2003a, 2003b). However, it has an associate effect on calcium metabolism and this limits its clinical and cosmetic use (Mitani *et al.*, 2004). Vitamin D (cholecalciferol) is not approved for use in the cosmetic industry in Europe.

VITAMIN E

The term vitamin E covers eight different compounds found in nature. Four of them are called tocopherols and four tocotrienols, and they are identified by the prefixes—α, *β*, *γ* and *δ*.

α-Tocopherol is the most common and biologically the most active of these naturally occurring forms of vitamin.

The most important sources of vitamin E in nature are vegetable oils (peanut, soya, palm, corn, safflower, sunflower, etc.) and wheat germ. Secondary sources are nuts, seeds, whole grains and leafy green vegetables.

Measurement units

The mass equivalents of 1 IU for vitamin E is the biological equivalent of about 0.667 mg of D-α-tocopherol (2/3 mg exactly), or of 1 mg of DL-α-tocopherol acetate.

Functions

The primary role of vitamin E is to protect the body tissues from damaging reactions (peroxidation) that arise from many normal metabolic processes and exogenous toxic agents. Specifically, vitamin E protects biological membranes such as those found in the nerves, muscles and cardiovascular system, helps to prolong the life of erythrocytes (red blood cells) and helps the body to make optimal use of vitamin A.

Vitamin E has been used successfully in the therapy of progressive neuromuscular disease in children with liver or bile dysfunction and a number of diseases, which afflict prematurely born infants, such as haemolytic anaemia, intraventricular haemorrhage and retrolental fibroplasias, which can produce blindness.

There is evidence indicating that vitamin E may play an important role in intermittent claudication, thrombotic diseases, immune function, cancer prevention, cardiovascular disease and prevention and protection of lipoproteins from oxidation.

Dietary deficiency of vitamin E is rare. Deficiency symptoms are seen in patients with fat mal-absorption and in newborn infants, particularly premature infants. Results are a rare type of progressive neuromuscular disease; symptoms include a loss of coordination and balance and in severe cases loss of the ability to walk.

Cosmetic applications

Vitamin E is also called the "protecting vitamin" and is used in the cosmetic industry as antioxidant for either the skin or the formulation. It also softens the skin and alleviates dry skin conditions. Esterified forms such as vitamin E acetate are used because of their superior stability. Unesterified α-tocopherol has antioxidant properties *in vitro* and is a physiological antioxidant *in vivo*. The effective antioxidant form DL-α-tocopherol is released, by enzymatic hydrolysis of the acetate form. This was shown in the study by Norkus *et al.* (1993). The study shows the bioconversion of vitamin E acetate to tocopherol in the skin over a period of 10 days. This conversion can be increased by about 70% by the influence of UVB irradiation.

The effects of the topically applied vitamin E acetate are very well documented in published studies: increased moisturization of the horny layer; improvement in skin surface relief; evident anti-inflammatory properties; increased epithelization of surface wounds; improved enzyme activity in the skin; prevention of skin damage induced by free radicals; protection of properties against sunburn. Vitamin E acetate can increase the natural protection of the skin against UV radiation; reduce the amount of the UV damaged cells and protect against damage by reactive oxygen radicals.

A number of studies in the past few years have shown that vitamin E acetate is responsible for the previously mentioned complex protective functions within the skin. First of all, its suggested anti-inflammatory action was published by Kamimura (1972), whose results showed that vitamin E reduced erythema and swelling. Miyamoto *et al.* (1986) determined the improvement of the epithelization and an increase in enzyme activity in the skin.

Two published studies (Pugliese *et al.*, 1985, 1986) showed the protective effect of vitamin E acetate on lipid peroxidation, which occurs after UV irradiation. Vitamin E acetate also reduces premature skin ageing caused by UV irradiation and it has been proved that a sufficiently high dose of vitamin E acetate has a positive effect in reducing erythema production after sunburn.

Studies carried out in 1990, commissioned by Hoffmann-La Roche USA, showed that vitamin E acetate can improve natural skin protection against UV radiation. The SPF of a suncare product was determined *in vivo* using several volunteers whose skins were

pre-treated for 10 days with a 2.5% vitamin E gel; the obtained mean SPF was 2–4 times higher than the means recorded for volunteers without skin pre-treatment.

Junginger (1989) showed the protective effect of α-tocopherol and α-tocopheryl acetate during UV irradiation. Results demonstrated higher skin protection was achieved with vitamin E acetate in comparison with α-tocopherol.

All these effects together with the antioxidative properties make vitamin E a valuable additional ingredient for all kinds of cosmetic products.

Processing

Vitamin E acetate is easily incorporated into cosmetics and has good stability. Vitamin E is soluble in alcohol, fats and oils, and like vitamin A palmitate, can be solubilized in aqueous solutions with solubilisers such as polysorbate 80. Vitamin E acetate can be added to the oil phase of topical formulations without special precautions; however, strong alkaline solution will specify the ester.

The unesterified α-tocopherol darkens on exposure to air, but dilution in oils will minimize this. Trace metals accelerate decomposition and chelating agents are recommended in emulsion or alcoholic-solution based preparations. They should be added at a temperature not higher than 40°C.

Principal synergists

The presence of other antioxidants, such as vitamin C and *β*-carotene, supports the antioxidative, protective action of vitamin E.

VITAMIN K

Vitamin K occurs in three forms: vitamin K1 (phylloquinone, phytonadione, phytomenadione) is found in plants; vitamin K2 (menaquinone) is synthesized by bacteria in the human and animal intestine; and vitamin K3 (menadione) is a synthetic compound that can be converted to vitamin K2 in the intestinal tract.

Vitamin K is needed primarily for the blood-clotting mechanism that prevents bleeding to death from cuts and wounds or internal bleeding. Vitamin K is found naturally in plants and can be produced by bacteria in the intestine. The best dietary sources are leafy green vegetables, such as spinach, sprouts, broccoli and cauliflower. Lower levels are found in liver, lean meat, cow milk, egg yolk and whole-wheat products.

Functions

The vitamin's main function is as part of the blood-clotting system that prevents excessive blood loss due to cuts, wounds or following an operation. Vitamin K is needed to make a protein called prothrombin, which is the first stage in blood clotting and wound healing. Scientists also believe that a vitamin K dependant protein may be needed for healthy bone mineralization.

Shortages of vitamin K are very rare but individuals suffering from liver disease or who are unable to absorb fat are at risk. New-born babies have low stores of vitamin K. They may be at risk of vitamin K shortage since breast milk contains low levels and new babies are unable to make vitamin K in their intestines.

Cosmetic application

Dermatologists have recently found vitamin K to be successful for treating dark circles under the eyes and bruising on the face. Dark circles may be hereditary for some people or simply a part of the aging process. When the fat pad beneath the eye begins to thin with age, it can create a sunken look to the under eye area. Studies have shown that sluggishness of blood flow underneath the eyes may also contribute to dark circles. Vitamin K has been found to diminish the appearance of these dark circles.

Vitamin K has also recently been studied for its effects on reducing bruising following certain dermatologic procedures. More recently, it has also been used in patients undergoing laser treatments to lessen the appearance of spider veins on the face.

B GROUP VITAMINS

The B group vitamins comprise B1, B2, B3, B5, B6, B8, B9 and B12.

Vitamin B1

Also called thiamine, occurs widely in food, but mostly in small amounts. The best source of thiamine is dried brewers yeast. Other good sources include meat (pork, lamb and beef), poultry, whole-grain cereals, nuts, pulse and dried legumes. Because thiamine has a high turnover rate and is not appreciably stored in the body, a continuous supply is required. The heart, kidney, liver and brain have the highest concentrations, followed by the leukocytes and red blood cells.

Functions

Thiamine is essential for the breakdown of foods, especially carbohydrates, to release energy and for healthy nerve and muscle function.

Deficiency causes growth retardation and disorders of the nervous and cardiac systems (well known under the name ‘beri-beri’). Historically, it occurred in people living on diets of mainly white rice (where the thiamine in the whole grain has been removed or destroyed). In animals, progressive paralysis and neck retraction has been observed.

Cosmetic application

Vitamin B1 (thiamine HCl) acts as a co-enzyme in amino acid metabolism and maintains healthy skin.

Vitamin B2

Also called riboflavin, is one of the most widely distributed vitamins. All plants and animal cells contain it, but there are very few rich sources. Yeast and liver have the highest concentrations, but the commonest dietary sources are milk and milk products, meat, eggs and leafy green vegetables. Cereal grains, although poor sources of riboflavin are important for those who rely on cereals as their main dietary component. Animal sources of riboflavin are better absorbed than vegetable sources.

Functions

Riboflavin is vital for the release of energy from foods and for healthy skin, eyes and growth. It plays a major role in oxidation and reduction processes in cells. Deficiency is rare, and usually occurs in combination with deficiencies of other water-soluble vitamins. Since cereals are poor sources of vitamin B2, virtually all types of compound animal feed must contain vitamin B2 supplements. In farm animals, even marginal vitamin B2 deficiency leads to loss of appetite and impaired growth rate. Riboflavin deficiency also affects the nervous system, gastrointestinal tract and reproductive organs. To prevent deficiency, most feed mixes (except those intended for ruminants) are fortified with riboflavin.

Cosmetic application

Vitamin B2 (riboflavin 5′-phosphate sodium) has a bright deep yellow colour, although is not used in skin care, it could be used as a colouring agent in cosmetic products.

Vitamin B3

Also called niacin refers to both nicotinic acid and its derivative nicotinamide (also called niacinamide). Both occur widely in nature, with nicotinic acid being more prevalent in plants, and nicotinamide in animals. Yeast, liver, poultry, lean meats, nuts and legumes contribute most of niacin in food. Milk and leafy green vegetables contribute lesser amounts. Niacin belongs to the group of B vitamins.

Functions

Niacin is vital for energy release in tissues and cells. Working with riboflavin and thiamin, it helps to maintain healthy nervous and digestive systems. It is essential for growth and is involved in the synthesis of hormones.

Deficiency in animals affects the skin and digestive tract. Ruminants on green fodder usually do not require extra niacin, but niacin supplements improve milk yield in cows. Pellagra is a disease resulting from a combined deficiency of niacin and tryptophan. The symptoms of pellagra include dermatosis, dementia, diarrhoea and nervous disorders. Pellagra is rarely seen in industrialized countries and is associated with alcohol abuse. In other parts of the world where maize is the major staple diet, pellagra persists.

Cosmetic application

The multiplicity of effects and formulation benefits seen with niacinamide make it a good choice for a variety of cosmetic products: niacinamide can help normalize the imbalance of nicotinamide coenzymes in skin depleted with age. It increases biosynthesis of ceramides as well as other stratum corneum lipids to improve the epidermal permeability barrier (Tanno *et al.*, 2000). Aged fibroblasts secrete less collagen than young cells, thus niacinamide can stimulate new collagen synthesis. Other properties of niacinamide include up-regulation of biosynthesis of keratinocyte differentiation markers; helps prevent UV-induced deleterious molecular and immunological events; inhibits transfer melanosomes from melanocytes to keratocytes; reduces human skin hyperpigmentation; regulates sebaceous lipid and acne; and also exerts multiple benefits on the appearance of ageing and photodamaged skin.

Vitamin B5 (Pantothenic acid)

Pantothenic acid is present in almost every type of food. It is particularly abundant in yeast and organ meats (liver, kidney, heart, brain); however, eggs, milk, vegetables, legumes and whole-grain cereals are probably more common sources. Processed foods contain smaller amounts, unless the vitamin B5 lost during processing is replaced afterwards. Pantothenic acid is synthesized by intestinal microorganisms, but the amount produced and its role in human nutrition is unknown.

Functions

Pantothenic acid is vital for the release of energy from food, for healthy growth and for the production of antibodies. Pantothenic acid requires vitamin A, vitamin B6, vitamin B12, vitamin B9 and vitamin B3 in order to function properly.

Cosmetic application

Panthenol is broadly used in cosmetic products and has become an essential ingredient in skin- and hair-care products. Its main functions are improved wound healing, anti-inflammation and hydrating activities. Panthenol is often called “pro-vitamin B5” in advertising literature. Panthenol is the stable biologically active form of vitamin B5 or pantothenic acid. This vitamin is essential for growth and normal maintenance of skin and hair. Pantothenic acid is an essential constituent of coenzyme A, which plays a central role in the metabolism.

Ethyl panthenol, like panthenol, is metabolized into pantothenic acid in the skin. The pantothenic acid is then incorporated as an important component in the coenzyme A.

The use of panthenol as a moisturizer and conditioner in hair-care products imparts several effects: gives hair long-lasting moisturization, improves the manageability, protects and repairs damage due to chemical and mechanical procedures (brushing, combing, shampooing, perming, colouring, etc), reduces the formation of split ends, improves the condition of damaged hair, thickens the hair and imparts shine and lustre.

In hair-strengthening products, like setting lotions and hair sprays, polymer softeners can often be replaced with D-panthenol. In contrast to the normal softeners D-panthenol is released from the film coating the hair and penetrates the hair slowly. The panthenol, which penetrates the hair, is replaced with sebum so the hair does not get heavy and the style lasts longer.

Studies carried out in Hoffmann-La Roche have shown that although single applications of panthenol have an effect, multiple applications give better results. Panthenol is deposited on the hair and also penetrates the hair shaft accumulating in the hair.

D-Panthenol in shampoos, conditioners, hair and scalp treatments and hair tonics not only improves the shine, the feel and the flexibility of the hair, it also protects the hair against mechanical damage and in general makes the hair more resistant to environmental stresses.

In skin-care products, it helps to keep the skin moist and supple, stimulates cell growth and tissue repair and inhibits inflammation and reddening. It improves and increases the humidity properties of the skin (moisturizing effect); it also makes dry skin softer and more elastic, has anti-inflammatory effects and soothes irritated skin, stimulates epithelization and helps to heal minor wounds (shaving, skin grazes and blisters).

Weiser and Erlemann (1986) have shown that even low concentrations of D-panthenol have a positive influence on epithelization. The studies were carried out using a commercially available W/O-cream with varying concentrations of D-panthenol. The effect of D-panthenol in a 5% formulation on epithelization showed improved wound healing.

Panthenol can also be used in nail care. The elasticity of fingernails depends on the water storage capacity of the nail keratin. Panthenol can substantially increase the water storage capacity of nails and by this mechanism the flexibility and stability of nails are improved.

Processing. Panthenol is added to the water phase and is stable to heat during manufacturing (up to 75°C), although prolonged heat should be avoided. It is stable in the pH range from 4.0 to 7.5 (optimum pH 6.0). The above-mentioned panthenol product forms are miscible with water, alcohol, propylene glycol and glycerin, but do not mix with fats or mineral oils.

Vitamin B6

Also called pyridoxine, occurs widely in foods like wholemeal bread, bananas, yeast extract, nuts, liver and pulses.

Functions

Vitamin B6 plays a role in the metabolism of protein, carbohydrates and fats, the production of neurotransmitters and the formation of nicotinic acid. It is vital for maintaining a healthy nervous system, skin, muscles and blood. One of the central roles of this vitamin is in protein metabolism where it helps regulate the balance of amino acids in the body. It is also closely involved in hormone production.

Severe deficiency is rare, but surveys have revealed that marginal deficiency might be quite common. The need for this vitamin increases during pregnancy and lactation due to

the additional demands made by foetus or infant. In animals, adult ruminants are self-sufficient in vitamin B6, but young animals require supplements during the growth period.

Cosmetic application

Vitamin B6 (pyridoxine pure crystalline powder or pyridoxine hydrochloride forms) acts as a coenzyme in amino acid metabolism and maintains healthy skin; it also controls oily skin.

Vitamin B8

Also called D-biotin, is one of the more recently discovered vitamins. It belongs to the group of B vitamins and is found in most foods in small amounts. The richest sources are yeast, liver and kidney. Egg yolk, soybeans, nuts and cereals are also good sources. Biotin is found in most feedstuffs because its bioavailability is low it is added to most animal feeds to improve reproductive functions and general health.

Functions

There are eight different forms of biotin, but only one, D-biotin, has full vitamin activity. It is vital for the production of energy from carbohydrates and fats, and for healthy skin and hair. It forms part of several enzyme systems and is necessary for normal growth and body function. It plays a key role in carbohydrate, fat and protein metabolism.

Human biotin deficiency is extremely rare. Deficiency symptoms include anorexia, nausea, vomiting, glossitis, pallor, mental depression, dry scaly dermatitis and, after long-lasting, severe biotin deficiency, hair loss (alopecia). Biotin is extremely important in animal production. Spontaneous biotin deficiency has led to heavy losses in certain livestock species. Consequently, biotin is added to feed mixes for poultry, pigs and fish in order to ensure optimal growth, healthy skin and bones and efficient reproduction.

Cosmetic application

New research by DSM Nutritional Products has led to findings for topically applied D-biotin (coenzyme R). Furthermore, it has been shown that vitamin C has a boosting effect on the D-biotin activity on the skin. D-biotin can be used in the following applications: anti-aging products because it reduces wrinkles and smoothes the skin surface; anti-age-spot treatments, because it lightens age-spots and therefore evens skin tone; skin protection and regeneration products, because it enhances the recovery of skin-barrier damage. Other applications are also possible, such as lip care, hair care, nail care, men's care, decorative cosmetics, etc.

Vitamin B9 (folic acid)

Folic acid belongs to the group of B vitamins and is found primarily in leafy green vegetables, oranges, fortified cereals, wholemeal bread, liver and potatoes. Folic acid was the

first member of a class of compounds to be discovered, and the name is still used for the whole group, also known collectively as folates or folacin.

Functions

Folic acid is vital for healthy blood cells, the formation of new body cells and for healthy growth. It plays an important role in the metabolism of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), the carriers of genetic information in all living things.

Folic acid deficiency may be the consequence of disease or treatment with certain drugs and may also occur during pregnancy. Abnormalities in the production of blood cells are the main clinical sign of deficiency. The hematological changes associated with folic acid deficiency are similar to those encountered in vitamin B12 deficiency, and patients are often deficient in both vitamins. Folic acid reduces the risk of neural tube birth defects when consumed in adequate amounts by women before and during early pregnancy. For this reason, the enrichment of cereal products with folic acid is compulsory in the USA. Folic acid is easily destroyed during prolonged storage and up to 50% can be lost during cooking. In animals, folic acid deficiency results in anaemia and impaired reproduction, and in poultry with bad feathering and reduced laying performance and hatchability.

Cosmetic Application

Very few cosmetic products (e.g. lip glosses) include folic acid in their formulations. It may have skin repair applications because it plays a role in the production of DNA and RNA (Debowska, 2005).

With regard to other B-group vitamins, such as vitamin B12 (cyanocobalamin), to our knowledge no cosmetic applications have been described in literature.

VITAMIN C

Vitamin C (ascorbic acid) is found in citrus fruits, blackcurrants, sweet peppers, parsley, cauliflower, potatoes, sweet potatoes, broccoli, Brussels sprouts, strawberries, guava and mango.

Measurement units

The mass equivalents of 1 IU for vitamin C is the biological equivalent of 50 μg ascorbic acid.

Functions

Vitamin C is important for the production of collagen, connective tissue and protein fibres that give strength to our teeth and gums, muscles, blood vessels and skin. In the immune

system, vitamin C helps the white blood cells to fight infection. It helps the body to absorb iron. Iron is needed to make haemoglobin, the red pigment in the blood, which transports oxygen from the lungs to the rest of the body. Research has indicated its role in a wide range of other functions, such as the synthesis of hormones and neurotransmitters, as well as in the immune system. It is believed that the so-called "antioxidant" properties of vitamin C help protect the body from the harmful effects of too many free radicals. These are potentially damaging molecules in our bodies that may harm healthy cells. Together with vitamin A and vitamin E it forms the trio of antioxidant vitamins now believed to have a preventive effect on degenerative diseases such as cardiovascular disease and cancer. Vitamin C is also commonly used as a natural antioxidant, i.e. it prevents foods and beverages spoilage caused by oxygen in the air.

The early symptoms of vitamin C deficiency are fatigue, lassitude, loss of appetite, drowsiness and insomnia, feeling run-down, irritability and low resistance to infections. Severe deficiency causes a weakening of these tissues (scurvy), resulting in capillary bleeding. Fish, like humans, are dependent on a vitamin C supply via the diet. The other food-producing animals are able to synthesize vitamin C themselves. However, under conditions of stress, this production may not be sufficient to support optimum health and performance.

Cosmetic application

Several benefits of ascorbic acid topical application have been described. However, due to its pure stability in aqueous systems, until the last years these benefits were not exploited by the cosmetics industry on a large scale.

Some derivatives of vitamin C, e.g. sodium ascorbyl phosphate, have the benefit of being stable in cosmetic formulations and exerting the favourable effects of vitamin C when applied on the skin. The most commonly used are salts of ascorbic acid: sodium or magnesium ascorbate, sodium or magnesium ascorbyl phosphate, ascorbyl glucoside and ascorbyl palmitate.

One of the most useful is sodium ascorbyl phosphate (SAP); some major cosmetic activities of this compound are described briefly below.

It is the sodium salt of the monophosphate ester of ascorbic acid. It is a white powder, easily soluble in water to concentrations up to 50%. In contrast to ascorbic acid, SAP is stable in aqueous solutions at a pH above 6.5.

SAP can be used in skin-care products to prevent the formation of free radicals and to increase the firmness of the skin. Furthermore, SAP has skin lightening activities and inhibits the growth of harmful oral bacteria. SAP is also ideal in combination with vitamin E acetate to enhance the protection of sun-care products.

Sodium ascorbyl phosphate alleviates oxidative stress to the skin. Lipid peroxidation induced by UV-A light leads to the formation of peroxide radicals along the side-chains of polyunsaturated fatty acids in lipids. A single initiating event can lead to many cycles of peroxidation, leading in turn to breakdown products that can damage DNA or protein. A study conducted by DSM Nutritional Products on 20 healthy volunteers showed that the application of a cream containing SAP prevents the formation of UV-A-light-induced

squalene hydroperoxide. This inhibition was even more pronounced when SAP was combined with DL-α-tocopheryl acetate.

Dermatologists have long observed that skin fibroblasts synthesize less collagen as they age. Vitamin C can counteract this decline in two ways. It is an essential cofactor in the hydroxylation of lysine and proline, which contribute to the stability of collagen. It also stimulates the synthesis of collagen, thus increasing skin firmness. Regular skin-treatment with 3% SAP leads to significantly firmer skin.

Ascorbic acid plays an important role in the regeneration of vitamin E from vitamin E radical. This recycling effect has been specifically shown in skin. Therefore, sun-care products should ideally combine vitamin E acetate and sodium ascorbyl phosphate to protect the skin from harmful UV irradiation. To test this, a new method was developed by Hanson (2003) to visualize UV-induced free radical formation in human skin. Results show that SAP in combination with vitamin E acetate reduces the formation of free radicals in the skin by almost 50% compared to a placebo sun-care formulation having an SPF of 8.

Ascorbic acid and its derivatives are widely used in skin-lightening products and skin-care formulations which prevent and attenuate age spots.

Daily teeth brushing is important but often not enough alone to prevent caries, gingivitis and periodontitis. Bacterial infections are the main cause of dental problems. Traditional toothpaste formulations use rather strong anti-bacterial agents, which are not well accepted by consumers. Furthermore, there is a lack of effective natural substances which inactivate oral bacteria and which are stable in oral-care formulations. SAP is the ideal ingredient for oral-care products because it inhibits the growth of several oral bacteria, such as *Streptococcus mutans;* the caries bacteria, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, the principal bacteria causing gingivitis and periodontitis.

Processing

The following recommendations should be followed when formulating with SAP: it is compatible with almost all cosmetic ingredients (check stability with acrylic acid thickeners and some ionic emulsifiers); use always chelating agents (e.g. disodium EDTA); maintain the formulation at a pH above 6.8; dissolve SAP in water and add it to an emulsion as an aqueous solution below 35–40 °C.

Very good stability levels can be achieved by keeping the pH at 6.8. While the content remains essentially unchanged at room temperature, a slight decrease can be observed at 43 °C. Finished products should therefore be stored at temperatures below 25 °C.

VITAMIN F OR POLYUNSATURATED FATTY ACIDS (PUFAs)

Polyunsaturated fats are known as essential fats. Like vitamins they are essential for life, they cannot be made in the body and must be obtained from the diet. These good fats have actually been shown to counter the effects of "bad" fats in our blood and are important for maintaining good health of our hearts, cells and nervous systems. In contrast to saturated and mono-unsaturated fatty acids, PUFAs have at least two double bonds, a feature which crucially affects their structural, physical and chemical properties.

There are two large families of PUFAs, Omega-3, which has a fluidic effect on the blood and Omega-6 involved in blood coagulation. A healthy body should maintain a balance between the tendency of the blood to clot and its tendency to flow. Both families can be metabolized to long-chain PUFAs. They have been shown to have an important role in health and disease. Diets rich in Omega-3 fatty acids contribute to reduced risk of cardiovascular disease.

Cosmetic applications

PUFAs are just as essential as vitamins, mineral salts and proteins. Omega-6 oils enhance the barrier function of the skin and are therefore ideal for cosmetic products designed to combat dry and scaly skin. When applied topically, unsaturated fatty acids, as contained for example in borage and evening primrose oil (both especially rich in gamma-linolenic acid (GLA)), are characterized by many positive effects.

Studies conducted in recent years have revealed that although water plays an important role in keeping the skin moist and supple, the ability of the top layers of the skin (stratum corneum) to resist moisture loss depends on the presence of certain long-chain polyunsaturated lipids known as essential fatty acids (EFA). These lipids classified as Omega-3 and Omega-6 lipids cannot be synthesized by the body, although when topically applied, they can be metabolized in the skin and directly incorporated into the structural lipids of the epidermis (ceramides): the building blocks of the water barrier of the stratum corneum. Evening primrose oil and borage oil (the latter also known as starflower oil) are rich sources of Omega-6 lipids and in particular GLA. Insignificant levels of GLA are detected in mammalian skin, because the epidermis lacks the enzymes necessary to convert linoleic acid into GLA. Thus, there is a need to provide the body with products containing this material. Topical application of borage oil and evening primrose oil significantly increases the level of GLA in the stratum corneum.

The structure and properties of the cell membranes and lamellar skin lipids depend greatly on the type and composition of the phospholipids or ceramides forming the double lipid layers.

If only saturated fatty acids are present, stiff and rigid cell membranes or skin lipids are formed. In contrast to this, phospholipids and ceramides with a high content of unsaturated fatty acids, such as linoleic acid or GLA, form structures which are more mobile and flexible. The result is an increase in the elasticity of the skin since unsaturated fatty acids are incorporated in the cell membranes of the skin. PUFA-nourished skin looks younger and smoother.

Processing

PUFA oils have to be added to the oily phase of an emulsion and can be heated for a short time up to 75 °C. When formulating with PUFA oils, one should add antioxidants (e.g. tocopherol, BHT) to prevent them from decomposing in the formulation over time. It is also recommendable to add a chelating agent (e.g. EDTA) and to avoid contact with oxygen.

PUFA oils can be used in skin-care products against dry skin, as well as in baby-care cosmetics, soaps, cleansers and shampoos.

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8.7. Bioactive Ingredients in Cosmetics

I. Vivó-Sesé* and M.D. Pla

Departamento I&D, Germaine de Capuccini S.A., 86 Carretera Alicante, 03801-Alcoi, Spain

Today, the concept of "cosmetic" is perceived by part of society as a necessity, a way to feel better about oneself and about others. However, cosmetic products are valued not simply for their pleasant textures and elegant feel, but also, very fundamentally, for their effectiveness. The idea that they are inert substances has totally disappeared. This new perception is largely due to the biological activity exerted by their innovative active ingredients.

Advances in our knowledge of biochemistry and skin physiology (such as the discovery of aquaporins or the function of heat shock proteins), as well as the development of technologies, such as combinatorial chemistry or biotechnology, have made it possible to develop new molecules capable of offering effective and specific solutions for skin conditions. All this is most useful in the field of cosmetics.

The world of cosmetics has also received a clear input from other fields, such as pharmaceuticals and nutrition. This has led to the synthesis of molecules that already existed in nature in order to achieve reproducible effects and avoid secondary effects, or the use of products that are typically foods, for their beneficial topical application.

As a result of all this, a multitude of active substances have appeared, with different origins and effects and which offer, when correctly combined, almost infinite possibilities to the cosmetic formulator.

This innovation is accompanied by increasingly complex and strict legislation whose purpose is to protect and inform the consumer about the product to be used. This affects both formulation and the development of new active ingredients.

Thanks to both new scientific advances and more in-depth knowledge of the causes underlying processes such as skin aging, acne, cellulite and body flaccidity, many new active ingredients have been designed to solve these problems.

However, the formulator must take into account the fact that all such problems generally have multiple causes and, for a product to be effective, it must be made up of a mixture of substances that act synergistically and attack the problem from all possible angles.

In this section, the most significant and innovative groups of bioactive ingredients are commented, taking into account the methods by which they are synthesized and obtained, their activity on a biochemical level and their cosmetic use.

*Corresponding author. E-mail: inmaculada.vivo@germaine-de-capuccini.com

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

SYNTHETIC PEPTIDES

Specific peptides have become one of the most common sources of active ingredients in cosmetics. To synthesize peptides today, combinatorial chemistry methodologies (Nielsen, 2002) are employed making it possible to use different processes to rapidly and simultaneously synthesize a large number of compounds (combinatorial libraries) and subsequently to determine their potential usefulness. This working method is more and more commonly used in the pharmaceutical industry, since it makes it possible to identify and isolate active molecules rapidly.

Solid-phase peptide synthesis (SPPS) (Lehninger *et al.*, 1993) applied to combinatorial chemistry has made it possible to synthesize peptides effectively and rapidly. SPPS involves synthesizing a peptide with a pre-established sequence while it remains connected to a solid support. This support is an insoluble polymer contained inside a column, similar to the columns used in chromatography. The peptide is constructed on this support by adding amino acid after amino acid and using a group of reactions repeatedly. This technique enables simultaneous synthesis of peptides measuring around one hundred amino acids long. Thus, greater product purity, as well as time-saving automation can be achieved (Gette and Merrifield, 1971; Merrifield, 1984, 1996). From among the numerous peptides used in cosmetics at present, those that have the most interesting and latest cosmetic functions are chosen.

Competitive inhibitors

Competitive inhibition implies, in this case, the use of peptides that imitate other peptides found in the skin and which compete with them in a specific biochemical reaction.

Expression wrinkle reducers

Expression wrinkles are the result of excessive stimulation of muscle cells. With each facial movement, facial muscles contract and relax excessively, leading to this type of wrinkle. Avoiding the formation of wrinkles by impeding muscle contractions is possible through botulinum neurotoxin injections. However, apart from the fact that this technique is not strictly cosmetic, one runs the risk of it being irreversible. An alternative can be the use of a hexapeptide (Glu–Glu–Met–Gln–Arg–Arg) capable of inhibiting the release of muscle cell-stimulating neurotransmitters, with an effect similar to that of the botulinum neurotoxin. Neurotransmitter release inhibition is caused by the interaction of this hexapeptide with the protein complex: Soluble NSF-attachment protein receptors (SNARE complex) which is a mediator of exocytosis and thus, the release of neurotransmitters. This interaction is caused by the hexapeptide imitating the amino acid sequence of a protein soluble NSF-attachment protein-25 (SNAP-25) in the SNARE protein complex. The resulting complex, when connected to the hexapeptide in the place of the SNAP-25 protein, is unstable, and no neurotransmitter release takes place. If the neurotransmitters are not released, the muscle cells are not stimulated and the muscle contraction process is reduced (Blanes *et al.*, 2002). SPPS has led to the synthesis of the hexapeptide capable of imitating part of the SNAP-25 protein and acting in competition with it, to create unstable SNARE complexes, although the blockage is not permanent, as is the case with botulinum toxin (Blanes *et al.*, 2002). Topical application of this competitive peptide enables expression-wrinkle formation to be lessened, reducing the process of muscle contraction in a reversible and controlled way.

Biomimetic Peptides

Biomimetic molecules copy the working mechanisms of molecules that are already found in our bodies. By synthesizing biomimetic molecules, effective and directed functions are achieved without side effects, since only the part of the molecule with the desired effect is copied, and the rest, which is generally responsible for undesired effects, is discarded.

Currently there are a large number of biomimetic molecules with very different effects, some of them are cited in the following examples.

Extracellular matrix modifiers

Biomimetic molecules of two of the most abundant proteins in the extracellular matrix have been synthesized: collagen and laminin. The slowing of the synthesis of these two proteins is one of the basic causes of the loss of skin firmness and the appearance of wrinkles. Working to assist synthesis of these proteins is fundamental to the struggle against skin aging (Katayama *et al.*, 1993).

In the first case, a synthetic pentapeptide, corresponding to a micro-fragment of collagen, was shown to activate the *de novo* synthesis of collagen, fibronectin and other components of the extracellular matrix in fibroblasts (Guttman, 2002). *Ex vivo, in vitro* studies, as well as *in vivo* measurement of wrinkle surface, volume, density and depth, demonstrate the improvement in cutaneous relief attained after topical application, confirming the effects of this peptide (Sederma SAS, Societé d'études dermatologiques, *Matrixyl*™).

Laminin is a glycoprotein made up of three peptide chains called alpha, beta and gamma joined by disulphide bonds. Laminin and integrins (receptors responsible for interactions in the extracellular matrix and involved in recognizing laminin) intervene in the cellular adhesion and angiogenesis processes, allowing nutrition, cellular oxygenation and other processes to function properly (Ruoslahti and Engvall, 1997). A hexapeptide found in the alpha chain of laminin, which reinforces the synthesis of laminin and integrins in human fibroblasts, has been obtained, improving the adhesion, migration, proliferation and nutrition of skin cells.

Neurocosmetics

A state of well-being is associated with the presence of β-endorphins, endogenous chemical substances (31-aminoacid neurohormones), that act as mini "anti-stress" peptides and transmit a message of calm and relaxation by activating opiate receptors. Besides this calming, relaxing effect, these hormonal mediators can act on other processes, such as lipolysis. In this case, the concept of neurocosmetics is based on the reciprocal relationship between the neural endocrine system and adipose tissue. The lipolytic effect of β-endorphins (Vettor *et al.*, 1993) depends on a short sequence of amino acids within it: enkephalin (Nencini and Paroli, 1981). The peptide Tyr–Gly–Gly–Phe–Leu, which is biomimetic of enkephalin, is capable of acting on the lipolytic process with no hormonal side effects, since only this fragment of β-endorphin is used rather than the whole molecule (Sederma SAS, Societé d'études dermatologiques, *Kephaslim*®).

Melanogenesis activators

The process of melanogenesis or melanic pigment synthesis takes place in organelles in the melanocytes called melanosomes. Melanogenesis is regulated by ultraviolet (UV) light, hereditary factors and hormonal stimulation. Alpha-melanotrophin, a hormone made up of 13 amino acids, is responsible for stimulating melanosome synthesis and accelerating their transfer to keratinocytes. Injecting this hormone causes skin tanning and darkening of the skin in lightly pigmented individuals. A biomimetic hexapeptide has been synthesized based on this hormone and when applied topically, it can stimulate melanogenesis and tan the skin with no hormonal effects (Pons and Parra, 1995).

ACTIVE INGREDIENTS OF BIOTECHNOLOGICAL ORIGIN

One of the largest groups of cosmetic active ingredients is now available, thanks to biotechnology. Cell cultures, fermentation and subsequent selection make it possible to obtain cellular and microorganism biomolecules of great interest in the field of cosmetics. The applications of two kinds of cultures are briefly described below.

Bacterial and yeast cultures. Fermentation processes. Metabolite extraction and selection

Anti-glycation effect

Glycation (Maillard reaction) of proteins is one of the factors that contribute to skin aging; which leads to cross-linking in the protein structure, especially the collagen structure (Araki *et al.*, 1992; Frye *et al.*, 1998; Ulrich and Cerami, 2001). In the search for methods to inhibit protein glycation, one of the most interesting has involved resorting to a *Saccharomyces* yeast and a *Xylinum* bacterium that act in symbiosis. Sweet black tea is used as the medium and after the fermentation process a beverage typical in some parts of Russia and rich in B-group vitamins, etc. is obtained (Frank, 1999). *In vitro* studies have been carried out to evaluate the anti-glycation potential of the product obtained through this symbiotic process compared to the results of those carried out with aminoguanidine (a molecule that inhibits glycation) (Brownlee *et al.*, 1986). These studies showed a powerful anti-glycation effect of the product that, together with other beneficial effects, justified its subsequent topical application as a cosmetic active ingredient (Sederma SAS, Societé d'études dermatologiques, *Kombuchka*™).

Proteolytic agents

Renewing the stratum corneum is not a new concept, since alphahydroxy acids (AHAs) have been in general use for some time to eliminate superficial cells from the epidermis, leaving the skin with a finer texture. However, AHAs are aggressive and can be irritating. A protease obtained by fermenting the *Bacillus subtilis* microorganism was selected for its proteolytic effect (Sederma SAS, Societé d'études dermatologiques, *Keratoline*™). This

new concept of biotechnological origin promotes cell renewal in a less aggressive but effective way both *in vitro* and *in vivo*.

Cell protectors and heat shock protein stimulators

In the search for new substances to protect cells against external environmental effects such as UV radiation, some research has centered on the study of organisms capable of surviving under extreme conditions. These studies show that certain halophilic bacteria, such as *Ectothiorhodospira halochloris,* contain molecules (ectoins) capable of protecting cells against extreme conditions, maintaining osmotic equilibrium and stabilizing the protein, nucleic acid and biomembrane structures (Beyer, 2000).

Various studies carried out by researchers at Merck KGaA Darmstadt (Beyer, 2000) demonstrate that these molecules can also be used to protect human skin cells against external aggressions such as UV radiation, which cause the destruction of Langerhans cells. These cells are the most important antigen-presenting cells in the skin. The immunoprotecting potential of the ectoins is based on their ability to prevent destruction of the Langerhans cells in human skin affected by UV radiation (Beyer, 2000). The protective effect of ectoins goes beyond this, since they can increase the expression of heat shock proteins (HSP) 72/73 (Beyer, 2000), proteins found in our bodies naturally, the function of which is to defend and protect other proteins.

Another example of a cell protector of biotechnological origin is the one obtained by fermenting *Thermus thermophilus*, whose natural habitat is in ocean rifts in the Gulf of California. This is a highly toxic environment, with a very high concentration of free radicals and temperatures of up to 75 °C. The resulting extract can protect deoxyribonucleic acid (DNA) against free radicals and cellular membranes against lipid oxidation. This protective effect depends on the temperature—the higher the temperature the more effective its action (Sederma SAS, Societé d'études dermatologiques, *Venuceane*™).

Restructuring agents for the dermal–epidermal junction

The dermal–epidermal junction, which aids exchanges between the epidermis and the dermis, is responsible for cohesion between the two layers and for resisting external traction (Marinkovich *et al.*, 1992; Burgeson, 1993; Burgeson and Christiano, 1997; Spirito *et al.*, 2001). With age, the synthesis of the anchoring proteins is reduced and the dermal–epidermal junction is weakened and becomes less effective. As a result, the skin microrelief becomes irregular and wrinkles appear. Following topical application of an active ingredient obtained from a yeast extract, the expression of the anchoring dermal–epidermal junction proteins (collagen IV, collagen VII, laminins and integrins, etc.) increases and the integrity of this union is preserved (Castagne *et al.*, 2000).

Phytoplankton and algae cultures

Phytoplankton is made up of millions of single-celled organisms capable of living under varying conditions of environmental stress. As a defense mechanism against environmental aggressions, they synthesize different biomolecules (antioxidants, peptides, polysaccharides, amino acids, vitamins, fatty acids, polyunsaturated fats, etc.) that have been found especially

useful in skincare. As a result, industrial phytoplankton culture systems have been devised to make it possible to obtain these metabolites of interest, by varying culture conditions, including osmotic pressure, oxidative environment, temperature, salinity, etc. Cultivating macroscopic algae is also possible, in order to obtain the whole plant or to extract molecules whose effects are of interest. To begin with this cultivation takes place in tanks of seawater in order to aid reproduction and subsequently, the plantlets grow in the sea (BiotechMarine®, *The Sea, the Source of Life*). An enormous number of active substances with different cosmetic effects are obtained from phytoplankton and algae. Two of these thousands of cosmetic applications are described below as examples.

Lipolytic stimulants

In cosmetics for the body, active ingredients that have a lipolytic effect are generally the most sought after. Given that the metabolism of algae also involves the storage and release of lipids, algae are often an excellent source for obtaining them. To satisfy their energy requirements, algae store lipids that are later transformed into energy through a series of metabolic reactions triggered by a group of messenger molecules that are largely sterols or derivatives. In the case of red algae belonging to the *Gelidium sp.* genus, the signal molecule responsible for activating the lipolytic process through a cascade of reactions has been identified. It is a sterol that, like the hormones adrenaline or glucagon, activates the adenylate cyclase enzyme found in adipocyte membranes. This leads to an increase in the intracellular concentration of cyclic adenosin monophosphate (cAMP), activating the transformation of the triglycerides stored in the adipocytes into fatty acids and free glycerol. This sterol, extracted from a culture of *Gelidium cartilagineum* algae, has been used as a cosmetic active ingredient and its lipolytic activity has been demonstrated in both *in vitro* and *in vivo* trials (BiotechMarine®, *Rhodysterol*®, 1998). The active ingredient was added to *in vitro* culture plates that contained human adipocytes and the amount of glycerol released to the culture medium was determined. It was in the same range as the result obtained with the traditional lipolytic agents, such as xanthins. Its effectiveness was measured *in vivo* in clinical tests using non-invasive techniques, such as measuring the circumference of the thighs before and after application, or the reduction in the thickness of the adipose layer in the area to be treated through measurements by means of three-dimensional ultrasounds (BiotechMarine®, *Rhodysterol*®, 1998).

Free-radical scavengers and DNA protectors

Protecting DNA and slowing the devastating effects of free radicals are two of the most common actions in anti-aging cosmetics. *Corallina officinalis* algae, which is a heat-resistant organism that forms calcium-rich structures similar to coral, are a tremendously interesting example of a cosmetic active ingredient with DNA-protecting properties. In spite of its resemblance to coral, *Corallina officinalis* is classified as a plant and the special porous skeleton that forms tunnels and pores makes it one of the most complex marine structures in the world of algae. Its usefulness in cosmetic science is based on its particular ability to act as a filter of UV and infrared radiation, which can produce damage to cellular DNA. UV radiation can produce the dimerization of pyrimidines and other changes to the chemical structure of DNA (Kvam and Tyrrell, 1997) as well as affecting its repair mechanism, leading to the formation of cancer. The filtering capacity of *Corallina officinalis* is due to

the fact that the carbonates it generates crystallize in rhombohedrons with magnesium ions. The absorption spectrum of these structures includes a section of the spectrum that enables it to protect against infrared and UV radiation. Its ability to protect DNA was determined by measuring its ability to repair previously damaged DNA (BiotechMarine®, *Oligophycocorail*®, 2002). Its activity as a protector against the aggression caused by oxygen free radicals has also been evaluated. UV radiation causes the appearance of oxygen free radicals named reactive oxygen species (ROS) which induce the degradation of lipids in cellular membranes, thereby affecting basic cellular functions such as nutrition, transport and inter and extracellular communication. Using vitamin C and α-tocopherol as references, the ability of the active ingredient to prevent lipid oxidation was determined. The results obtained showed that it is capable of offering protection both to the DNA, from damage caused by UV radiation, and the lipids in the membrane, from the oxidation caused by free radicals (BiotechMarine®, *Oligophycocorail*®, 2002).

PROTEIN EXTRACTS

Cytokines

The word cytokine refers to a complex group of low-molecular weight glycoproteins that act as humoral regulators, which are in charge of regulating cellular and tissue processes. They play a role in many organic functions, such as the development and regulation of immunity, inflammatory processes, cell growth, tissue remodeling and embryo development. Stimulating certain cells induces the expression and secretion of cytokines and of their membrane-bound receptors. Through these receptors, the cytokines can provoke cellular responses in the very cell that secretes them (autocrine response) or in adjacent cells (paracrine response). This is the main difference between cytokines and hormones, since hormones are produced by specific glands or glandular tissues, and circulate in the bloodstream as endocrine agents.

Colostrum, the milk secreted by mammals after the birth of their young, has a large variety of immunocompetent cells such as macrophages and lymphocytes which help develop and reinforce the immune system of the newborn (Britton and Kastin, 1991). Given the high density of these cells present in this type of milk, it could be defined as an extracellular matrix. The cells in this matrix are capable of synthesizing cytokines of two types: (1) Inflammatory cytokines that act at an immunological level, such as interferon gamma (IFN-gamma) and tumor necrosis factor alpha (TNF-alpha). (2) Non-immunological cytokines that activate tissue-supportive functions, such as the epidermal growth factor (EGF) and some kind of interleukins. Activation of defense functions occurs at the same time as tissue-supportive function inhibition. This regulation is possible through the interaction of the two types of cytokines found in milk. The lifetime of cytokine activity in colostrum is very short; days after the birth this activity is lost and the cytokines remain in a latent state. In order to take advantage of their modulating effect on cells, it is necessary to separate the perishable parts of the milk (fat, casein, and other enzymes) and activate the cytokines. CLR (Chemisches Laboratorium Dr. Kurt Richter GmbH) obtained a preparation with an optimal biological activity that, apart from the cytokines it contains, is rich

in other components such as lactoglobulin, lactalbumin, lactoferrin, etc., which help keep the cytokines within it stable and active (Petersen *et al.*, 1997). In order to demonstrate that cytokines remain active after they have been extracted, *in vitro* studies were carried out (migration-assay, proliferation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, crystal violet assay and activation of macrophages) and the cellular response was quantified. In the same way, *in vivo* studies were done to demonstrate the cytokine activity when applied topically: increase in the firmness and suppleness of the skin, increase in the density of the extracellular matrix, reduction in wrinkle depth and reduction in the irregularity of skin surface in areas affected by cellulite.

Film Formers

The anti-aging cosmetics sector is one of the most promising within this industry. Consumers of these products increasingly demand not just long-term effects, but also effects that are immediately visible after application. This is the reason why there has been a development in products with a "soft focus" effect and products that offer a "lifting" effect. The "lifting" effect is achieved by forming a film on the skin that gives a sensation of tautness to work against flaccidity and the effects of gravity. In order to form a film on the skin, both protein extracts and synthetic polymers have been used; however, there have been constant advances in this field overcoming problems such as stickiness, water solubility, skin discomfort, etc.

Such improvement has been possible thanks to the development of a new technology (patented by the cosmetics enterprise SILAB®), which has managed to obtain a tautening active ingredient through cross-linking, using a polymerizing agent (SILAB®, *Polylift*®, 2002). The polymerizing process is carried out using protein monomers from sweet almonds, selected using enzymatic hydrolysis. The protein complex obtained improves the tautening power of the native proteins, the degree of extensibility when the film is formed and also, its solubility in an aqueous medium, which makes it easier to use in formulas. These properties are determined by the degree of enzymatic hydrolysis and the polymerization process used (SILAB®, *Polylift*®, 2002). Moreover, the cross-linking process increases the number of hydrophobic groups in the protein chains, hydrophobic interactions are enhanced and their affinity with the lipids in the skin is increased. This affinity helps the tautening effect last longer (SILAB®, *Polylift*®, 2002). The "lifting" effect obtained on the skin is noticeable without being uncomfortable; the skin microrelief is smoother and softer, making imperfections less visible.

LIPID COMPLEXES

Protectors of the epidermal barrier. Stratum corneum components

Ceramides

The skin's barrier function relies on the stratum corneum. This outermost layer of the epidermis is made up of different types of keratinocytes, called corneocytes, which are found

in a lipid matrix. These lipids form multilamellar double layers and are composed of 50% ceramides, 25% cholesterol and the balance, fatty acids and esters (Grubauer *et al.*, 1989a; Pons and Parra, 1995; Pilgram *et al.*, 1998). Ceramides form part of this union through an amide link of different fatty acids with sphingosine- or phytosphingosine-type amino alcohols. As skin ages, the amount of ceramide decreases, so the ceramide levels are 50% lower in the skin of a 50-year-old person than in that of a 20-year-old individual (Rawlings *et al.*, 1994). As one ages, the keratinocytes lose their ability to synthesize them, especially ceramides of the type 1, 3 and 6 (Bouwstra *et al.*, 1998). Moreover, soaps and other products with surface-active agents progressively reduce the quantity of ceramides, especially ceramide type 3 (Grubauer *et al.*, 1989b). In order to help restore the original level of lipids in the skin's lipid barrier, enriched ceramide complexes have been developed that reproduce the lipid content of the skin. Topical application of these complexes makes it possible to maintain the skin's barrier function, preventing external aggressions and trans-epidermal water loss, and raising the possibility of a new topical therapy for different dermatoses (Coderch *et al.*, 2003).

Essential Fatty Acids

Mammals are not capable of synthesizing certain unsaturated fatty acids, which are, nevertheless, fundamental to maintain different functions, such as the stratum corneum and the skin's barrier function (Pons and Parra, 1995). Linoleic and linolenic fatty acids are considered essential. Linoleic acid is vital for maintaining epidermal thickness and the barrier function that impedes trans-epidermal water loss. A diet lacking in these fatty acids leads to dryness and desquamation, topical application can correct these skin conditions. Linolenic acid gives rise to the prostaglandins and leukotrienes involved in inflammatory processes, which explains why it is applied topically in treatments for sensitive skin. The combination of these two fatty acids is commonly called vitamin F and it is frequently applied topically to improve dryness and dehydration in mature skin.

The way in which advanced research in pharmaceutical, nutraceutical and chemical products has influenced the cosmetics industry is increasingly significant and will continue to grow. This brief review of just a few of the many examples of innovative active ingredients used in cosmetics is only the tip of the iceberg as this exciting research will lead to huge advances in the effectiveness and safety of cosmetic products.

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8.8. Analytical Methods for Actives used in General and Specific Skin-Care, Personal Hygiene and other Toiletry Products (*Excluding those Mentioned in Previous Chapters*)

A. Balaguer[1], A. Chisvert[2], J. Sisternes[1], J.G. March[3] and A. Salvador[1*]

[1]Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Doctor Moliner 50, 46100 Burjassot, Valencia, Spain
[2]Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Sciences, University of Alicante, Alicante, Spain
[3]Department of Chemistry, Faculty of Sciences, University of Islas Baleares, Palma de Mallorca, Spain

INTRODUCTION

Previous chapters revised published analytical methods for:

- UV filters (Sections 3.1 and 3.2);
- tanning and whitening agents (Section 3.3);
- general colouring agents (Section 4.2);
- hair dyes (Section 4.3);
- preservatives (Section 5.1);
- fragrances (Sections 6.1, 6.2 and 6.3); and
- surfactants (Section 7.1).

In the present section, analytical methods for other actives used in general and specific skin-care, personal hygiene or other toiletry products are considered.

Our aim is to make it easier for readers to find the most suitable method for the analyte they are interested in. Information about official and non-official methods is given here.

The compiled data for non-official methods have been obtained from the analytical literature published between January 1980 and June 2006. The Analytical Abstracts (Royal Society of Chemistry) database has been used.

A great many analytes have been studied in published articles. Here we have classified the different analytes into groups (according to their chemical family and/or other features),

*Corresponding author. E-mail: amparo.salvador@uv.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

GROUPS
Alcohols, phenols and polyphenols
Amines, amides, aminoacids and other nitrogenated compounds
Drugs and forbidden biological ingredients
Elements
Heterocyclic compounds
Hydroxides, peroxides and other oxygenated compounds
Lipids: fats, oils, waxes
Inorganic anions
Organic acids and its derivatives
Polymers (naturals and synthetics)
Thiocompounds
Vitamins
Other organic compounds

Figure 8.8.1 Classification of the analytes considered in this section.
Note: UV filters, tanning and whitening agents, colouring agents, preservatives, fragrances and surfactants are not included.

in order to help readers locate them more easily. Figure 8.8.1 shows how they have been classified.

OFFICIAL METHODS OF ANALYSIS

Section 2.1 gave a general overview of the official analytical methods for cosmetics worldwide. As was seen there, European Union (EU) has published a short number of official analytical methods that have been also adopted by other countries, whereas no analytical methods have been published as official in the United States (US). In Japan, authorities have approved some analytical methods for cosmetics but they are not published as official methods. That is why we have tried to give enough information on the EU official methods of analysis through this book.

Table 8.8.1 shows details on the EU official methods for analytes that were not dealt with in previous chapters.

Table 8.8.1

EU official methods of analysis for the analytes considered in this section[a]

Group	Analyte	Sample	Technique[b]
Alcohols, phenols and polyphenols	Methanol	All kinds of cosmetics products	GC-TCD (for aerosols) and GC-FID (for the rest)
	Hexachlorophene	All kinds of cosmetics products	GC-ECD
Amines, amides, aminoacids and other nitrogenated compounds	Ammonia	All kinds of cosmetics products	Potentiometry (titrant: sulphuric acid)
Drugs and forbidden biological ingredients			
Elements	Zinc (present as chloride, sulphate or 4-hydroxybenzenesulphonate)	Deodorants, antiperspirants and other cosmetic products	Gravimetry
	Silver nitrate (Ag determination)	Cosmetics intended to dye eyelashes or eyebrows	FAAS
	Selenium disulphide (Se determination)	Anti-dandruff shampoos	FAAS
	Zirconium (present as aluminium zirconium chloride hydroxide complexes)	Antiperspirants	FAAS
	Aluminium (present as aluminium zirconium chloride hydroxide complexes)	Antiperspirants	FAAS
Heterocyclic compounds	Oxyquinoline and its sulphate	All kinds of cosmetics products	UV/VIS
	Quinine and its salts	Shampoos and hair-lotions	LC-UV/VIS
Hydroxides, peroxides and other oxygenated compounds	Sodium and potassium hydroxides	Hair straightener products and nail-care products	Potentiometry (titrant: hydrochloric acid)
	Hydrogen peroxide	Hair products (without presence of other oxidation agents)	Volumetry: reaction with iodure and iodine determination (titrant: sodium thiosulphate)
Lipids: fats, oils, waxes			
Inorganic anions	Chlorides	Antiperspirants	Potentiometry (titrant: silver nitrate)
	Nitrites	All kinds of cosmetics products	UV/VIS (formation of an azoic colorant)

(*Continued*)

Table 8.8.1 (Cont.)

Group	Analyte	Sample	Technique[b]
	Fluoride derivatives	Tooth-cleaning	GC-FID
	Alkali and alkaline-earth sulphides	All kinds of cosmetics products	Volumetry: reaction with iodine and excess determination (titrant: sodium thiosulphate)
	Sulphites	All kinds of cosmetics products (containing aqueous or alcoholic phase)	Volumetry (titrant: sodium hydroxide)
	Alkali chlorates	Tooth-cleaning	Potentiometry (titrant: silver nitrate)
	Iodate	Rinse of cosmetics	LC-UV/VIS
Organic acids and its derivatives	Phenosulfonic acid	Aerosols and face-lotions	Bromoiodimetry
	Oxalic acid and its alkali salts	Hair-care products	Redox volumetry (titrant: permanganate)
Polymers (naturals and synthetics)			
Thiocompounds	Thioglycolic acid and its salts and esters	Hair products or depilatories	GC-FID
Vitamins			
Other organic compounds	Chloroform	Tooth-cleaning	GC-FID
	Dichloromethane and 1,1,1-trichloroethane	All kinds of cosmetics products	GC-TCD
	Nitromethane	Aerosols	GC-FID

[a]UV filters, tanning and whitening agents, colouring agents, preservatives, fragrances and surfactants are not included.
[b]**ECD**, electron capture detector; **FAAS**, flame atomic absorption spectrometry; **FID**, flame ionization detector; **GC**, gas chromatography; **LC**, liquid chromatography; **TCD**, thermal conductivity detector; **UV/VIS**, ultraviolet/visible spectrometry.

As can be seen, some groups of analytes considered in this section have no EU official method for analysis.

Next, analytical techniques and sample preparation procedures are summarized.

Analytical techniques

Alcohols, phenols and polyphenols

The official analytical method to determine methanol in aerosol is based on gas chromatography (GC) with a thermal conductivity detector (TCD). For all other cosmetic formats, GC with a flame ionization detector (FID) is proposed.

The determination of hexachlorophene is performed by GC with electron capture detection (ECD).

Amines, amides, aminoacids and other nitrogenated compounds

The method for the determination of ammonia in cosmetics is based on the use of potentiometry by using sulphuric acid as titrant.

Elements

Flame atomic absorption spectrometry (FAAS) is proposed to determine silver nitrate (by means of silver determination), selenium disulphide (by means of selenium determination) zirconium and aluminium. Zinc is determined gravimetrically.

Heterocyclic compounds

A method based on ultraviolet/visible spectrometry (UV/VIS) is proposed for the determination of oxyquinoline and its sulphate. On the other hand, liquid chromatography (LC) with UV/VIS detector is used for the determination of quinine and its salts.

Hydroxides, peroxides and other oxygenated compounds

Official methods to determine potassium and sodium hydroxides are acid–base volumetries. Hydrogen peroxide is determined by a redox volumetry, where the iodine generated from the reaction within hydrogen peroxide and iodide is titrated by using sodium thiosulphate.

Inorganic anions

The employed analytical techniques are potentiometry for the determination of chloride and alkali chlorates, UV/VIS for nitrite determination, GC-FID for fluoride derivatives, and LC with UV/VIS detector for iodate. Volumetry has also been applied for the determination of sulphides and sulphites.

Organic acids and its derivatives

Volumetry is proposed in both cases for the determination of oxalic (and its alkali salts) and phenosulphonic acids.

Thiocompounds

GC-FID is proposed to determine thioglycolic acid and its salts and esters.

Other organic compounds

Table 8.8.1 also shows the official methods to be applied in the EU framework for the determination of chloroform and nitromethane (GC-FID), and dichloromethane and 1,1,1-trichloroethane (GC-TCD).

Sample preparation

Before analysis, official methods shown in Table 8.8.1 propose different pretreatments according to analyte, sample and technique. Most commonly used are:

- Simple steps such as dissolution of samples in a suitable solvent (or mixture), filtration or centrifugation, evaporation of solvent, distillation, etc.

- Precipitation of analyte (e.g. gravimetric determination of zinc by precipitation using 2-methyl-8-quinolyl oxide as reagent) or interferent (e.g. precipitation of anions causing interferences in ammonia determination by using $BaCl_2$).
- Dispersion of sample and lixiviation of the analyte (e.g. before GC determination of chloroform).
- Liquid–liquid extraction of analyte or interferents (e.g. before GC determination of fluoride).
- Redox reactions before titration (e.g. those carried out for the determination of hydrogen peroxide, sulphide or chlorate).
- Reactions to form a coloured compound before UV/VIS (e.g. those performed in the nitrite or oxyquinoline determinations).
- Derivatization before GC determination (e.g. those carried out for thioglycolic or hexachlorophene determination).
- Dry or wet sample digestion before FAAS determination (e.g. in organomercurial compounds determination as mercury or selenium sulphide determination as selenium).

PUBLISHED METHODS

Published articles have been classified according to which group analytes are considered. Table 8.8.2 gives some details of the procedures. The complete references of the articles are given at the end of the section.

Next, analytical techniques and sample preparation procedures are summarized. It should be emphasized that some publications (particularly in the case of non-recent and non-English publications) were reviewed on the basis of their respective abstracts, and thus, several data may be incomplete as shown in the aforementioned table.

Analytical techniques

Alcohols, phenols and polyphenols

Methods to determine alcohols and phenols in different cosmetic products can be found in the literature. LC and GC techniques are the most commonly used.

Less studied are polyphenols, which have interesting cosmetic properties. LC is the technique used for their determination.

Amines, amides, amino acids and other nitrogenated compounds

Different types of amines have been determined in shampoos and other cosmetics. LC and GC are the most frequently used techniques. The determination by fluorimetry (FL) requires the derivatization of the analytes.

Urea and allantoine have been analyzed in different types of cosmetics.

Amino acids such as carbocysteine, cysteine, glycine or lysine have been determined by LC or UV/VIS spectrometry. Electroanalytical techniques and capillary electrophoresis (CE) have also been proposed.

Nitrosamines have been widely studied as they are toxic by-products. LC and GC are the most frequently used techniques. Most studied compounds are *N*-nitrosodiethanolamines.

Table 8.8.2

Published methods for the analytes considered in this section[a]

Analyte	Sample	Technique[b]	Reference
Alcohols, phenols and polyphenols			
Alcohols			
■ 2-Chloroethanol	Shampoos	GC	Sasaki *et al.* (1993)
■ Ethanol		IR	Bernardini *et al.* (1986)
■ 2-Ethoxyethanol	Emulsions, lotions, nail lacquers	LC	Mariani *et al.* (1999)
■ Ethylene glycol		HS-GC	Wala Jerzykiewicz and Szymanowski (1998)
■ Glycerin	Hair-care products	Volumetry	Loginova *et al.* (1988)
	Toothpaste	CZE	Fang *et al.* (1996)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
■ Hinokitiol		LC	Hanafusa *et al.* (1989)
	Toothpaste	LC	Endo *et al.* (1988)
■ Methanol		HS-GC	Shi (1993)
		HS-GC	Yan (1989)
■ Sorbitol	Toothpaste	CZE	Fang *et al.* (1996)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
Phenols			
■ Aloenin	Skin care	GC-MS	Nakamura *et al.* (1989)
	Skin care	GC-MS	Nakamura and Okuyama (1990)
■ Aloins		CE	Girelli *et al.* (2001)
■ Barbaloin	Skin care	GC-MS	Nakamura and Okuyama (1990)
■ 4-*t*-Butylphenol	Cream	LC	Gagliardi *et al.* (1989)
■ Triclosan	Toothpaste	LC	Demkowicz *et al.* (1994)
■ Others/several	toothpaste, shampoo	LC	Dondi *et al.* (1984)
Polyphenols			
■ Biochanin-A	Creams	Sensors	Beissenhirtz *et al.* (2003)
■ Catechins		LC-MS	Frauen *et al.* (2002)
■ Flavonoids	Shaving cream	LC	Bruschi *et al.* (2003)
Amines, amides, aminoacids and other nitrogenated compounds			
Alkanolamines			
■ Alkanolam-ines	Shampoo	LC	Nakae *et al.* (1981)
	Shampoo	LC	Fukui *et al.* (1992)
■ Diethanolamine		GC, LC	Chou (1998)
Amides			
■ Allantoin and its derivatives		LC	Liu (2003)
	Cream	LC	Dallet *et al.* (2000)
	Lotion, gel	CZE	Geise *et al.* (1994)
		LC	Trivedi (1988)
	Toothpaste, shampoo	LC	Kawase *et al.* (1982)
	Cream, lotion	LC	Nakao *et al.* (1982)
■ Triclocarban	Personal care	TLC	Marijan and Marinan (2002)
■ Urea	Cream	LC	Dallet *et al.* (2000)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
Amines			
■ Alkylamines, diamines		GC	Anon (1994)
■ Secondary amines		LC-FL	Khalaf and Steinert (1996)
Aminoacids			
■ Carbocysteine	Shampoo	UV/VIS	Zaia *et al.* (1999)
	Shampoo	LC	Hoffmann *et al.* (1981)
■ Cysteine	Hair care	Electrochemical	Wang and Chen (1997)
	Hair care	UV/VIS	Teshima *et al.* (2001)
	Shampoo	UV/VIS	Zaia *et al.* (1999)
■ Glycine	Anti-perspirant	LC	Chin and Achari (1982)
■ Lysine	Cream	LC	Dallet *et al.* (2000)
Ammonia derivatives			
■ Domiphen bromide		LC	Wyhowski de Bukanski (1987)
■ Quaternary ammonium compounds	Hair care	LC	Toomey *et al.* (1997)
Nitrosamines			
■ Methylalkylnitrosamines		GC	Kamp and Eisenbrand (1991)
■ *N*-nitrosodiethanolamine		LC	Takeuchi *et al.* (1979)
		LC	Billedeau *et al.* (1994)
		CL	Brennan and Frank (1983)
		LC	Diallo *et al.* (1996)
	Emulsion, shower gel	GC	Sommer *et al.* (1989)
		Polarography	Chou *et al.* (1982)
	Shampoos, creams	UV/VIS	Telling and Dunnett (1981)
		LC	Fukuda *et al.* (1981)
		LC	Ho *et al.* (1981)
		LC	Klein *et al.* (1981)
		GC	Black *et al.* (1981)
		GC, LC	Takeuchi *et al.* (1980)
		GC	Rollmann *et al.* (1981)
		GC, LC	Chou (1998)
■ Others/several		UV/VIS	Baptist and Brown (1980)
	Care products	GC	Sine (1986)
		LC	Rosenberg *et al.* (1980)
		LC	Rosenberg *et al.* (1979)
		LC	Collier *et al.* (1988)
		LC	Vohra and Harrington (1981)
		CL	Chou *et al.* (1987)
	Shampoo	GC	Sommer and Eisenbrand (1988)
Others			
Chlorhexidine	Toothpaste	LC	Kishi *et al.* (1989)
	Toothpaste	UV/VIS, titration	Borissova and Mandjukova (1997)
Drugs and forbidden biological ingredients			
■ Clobetasol propionate	Anti-dandruff shampoo	LC	Gagliardi *et al.* (2000)
		LC-MS	Reepmeyer *et al.* (1998)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
■ Corticosteroids (several)		LC	Kamata *et al.* (1982)
	Lotion, shampoo, Cream, spray	LC, TLC	Gagliardi *et al.* (2002)
	Shampoo	LC-MS	Panusa *et al.* (2004)
■ Ethinyloestradiol		LC	Kamata *et al.* (1980a)
■ Oestradiol		LC	Kamata *et al.* (1980a)
■ Lidocaine		TLC	Schmahl (1980)
■ Methylprednisolone acetate	Lotion	LC-IR, LC-MS	Zhang *et al.* (2003)
■ Sexual hormones (several)		LC	Zhao *et al.* (2004)
■ Stilboestrol		LC	Kamata *et al.* (1980b)
Elements			
■ Aluminium	Antiperspirants	NAA	Kanias (1984)
	Toothpaste	FL	Simeonov *et al.* (1982)
	Oral products	PIGE, PIXE	Olabanji *et al.* (1996)
	Deodorants	LC	Palmieri and Fritz (1987)
■ Antimony		XRFS	Wakisaka (1998)
	Shampoos, shaving Preparations, Deodorants	UV/VIS, electrochemical	Demanze *et al.* (1984)
	Deodorants	LC	Palmieri and Fritz (1987)
■ Arsenic		FAAS	Zhu (2002)
		ICP-AES	Song *et al.* (2000)
		ICP-AES	Mo *et al.* (1999)
		XRFS	Wakisaka *et al.* (1996a)
	Shampoos, shaving preparations, deodorants	UV/VIS, electrochemical	Demanze *et al.* (1984)
		Voltammetry	Kotoucek *et al.* (1993)
■ Bismuth		UV/VIS	Hasebe and Taga (1982)
		ICP-AES	Mo *et al.* (1999)
■ Boron		γR	Rajesh and Subramanian (1995)
■ Cadmium		FAAS	Hou *et al.* (2003)
		FAAS	Maslowska and Legedz (1984)
		ICP-AES	Mo *et al.* (1999)
		voltammetry	Xie (1997)
		FAAS	Vondruska (1995)
	Shampoos, shaving preparations, deodorant	UV/VIS, electrochemical	Demanze *et al.* (1984)
	Toothpaste	Voltammetry	Gevorgyan *et al.* (1998)
■ Chromium		FAAS	Hou *et al.* (2003)
	Shampoos, shaving	ICP-AES	Mo *et al.* (1999)
		UV/VIS,	Demanze *et al.* (1984)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
	preparations, deodorants	electrochemical XRFS	Wakisaka (1998)
■ Cobalt		FAAS	Hou *et al.* (2003)
■ Copper		FAAS	Maslowska and Legedz (1984)
	Nail-lacquers	XRFS	Misra *et al.* (1992)
		UV/VIS	Maslowska and Szmich (1983)
	Toothpaste	Voltammetry	Gevorgyan *et al.* (1998)
	Toothpaste	UV/VIS	Zelenetskaya *et al.* (1983)
■ Gold	Cream	UV/VIS	Balcerzak (2004)
■ Hafnium	Deodorants	LC	Palmieri and Fritz (1987)
■ Iron	Nail-lacquers	XRFS	Misra *et al.* (1992)
		NAA	Kanias (1987)
	Eye shadows, blushes, compact powders	Voltammetry	Cepria *et al.* (2003)
	Deodorants	LC	Palmieri and Fritz (1987)
■ Lead		FAAS	Hou *et al.* (2003)
		UV/VIS	Fan and Dong (2001)
		FAAS	Maslowska and Legedz (1984)
		Polarography	Maslowska and Wojtysiak (1982)
		UV/VIS	Li (2003)
		FAAS	Zhu (2002)
		Polarography	Li *et al.* (2000)
		ICP-AES	Song *et al.* (2000)
		ICP-AES	Mo *et al.* (1999)
		FAAS	Xu *et al.* (1997)
	Care products	Polarography	Liang and Li (1991)
		FAAS	Wu *et al.* (1985)
	Talc or body powders	Electrochemical	Yang and Leng (1988)
	Toothpaste	UV/VIS	Fu *et al.* (2000)
	Shampoo	FI-UV/VIS	Bao *et al.* (2000)
	Toothpaste	ETAAS	Zhang *et al.* (1999a)
	Shampoo	FAAS	Luo and Liu (1998)
	Toothpaste	ETAAS	Khammas *et al.* (1989)
	Shampoos, shaving preparations, deodorant	UV/VIS, electrochemical	Demanze *et al.* (1984)
	Toothpaste	FAAS	Ma (1988)
■ Magnesium	Oral products	PIGE, PIXE	Olabanji *et al.* (1996)
■ Mercury		AFS	Qin *et al.* (2004)
		UV/VIS	Zhang *et al.* (2004)
		FAAS	Zhu(2002)
		ICP-AES	Song *et al.* (2000)
	Eye shadow, eye-pencil, eye-liner	FI-AFS	Gamiz and Luque (1999b)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
		ETAAS	Zhang *et al.* (1999b)
		XRFS	Wakisaka (1998)
		Voltammetry	Wang *et al.* (1996a)
		FAAS	Ma *et al.* (1990)
		FAAS	Zhang *et al.* (1998)
	Shampoos, shaving preparations, deodorant	UV/VIS, electrochemical	Demanze *et al.* (1984)
■ Nickel		FAAS	Hou *et al.* (2003)
■ Niobium	Deodorants	LC	Palmieri and Fritz (1987)
■ Phosphorus	Oral-care products	PIGE, PIXE	Olabanji *et al.* (1996)
■ Selenium			Revanasiddappa
		UV/VIS	and Kumar (2001)
	Shampoo	ICP-AES	Lopez Molinero *et al.* (2002)
		UV/VIS	Revanasiddappa and Kiran-Kumar (2002)
		ICP-AES	Mo *et al.* (1999)
		UV/VIS	Agrawal *et al.* (1998)
	Shampoo	UV/VIS	Ensafi and Lemraski (2004)
	Shampoo	UV/VIS	Gurkan and Akcay (2003)
	Shampoo	UV/VIS	Parham and Jafarpoor (2001)
	Shampoo	UV/VIS	Afkhami and Mosaed (1999)
	Shampoo	ICP-AES	Salvador *et al.* (2000)
	Shampoo	AFS	Gamiz and Luque de Castro (1999a)
	Shampoo	UV/VIS	Safavi *et al.* (1999)
	Shampoo	UV/VIS	Amin and Zareh (1996)
	Shampoo	UV/VIS	Safavi and Afkhami (1995)
	Shampoo	UV/VIS	Ensafi and Dehaghi (1995)
	Shampoo	UV/VIS	Pathare and Sawant (1995)
	Shampoo	LSC	Ramesh *et al.* (1994a)
	Shampoo	UV/VIS	Ramesh *et al.* (1994b)
	Shampoo	LC	Khuhawar *et al.* (1992)
	Shampoo	UV/VIS	Afkhami *et al.* (1992)
	Shampoo	UV/VIS	Sanz *et al.* (1988)
	Shampoo	Gravimetry	Bertini *et al.* (1982)
■ Silver		Coulometry	Demkin (2003)
■ Sodium	Oral products	PIGE, PIXE	Olabanji *et al.* (1996)
■ Strontium		ICP-AES	Mo *et al.* (1999)
■ Sulphur		LC	Tsuji *et al.* (1996)
		ICP-AES	Bettero *et al.* (1984a)
	Shampoo, lotions	ICP-AES	Betttero *et al.* (1984b)
		LC	Nakamura and Morikawa (1983)
■ Tellurium	Shampoo	UV/VIS	Amin and Zareh (1996)
■ Uranium	Toothpaste	Nuclear track detector (fission-track density measurement)	Singh and Virk (1983)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
■ Vanadium		UV/VIS	Sikalos *et al.* (2000)
		XRFS	Wakisaka (1998)
	Shampoo	UV/VIS	Safavi *et al.* (1999)
■ Zinc		FAAS	Maslowska and Legedz (1984)
		UV/VIS, FAAS	Maslowska and Legedz (1982)
		FI-UV/VIS	Ayora Canada *et al.* (1998)
		NAA	Kanias (1987)
	Shampoo	ICP-AES	Salvador *et al.* (2000)
	Toothpaste	Voltammetry	Gevorgyan *et al.* (1998)
■ Zirconium	Deodorants	LC	Palmieri and Fritz (1987)
■ Others/several	Face creams	XRFS	Yap and Leenanupan (1986)
		FAAS, ICP-AES	Kubrakova *et al.* (1997)
		NAA	Kanias (1985)
		ICP-AES	Besecker *et al.* (1998)
	Shampoo	ICP-MS	LeBlanc *et al.* (1999)
		XRFS	Wakisaka *et al.* (1996b)
	Cream	FAAS	Heisz (1990)
		FAAS	Weber and Beil (1981)
		XRFS	Wakisaka *et al.* (1996b)
Heterocyclic compounds			
■ 1,4-Dioxane	Shampoo	GC-MS	Rastogi (1990)
		LC	Scalia *et al.* (1990)
		GC	Helms (1988)
	Shampoo	GC	Wala Jerzykiewicz and Szymanowski (1998)
■ 3-Pyridinemethanol	Oral-hygiene products	LC	De Orsi *et al.* (2000)
■ Potassium acesulfame		LC	Grosspietsch and Hachenberg (1980)
■ Nitrogen containing heterocyclic compounds		GC	Anon (1994)
■ Cetylpyridinium chloride		LC	Benassi *et al.* (1989)
■ Ciclopirox olamine	Anti-dandruff	LC	Gagliardi *et al.* (1998)
■ Ketoconazole	Shampoo	FL	El-Bayoumi *et al.* (1997)
■ Quinine	Shampoo	Sensors	Ortega-Algar *et al.* (2004)
	Shampoo	LC	Cavazzutti *et al.* (1983)
■ Cinchonidine, cinchonine, quinidine, quinine, their dihydroderivatives	Shampoo, balsam	LC	Gatti *et al.* (2004)
■ Saccharin	Toothpaste, lip-gloss, mouth-wash	TLC	Piekacz and Kiss (1983)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Toothpaste	UV/VIS	Hernandez-Cordoba *et al.* (1985)
■ Sanguinarine	Oral care	LC	Thorne *et al.* (1986)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
■ Zinc pyrithione	Hair-care products	Voltammetry	Wang (2000a)
	Shampoo, hair rinses	LC	Kondoh and Takano (1987)
	Hair care	FI-electrochemical	Shih *et al.* (2004)
	Shampoo	LC	Ferioli *et al.* (1995)
	Shampoo	LC	Nakajima *et al.* (1993)
	Shampoo	LC	Nakajima *et al.* (1990)
	Shampoo	LC	Fenn and Alexander (1988)
	Shampoo	LC	Cheng and Gadde (1984)
		LC	Zubata *et al.* (2000)
	Shampoo	CE	Pena-Mendez *et al.* (1997)
	Shampoo	LC	Gagliardi *et al.* (1998)
Hydroxides, peroxides and other oxygenated compounds			
■ Hydrogen peroxide		Sensors	Campanella *et al.* (1998)
		Sensors	Wang *et al.* (1993)
	Dental gels	NIR	Woo and Kim (2004)
	Toothpaste	Amperometry	Mulchandani *et al.* (1995)
■ Hydroperoxides		Sensors	Campanella *et al.* (2003)
■ Organic peroxides		Sensors	Garcia Moreno *et al.* (2001)
		TLC	Wang *et al.* (1992)
■ Peroxides		Electrochemical	Li *et al.* (1998)
■ Oxygenated compounds		GC	Lakszner and Szepesy (1988)
Lipids: fats, oils, waxes			
■ Ceramides		LC-MS	Lee *et al.* (2002)
■ Lanolin and its derivatives	Lanolin	GC-MS, TLC	Domínguez *et al.* (2003)
		GC	Reiter-Reimers and Baltes (1986a)
		SFC	King (1998)
■ Waxes and greases		GC	Giles (1987)
Lanolin (wool wax)	Ointment or lotion	GC	Reiter Reimers and Baltes (1986b)
		Enzymatic	Orlick and Montag (1982)
■ Jojoba-wax	Creams	TLC	Studer and Traitler (1985)
■ Oil	Emulsions	NMR	Brosio *et al.* (1982)
■ Phosphatidylcholine		GC	Liem *et al.* (1980)
Inorganic anions			
■ Bromate	Toothpaste	CE	Harakuwe and Haddad (1996)
■ Carbonate	Toothpaste	Electrochemical	Takano *et al.* (1985)
	Toothpaste, tooth-powder	TGA	Wesolowski (1986)
	Toothpaste	CE	Harakuwe and Haddad (1996)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Toothpaste	Electrochemical	Takano *et al.* (1985)
■ Chloride	Oral products	PIGE, PIXE	Olabanji *et al.* (1996)
	Toothpaste	CE	Harakuwe and Haddad (1996)
	Hair care, toothpaste	LC	Dionex (2000)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
	Toothpaste	LC	Alltech (2000)
■ Cyanide		Electrochemical	Jovanovic *et al.* (1987)
■ Fluoride	Mouth washes	TGA	Salman *et al.* (1997)
	Toothpaste	UV/VIS	Zhang and Jia (2003)
	Toothpaste	FL	Fan *et al.* (2001)
	Toothpaste	FI-UV/VIS	Themelis and Tzanavaras (2001)
	Toothpaste	Potenciometry	Metrohm (2001b)
	Toothpaste	Electrochemical	Metrohm (2001a)
	Toothpaste	FI-UV/VIS	Zhou *et al.* (2000)
	Toothpaste	SI-ISE	Van-Staden *et al.* (2000)
	Toothpaste	LC	Hummrich *et al.* (1998)
	Toothpaste	CE	Wang and Li (1997)
	Toothpaste	CE	Wang *et al.* (1997)
	Toothpaste	CZE	Harakuwe and Haddad (1997)
	Toothpaste	UV/VIS	Sandulescu *et al.* (1996)
	Toothpaste	FL	Ji *et al.* (1996)
	Toothpaste	Electrochemical	Ivanova and Christova (1995)
	Toothpaste	LC	Demkowicz *et al.* (1994)
	Toothpaste	Electrochemical	Heidbuechel (1991)
	Toothpaste	LC	Sendra *et al.* (1990)
	Toothpaste	Electrochemical	Hattab (1989)
	Toothpaste	UV/VIS, GC	Black and Matthews (1989)
	Toothpaste	LC	Potter *et al.* (1986)
	Toothpaste	Electrochemical	Powley and Nieman (1982)
	Toothpaste	Electrochemical	Avsec and Kosta (1981)
	Toothpaste	FI-potenciometry	Landry *et al.* (1981)
	Oral products	PIGE-PIXE	Olabanji *et al.* (1996)
	Toothpaste	UV/VIS	Gomez *et al.* (1993)
	Toothpaste	LC	Alltech (2000)
	Toothpaste	CE	Harakuwe and Haddad (1996)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Hair care, toothpaste	LC	Dionex (2000)
	Toothpaste	FL, potenciometry	Simeonov *et al.* (1982)
■ Iodate	Toothpaste	CE	Harakuwe and Haddad (1996)
■ Iodide	Toothpaste	CE	Harakuwe and Haddad (1996)
■ Monofluorophosphate	Toothpaste	CE	Wang and Li (1997)
	Toothpaste	CE	Wang *et al.* (1997)
	Toothpaste	Titration	Borissova *et al.* (1993)
	Toothpaste	CE	Skocir *et al.* (1993)
	Toothpaste	FI	Tzanavaras and Themelis (2001)
	Toothpaste	FI	Themelis and Tzanavaras (2001)
	Toothpaste	Electrochemical	Pavic *et al.* (1999)
	Toothpaste	LC	Talmage and Biemer (1987)
	Toothpaste	LC	Alltech (2000)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
	Hair care, toothpaste	LC	Dionex (2000)
	Toothpaste	LC	Potter *et al.* (1986)
	Toothpaste	CE	Compiano *et al.* (1993)
■ Nitrate	Toothpaste	LC	Chen *et al.* (1985)
	Oral products	Electrochemical	Perez Olmos *et al.* (2000)
	Toothpaste	LC	Lulla *et al.* (1984)
	Toothpaste	LC	Chen *et al.* (1985)
		CL	Chou and Yates (1998)
		LC	Rosenberg *et al.* (1979)
	Toothpaste	UV/VIS	Geetha and Balasubramanian (2001)
	Toothpaste	LC	Lulla *et al.* (1984)
	Toothpaste	CE	Harakuwe and Haddad (1996)
	Hair care, toothpaste	LC	Dionex (2000)
	Hair care, toothpaste	LC	Dionex (2000)
	Toothpaste	LC	Talmage and Biemer (1987)
■ Nitrite	Toothpaste	CE	Harakuwe and Haddad (1996)
■ Phosphate		Titration	Borissova *et al.* (1993)
	Toothpaste	CE	Skocir *et al.* (1993)
	Toothpaste	CE	Wang and Li (1997)
	Toothpaste	CE	Shamsi and Danielson (1995)
	Toothpaste	LC	Alltech (2000)
	Toothpaste	CE	Harakuwe and Haddad (1996)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Hair care, toothpaste	LC	Dionex (2000)
	Hair care, toothpaste	LC	Dionex (2000)
	Toothpaste	LC	Demkowicz *et al.* (1994)
■ Pyrophosphate	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Hair care, toothpaste	LC	Dionex (2000)
■ Sulphate	Toothpaste	CE	Harakuwe and Haddad (1996)
	Hair care, toothpaste	LC	Dionex (2000)
	Toothpaste	LC-MS	Cavalli *et al.* (2004)
■ Sulphide	Toothpaste	Potenciometry	Sun (1988)
■ Thiocyanate	Toothpaste	CE	Harakuwe and Haddad (1996)
■ Triphosphoric acid	Toothpaste	LC-MS	Cavalli *et al.* (2004)
	Hair care, toothpaste	LC	Dionex (2000)
■ Others/several	Toothpaste	CZE	Compiano *et al.* (1993)
	Toothpaste	LC	Saari Nordhaus *et al.* (1992)
	Toothpaste	LC	Gennaro and Bertolo (1990)
	Toothpaste	LC	Hayakawa *et al.* (1989)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
Organic acids and derivatives			
Alpha-hydroxyacids			
■ Alpha-hydroxyacids (several)		LC	Huang *et al.* (2002)
		LC	Hempel (1998)
■ Citric acid		LC	Irache *et al.* (1993)
		LC	Nakamura *et al.* (1980)
	Hair care, toothpaste	LC	Dionex (2000)
■ Glycolic acid		Sensors	Tsiafoulis *et al.* (2002)
	Day cream, gel, shampoo	LC	Scalia *et al.* (1998)
	Cream	LC	De Villiers *et al.* (1998)
	Hair care, toothpaste	LC	Dionex (2000)
	Cream, gel	IR	Salvador *et al.* (2001)
■ Isocitric acid	Hair care, toothpaste	LC	Dionex (2000)
■ Lactic acid	Cream	LC	De Villiers *et al.* (1998)
		LC	Irache *et al.* (1993)
		LC	Nakamura *et al.* (1980)
	Hair care, toothpaste	LC	Dionex (2000)
	Cream, gel	IR	Salvador *et al.* (2001)
■ Malic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Tartaric acid		LC	Irache *et al.* (1993)
	Hair care, toothpaste	LC	Dionex (2000)
Others			
■ Acetic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Aconitic acid	Hair care, toothpaste	LC	Dionex (2000)
■ 4-Aminosalicylic acid		ITP	Goto *et al.* (1985)
■ Asiatic acid		LC	Morganti *et al.* (1999)
■ Asiaticoside		LC	Morganti *et al.* (1999)
■ Benzoic acid		ITP	Goto *et al.* (1985)
■ Ethylenediaminetetraacetic acid (EDTA)		LC	Irache *et al.* (1993)
■ Fatty acids		GC	Challinor (1996)
■ Fatty acids and their esters		TLC	Pore and Rasori (1982)
■ Formic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Fumaric acid		ITP	Goto *et al.* (1985)
	Hair care, toothpaste	LC	Dionex (2000)
■ Glutaric acid	Hair care, toothpaste	LC	Dionex (2000)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
■ Glycyrrhetinic acid	Toothpaste	LC	Andrisano *et al.* (1993)
	Hair care	LC	Mori *et al.* (1999)
	Toothpaste	LC	Andrisano *et al.* (1995)
■ Glycyrrhetinic acid glycyrrhetinyl stearate	Toothpaste	LC	Li (1999)
		LC	Okaya and Haruyama (1993)
		LC	Haruyama and Okaya (1994)
■ Glycyrrhizic acid	Care products	LC	Okaya and Haruyama (1993)
		LC	Tsubone *et al.* (1982)
		LC	Mikami *et al.* (1988)
	Toothpaste	LC	Andrisano *et al.* (1993)
■ Linoleic acid	Shampoo	GC	Bertini *et al.* (1982)
■ Liquiritic acid	Toothpaste	LC	Andrisano *et al.* (1995)
■ Madecassic acid		LC	Morganti *et al.* (1999)
■ Malonic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Oxalic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Phthalates		GC, GC-MS	Chen *et al.* (2004a)
		LC	Chen *et al.* (2004b)
		GC-MS	George and Prest (2002)
	Nail cosmetics	LC	De Orsi *et al.* (2006)
	Nail lacquers, gels and creams	GC GC-MS	Chen *et al.* (2005)
	Branded hair sprays, deodorants, nail polishes	LC	Koo and Lee (2004)
■ Pyroglutamic acid		LC	Nakamura *et al.* (1980)
■ Retinoic acid		Voltammetry	Wang (2000b)
■ Salicylic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Succinic acid	Hair care, toothpaste	LC	Dionex (2000)
■ Sucrose fatty acid esters		LC	Jaspers *et al.* (1987)
		LC	Kaufman and Garti (1981)
■ Carboxylic esters	Toothpaste	LC	Hayakawa *et al.* (1989)
Polymers (naturals and synthetics)			
■ Carrageenan	Toothpaste	UV/VIS	Guvener *et al.* (1988)
■ Caseinoglycomacropeptide	Lotion	CE	Cherkaoui *et al.* (1999)
■ Collagen		SDS-PAGE	Valenta and Gabor (1992)
■ DNA		PCR	Waiblinger and Pietsch (1999)
■ Dimethicone	Lotion	IR	Sabo *et al.* (1984)
■ Gums	Toothpaste	TLC	Mann (1987)
■ Polyethyleneglycol (PEG)		MEKC, CZE	Oudhoff *et al.* (2003)
■ Polydimethylsiloxane		GC-IR	Clarke and Willis (1999)
■ Polypropyleneglycol (PPG)		MEKC, CZE	Oudhoff *et al.* (2003)
■ Silicones		FAB-MS, GC-MS	Facino *et al.* (1997)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
		IR, ICP-AES, GC-MS	Watanabe *et al.* (1985)
■ Sodium chondroitin sulphate		ITP	Yamamoto *et al.* (1982)
Thiocompounds			
■ 2,2′ -Dithiobis related compounds	Shampoo	LC	Fenn and Csejka (1979)
■ Aliphatic thiols		TLC	Roveri *et al.* (1993)
■ Taurine		LC-ICP-AES	Yoshizawa *et al.* (1995)
■ Thioglycolic acid		Potenciometry	Gerakis and Koupparis (1993)
	Hair care	Electrochemical	Wang and Chen (1997)
		LC	Cavrini *et al.* (1990)
	Depilating creams	LC	Rooselaar and Liem (1982)
	Hair care, toothpaste	LC	Dionex (2000)
Vitamins			
Vitamin A related compounds			
■ Beta-carotene		LC	Wang and Huang (2002)
■ Retinal		LC	Haruyama *et al.* 1(995)
	Cream, gel	Voltammetry	Wang (2000a)
■ Retinal isomers, retinol and photo-oxidation products		LC	Ceugniet *et al.* (1998)
■ Retinol		Raman spectrometry	Failloux *et al.* (2003)
	Cream, gel	Voltammetry	Wang (2000a)
■ Retinyl palmitate		LC	Wang and Huang (2002)
	Cream, lotion	LC	Scalia *et al.* (1995)
		LC	Scalzo *et al.* (2004)
Vitamin B related compounds			
■ Panthenol	Lotion	ITP	Goto *et al.* (1985)
	Shampoo, hair gel, hair cream, hair tonic, skin cream	Voltammetry	Wang and Tseng (2001)
		LC	Liu (2003)
		FL, UV/VIS	Shehata *et al.* (2002)
		LC	Haruyama *et al.* (1995)
■ Panthenyl ethyl ether		LC	Haruyama *et al.* (1995)
	Cream, gel	Voltammetry	Wang (2000a)
■ Pantothenic acid (calcium salt)	Shampoo, hair gel, hair cream, hair tonic, skin cream	Voltammetry	Wang and Tseng (2001)
■ Pyridoxine	Shampoo, balsam	LC	Gatti *et al.* (2004)
Vitamin C related compounds			
■ Ascorbic acid		LC	Irache *et al.* (1993)
	Cream	Sensors	Beissenhirtz *et al.* (2003)
		LC	Baruffini *et al.* (1992)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
Vitamin E related compounds			
■ Alpha-tocopheryl acetate	Hair care	LC	Mori *et al.* (1999)
	Cream, lotion	LC	Scalia *et al.* (1995)
■ Alpha-tocopheryl nicotinate		LC	Baruffini *et al.* (1992)
■ Tocopherols	Toothpaste	LC-MS, LC-NMR	Lienau *et al.* (2002)
	Cream	GC	Yu *et al.* (2000)
Other organic compounds			
Pesticides			
■ Allethrin	Aerosol, shampoo	UV/VIS	Abuirjeie *et al.* (1991)
■ Cinerin	Shampoo	LC	Wang *et al.* (1996b)
■ Lindane	Shampoos	GC	Miles and Mount (1984)
■ Jasmolin	Shampoo	LC	Wang *et al.* (1996b)
■ Organochlorine pesticides	Skin care	GC	Cetinkaya (1988)
■ Organophosphorus pesticides	Skin care	GC	Cetinkaya (1988)
	Toothpaste	LC	Marini and Balestrieri (1988)
■ Permethrin	Shampoo	UV/VIS	Kazemipour *et al.* (2002)
	Shampoo	LC	Manadas *et al.* (1999)
■ Phenothrin	Shampoo	LC	Sakaue *et al.* (1985)
■ Piperonyl butoxide	Shampoo	LC	Manadas *et al.* (1999)
	Shampoo	LC	Wang *et al.* (1996b)
	Shampoo, aerosols	UV/VIS	Abuirjeie *et al.* (1991)
	Shampoos, lotions	GC	Richard and Andermann (1984)
■ Pyrethrin	Shampoo	LC	Wang *et al.* (1996b)
■ Pyrethroids	Shampoo	GC-MS	Krappe *et al.* (1999)
		GC, GC-MS	Zehringer (2001)
■ Tetramethrin	Shampoos, lotiocons	GC	Richard and Andermann (1984)
■ Others/several		GC, GC-MS	Zehringer (2001)
		GC	Mariani *et al.* (1995)
Other			
■ Anethole	Toothpaste	GC	Grob *et al.* (1984)
■ Benzopyrene	Paraffin-wax	GC	Radecki *et al.* (1980)
■ Camphor pentones		MS	Glish and Cooks (1980)
■ Ethylene oxide	Shampoo	GC	Sasaki *et al.* (1993)
		GC	Helms (1988)
		HS-GC	Wala Jerzykiewicz and Szymanowski (1998)
■ Gases	Deodorants	Sensors	Fujimoto *et al.* (1996)
■ Diethyl toluamide, dimethyl phthalate (insect repelents)	Emulsion, gel stick	TLC	Markovic *et al.* (1999)
■ Propellents and solvents		GC	Wyhowski de Bukanski and Masse (1985)
■ Ubiquinone (coenzyme Q10)		LC	Jiang *et al.* (2004)
■ Vinyl chloride	Shampoo	GC	Watson *et al.* (1979)

(*Continued*)

Table 8.8.2 (Cont.)

Analyte	Sample	Technique[b]	Reference
■ Volatile components		IR	Gruber *et al.* (1982)
■ Water		Karl-Fischer	Metrohm (2001c)
		IR	Bernardini *et al.* (1986)
		NMR	Brosio *et al.* (1982)
	Toothpaste	Karl-Fischer	Isengard and Schmitt (1995)
		GC	Feng *et al.* (1996)
■ Others/several	Toothpaste	GC	Takeoka and Jennings (1984)
	Oil-rich cosmetics	LC	Yamamoto *et al.* (1987)

[a]UV filters, tanning and whitening agents, colouring agents, preservatives, fragrances and surfactants are not included.

[b]**AES**, atomic emission spectrometry; **AFS**, atomic fluorescence; **CE**, capillary electrophoresis; **CL**, chemiluminescence; **CZE**, capillary zone electrophoresis; **ETAAS**, electrothermal atomic absorption spectrometry; **FAAS**, flame atomic absorption spectrometry; **FAB**, fast atom bombardment; **FI**, flow injection; **FL**, fluorimetry; **GC**, gas chromatography; **γR**, gamma-ray spectrometry; **HS**, head-space; **ICP**, inductive coupled plasma; **IR**, infrared spectrometry; **ISE**, ion selective electrode; **ITP**, isotacophoresis; **LC**, liquid chromatography; **LSC**, liquid scintillation counting; **MEKC**, micellar electrokinetic chromatography; **MS**, mass spectrometry; **NAA**, neutron activation analysis; **NIR**, near infrared spectrometry; **NMR**, nuclear magnetic resonance; **PCR**, polymerase chain reaction; **PIGE**, particle-induced gamma-ray emission spectrometry; **PIXE**, particle-induced X-ray emission spectrometry; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **SFC**, supercritical fluid chromatography; **SI**, sequential injection; **TGA**, thermogravimetric analysis; **TLC**, thin layer chromatography; **UV/VIS**, ultraviolet/visible spectrometry; **XRFS**, X-ray fluorescence spectrometry.

Drugs and forbidden biological ingredients

Corticosteroids in shampoos and other cosmetics are determined by LC-UV/VIS or LC with mass spectrometry (MS) detection. Thin layer chromatography (TLC) has also been proposed. Drugs such as lidocaine are determined by TLC. Hormones are determined by LC procedures.

Elements

In spite that some elements can be used as colouring agents in cosmetics, we have treated all the literature on elements determination in this section.

Analytical procedures have been described for a high number of elements. Se, Pb, Zn, Cd and Hg, among others, are the most commonly studied. Analyzed samples are: creams, shampoos, deodorants, antiperspirants, toothpaste, other oral products and other cosmetics.

The most commonly used are the atomic spectrometric techniques, especially FAAS, electrothermal atomic absorption spectrometry (ETAAS) and inductive coupled plasma with atomic emision spectrometry (ICP-AES). ICP-MS, X-ray fluorescence spectrometry (XRFS), electroanalytical techniques, UV/VIS spectrometry and FL have also been used among others.

Heterocyclic compounds

Table 8.8.2 shows the published procedures to determine heterocyclic compounds. The most commonly studied are saccharin, which is analyzed in different types of cosmetics for lips, teeth and mouth as sweater.

1,4-Dioxan is a contaminant arising from the manufacturing process and has been widely studied.

Zn pyrithione is a compound specially used in hair-care cosmetics as anti-seborrheic. It has been well studied. LC has been the most used technique, although other procedures based on flow injection with electroanalytical detection and CE have also been employed. Other anti-seborrheic, ciclopirox olamine, has been also determined by LC.

Quinine has been determined by LC and also by methods based on the use of sensors. Ketoconazole has been determined by spectrofluorimetry.

Hydroxides, peroxides and other oxygenated compounds

Peroxides have been studied in dental and other products. Most of the articles are based on the use of sensors. Some electroanalytical techniques such as amperometry have also been used.

Lipids: fats, oils, waxes

The most commonly used techniques to determine some of these compounds are GC and TLC. Supercritical fluid chromatography (SFC) and nuclear magnetic resonance (NMR) have been used in some cases. Enzymatic procedures have been also proposed.

Lanolin and its derivatives are the most studied ingredients.

Inorganic anions

A wide number of anions have been studied in the different analytical articles. Those most frequently studied are fluoride, monofluorophosphates, phosphate, nitrate, etc. They have been determined in toothpaste, mouthwashes, other oral-care products, shampoos, lotions and other cosmetics. A large variety of analytical methods have been proposed, with the most commonly used being LC, CE, electroanalytical methods, UV/VIS and FL. Some automated techniques such as flow injection and sequential injection analysis have been proposed with different detectors. Electroanalytical and other techniques have been also used as can be seen in Table 8.8.2.

Organic acids and its derivatives

The most frequently studied organic acids are α-hydroxyacids, such as glycolic, citric, lactic, tartaric, etc. Glycyrrhizic and glycyrrhetinic and others can also be determined. Most of the methods are based on the use of LC. CE, GC, infrarred spectrometry (IR) and other techniques are sometimes used. Fatty acids and derivatives are determined by GC or LC.

Polymers (naturals and synthetics)

Different polymers are sometimes used as thickening agents to change the consistency of the cosmetics. Some articles are devoted to polymers determination. Different techniques are used according to the polymer to be determined: TLC, LC, micellar electrokinetic chromatography (MEKC), CE, IR, etc.

Analytical methods for determination of natural polymers, such as proteins and DNA are also proposed.

Thiocompounds

Thiocompounds have been determined using LC or TLC methods.

Vitamins

Vitamins have been widely studied. LC methods are the most common.

Other organic compounds

Some solvents, including water have also been determined in deodorants and other cosmetics by different techniques.

Gases, volatile compounds and other substances have also been determined, as can be seen in Table 8.8.2.

Pesticides and other toxic compounds, such as ethylene oxide, have also been studied.

Sample preparation

Publications shown in Table 8.8.1 refers to analytical methods applied to all types of cosmetic products. Direct dissolution of samples in a suitable solvent (or mixture) was used only for some samples in which dissolution is easy, such as shampoos, lotions and gels. Most part of these analyses required specific sample preparation techniques. Most commonly used are:

- Simple steps as filtration or centrifugation, after the sample dispersion in a suitable solvent are used in most procedures. These techniques allow analyte separation after lixiviation from the matrix.
- Filtration through paper is the most common way for solid separations. Solid separation is usually required before using some techniques, moreover chromatographic and related techniques require sample injection through a microfilter to avoid solid particles which could be dispersed in the sample solution. Sometimes only this last type of filtration is required; an example is the LC (with conductivity detection) determination of nitrate and monofluorophosphate in toothpaste after dispersion of sample in water (Talmage and Biemer, 1987) in which only injection through a 0.45 μm microfilter was needed.
- Centrifugation after dispersion of sample in a suitable solvent has also been used; sometimes followed by filtration. Centrifugation can be found in articles related to fluoride determination in toothpaste by selective ion electrode (Ivanova and Christova, 1995) or by LC (with conductivity detection) (Demkowicz *et al.*, 1994).
- Sonication of the sample dispersions has been used to favour the lixiviation of analytes, as a previous step to the determination, as for α-hydroxyacids determination by LC-UV/VIS (Hempel, 1998; Huang, 2002). It has also been used as a unique treatment or followed by centrifugation and/or filtration.
- Conventional heating of samples has been used in most cases to make easier sample dissolution or analyte lixiviation. Dry or wet mineralization of samples are used specially before elements determination by atomic spectrometry techniques.
- Sometimes digestion or lixiviation can be excesively time-consuming and microwave irradiation can accelerate these processes; some examples are the determination of Pb,

As, Cr, Cd, Sr, Bi and Se in solid cosmetics by ICP-AES (Mo *et al.*, 1999) or the determination of Se and Zn in shampoos by ICP-AES (Salvador et al., 2000).

- Emulsification of samples can be used in liposoluble samples analysis to avoid mineralization, as for example before Cd determination in cosmetic oils (Vondruska, 1995).
- A high number of researchers propose the use of liquid–liquid extraction as a separation step. Solid-phase extraction (SPE) is also used; for instance, organic acids as 18β-glycyrrhetinic and glycyrrhizinic acids (Andrisano *et al.*, 1993) have been determined in toothpaste by LC and glycolic acid (Scalia, 1998) has been determined in shampoo, day cream and gel by LC, after SPE. Solid-phase microextraction (SPME) is still less used in cosmetic analysis, an example is the determination of toxic contaminants in shampoos by GC (Wala Jerzykiewicz and Szymanowski, 1998).
- Some proposed analytical methods are based on the use of distillation or volatilization steps. Head-space technique has been extensively used for GC determination (as for example in the above-mentioned article by Wala Jerzykiewicz and Szymanowski, 1998).
- Sometimes, analytical techniques require a derivatization of the initial form of the analyte to other which provides better analytical properties. Thus, different types of reactions have been used, as hydrolysis or saponification (as for example for hormone determination in cosmetics by LC, Zhao *et al.*, 2004), formation of a coloured complex before UV/VIS spectrometry (as for example for Cu determination in cosmetics, Maslowska and Szmich, 1983), formation of a fluorescent compound (as for example for secondary amines LC determination with fluorimetric detection, Khalaf, 1996).

SUMMARY

From the above mentioned, it can be deduced that new analytical methods have been published during the last years which have incorporated a great number of instrumental techniques and improved the classical sample preparation procedures.

Sample preparation strategies such as sonication, microwave heating, SPE, SPME, etc., analytical techniques such as CE, GC-MS, sensors based on, etc. and automated flow methods should be taken into account in order to improve the official methods. Moreover, toxic organic solvents should be avoided or reduced.

All this new possibilities derived from research in Analytical Chemistry should be studied in-depth and new official methods should be approved covering a high number of analytes. This will allow cosmetic enterprises to carry out their quality control by simple, rapid and environmentally friendly and safe methods.

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– 9 –

Alternative Methods to Animal Testing for Cosmetic Products Evaluation

9.1. Safety Evaluation

M. Herráez Dominguez* and O. Diez Sales

Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Valencia

INTRODUCTION

The EU Cosmetics Directive (76/768/EEC) demands that: "*A cosmetic product put on the market within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use …*". Thus the responsibility for the product safety lies with the cosmetic manufacturer or the person placing a cosmetic product on the Community market, who must be able to demonstrate that this product is safe for consumer use. Concerning assessment of the safety for human health of the finished product, Article 7a (d) states: "*To that end the manufacturer shall take into consideration the general toxicological profile of the ingredients, their chemical structure and their level of exposure*".

On the other hand, the 7th Amendment to the Cosmetics Directive (2003/15/EC) introduces new provisions related to non-animal testing of finished cosmetic products and ingredients. In particular, it bans the testing of finished cosmetic products and cosmetic ingredients on animals and prohibits marketing such products in the European Community. The safety assessment of finished cosmetic products without animal testing is only possible provided that an adequate toxicological data package on the ingredients is available.

*Corresponding author. E-mail: marina.herraez@uv.es

Analysis of Cosmetic Products
Amparo Salvador and Alberto Chisvert

For this reason, recent years have witnessed important efforts being made to develop new alternative methods to ensure the safety of cosmetic preparations without using laboratory animals. These alternative methods involve new techniques to determine a toxicological endpoint that results in "Reduction" (of the number of animals per test), "Refinement" (of the methodologies by reducing the pain and distress of the animals) or "Replacement" (of the animals by non-sentient material). The "Three Rs" provide a strategy for a rational and stepwise approach to minimising animal use without compromising the quality of the scientific work being done, while having, as the ultimate aim, total replacement of animal models with non-animal alternatives (Russell *et al.*, 1959).

In Europe, the validation of alternative methods is officially co-ordinated by the European Centre for the Validation of Alternative Methods (ECVAM), while the USA counterpart is the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The tendency for close collaboration between both organisations exists and joint reports are generated. When an alternative method has been formally validated and accepted by the EU, its use becomes mandatory. Consequently, this implies that animal testing for the same endpoint is prohibited.

Nevertheless, it is important to point out that the laboratories where the different validation methods are carried out should be certified in the execution of the good laboratory practice (GLP). These principles of GLP are a group of rules, procedures and established practices and are developed by the OECD (Organisation for Economic Cooperation and Development). This normative has been assumed by the EU in the directives 87/18/CEE and 99/11/CE: "*The purpose of these principles of good laboratory practice (GLP) is to promote the development of quality test data. Comparable quality of test data forms the basis for the mutual acceptance of data among countries. If individual countries can confidently rely on test data developed in other countries, duplicative testing can be avoided, thereby saving time and resources. The application of these principles should help to avoid the creation of technical barriers to trade, and further improve the protection of human health and the environment*".

On the other hand, the Cosmetics Directive does not specify a fixed data set or methods needed to assess the safety of cosmetic ingredients. Guidelines are given by the SCCNFP (Scientific Committee on Cosmetics and Non Food Products) with regard to the areas of potential toxicity to be addressed (SCCNFP/0690/03). The areas in which important advances have taken place in the use of new alternative methods are:

- Acute systemic toxicity
- Skin corrosion/irritation
- Eye irritation
- Skin sensitisation
- Skin absorption
- Subacute and subchronic toxicity
- Genotoxicity/mutagenicity
- UV-induced toxic effects (phototoxicity, photogenotoxicity, photoallergy)
- Carcinogenicity
- Reproductive and developmental toxicity.

This chapter revises the methods recommended for the different areas listed above, which are included in the EU legislation (Annex V, Part B Council Directive 67/548/EEC and successive amendments on the approximation of the laws in Member States relating to cosmetic products) and in the "OECD testing guidelines (TG) for chemicals" as well as of other alternative methods that, given the current state of the art, should be validated in the near future.

ACUTE TOXICITY

The main objective of acute toxicity testing is basically to classify chemicals according to their intrinsic toxicity as required by the EEC directive on classification, packaging and labelling of dangerous substances (Council Directive 67/548/EEC and subsequent amendments). This requirement aims to protect public health by regulating exposure to potentially dangerous materials.

Chemicals are classified on the basis of the medium lethal dose (LD_{50}) value, defined as "the statistically derived single dose of a substance that can be expected to cause death in 50% of the animals in an experimental group". The LD_{50} test procedure has been modified in various ways to reduce the number of animals required, and to reduce the suffering caused to any animal used (Balls and Fentem, 1993). This modification to the classical LD_{50} test includes:

- the fixed-dose method (B.1 bis, OECD TG 420, 2001a),
- the acute toxic class method (B.1 tris, OECD TG 423, 2001b),
- the up and down procedure (OECD TG 425, 2001c),
- the acute inhalation toxicity (B.2 , OECD TG 403, 1981) and,
- the acute inhalation toxicity—fixed dose procedure, as an alternative to OECD TG 403.

The list of *in vitro* tests cannot be predictive for acute oral toxicity as single methods, but they become a good alternative if integrated in a tiered approach and/or in a test battery.

At present there are no validated alternative methods able to completely replace the use of animals in the field of acute toxicity. Validation of an alternative model for acute oral toxicity is very complex and the time estimated to achieve complete animal replacement for acute toxicity is not clearly defined, nor can it be estimated at anything less than 10 years. However, there are some alternative methods currently available in different validation status: prevalidated (MEIC, Multicenter Evaluation of *in vitro* cytotoxicity tests), under prevalidation (ECVAM), and validation (ECVAM, ICCVAM).

Currently, the methods prevalidated in the MEIC programme (Clemedson *et al.*, 1996) are carried out by means of different cellular lines, such as

- The hepatoma cell line Hep G2 (HepG2 cell/protein content, 24 h), the cells are exposed to the test substances and cytotoxicity is measured as changes in protein content (Dierickx, 1989).
- The human acute promyelocytic leukaemia (HL-60/ATP content, 24 h), after it is exposed to the test substance the ATP content is measured by means of a Lucifer-LU plus kit as the bioluminescence generated from the enzymatic luciferin–luciferase reaction (Wakuri *et al.*, 1993).

– The Chang liver cells are cultured in paraffin-sealed 96-well microtitre plates (24 h). Deficient outgrowth of fusiform or spindle-shaped cells is used as a criterion of cytoinhibition (Ekwall and Sandström, 1978), after the cells have been in contact with the test substance.

Moreover, primary cultures of rat hepatocytes, bovine kidney cells (MDBK) and human epithelial cells to predict the maximum tolerated dose are used. After exposing the cells (24 h for hepatocytes, 24, 48 and 72 h for cell lines) to the test compound, the following parameters of cell growth and morphology are scored: surface occupied by growing cells (cell lines only), changes in cell size and shape, presence of cytoplasmic vacuoles, cell detachment, and dead and dying cells (Shrivastava *et al.*, 1992).

Under prevalidation (ECVAM) we find the transepithelial resistance (TER) and paracellular permeability (PCP) in two renal cell lines (LLC-PK1, epithelial proximal tubular cells and MDCK, epithelial distal cells). TER measurement and the transepithelial transport of uncharged small molecules like FITC-inulin (PCP) are reliable parameters for characterising the intactness of an epithelial barrier. Cells are seeded onto 24-well polycarbonate filter plates and then exposed to test substances. Barrier damage is measured for TER assessment and with fluorescence measurement in the base plate for PCP (Duff et al., 2002).

Under validation (ECVAM, ICCVAM) we have the BALB/c 3T3 neutral red uptake (NRU) cytotoxicity assay and the normal human keratinocyte NRU cytotoxicity assay. Both methods are based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR penetrates cell membranes and accumulates in lysosomes. Alterations in the cell surface lead to decreased uptake and binding of NR (Nottingham and Spielmann, 1995; Spielmann *et al.*, 1999).

Finally, under research and development, there are a number of software packages (quantitative structure activity relationships, QSAR's models) to predict effects on human health and related toxicities (e.g. Toxicity Prediction by Komputer Assisted Technology, TOPKAT model, Accelerys Inc., 2002). These systems are used because they are user friendly and fast and they can also predict toxicity directly from chemical structure (Cronin *et al.*, 2003).

SKIN CORROSION/IRRITATION

The skin is often exposed, either intentionally or unintentionally, to cosmetic products. It is clear that the potential for a particular product/ingredient to cause skin irritation or corrosion needs to be carefully evaluated as part of the overall safety assessment process.

Skin corrosion

Skin corrosion is defined as the production of irreversible tissue damage to the skin: visible necrosis through the epidermis and into the dermis, following the application of a test substance for up to 4 h. Corrosivity is not a feature one expects to find with cosmetics, but can occasionally occur after a manufacturing error or misuse by the consumer. On the

Table 9.1.1

Summary of validated alternative methods for skin corrosion, accepted by regulatory authorities in EU legislation (Annex V of the Dangerous Substances, Directive 67/548/EEC) and in the OECD testing guidelines (TG) for chemicals

Alternative test	Test system	Endpoints	Status (Annex V, EU/ OECD TG)
Rat skin resistance transcutaneous electrical (TER) assay	Rat skin	Stratum corneum integrity and barrier function	B.40/OECD TG 430
EPISKIN™ human skin model (commercial system)	Reconstructed human epidermal	Cell viability (MTT-test)	B.40/OECD TG 431
EpiDerm™ human skin model (commercial system)	Reconstructed human epidermal	Cell viability (MTT-test)	B.40/OECD TG 431
CORROSITEX™ (commercial system)	Biobarrier membrane	Visually detectable change	Validated and endorsed (US and EU) method for skin corrosion testing of acids and bases

other hand, a cosmetic ingredient that has an intrinsic corrosive property is not necessarily excluded for use in cosmetics. It very much depends on its final concentration in the cosmetic product as well as the presence of "neutralising" substances, the excipients used, the exposure route, the conditions of use, etc.

In the past, skin corrosion was assessed using animal studies (OECD TG 404, 2002) but recently three *in vitro* tests for skin corrosivity are used. The rat skin transcutaneous electrical resistance (TER) assay and a test employing a human skin model are included in Annex V of the Dangerous Substances Directive (Directive 2000/33/EC, B.40. Skin corrosion) and also proposed as an OECD draft for testing guidelines TG 430 (OECD, 2004a) and TG 431 (OECD, 2004b) (Table 9.1.1).

The Corrositex™ model was prevalidated and validated by ECVAM Scientific Advisory Committee (ESAC) and had an unacceptably high underprediction rate. Consequently, it was endorsed by ECVAM only for skin corrosion testing of acids, bases and their derivatives (NIH, 1999; ECVAM, 2001). It has not been taken up in the UE legislation, but is, nevertheless, a legal test adopted by the US Department of Transport (US DOT).

Consequently, alternative methods for skin corrosion have been validated and accepted for regulatory use in the EU and the OECD Member Countries, so animal testing should not be performed to this end.

Rat skin transcutaneous electrical resistance test

This *ex vivo* test is used to assess the skin corrosivity of a test substance following topical application to the epidermal surface of skin discs taken from a single rat (Figure 9.1.1).

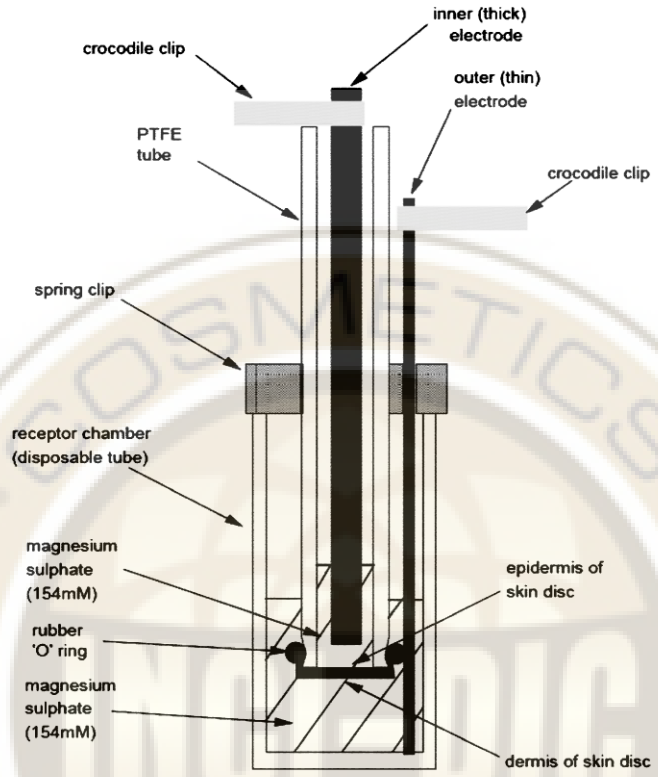

Figure 9.1.1 Device to determine the transcutaneous electrical resistance (TER) test through the rat skin disc (B.40. Skin corrosion, Directive 2000/33/EC).

Table 9.1.2

Values of resistance for positive and negative control (TER test)

Control	Substance	Resistance range (kΩ)
Positive	10 M hydrochloric acid (36%)	0.5–1.0
Negative	Distilled water	10–25

The contact period between the test substance and the skin is 24 h. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level of 5 kΩ (Oliver *et al.*, 1986; Botham *et al.*, 1992). The mean TER results are accepted on condition that concurrent positive and negative control values fall within the acceptable ranges for the method (Table 9.1.2).

Table 9.1.3

Values of dye content range for positive and negative control (TER test)

Control	Substance	Dye content range (μg/disc)
Positive	10 M hydrochloric acid (36%)	40–100
Negative	Distilled water	15–35

If the TER values of test substances, which are either surfactants or neutral organics, are less than or equal to 5 kΩ, an assessment of sulforhodamine B dye penetration can be carried out on the tissues (skin disc) in order to reduce false positives obtained specifically with these types of chemical (Botham *et al.*, 1995; Fentem *et al.*, 1998). The sulforhodamine B dye content is determined for each skin disc (n=3). The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method (Table 9.1.3).

Human skin model assay

The reconstructed human skin models are three-dimensional models generated by growing keratinocyte cultures at the air–liquid interface on various substrates and that enable the topical application of either neat or diluted test materials.

Corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels at specified exposure periods, as determined using the MTT dye [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromid] reduction assay. Skin models such as EPISKIN™ (NIH, 2002) and EpiDerm™ (ECVAM, 2001) are used.

The test material is applied topically for up to 4 h to a three-dimensional human skin model, comprising a reconstructed epidermis with a functional stratum corneum (Figure 9.1.2). For liquid (a minimum of 25 μl/cm²) and solid materials, sufficient test substance must be applied to cover the skin surface.

The principle of the assay is in accordance with the hypothesis that corrosive chemicals are those that are able to penetrate the stratum corneum (by diffusion or erosion) and are sufficiently cytotoxic to cause cell death in the underlying cell layers.

The viability of the living cells in the model must be high enough to discriminate properly between the positive and negative control substances. Cell viability is measured by the amount of MTT reduction: water-soluble yellow dye is converted to insoluble purple-coloured formazan within cells. This technique has been shown to give accurate and reproducible results in various laboratories. The skin disc, after treatment with the material test, is placed in an MTT solution of 0.3 mg/ml at 20–28 °C for 3 h. The precipitated blue formazan product is then extracted (solvent extraction) and the concentration of the formazan is measured with a wavelength of between 545 and 595 nm.

Corrositex™ assay

The Corrositex™ test (*In Vitro* International, Irvine, CA, USA) is a standardised, quantitative *in vitro* test for skin corrosivity, based upon determination of the time required

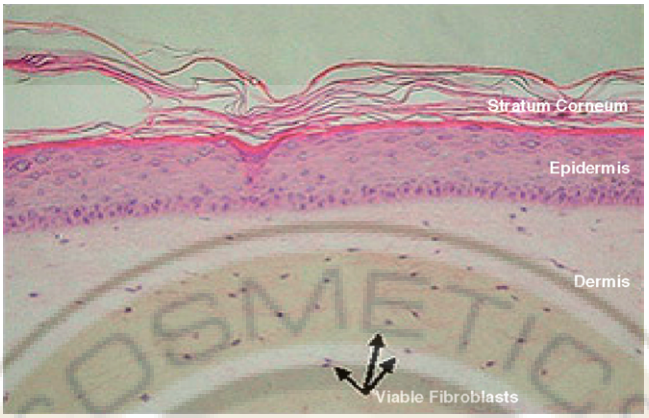

Figure 9.1.2 Full thickness skin model (adapted from MatTek Corporation, Ashland, MA, US, in <www.mattek.com>).

for a test material to pass through a biobarrier membrane (a reconstituted collagen matrix, constructed to have physico-chemical properties similar to rat skin), and produce a visually detectable change. The time required for this change to occur (the break-through time) is reported to be inversely proportional to the degree of corrosivity of the test material.

The Corrositex™ can be used to assess corrosivity of seven categories of chemicals: acids, acid derivatives, acyl halides, alkylamines/polyalkylamines, bases, chlorosilanes, metal halides and oxyhalides. Therefore, if the test substance is found to be corrosive it can be assigned packing group criteria according to the US DOT regulations.

Skin irritation

Dermal irritation is defined as the production of "reversible damage of the skin following the application of a test substance for up to 4 hours". It is generally assessed by the potential of a certain substance to cause erythema/eschar and/or oedema after a single topical application on rabbit skin and based on the Draize score (OECD TG 404, 2002). The main overall objective is to identify those *in vitro* alternatives capable of discriminating skin irritants from non-irritants.

In vitro alternatives (Table 9.1.4) in the field vary from more simple models such as keratinocyte cultures to more complex reconstituted human skin models.

A major advantage of these *in vitro* models is that the test substance can be applied directly (topically) to the culture surface (stratum corneum) at the air interface, thereby closely mimicking dermal exposure in humans. The models are therefore particularly appropriate for irritancy testing of products intended for topical exposure in humans such as treatments for skin conditions, cosmetics, wound dressings, transdermal delivery systems

Table 9.1.4

Summary of some alternative methods for skin irritation

Alternative test	Test system	Endpoints	Status
EPISKIN™ human skin model (commercial system)	Reconstructed human epidermal	Cell viability (MTT-test)	Under validation (ECVAM)
EpiDerm™ human skin model (commercial system)	Reconstructed human epidermal	Cell viability (MTT-test)	Under validation (ECVAM)
SkinEthic™ human skin model (commercial system)	Reconstructed human epidermal	Cell viability (MTT-test assay)	Under validation (ECVAM)
Mouse skin integrity function test (SIFT)	Excised mouse skin	Transepidermal water loss (TEWL) and electrical resistance	Under validation (ECVAM)
Non-perfused pig ear test	Pig ear	TEWL	Further development

and medical devices, but can also be used for irritancy testing of chemicals and agrochemical formulations (Botham *et al.*, 1998; Van de Sandt *et al.*, 1999; Faller *et al.*, 2002; Fentem and Botham, 2002).

Two skin models are under validation (ECVAM):

– The EpiDerm™ model (MatTek, Ashland, MA, USA) is a reconstituted human skin model, in which human skin-derived keratinocytes (Figure 9.1.2) are grown on specially prepared Millicell cell culture inserts, forming a multi-layer (Earl *et al.*, 1999). The EPI-200 epidermal tissue model is used to assess the skin irritation potential of test substances following a timed exposure of the tissues to the test substance. Irritant substances are identified by their ability to produce a decrease in cell viability (as determined by using the MTT reduction assay). The endpoint of the assay is the ET_{50}, effective time of exposure to reduce tissue viability to 50% in the treated tissues as compared to untreated controls (Fentem *et al.*, 2001; Zuang *et al.*, 2002).

– The EPISKIN™ model (EPISKIN-SNC, Gerland, France) is another three-dimensional human skin model (Tinois *et al.*, 1991). Its use for skin irritation testing involves topical application of test materials to the surface of the skin, and the subsequent assessment of their effects on cell viability by using the MTT assay. The endpoint used to distinguish between potential skin irritants and non-irritants is the percentage (%) of cell viability (Roguet *et al.*, 1998). However, the use of other, more mechanistic, endpoints such as interleukin 1-α, lactate dehydrogenase (LDH) has also been evaluated (Faller *et al.*, 2002). Following results from a prevalidation study, the protocol was refined in order to improve the specificity of the method (Fentem *et al.*, 2001; Zuang *et al.*, 2002). This refinement consisted in reducing the time that the epidermis is exposed to chemicals. Sensitivity, specificity and accuracy of the new method were improved and the EPISKIN™ model is now ready to enter a validation study of *in vitro* tests for acute skin irritation (Portes *et al.*, 2002).

– Other models exist where the keratinocyte cultures grown at air–liquid interface are cultured in different substrates: the SkinEthic™ model (Rosdy and Clauss, 1990), the Cosmital in-house model (Wella, Switzerland) (Faller *et al.*, 2002), and the Apligraf® model (Organogenesis Inc., Novartis Pharmaceuticals Corporation, Canton, MA USA) (Medina *et al.*, 2000). The SkinEthic™ is not a validated method and the other models need further development.
– Other methods, such as the mouse skin integrity function test (SIFT) and the non-perfused pig-ear test, have been assayed, but only the former has reached the validation step (Table 9.1.4):
– The mouse SIFT (Syngenta CTL, Macclesfield, UK) is based on assessment of mouse skin integrity following exposure to test material. Two methods are used to assess stratum corneum integrity: transepidermal water loss (TEWL) and electrical resistance (ER) (Heylings *et al.*, 2001). The SIFT protocol was developed as a pre-screen to assess the skin irritation potential of industrial chemicals. The basis of the SIFT prediction model is as follows: if the ratios of the pre- and post-application values for either TEWL or ER are greater or smaller than five-fold, then the test chemical is deemed irritant or non-irritant, respectively (Zuang *et al.*, 2002; Heylings *et al.*, 2003).
– The non-perfused pig-ear test is based on determining the absolute increase in TEWL from the skin surface, following exposure of the pig ear to test material, as the endpoint to distinguish between irritants and non-irritants (Fentem *et al.*, 2001; Zuang *et al.*, 2002) and needs further development to achieve the status of the prevalidation studies.

Consequently, the human skin model assays (e.g. EpiDerm™ and EPISKIN™) and the mouse SIFT appear to be the most promising *in vitro* methods for skin irritation testing. However, there is a need to develop new endpoints that are more predictive of skin irritation than simple cytotoxicity determinations.

EYE IRRITATION TESTS

The eye can be exposed to cosmetic products and their ingredients (e.g. mascaras, eye creams) or through accidental exposure (e.g. shampoos). Therefore, the evaluation of eye irritation potential for a cosmetic product and its ingredients is essential to provide reassurance that a product is safe for consumers.

The conventional test for the irritant and corrosive potential of chemicals is the rabbit eye test, which was developed by Draize *et al.* (1944), and has become the international standard assay for acute eye irritation and corrosion (EC B.5, Directive 2004/73/EC; OECD TG 405, 2002). The test material is applied to the conjunctival sac of the animal's eye and subsequent grading of ocular lesion is established: cornea opacity, iris lesion, redness of conjunctivae, and oedema of conjunctivae (chemosis).

Major validation studies took place in the 1990s to replace the Draize test for eye irritation testing (Balls *et al.*, 1995; Gettings *et al.*, 1996; Spielmann *et al.*, 1996; Bradlaw *et al.*, 1997; Brantom *et al.*, 1997; Ohno *et al.*, 1999). Good reproducibility and reliability of the most valuable alternative methods have been demonstrated, but so far it has not been possible to identify a single method able to replace the Draize rabbit eye test. This is due to different factors including the limited quality of the existing *in vivo* data, limitations of

Table 9.1.5

In vitro eye irritation alternative methods for achieving animal replacement

Alternative tests	Test system	Endpoint	Status
Bovine corneal opacity and permeability (BCOP) test	Excised cornea from the bovine eye	Opacity and permeability of the cornea	Optimised validation
Isolated rabbit eye (IRE) test	IRE corneal swelling	Corneal opacity and fluorescein retention	Optimised validation
Chicken enucleated eye test (CEET)	IRE corneal swelling	Corneal opacity and fluorescein retention	Optimised validation
Hen's egg test-chorioallantoic membrane (HET-CAM)	Hen's egg CAM	Damage to chicken CAM	Optimised validation
EpiOcular™ assay	Reconstituted human-derive epidermal keratinocytes	Cytotoxicity	Under validation
SkinEthic (HCE)	Reconstituted human corneal epithelium (HCE)	Cytotoxicity and histology	Under prevalidation
HCE-TTP	Reconstituted HCE	Transepithelial permeability (TEM) to sodium fluorescein, transepithelial electrical resistence (TER), cytotoxicity and histomorphology	Under prevalidation
NRU assay	Cell membranes	Cytotoxicity	Optimised validation
NRR assay	Cell membranes	Cytotoxicity	Optimised validation
Red blood cell (RBC)	Mammalian erythrocytes	Haemolysis, denaturation of oxyhaemoglobin	Optimised validation
Fluorescein leakage (FL)	Monolayers or multi-layers of epidermal keratinocytes	Permeability	Prevalidated
Irritation assay	Macromolecular reagent	Turbidity	Additional data are required for its validation

the animal test method and the fact that the range of criteria for injury and inflammation covered by the Draize rabbit eye test is unlikely to be replaced by a single *in vitro* test.

This section includes some of the most promising methods that may replace animals in the eye irritation test in the future (Table 9.1.5).

The alternative methods in this field comprise isolate organs, chorioallantoic membrane (CAM) methods, tissue and cell culture systems and physico-chemical tests (Christian and Diener, 1996; Chamberlain *et al.*, 1997; Spielmann, 1997).

Three methods using isolated organs can be used:

– The bovine corneal opacity and permeability (BCOP) test uses freshly isolated cornea from slaughterhouse material. The cornea is mounted horizontally on a holder, which is placed inside a specially modified opacitometer. This cornea divides the test chamber into two compartments with controlled temperature and the test compound is added to the compartment enclosing the epithelial surface of the cornea (Gautheron *et al.*, 1992). After measuring opacity, a fluorescein-containing solution is added to the epithelial side (i.e. the upper compartment) in order to determine cornea permeability by assessing the optical density of the medium in the lower compartment. The measured numerical values for opacity and permeability can be used to calculate a so-called *in vitro* score (Gautheron *et al.*, 1994; Gautheron, 1996; Sina *et al.*, 1995). Test materials can be classified according to this score (Kay and Calandra, 1962). Better prediction of certain chemical classes can be obtained by including histological evaluation of the corneas (Curren *et al.*, 1999).

The BCOP test is well suited to classifying the moderate, severe and very severe eye irritants since the depth of injury can be measured in this *ex vivo* tissue model. However, the resolution of mild to very mild levels of irritancy would be better suited to an epithelial tissue construct or cytotoxicity assay. For these reasons, the BCOP assay in tandem has been put forward with assays measuring other endpoints such as cytotoxicity (Rachui *et al.*, 1994; Swanson *et al.*, 1995) or as part of a test battery (Sina and Gautheron, 1994; Harbell and Curren, 2001).

– The isolated rabbit eye (IRE) test (Burton *et al.*, 1981) determines the opacification of the cornea and the increase in corneal thickness (corneal swelling) after exposure to irritant substances. Whole eyeballs obtained by immediate dissection from humanely killed laboratory rabbits with healthy eyes are mounted and maintained in a vertical position in a superfusion chamber, with controlled temperature and humidity. This ensures that the eyes remain viable throughout the duration of the test. Pre-warmed saline solution is applied drop by drop directly onto the cornea at regular intervals to keep it moist.

Prior to treatment with the test sample in question, the eye is checked visually for opacity together with an evaluation of fluorescein penetration and cornea swelling, and damaged tissues are excluded. The eyeball is then either taken out of the chamber or left *in situ* (depending on the type of test material) and exposed to the test chemical; for example, 10 sec for identification of severe irritants and 1 min (or longer) for the ranking of less severely damaging materials (Whittle *et al.*, 1992; York *et al.*, 1994). After removal of the chemical, the eye is repositioned in the chamber and the cornea is examined for evidence of opacification and corneal thickness is measured. Further assessments are made at different times (30 min, 1, 2, 3, and 4 h) after dosing. A check on fluorescein penetration is carried out 4 h after treatment (Figure 9.1.3).

Scores for corneal opacity (similar to Draize scores) and fluorescein penetration are recorded (qualitative assessments). For each test sample the mean percentage of corneal swelling of three eyes is calculated and compared to an untreated control eye. The preparation and examination of histological sections of the treated corneas can be used to confirm the level and depth of corneal damage.

Overall damage is assessed by means of combining the different parameters scored, depending on the nature of the effects observed and in-house classification systems may

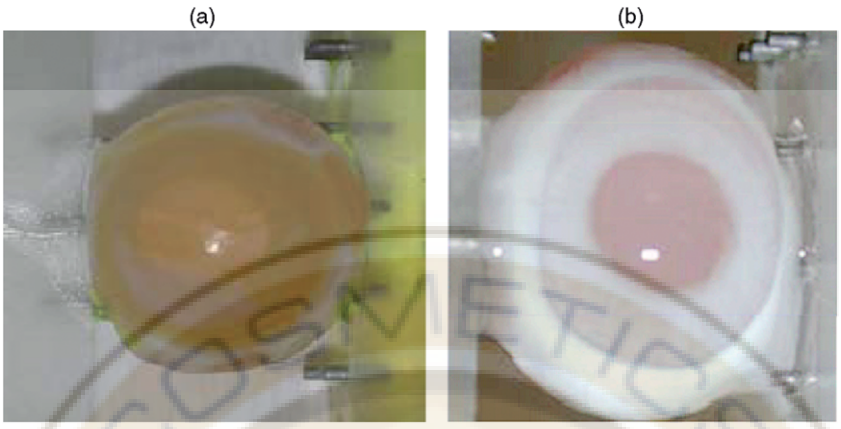

Figure 9.1.3 Visual assessment of corneal opacity (a) and uptake of sodium fluorescein dye by the cornea (b) in isolated rabbit eye test (adapted from Safepharm Laboratories, Derbyshire UK, in <www.safepharmlabs.com/safepharm_contact.php>).

vary (Whittle *et al.*, 1992). Chemical substances causing the cornea to swell by more than 15% were considered to have the potential to cause severe eye irritation *in vivo* (Lewis *et al.*, 1994); however, more recently a more complex classification model combining opacity, corneal swelling and histological observations of the corneal epithelium has been published (Cooper *et al.*, 2001; Jones *et al.*, 2001).

The IRE test is considered to be well suited to identifying substances that are "severely irritating to the eye" according to EU classification R 41. It is considered to be a valid test for the (pre)screening of severely irritating materials/formulations (Chamberlain *et al.*, 1997). The assay is useful for in-house comparative testing of materials/formulations to obviate the need for any animal testing during product development.

– The chicken enucleated eye test (CEET) assesses the ocular irritation potential of a test substance following topical application onto the surface of the cornea of enucleated chicken eyes (obtained from freshly slaughtered chickens). The eyes (pre- and post-treatment) are maintained in a superfusion chamber at 32 ºC. The direct effect of the test substance on the cornea can be assessed using multi-endpoint analysis following evaluation of changes in corneal thickness, corneal opacity, alteration of the corneal epithelium and fluorescein uptake up to 240 min following treatment. The CEET has been shown to reliably differentiate between negligible and moderate-to-severe irritants (Prinsen and Koëter, 1993; Prinsen, 1996).

The CAM of the fertilised chicken egg is considered a suitable model to establish what effects substances have on the conjunctival tissues of the eye (e.g. Christian and Diener, 1996).

– The hen's egg test on the chorioallantoic membrane (HET-CAM assay) enables irritant reactions to be identified that are similar to those which occur in the eye using the standard Draize rabbit eye test. In the HET-CAM test system, three reactions are determined, namely, haemorrhage, lysis and coagulation (sometimes hyperemia is also used as a parameter) of the CAM on the ninth day of embryonation when nerve tissue and pain perception have not yet developed (Figure 9.1.4).

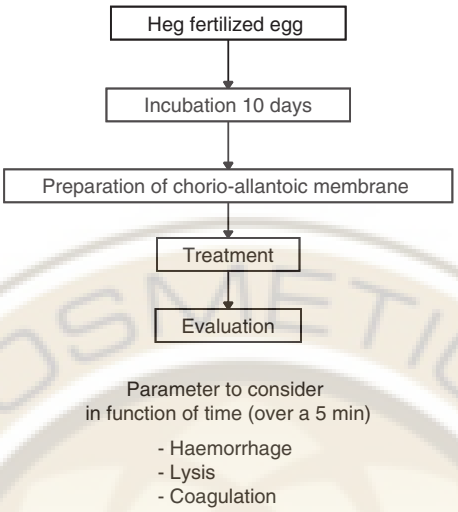

Figure 9.1.4 Hen's egg test on the chorioallantoic membrane (HET-CAM assay) permits the identification of irritative reactions. Three parameters are determined, namely, haemorrhage, lysis and coagulation.

After placing the test sample directly onto the CAM, the above-mentioned parameters are evaluated over a 5-min observation period. The most widely used approach is the reaction time method, which determines the time taken for each of the three endpoints to appear. Another approach is the irritation threshold method, which determines the test material concentration at which effects on these parameters are first observed. While these approaches are mainly used for transparent test materials, a third approach for non-transparent insoluble and solid materials can be used by exposing the CAM to test samples for a fixed time (e.g. 30 sec or 5 min) and examining the membrane after careful rinsing to remove the sample. The majority of the validation studies carried out, showed a useful correlation between the HET-CAM test and the Draize rabbit eye test for the assessment of raw materials and cosmetic products. These *in vivo* versus *in vitro* correlations revealed good results in the area of mild and non-irritating test materials as well as for surfactants and surfactant-based formulations (Spielmann *et al.*, 1993). As a consequence, the HET-CAM test can be regarded as an alternative, which is already leading to a reduction in animal experiments.

Three different epithelium models like the EpiOcular™ model (MatTek, Ashland, MS, USA), the SkinEthic HCE model (SkinEthic, Nice, France) and HCE-T Tissue Construct (Gillete) are used:

– The EpiOcular™ corneal model involves culturing normal, human-derived epidermal keratinocytes to form a stratified, squamous epithelium similar to that found in the cornea. The epidermal cells, which are cultured on specially prepared cell-culture inserts using serum-free medium, differentiate to form a multi-layered structure that closely parallels the corneal epithelium. EpiOcular is mitotically and metabolically active and releases many of the pro-inflammatory agents (cytokines) known to be important in ocular irritation and

inflammation. Comparison with *in vivo* animal data has been carried out, using the ET_{50} value determined by MTT assay. Using the variable of time rather than dose allows ingredients and formulations to be tested without dilution in medium. Thus both hydrophobic and hydrophilic materials may be tested.

As a stratified epithelium, the EpiOcular system is intended to model damage to the corneal epithelium and conjunctiva (with its very thin epithelium). Therefore, it can be used to resolve degrees of irritancy potential (cellular damage) in the moderate to very mild irritancy range (Stern *et al.*, 1998). The model is also capable of identifying high moderate and severe irritants by their very short ET_{50} values. However, based on the stromal changes associated with severe irritation, an epithelial construct would not be expected to provide the degree of resolution in the severe range that a full thickness cornea (e.g. *ex vivo* cornea) would provide (Blazka *et al.*, 2003). The EpiOcular can be used to differentiate between mild and moderate irritants and identify potentially severe irritants.

– The SkinEthic *in vitro* reconstituted human corneal epithelium (HCE model) consists of immortalised human corneal epithelial cells (HCE cell line, LSU Eye Centre, New Orleans, USA) that are cultivated at the air–liquid interface in a chemically defined medium on a polycarbonate substrate and form an air-epithelial tissue, devoid of stratum corneum, morphologically resembling the corneal mucosa of the human eye (Figure 9.1.5).

Triplicate *in vitro* reconstituted human corneal epithelial tissues (size 0.5 cm²) are topically dosed with a small amount of test agent for different time points:

- For finished products, tissues are dosed for 10 min, 1, 3 and 24 h.
- For chemical raw materials, tissues are dosed for 10, 20, 30 and 60 min.

Negative control (phosphate-buffered saline solution) as well as positive controls (sodium dodecyl sulphate 0.5% and sodium dodecyl sulphate 1%) are run in parallel. At

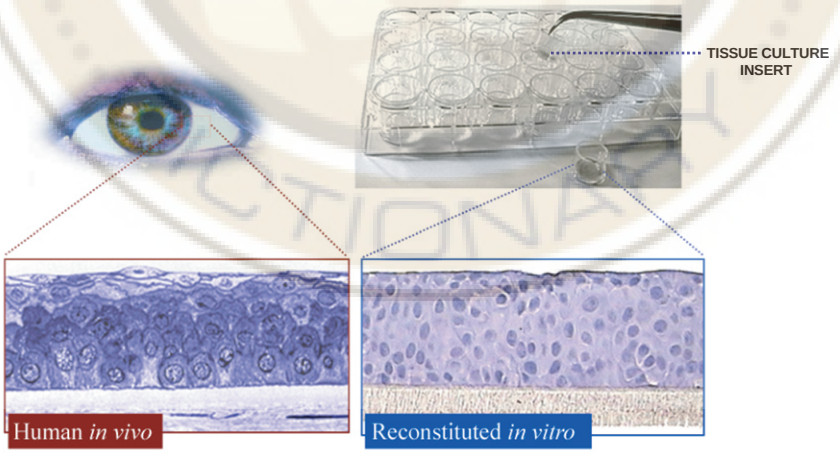

Figure 9.1.5 Transversal section of human corneal epithelium (HCE) *in vivo* (left), and reconstituted *in vitro* on a polycarbonate membrane in tissue culture inserts (right) (adapted from Skinethic Laboratories, Nice, France, in <//pros.orange.fr/skinethic/Home%20Page.htm>).

each time point, duplicate tissues are assessed for tissue viability (MTT assay), and one culture is fixed in a balanced 10% formalin solution for histological analysis, which is performed when the MTT assay data show no tissue toxicity. Meanwhile, the culture media underneath the tissues is stored at −20 °C for pro-inflammatory mediator analysis. Possible endpoint measurements are: tissue viability using the MTT assay or LDH release, histology, and quantification of cytokine release (e.g. IL-1α, IL-6, IL-8, PGE_2). Until this test has passed formal validation, it is suitable as a pre-screen for eye irritation of chemicals and finished products (Nguyen *et al.*, 2003).

– The HCE-T Tissue Construct (Gillette model) uses a transfected human corneal epithelial cell line (Kahn *et al.*, 1993) cultured on collagen-membrane cell culture inserts, which, at the air–liquid interface, stratify to form a four- to six-layer epithelium, known as the HCE-T model. Transepithelial permeability (TEP) to sodium fluorescein and transepithelial electrical resistance (TER) have been identified as physiologically relevant parameters to evaluate the barrier function of the corneal epithelium. Cell viability can be determined by the MTT assay, and histomorphology can also be used as an endpoint (Kruszewski *et al.*, 1997).

The application of cytotoxicity tests to assess eye irritation potential is based on the observation that some materials, which are damaging to the eye, can produce cytotoxic effects in various cell types, including those present in ocular epithelial and endothelial tissues.

– One of the most widely used cytotoxicity tests, the NRU assay, has been conducted on primary cell cultures as well as on established cell lines: 3T3 Balb/c mouse fibroblast (Harbell, 1994) and SIRC cell line derived from rabbit corneal cells (Blein-Shella and Adolphe, 1995). For the test, monolayer cell cultures are grown on microtitre plates and incubated with serial dilutions of the test substance yielding a wide range of concentrations. After exposure (24 h or 48 h) the cells are washed and incubated for 3 h with medium containing NR dye. Afterwards, the cells are treated with wash/fixation solution and then with appropriate solvent to elute the dye. The optical density of the resulting solution is measured at 540 nm. The concentration of the test substance producing a 50% inhibition of NRU in comparison to control samples, which have not been exposed to the test substance, is obtained by extrapolation from the dose–response curve. This so-called NRU_{50} or IC_{50} value serves as a toxicological endpoint. It has been shown that, when used in combination with the HET-CAM assay (Spielmann *et al.*, 1996; Spielmann, 1997) or the IRE test, the NRU assay can be employed successfully as a part of a test battery to identify materials that are severely irritating to the eye.

– A variant of the NRU assay is the neutral red release assay (NRR assay) (Reader *et al.*, 1990). The NRR assay is fast to perform and easy to standardise. It is not designed to be a test of general cytotoxicity, but has been developed to identify substances potentially capable of causing toxic responses when in brief contact with the eye (such as may occur by accident). The NRR assay is therefore appropriate to evaluate surface-active agents such as surfactants and detergents. High concentrations of test material need to be tested due to the very short exposure time. A standard protocol with Balb/c 3T3 mouse fibroblasts has been developed (Clothier, 1992) as well as a commercial assay kit using the SIRC cell line derived from rabbit cornea (Guyomard *et al.*, 1994).

– Another method is the red blood cell (lysis) test (RBC test) based on the (cytotoxic) potential of a chemical substance to disrupt cell membranes. The RBC test contributes to the reduction of animal numbers used for eye irritation testing. Access to mammalian erythrocytes is easy (e.g. slaughterhouse material). The RBC test is used in industry as part of an *in vitro* test battery, which is routinely used as a pre-screen (Brantom *et al.*, 1997; Harbell *et al.*, 1997). Membrane damage is assessed by photometrically measuring the leakage of haemoglobin from freshly isolated red blood cells incubated with test materials under standard conditions (Pape and Hoppe, 1991; Lewis *et al.*, 1994).

Other eye irritation assays

– The fluorescein leakage test is based on the principle that the corneal epithelium has the ability to function as an impermeable barrier to potentially hazardous chemical substances and that eye irritation can ensue when its structure is damaged. In the fluorescein leakage test the corneal epithelium is mimicked *in vitro* by adherently growing cells which form tight junctions and desmosomes in culture (Tchao, 1988; Shaw *et al.*, 1990). The test is performed in a variety of formats using monolayers or multi-layers of epidermal keratinocytes (NHEK) or human corneal cells (Cook *et al.*, 1992; Kruszewski *et al.*, 1995). Cells are grown on inserts so that, when confluent, the cell layer separates the medium into two compartments. The cells are exposed to the test substance in the upper compartment for a set period. Then the sample solution is removed and, after a washing step, the cells are incubated with a sodium fluorescein solution (usually for 30 min). Disruption of the cell layer permits the diffusion fluorescein into the lower compartment, which is measured spectrophotometrically.
– The irritation assay system (*In Vitro* International, Irvine CA, USA) is based on the premise that eye irritation and corneal opacity after exposure to irritating chemicals is the result of perturbation or denaturation of corneal proteins (Kelly, 1989). This test consists of two essential components: a membrane disc that permits controlled delivery of the test material to a reagent solution; and a proprietary reagent solution that is composed of proteins, glycoproteins, lipids and low-molecular-weight components that self-associate to form a complex macromolecular matrix that mimics the highly ordered structure of the transparent cornea. Application of an irritant chemical to the membrane disc disrupts the ordered structure of keratin and collagen and causes a release of the bound indicator dye. The extent of dye release and protein denaturation may be quantitated by measuring the changes in optical density of the reagent solution at 450 nm (OD_{450}). Comparison of these optical density measurements to those produced by standard chemical irritants permits calculation of an "irritancy score" that has been shown to be directly related to the potential dermal irritancy of the test material. The turbidity measured spectrophotometrically is compared with that produced by eye irritant standards of known Draize score (Curren *et al.*, 1997). Chemical irritants typically produce a linear or sigmoidal dose–response (Christian and Diener, 1996).

Finally, eye irritation is a difficult endpoint to model "*in silico*" because of the complexity of the biological mechanisms that may be involved. Therefore, most approaches

have modelled eye irritation resulting from physical effects, such as ocular penetration or corrosion (Cronin *et al.*, 2003).

SKIN SENSITISATION

In the last 50 years, skin sensitisation potential assessment has been of paramount importance for ensuring the safety of cosmetic products. Different human sensitisation tests, such as Schwartz–Peck test (Schwartz, 1969), human repeated insult patch tests (Marzulli and Maibach, 1973; Griffith and Buehler, 1976) and human maximisation test (Kligman and Epstein, 1975), have been used; but standardised test guidelines are not available and the tests have not undergone an official validation process.

On the other hand, skin sensitisation is a very complex biological process, involving a series of interrelated events, many of which are either not understood or only partially understood. Therefore, it is not surprising that at the time of writing, there are no validated non-animal tests for assessment of skin sensitisation potential.

However, refinement and reduction methods have been developed and are widely used. Nevertheless, there is no single test method which will adequately identify all substances with a potential for sensitising human skin and which is relevant for all substances. Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, must be considered on selecting a test.

Animal tests for identifying skin sensitising chemicals are used and test guidelines have also been developed:

– Local lymph node assay (LLNA) has already been validated and adopted for regulatory use in the EU (Method B.42 Directive 2004/73/EC) and equivalent to the OECD TG 429 (OECD, 2002). The LLNA has been accepted by the ICCVAM in USA as a stand-alone alternative to the current guinea-pig tests. This is a method to assess skin sensitisation potential of chemicals in animals (Kimber *et al.*, 1994, 1998). Therefore, the LLNA is an *in vivo* method (Kimber *et al.*, 2002) and, consequently, will not eliminate the use of animals in assessing contact sensitising activity. It can, however, potentially reduce the number of animals required for this purpose. The basic principle underlying the LLNA is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose–response relationship in which the proliferation in test groups is compared to that in vehicle-treated controls. The method is based on the use of radioactive labelling (^{3}H-methyl thymidine) to measure cell proliferation. The ratio of the proliferation in treated groups to that in vehicular controls, stimulation index (SI), is determined. The test is positive when the SI$\leq$3.

– Magnusson Kligman guinea-pig maximisation test (GPMT) and Buehler guinea-pig test, (Buehler, 1965) are available as Number B.06 (Directive 96/54/EC, 1996) and OECD Test Guideline No. 406 (OECD, 1992). These methods are an accepted test for hazard identification of skin sensitising substances. The GPMT is a highly sensitive method using Freund's complete adjuvant (FCA) as an immune enhancer (Magnusson, 1980), but the

Buehler test is less sensitive and may underestimate the sensitisation potential of a substance. The limitation of the tests is that the evaluation is based on visual inspection of erythema and personal judgement. For these reasons, evaluation of coloured test substances, e.g. pigments and dyestuffs, is often impossible due to staining of the skin by the test substance.

– Mouse ear swelling test (MEST) is a useful model for identifying strong contact sensitisers (Asherson and Ptak, 1968; Gad, 1994). Several weeks prior to and during the test period mice are fed a diet enriched in vitamin A for enhancement of contact sensitisation. The induction phase comprises clipping off the fur on the belly region and removal of the outer layers of the epidermis by tape stripping. FCA is injected intradermally before the test substance is applied topically, in vehicle (test mice) or vehicle alone (control mice). Ear thickness of test and control ears is measured under ether anaesthesia with a micrometre 24 and 48 h after the test substance is applied. Ear swelling is expressed as the difference between test and control ears in per cent.

On the other hand, different alternative models *in vitro* are being developed to assess the skin sensitising potential of chemicals and products. These methods are based on cell systems, which have been shown capable of distinguishing between sensitisers and non-sensitisers, depending upon the profile of cytokine release. However, they are still at a basic research level. Among these it is possible to use keratinocyte (Matsue *et al.*, 1992; Wakem *et al.*, 2000; Lozsekova *et al.*, 2002) and Langerhan cell cultures (Simon *et al.*, 1995; Neisius *et al.*, 1999), peripheral blood-derived dendritic cells (Pichowski *et al.*, 2001), different cell lines (Xu *et al.*, 1995; Yoshida *et al.*, 2003), co-culture systems with Langerhans cell and T-cells (Hauser and Katz, 1988; Régnier *et al.*, 1997; Facy *et al.*, 2004), human skin equivalent (Gerberick and Sikorski, 1998; Corsini *et al.*, 1999; Coquette *et al.*, 2003) and human skin explant cultures (Pistoor *et al.*, 1996) from breast or abdominal reduction surgery.

Finally, different "*in silico* models" are being developed to assess the skin sensitising potential of chemicals and products. Relationships between the structure and biological properties of chemicals can be programmed into knowledge-based expert systems (OECD, 2002; Walker *et al.*, 2002): DEREK ("deductive estimation of risk from existing knowledge"), TOPKAT (toxicity prediction by komputer-assisted technology), and CASE (computer automated structure evaluation) for the substitution of *in vivo* sensitisation comprise computer-based expert or QSAR systems.

SKIN ABSORPTION STUDIES

Skin-absorption testing of chemicals is normally not required by legislation governing dangerous chemical substances. However, cosmetic companies still require skin-absorption data from ingredient suppliers, in particular, in the case of "actives", e.g. colourants, preservatives, UV filters and substances with restrictions in concentration or site of application. For this purpose, knowledge of dermal absorption studies is essential.

The methods for measuring dermal absorption and dermal delivery can be divided into two categories: *in vivo* and *in vitro*. The methods study the diffusion of a test substance from a test preparation through the skin barrier and into the skin.

In vitro dermal penetration

In vitro testing is carried out on excised pig or human skin, according to OECD guideline 428 (OECD, 2004b). In general animal skin (e.g. rat skin) is more permeable and therefore may overestimate human percutaneous absorption (Diembeck *et al.*, 1999).

The use of *in vitro* dermal absorption studies on isolated skin is based on the fact that the epidermis, in particular the stratum corneum, forms the principal *in vivo* barrier of the skin against the penetration and uptake of xenobiotics in the body.

The test substance is applied in an appropriate formulation on the skin sample, which is usually placed in a diffusion cell (Figure 9.1.6) (Balaguer *et al.*, 2006). The diffusion cell consists of an upper donor and a lower receptor chamber, separated by a skin preparation. The cells are made preferably from an inert non-adsorbing material. Temperature control of the receptor fluid is crucial throughout the experiment. The skin surface temperature in the diffusion cell should be kept at the *in vivo* skin temperature of 32 °C.

The receptor fluid is well mixed throughout the experiment. The composition of the receptor fluid is chosen so that it does not limit the extent of diffusion of the test substance, i.e. the solubility and stability in the receptor fluid of the chemical under assay must be guaranteed. Saline or buffered saline solutions are commonly used for hydrophilic compounds. For lipophilic molecules, serum albumin or appropriate solubilisers/emulsifiers are added in amounts that do not interfere with membrane integrity. One must ensure that the amount of penetrant in the receptor fluid is less than 10% of its saturation level at any time. The substance must remain stable in the receptor fluid for the duration of the *in vitro* test and the subsequent analysis (SCCNFP/0750/03).

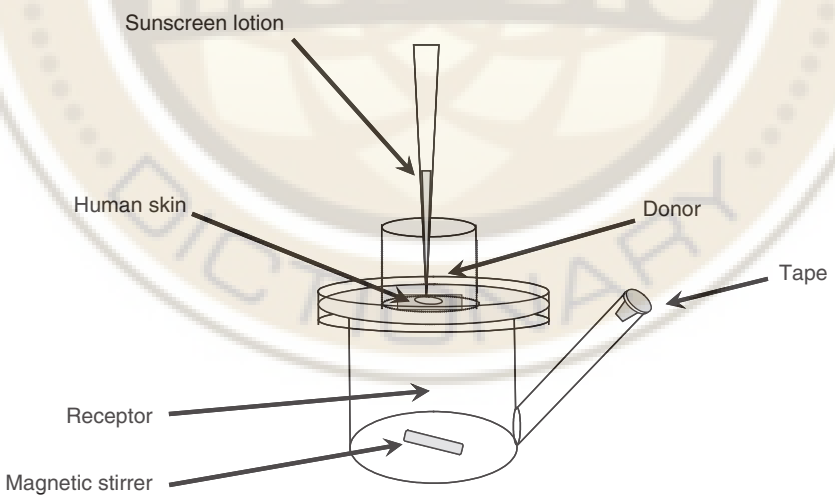

Figure 9.1.6 The Franz's diffusion cell consists of an upper donor and a lower receptor chamber, separated by a skin preparation. The test substances (e.g. sunscreen lotion) that are applied on the stratum corneum faces the donor compartment. The receptor fluid is well mixed (magnetic stirrer) throughout the experiment (adapted from Balaguer *et al.*, 2006).

Both the dose and the contact time (exposure) with the skin are chosen to mimic intended use conditions. The amount of the formulation to be applied is between 2 and 5 mg/cm^2 for solids and semi-solid preparations, and up to 10 µl/cm^2 for liquids. The volume of formulation used should be enough to spread the sample homogeneously over the skin surface. This strongly depends on the viscosity of the formulation.

The exposure time and sampling period should be defined in the protocol. The normal exposure time is 24 h. Longer duration may result in membrane deterioration and requires membrane integrity to be carefully checked. Barrier integrity should be checked using a suitable method. This is achieved by either measuring the penetration of a marker molecule, e.g. tritiated water, caffeine or sucrose, or by physical methods like TEWL or TER measurements. Data obtained should be reported.

Sampling frequency depends on the rate/extent of dermal absorption. Appropriate analytical techniques, e.g. scintillation counting, HPLC or GC, should be used. Their validity, sensitivity and detection limits should be documented in the report. When an increase in sensitivity is needed, the test substance should, whenever possible, be radiolabelled. Qualitative or semi-quantitative methods such as microautoradiography can be useful tools for skin distribution assessments.

Amounts of the test compound must be determined in (SCCNFP/0750/03):

- the surplus on the skin,
- the stratum corneum (e.g. adhesive tape strips),
- the epidermis without stratum corneum,
- the dermis, and
- the receptor fluid.

The mass balance of the applied dose must be determined. The overall recovery of the test substance (including its metabolites) should be within the range of 85–115%. If lower recoveries of the test substance are obtained, the reasons need to be investigated and explained. One should check for substance adsorption in the equipment.

The results of dermal absorption studies should be expressed as an absolute amount (µg/cm^{2}of skin surface) and as a percentage of the amount of test substance contained in the intended dose applied per square centimetre of skin surface.

The amounts of penetrated substance found in the receptor fluid are considered to be systemically available. The epidermis (except for the stratum corneum) and dermis are considered as a sink, therefore the amounts found in these tissues are equally considered as absorbed and are added to those found in the receptor fluid. The amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed and, thus, they do not contribute to the systemic dose. The absorption rate and mass balance should be calculated separately for each diffusion cell. Only then, the mean ± SD can be calculated.

In vivo studies

This type of study has some advantages over *in vitro* methods, which include the generation of systemic kinetic and metabolic information (Schaefer and Redelmeier, 1996). The

disadvantages are the use of live animals, the need for radiolabelled material to facilitate reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin (Sanco/222/2000).

Animal testing should be carried out in accordance with OECD test guideline 427 (OECD, 2004a). The rat is the most commonly used species for tests *in vivo*. In rats, the application site should be about 10 cm^2 and should be defined by a device, which is secured on the skin surface. The test preparation is applied to the skin surface, and remains there for a specified period of time, relating to potential human exposure. During exposure, animals are housed individually in metabolism cages from which excreta are collected. At the end of the study, the amount to be found in the skin, the carcass and the excreta, is determined. These data give an estimate of the total recovery of the test substance. The skin absorption of the test substance can be expressed as the percentage of dose absorbed per unit time or in terms of an average absorption rate per unit area of skin (e.g. $\mu g/cm^2/h$) (OECD, 2004c).

SUBACUTE AND SUBCHRONIC TOXICITY

Chronic toxicity is a consequence of the persistent or progressively deteriorating dysfunction of cells, organs or multiple organ systems, resulting from long-term exposure to a chemical.

In the case of developing cosmetic ingredients with specific biological properties and which will come into contact with human skin for long periods of time, evaluation of the systemic risk is a key element in evaluating the safety of these new ingredients (Annex V, Part B, of the Dangerous Substances Directive 67/548/EEC and in the OECD testing guidelines for chemicals). Therefore, in certain cases, long-term animal experimentation is still a legal requisite for the study of one or more potential toxic effects.

For cosmetic products the following tests are used:

- repeated dose 28-day oral toxicity study in rodents (OECD 407, EC B.7, 1995);
- repeated dose 90-day oral toxicity study in rodents (OECD 408, EC B.26, 1998);
- repeated dose dermal toxicity: 21/28-day study (OECD 410, EC B.9, 1981a);
- subchronic dermal toxicity: 90-day study (OECD 411, EC B.28, 1981b);
- repeated dose inhalation toxicity: 28-day or 14-day study (OECD 412, EC B.8, 1981c); and
- subchronic inhalation toxicity: 90-day study (OECD 413, EC B.29, 1981d).

Currently, no generally accepted alternative methods are available to replace repeat-dose *in vivo* testing. *In vitro* models are, however, object of research, in relation to five of the most common targets for toxicity (liver, kidney, CNS, lung and haematopoietic):

- *in vitro* liver preparations (Coecke *et al.*, 1999; Muller *et al.*, 2000; Gebhardt *et al.*, 2003),
- *in vitro* kidney preparations (Felder *et al.*, 2002),
- central nervous system (Schmuck and Ahr, 1997; Schmuck *et al.*, 2000; Hanisch, 2002; Deng and Poretz, 2003; Goldoni *et al.*, 2003),
- pulmonary epithelial systems (Dietl *et al.*, 2001; Gindorf *et al.*, 2001), and
- haematopoietic system (Breems *et al.*, 1994; Punzel *et. al.*, 1999).

Complete replacement of animal usage in these areas represents an enormous scientific and technical challenge. Many of the models for target organ toxicity are currently used to investigate toxicological issues (e.g. mechanisms of toxicity) and not for predictive purposes.

On the other hand, more efforts are needed to further evaluate the usefulness of QSARs approaches to predict toxicity. In particular, the statistically based system TOPKAT has been proposed as a model for rat chronic lowest observed adverse effect level (Cronin *et al.*, 2003).

GENOTOXICITY AND MUTAGENICITY

Genotoxicity and mutagenicity testings are an important part of the hazard assessment of chemicals for regulatory purposes. Genotoxicity is a broader term that refers to the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome, such as the spindle apparatus and topoisomerase enzymes. Mutagenicity refers to the induction of permanent transmissible changes in the structure of the genetic material of cells or organisms. These changes (mutations) may involve a single gene or a block of genes.

To assess genotoxicity and/or mutagenicity, different endpoints must be taken into considerations: beside point mutation induction, a compound can induce changes in chromosomal number (polyploidy or aneuploidy) or in chromosome structure (breaks, deletions, rearrangements). Owing to the diversity of the endpoints, it is then clear that the potential genotoxicity and/or mutagenicity of a compound cannot be assessed by a single assay system (SCCNFP/0755/03). As a consequence, a battery of tests is needed to determine the genotoxic and mutagenic profile of a compound.

Several *in vitro* tests are routinely used and accepted by regulatory authorities, but they present crucial limitations that affect the usefulness of the assays to predict mutagenicity/genotoxicity potential of a substance *in vivo* in mammals and especially in humans. Owing to these limitations, no single *in vitro* test can fully replace an existing *in vivo* animal test yet.

The *in vitro* methods to partially replace *in vivo* methods (laboratory animals) are summarised in Tables 9.1.6 and 9.1.7 (Annex V, Part B, Directive 67/548/EEC; OECD testing guidelines for chemicals), respectively.

– The Ames test (B13/14-TG 471, OECD, 1997a), *S. cerevisiae* gene mutation (B15-TG 480, OECD, 1986b) and mammalian cell gene mutation test (B17-TG 476, OECD, 1997e) are alternative tests available to partially replace the gene point mutations addressed in animal tests (B20/TG 477, OECD, 1984a; B22/TG 478, OECD, 1984b; B24/TG 484, OECD, 1986e).
– In the case of determining *in vivo* DNA damage (B39/TG 486, OECD, 1997g), the mitotic recombination in *S. cerevisiae* (B16-TG 481, OECD, 1986c), unscheduled DNA synthesis (B18-TG 482, OECD, 1986d) and sister chromatid exchange (B19-TG 479, OECD, 1986a) are used to partially replace the *in vivo* test.
– To detect chromosome aberrations, the mammalian chromosomal aberration assay (B10-TG 473, OECD, 1997b) is used to partially replace the *in vivo* methods (B22-TG

Table 9.1.6

In vitro genotoxicity and mutagenicity tests, accepted by regulatory authorities in EU legislation (Annex V of the Dangerous Substances, Directive 67/548/EEC) and in the OECD testing guidelines for chemicals

Methods	Test system	Endpoint	Status (Annex V, EU/OECD TG)
Bacterial reverse mutation test (Ames test)	Bacteria	Gene mutations in bacteria	B13-14/TG 471
Mammalian chromosome aberration test	Mammalian chromosome	Chromosome aberrations	B10/TG 473
Mammalian cell gene mutation test (mouse lymphoma test)	Mammalian cell	Gene mutations	B17/TG 476
Sister chromatid exchange assay in mammalian cells (SCE)	Mammalian cell	Mammalian DNA damage	B19/TG 479
Saccharomyces cerevisiae gene mutation assay	Yeast	Gene mutations in yeast	B15/TG 480
Saccharomyces cerevisiae mitotic recombination assay	Yeast	Recombination in yeast	B16/TG 481
Unscheduled DNA synthesis (UDS)	Mammalian cells	Mammalian DNA damage in liver cells	B18/TG 482

478, OECD, 1984b; B25-TG 485, OECD, 1986f; B11-TG 475, OECD, 1997d; B23-TG 483, OECD, 1997f).

– To determine the presence of aneugenes and clastogenes by means of *in vivo* method (B12-TG 474, OECD, 1997c), the micronucleus in cell lines is a method to partially replace laboratory animals. The purpose of the *in vitro* micronucleus assay is to identify agents that cause structural and numerical chromosome changes. The *in vitro* micronucleus test may employ cultures of established cell lines or primary cell cultures (Evans, 1976). However, until now OECD test guidelines for this method have not been submitted, nor has any formal valid method been found yet.

UV-INDUCED TOXIC EFFECTS

According to the current "Notes for Guidance" of the SCCNFP (2003), cosmetic ingredients and mixtures of ingredients absorbing UV light, in particular UV filter chemicals used, e.g. to ensure light stability of cosmetics, or used in sun protection products, need to be tested for acute phototoxicity and photogenotoxicity potential. Testing for photosensitisation potential (immunological photoallergy) is not specifically required, but is often performed nevertheless.

Acute phototoxicity is defined as a toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly

Table 9.1.7

In vivo genotoxicity and mutagenicity tests, accepted by regulatory authorities in EU legislation (Annex V of the Dangerous Substances, Directive 67/548/EEC) and in the OECD testing guidelines (TG) for chemicals

Methods	Test system	Endpoint	Status (Annex V, EU/OECD TG)
Mammalian erythrocyte micronucleus test	Mammalian erythrocyte	Structural and numerical chromosome aberrations in somatic cells	B12/TG 474
Mammalian bone marrow chromosome aberration test	Mammalian bone marrow	Chromosome aberrations	B11/TG 475
Sex-linked recessive lethal test in *Drosophila melanogaster*	*Drosophila melanogaster*	Gene mutations in germ line	B20/TG 477
Rodent-dominant lethal test	Germinal tissue of rodent	Chromosome aberrations and/or gene mutations in germinal tissue	B22/TG 478
Mammalian spermatogonial chromosome aberration test	Mammalian spermatogonial chromosome	Inheritable chromosome aberrations	B23/TG 483
Mouse spot test	Foetal cell of mouse	Mutagenicity in foetal cells	B24/TG 484
Mouse heritable translocation assay	Mouse heritable chromosome	Heritable chromosome aberrations	B25/TG 485
Unscheduled DNA synthesis (UDS) test with mammalian liver cells	Mammalian liver cells	Mammalian DNA damage in liver cells	B39/TG 486

by skin irradiation after systemic administration of a chemical. In this area there are three possible methods to use:

– The *In Vitro* 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) was included in Annex V Method No. 41 to Directive 86/906/EEC (2000) and accepted by OECD (Test Guideline 432, 2002) and the US FDA (2003). The 3T3 NRU PT test is used with a basic screen to identify acute phototoxic potential. It is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA/Vis light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye, NR, 24 h after treatment with the test chemical and irradiation.

A permanent mouse fibroblast cell line, Balb/c 3T3, is maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then pre-incubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/Vis light dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (−UV experiment). In both plates, the treatment

medium is then replaced by culture medium and after another 24 h of incubation; cell viability is determined by NRU for 3 h (Figure 9.1.7).

As previously described (Epigraph 3.2.1.4), the NR penetrates cell membranes and accumulates intracellularly in lysosomes. Alterations of the cell surface or sensitive lysosomal membrane lead to a decreased uptake of the NR. To discriminate between photo-irritant and non-photo-irritant chemicals, the photo-irritation factor (PIF) was defined as the ratio of the IC_{50} values, determined in the absence of UVA and the presence of UVA (PIF=IC_{50} (−UV): IC_{50} (+UV)) (Spielmann *et al.*, 1994, 1996). In a more sophisticated data analysis procedure, the mean photo-effect (MPE) is determined, which uses a complete comparison of the area under the curve AUC of the concentration response curves obtained with a chemical in the presence and absence of UV light (Peters and Holzhütter, 2002).

– Two additional tests, the red blood cell phototoxicity test (RBC PT) (Pape *et al.*, 1994) and the Human 3-D Skin Model *In Vitro* phototoxicity test (H3D PT) (Roguet *et al.*, 1994; Bernard *et al.*, 1999; Liebsch *et al.*, 1999; Jones *et al.*, 2003), are regarded useful and important adjunct tests to overcome some limitations of the 3T3-NRU-PT, like the fairly low UVB tolerance of 3T3 fibroblasts. Moreover, the RBC PT enables evaluation of the phototoxic mechanisms involved (Okamoto *et al.*, 1999) and the H3D PT model is qualified as an adjunct test to further investigate chemicals with (probably false) positive outcomes in the 3T3 NRU PT.

In conclusion, *in vitro* tests are regarded to sufficiently cover identification of acute phototoxic hazards, therefore, at present animal testing with this objective for that endpoint can be replaced 100%.

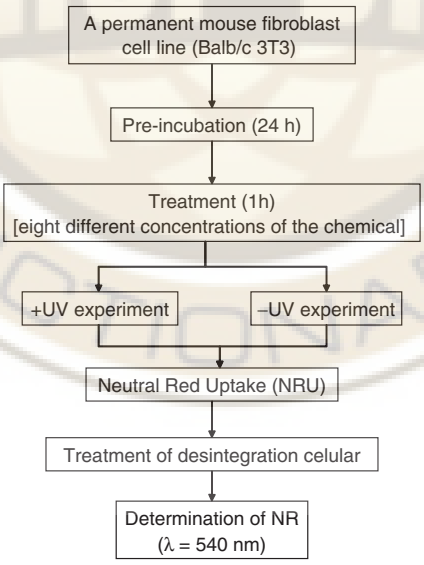

Figure 9.1.7 3T3 Neutral Red Uptake phototoxicity test (3T3 NRU PT) is used with a basic screen to identify acute phototoxic potential.

In the area of photochemical genotoxicity, almost the whole battery of *in vitro* genetic toxicity tests has been converted into test protocols of photogenotoxicity tests and several *in vitro* tests can be used.

– Photo-Ames test (P-AMES). Procariontic, bacterial mutation tests are easier and cheaper to perform than any photogenotoxicity tests with eucariontic mammalian cells (Chetelat *et al.*, 1993). Therefore, the P-AMES test was the first *in vitro* photogenotoxicity test adapted from the parallel use of light (Jose, 1979) and later proposed for safety testing of cosmetics (Loprieno, 1991). Nevertheless, the P-AMES test has never been validated formally. The critical point (Brendler-Schwaab *et al.*, 2004) of all P-AMES test protocols is the UV sensitivity of the *Salmonella typhimurium* or *Escherichia coli* strains used. Chemicals often have to be pre-irradiated in the absence of the bacteria to achieve doses of light necessary to activate the photogenotoxins.
– Photo-chromosome aberration test (P-CAT) is used to detect photochemically induced clastogenicity, the most relevant *in vitro* endpoint for the assessment of photocarcinogenic hazard potential of substances (Gocke *et al.*, 2000; Brendler-Schwaab *et al.*, 2004). The purpose of the *in vitro* P-CAT is to identify agents that cause structural chromosome aberrations in cultured mammalian cells in the presence of a non-clastogenic UV-Vis radiation. Structural aberrations fall into two categories, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations correspond to the chromatid type, but chromosome-type aberrations also occur. The *in vitro* P-CAT may employ cultures of established cell lines (mostly Chinese Hamster lines CHO, V79, CHL), or primary cell cultures (e.g. human lymphocytes). The P-CAT has not been formally validated so far. Despite this, the P-CAT is recommended and accepted by the SCCNFP for safety testing of cosmetic ingredients.
– The photo-micronucleus test (P-MNT) is an *in vitro* mutagenicity test system to detect chemicals, which induce the formation of small membrane-bound DNA fragments, i.e. micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from chromosome fragments lacking a centromere or whole chromosomes, which are unable to migrate with the rest of the chromosomes during the anaphase of cell division. Thus, the micronucleus assay is in principle suitable to detect *clastogenic* and *aneugenic* effects (the latter is not relevant for photogenotoxicity testing).

The method employing Chinese hamster V79 cells (Kalweit *et al.*, 1999) was successfully adapted to photogenotoxicity testing (Kersten *et al.*, 1999, 2002). The method is based on concentration–response experiments performed with and without irradiation with a UV-Vis sunlight simulation.
– The photo-COMET test (P-COMET) (Singh *et al.*, 1988) is a method for electrophoretic measurement of DNA strand breaks. The field of application of the P-COMET is to detect DNA strand breaks induced by a substance and subsequent/concurrent UV-Vis irradiation, using a small number of eukaryotic cells. The protocol by Singh (1988) has successfully been adapted to photogenotoxicity testing (Chetelat *et al.*, 1996; Bulera *et al.*, 1999; Marrot *et al.*, 2001). The P-COMET is an extremely sensitive test, but it has produced negative results with some compounds that provide positive results in other photogenotoxicity tests, like the P-MNT. Such chemicals are, for example, nalidixic acid and 8-MOP (8-methoxypsoralen) of which only the latter result is understood as DNA–DNA crosslinking under UV light (Brendler-Schwaab *et al.*, 2004).

For *in vivo* photoallergy testing (photosensitisation), no standard testing protocol exists. The frequently used protocol designs are similar to the GPMT, employing additional UV-Vis irradiation (Guillot and Martini, 1985). Currently, no promising *in vitro* methods to predict photosensitisation potential are in sight. The only promising stand-alone alternatives, currently under development, are *in vivo* refinements, like the photo-local lymph node assay, P-LLNA (Homey *et al.*, 1998; Ulrich *et al.*, 1998) or a combination of the P-LLNA with the photo-mouse ear swelling test, P-MEST (Vohr *et al.*, 2000).

CARCINOGENICITY

Substances are defined as carcinogenic if they induce tumours (benign or malignant), increase their incidence or malignancy, or shorten the time of tumour occurrence when they are inhaled, ingested, dermally applied or injected. The process of carcinogenesis is now recognised to be caused by the transition of normal cells into cancer cells, via a sequence of stages and complex biological interactions. It is generally accepted that carcinogenesis is a multi-step process that is strongly influenced by factors such as age, diet, environment, hormonal balance, etc. The complexity of this process makes it not only difficult to extrapolate findings in animals to humans, but also difficult to develop *in vitro* alternative test models.

Since the induction of cancer involves genetic alterations, which can be induced directly or indirectly, carcinogens have conventionally been divided into two categories according to their presumed mode of action: genotoxic carcinogens (=initiators) and non-genotoxic carcinogens (epigenetic carcinogens=promoters). Most potent mutagens are also carcinogens in animal experiments. Information about the genotoxic carcinogenic potential of a substance may be obtained from mutagenicity/genotoxicity studies.

Two *in vitro* tests, cell transformation assay and gap junction intercellular communication (GJIC) have been proposed as tests that may provide information on possible non-genotoxic as well as genotoxic carcinogens. In cases where the *in vitro* short-term tests suggest possible carcinogenic potential, carcinogenicity bioassay in animals are useful to determine potency and target organs.

– The cell transformation assay uses mammalian cell culture systems to detect phenotypic changes *in vitro* induced by chemical substances associated with malignant transformation *in vivo*. Widely used cells include SHE (Isfort *et al.*, 1996), C3H10T1/2 (Mondal and Heidelberger, 1970) and Balb/3T3 (Matthews *et al.*, 1993).

– The GJIC is the intercellular exchange of low-molecular-weight molecules (<1000–1500 Da) through gap junction channels between adjacent cells, and has been found to play an important role in the regulation of cell growth and differentiation. Several methods exist to determine GJIC in different cell types (Murray and Fitzgerald, 1979; Rivedal *et al.*, 2000; Rosenkranz *et al.*, 2000). Dysfunction in this type of communication has been observed to result in abnormal cell growth and behaviour, and associated with several pathological conditions in humans. Structure activity studies have shown a relationship between the ability of substances to inhibit GJIC, and to induce tumours in rodents, but not between inhibition of GJIC and genotoxic activity. This suggests that

GJIC inhibition is involved in non-genotoxic cancer induction, and is a candidate endpoint in screening assays for identification of non-genotoxic carcinogens and tumour promoters, which is not detected by conventional genetic toxicology tests.

Finally, a large number of systems and models dedicated to predicting carcinogenicity have been developed (QSARs and SARs). It has been demonstrated that carcinogenicity is generally only poorly predicted, and the best models tend to be those that can integrate mechanism-based reasoning with biological data (Cronin *et al.*, 2003).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle, including the impairment of reproductive function, the induction of adverse effects on the embryo, such as growth retardation, malformations and death. Owing to the complexity of the mammalian reproductive cycle it is not possible to model the whole cycle on one *in vitro* system in order to detect chemical effects on mammalian reproduction. However, the cycle can be broken down into its biological components, which can then be studied individually or in combination. This has the enormous advantage that the target tissue/organ of an agent can be identified.

Three embryotoxicity tests have been formally validated (ECVAM) in order to replace the OECD guideline 414 (developmental toxicity testing):

– The embryonic stem-cell test (EST). The test is composed of two procedures, a cytotoxicity test, which is conducted with the mouse embryonic stem (ES) cell line D3 and cells of the differentiated mouse fibroblast cell line 3T3, and a differentiation assay using D3 cells (Spielmann *et al.*, 1997; Genschow *et al.*, 2000). Three toxicological endpoints were identified to classify the embryotoxic potential of chemicals:

- The inhibition of differentiation of ES cells into cardiomyocytes (ID_{50})
- The decrease of viability of adult 3T3 cells (IC_{50}3T3)
- ES cells (IC_{50}D3) in an MTT cytotoxicity test. The validation of EST provides an overall accuracy of 78% (Genschow *et al.*, 2002).

– The whole embryo culture derived from mice, rats or rabbits, is used to detect developmental toxicants (Bechter and Schmid, 1987; Piersma *et al.*, 1996). Head-fold or early somite-stage embryos are dissected free from maternal tissue, parietal yolk sac and Reichert's membrane, leaving the visceral yolk sac and ectoplacental cone intact. The conceptus is cultured in medium under defined conditions for 24–48 h. Medium containing a high proportion of serum is usually used and test compounds are added to the cultures for appropriate periods of time. A defined number of endpoints are analysed after the incubation period, such as dysmorphogenic effects, embryonic growth, differentiation, yolk sac circulation and vascularisation, effects on the haematopoiesis, etc. (Genschow *et al.*, 2002).

– The micromass culture is making use of cell cultures of the limb bud and/or neuronal cells. The cells are isolated from the limb or the cephalic tissues of mid-organogenesis embryos (Whittaker and Faustman, 1994). After preparing a single cell solution, the cells

are seeded at high density and undergo differentiation into chondrocytes and neurons without additional stimulation. The differentiation after exposure to test chemicals is analysed by using defined toxicological endpoints (Rockley and Richold, 1990; Genschow *et al.*, 2002).

These tests can only be used in a test strategy that covers main manifestation of developmental toxicity.

CONCLUSIONS

Validation of an alternative model for acute oral toxicity is very complex and the time estimated to achieve complete animal replacement for acute toxicity testing is not clearly defined and cannot be estimated at any less than 10 years.

In the area of skin corrosion/irritation, alternative methods for skin corrosion have been validated and accepted for regulatory use in the EU and the OECD Member Countries, so animal testing should not be performed for this endpoint. Nevertheless, the human skin model assays (e.g. EpiDerm^{TM} and EPISKIN^{TM}) and the mouse SIFT appear to be the most promising *in vitro* methods for skin irritation testing. However, there is a need to develop new endpoints that are more predictive of skin irritation than simply being cytotoxicity determinations.

For eye irritation test, good reproducibility and reliability of the most valuable alternative methods have been demonstrated; however, no single method is able to replace the Draize rabbit eye test. This is due to different factors including the limited quality of the existing *in vivo* data, limitations of the animal testing method and the fact that the range of criteria for injury and inflammation covered by the Draize rabbit eye test is unlikely to be replaced by a single *in vitro* test.

No alternative method for predicting sensitisation has been validated. Refinement and reduction methods have been developed and are widely used. Nevertheless, there is no single test method which will adequately identify all substances with a potential for sensitising human skin and which is relevant for all substances.

The *in vitro* assessment of dermal absorption of cosmetic ingredients can be seen as a full alternative to the *in vivo* test. However, the quality of submitted *in vitro* data is still not always satisfying with respect to documentation and technical aspects, which have been published.

No generally accepted alternative methods are available to replace repeat-dose *in vivo* testing in subacute and subchronic toxicity. Complete replacement of animal usage will represent enormous scientific and technical challenge.

To determine the genotoxic and mutagenic profile of a compound, no single validated test can provide information on gene mutations. As a consequence, a battery of *in vitro* tests is needed.

In the field of UV-induced toxic effects, identification of acute phototoxic hazards is regarded sufficiently covered by *in vitro* tests, consequently, animal testing for that endpoint can be 100% replaced. On the other hand, the *in vitro* genetic toxicity tests have been converted into test protocols of photogenotoxicity tests and for *in vivo* photoallergy testing (photosensitisation) no standard testing protocol exists.

Two *in vitro* tests, cell transformation assay and GJIC have been proposed as tests that may provide information on possible carcinogenicity effects.

Finally, in order to replace the OECD (2001) guideline 414 about developmental toxicity testing, the ESCT, the whole embryo culture and the micromass culture have been formally validated (ECVAM).

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9.2. Efficacy Evaluation

A. del Pozo* and A. Viscasillas

Unitat de Tecnologia Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

EFFICACY OF A COSMETIC PRODUCT

A cosmetic product is effective when it fulfils the function for which it has been designed; thus for example, a moisturizing cream will have to improve the state of superficial cutaneous moisturizing quantitatively, and an antidandruff shampoo will have to significantly reduce (during the period of time stated) the incidence and morphological characteristics of the desquamation of the scalp.

Efficacy assessment involves designing and making tests able to quantify to what extent the product fulfils the effects claimed in its marketing and labelling, a stipulation required by the cosmetic legislation in force and demanded by the consumer (Council, Directive 3/35/CEE).

TYPES OF EFFICACY TESTS

They can be divided into two types:

Qualitative tests

They provide qualitative but subjective information, about the quality of the product because this information is obtained from a trained panel of possible consumers on the basis of perceived efficacy.

The most commonly used technique to assess this perceived efficacy is sensorial analysis, i.e. the scientific discipline used to measure, analyze and interpret characteristics of materials which are perceived by the human senses. This subject falls outside the scope of this book, but interested readers can find interesting literature on sensorial analysis of cosmetics (IFSCC, 1987; Civille and Dus, 1991; Piacquadio and Kligman, 1998; Civille and Meilgaard, 1999; Koehler and Maibach, 2000; Torres, 2001; Barkat *et al.*, 2003; Musnier *et al.*, 2004; Eisfeld *et al.*, 2005; Sang-Woong *et al.*, 2005; Coll, 2006).

*Corresponding author. E-mail: apozo@ub.edu

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Quantitative tests

They are based on the determination of different cutaneous experimental parameters before and after the use of the product, in order to evaluate the possible changes brought about by the cosmetic. The evaluation of previous results (without product application), is also of interest to characterize the skin type and also the different dysfunctions or dermatological pathologies.

Since alternative methods to animal testing should be used for cosmetics, according to the guidelines and/or rules set out in the legislation of most countries (see Section 1.1), to develop *in vitro* methods for efficacy studies constitutes an authentic challenge. Moreover, invasive *in vivo* techniques using human volunteers (such as the sun protection factor estimation by UV irradiation methods that was commented in Section 3.1) should also be changed for non-invasive *ex vivo* techniques or for *in vitro* methods.

Scientific tools, techniques and work protocols used for objective evaluation of cosmetic-product efficacy have their origin in the irruption of bioengineering in the dermatological field, the elements of which have contributed to an effective assessment of the state of skin, diagnosis and monitoring of a dermatological or cosmetic treatment (Takahashi, 2001; Sang-Woong *et al.*, 2005) and even of safety and tolerance to them (Rogiers *et al.*, 1999).

QUANTIFICATION OF COSMETIC EFFICACY: NON-INVASIVE BIOPHYSICS TECHNIQUES

The use of this type of techniques is based on measuring different physical properties of the skin (water transport, electrical capacitance, Doppler effect, elasticity, etc.), the experimental values of which can be correlated to certain biological properties (barrier effect, moisturizing, cutaneous microcirculation, etc.). These determinations enable conclusions to be drawn related to the state of skin, and/or the efficacy of applied cosmetic treatments.

These techniques were first proposed in 1976 at a meeting carried out in Miami Beach on non-invasive biophysical techniques. This meeting gave rise to the International Society of Bioengineering and the Skin (ISBS), founded in 1979, whose magazine "Skin Research and Technology" and later congresses have boosted the growth and development of such techniques.

The advantages of these techniques are that they are both easy to use and reproducible. It is particularly important to establish the environment in which to carry out the tests in order to obtain results with low standard deviations. In fact, there are many variables (psychological or heat stress, diet, etc.) with difficult controls (as they do not depend on the method but on the inter-individual variability of the trained panel of volunteers) and therefore it is necessary to limit these variables as far as possible. Moreover, other external variables have to be standardized and recommendations are to carry out the determinations in an isolated room (20 ºC and 40–60% relative humidity), with the panellist comfortably positioned and left to acclimatise for a minimum of 15 min before the measurements are taken. In some cases, special conditions may also be needed (relative humidity, temperature, ultraviolet or visible light).

Table 9.2.1 shows some of the parameters usually employed in the dermatological or cosmetic field, the techniques used to determine them and the properties measured (del Pozo, 2005).

Table 9.2.1

Some examples of biometrological techniques applied to cosmetic efficacy assessment

Features to be evaluated	Measured properties	Technique	Application fields[a]
Lipids content	Cutaneous lipids, sebaceous secretion	Sebumetry	EC, DD, TC
Skin surface moisture	Stratum corneum hydration Cutaneous pH, epicutaneous emulsion quality	Corneometry pHmetry, evaporimetry	EC, DD, TC EC, DD, TC
Roughness	Cutaneous relief	Image analysis of silicone replicas	CA, EC, DD
Transepidermal water loss Erythema	Perspiration Cutaneous microcirculation	Evaporimetry Laser Doppler velocimetry	EC, DD EC, DD
Cellulitis, strecht marks	Dimension and structure of the different layers from the skin	Echography	EC, DD, TC
Mechanical properties	Tone, elasticity, fatigue, recovery	Techniques based on viscoelastic properties	CA, EC, DD
Colour properties	Skin colour, phototype, sun protection factor	UV/Vis techniques	CA, EC, DD
Dryness, xerosis, seborrheic dermatitis, dandruff	Desquamation	Stripping and image analysis	EC, DD

[a]EC: cosmetic efficacy assessment; DD: dermatological diagnostic; TC: tipology cutaneous determination; and CA: cutaneous aging estimation.

Guides published by the European Group on the Efficacy Measurement on Cosmetics and Other Topical Products (EEMCO) (EEMCO, 2000) are very useful when carrying out these studies. Different articles revising characterization, reproducibility, interpretation criteria, instrumentation, work protocols and applicability of these techniques can be also useful (Serup a nd Jemec, 1995; Aust, 1998; Piérard, 2002; Agache and Humbert, 2004; Wallen and Maibach, 2005).

Here we will comment on some techniques used for effective evaluation of the most common cosmetic actions, with a special mention of those related to moisturizing and/or skin-barrier function maintenance. In all cases, and since official methods do not exist, researchers will have to select the most suitable method according to the specific cosmetic features to be evaluated.

Stratum corneum moisture

Unlike the corneous layer, the dermis and the keratinocytes in differentiation phase carry an electrical current. Free water (intercellular spaces, transpiration) is able to provide a characteristic conductance or capacitance to the skin surface, which can be determined

using a probe. The values recorded can then be related to the moisturized state of the cutaneous surface.

Estimation of the water content in stratum corneum from electric capacitance using a corneometer has been validated by different authors in terms of reproducibility and linearity, although it is important to emphasize that one cannot extrapolate the results obtained with different apparatus from the same laboratory, or that published by different laboratories (Wemer, 1986; Van Neste, 1991; Clarys and Barel, 1997; O'goshi and Serup, 2005).

The effects of room temperature and humidity conditions have been demonstrated, thus controlled conditions are recommended (18–20 ºC and 40–60% relative humidity) (Barel *et al.*, 1991; Mignini and Gagliardi, 1992).

Moreover, the typology of the patient (phenotype, phototype) and the body site to be studied (forearm, forehead, etc.) must be considered. In order to obtain statistically representative results (with low-variation coefficients), the study must be made using data obtained from individuals with similar typologies. However, other factors, such as sex, age, hour of the day or laterality (right or left forearm) do not seem to have significant effects on cutaneous hydration (Mignini and Gagliardi, 1992).

Bearing these considerations in mind, EEMCO recommends a general work protocol (Berardesca, 1997) in which the hydration level is evaluated after just one application, on the forearm (this zone is chosen because of its uniformity) at different experimental times, working under standard conditions.

Evaporation of water from the body through the skin

Transepidermal water loss (TEWL) includes a mixed phenomenon of passive diffusion (amount of water vapour passing the skin) and insensible perspiration (perspiration that evaporates before it is perceived as moisture on the skin). Mass or volume of water that evaporates in 1 h through 1 m^2 of skin is measured.

Literature describes different gravimetric methods used to determine the TEWL (Wilson and Maibach, 1989); however, these are practically in disuse given lack of reproducibility and discomfort of execution. At the moment, such determinations are made using computerized evaporimeters, some of which are very simple and provide fast readings.

Experimental determinations of the skin surface are made by applying a probe to the skin, with a cylindrical Teflon capsule open to the atmosphere. Two hygrosensors send measurements to a central unit, thus recording the amount of water vapour at two levels. These are then integrated and transformed into water transport values by applying Fick's law.

This technique has some limitations, such as high detection limits (around 0.1 g/m^2h). Moreover, it is highly susceptible to changes in environmental humidity, air turbulence in areas near to where samples are taken (effect of forced ventilation, operator's breathing, etc.), so it is recommendable to limit these phenomena to a minimum, even by using screens suitably placed in the zone where samples are taken (Scott *et al.*, 1982). On the other hand, variations were observed (sometimes up to 1 g/m^2h) among results obtained with different probes, even in studies made in parallel, by the same work team, but with different devices (Barel and Clarys, 1995).

The trend in technical development of evaporimeters focuses on improving measurement probes and software, enabling measurement time to be decreased with the consequent reduction in external agents interfering with the readings (De Paepe *et al.*, 2005).

Different work protocols based on determining TEWL variation before and after treatment can be found in the literature to estimate the efficacy of antiperspirants, moisturizing and emollient products, among others (Pinnagoda *et al.*, 1990; Rogiers and the EEMCO Group, 2001; Fröschle and Plüss, 2004).

Stratum corneum hydration and cutaneous barrier function: Combined use of corneometry and evaporimetry

The values obtained by corneometry and TEWL from a same panel of volunteers change over a period of time after applying a cosmetic formulation on their skin. A parallelism between both kinetic curves has been observed. The use of both techniques has been recommended for pre-formulation studies concerning the optimization of the nature and concentration of actives as well as excipients. On the other hand, comparative studies also showed the reproducibility and parallelism between the results obtained by evaporimetry and sensorial analysis (Blichmann *et al.*, 1989; Bimczok, 1992; Redoules *et al.*, 1993).

The emollient action of cosmetic formulations, which enables free water content to be increased in the corneous layer, may take place through two mechanisms: occlusion or water adsorption (hygroscopic effect) of the applied products. The physico-chemical features of the formulation have to be taken into account in order to interpret the results obtained by both corneometry and/or evaporimetry (Rawlings, 2003).

When the moisturizing preparation is an oil-in-water (O/W) emulsion, the values obtained by both corneometry and evaporimetry increase substantially during the first 15–30 min; nevertheless, these values do not correspond to an increase in the hydration state, but to the liberation of water provided during the application of the emulsion on the skin. Afterward, both values become stable. A slow recovery (4 h or more) of the basal value (defined as that obtained before the product was applied) indicates that the formulation allows humidity to be retained in the most superficial layers of the stratum corneum, which is a reason why it acts effectively for the purpose for which it was designed; however, short recovery times denote deficiencies in product formulation.

When, on the contrary, the preparation is a water-in-oil (W/O) emulsion and skin hydration is obtained only by an occlusion mechanism and reduction of perspiration to retain water that would otherwise be eliminated from the cutaneous structures, the interpretation is different: the TEWL values like those obtained by corneometry will be null (perspiration is reduced by occlusion) or they are lower than the basal ones (because of the difficulty of electrical transmission in low-polarity mediums). These values increase with time until reaching the basal value, either due to the dispersion or loss of the product by mechanical action (such as friction caused by clothes or other).

The so-called stratum corneum barrier function defines the ability of the skin to avoid external aggressions due to mechanical agents, temperature, etc. (Choi and Maibach, 2005).

The TEWL technique enables the state of the function barrier to be assessed (abnormally high TEWL values might indicate a deficient barrier function) (Savic *et al.*, 2003). For this reason, it is frequently used in dermatology as an element to confirm diagnoses

(Loden, 1995; Tokamura and Umekaga, 2005) as well as to study the efficacy and the cutaneous tolerance of cosmetics, such as shampoos, bath or shower gels, cleansing emulsions, make-up remover, etc. (Gloor *et al.*, 2004; Bornkessel *et al.*, 2005).

The water-holding capacity (WHC) or water-retention capacity of stratum corneum enables not only the state of skin hydration to be characterized, but more importantly, the integrity degree of barrier function. It can be determined by means of a sorption–desorption test based on studying the kinetic of water evaporation (Black *et al.*, 2000, 2002; del Pozo *et al.*, 2000, 2003).

Skin biomechanical (viscoelastic) properties

Determining the biomechanical properties (also qualified as rheological properties by some authors) of the skin enables the dermal state to be assessed. Parameters such as age, sex, thickness and hydration of the skin in the sample zone, and obviously the use of cosmetics influence the experimental values obtained (Black *et al.*, 1998; Piérard *et al.*, 1998; Rodrigues and Pinto, 2004). By evaluating the differences detected pre- and post-treatment allows one to judge the efficacy of the tested products.

Through time the cutaneous surface loses its elasticity and, at the same time its plasticity increases; consequently, the skin loses its smoothness and wrinkles appear. The extent to which one can improve this state by using refirming and antiaging preparations can be evaluated by applying different types of mechanical stress to the skin with later analysis of skin resistance. The same techniques also enable soothing preparations like after-sun to be evaluated as well as all those used to reduce cutaneous inflammation that affect skin turgidity (Piérard *et al.*, 1995; Rodrigues *et al.*, 2002).

EEMCO proposes protocols and techniques based on different types of stress (Rodrigues, 2001): traction, erosion (Piérard, 1993), drop impact resistance (Tosti *et al.*, 1977), torsion (Escoffier *et al.*, 1989), pressure (Osanai *et al.*, 2003), friction (Takahashi *et al.*, 2003; Tronnier *et al.*, 2003), and suction (Barel *et al.*, 1995). Of all these methods, and in agreement with specialized literature (Wickett and Murray, 1997; Dobrev, 2000; Pedersen *et al.*, 2003), those based on suction seem to be more useful and reproducible. The skin's capacity to recover once suction stops is also used as a parameter of its dynamic behaviour (Hermanns-Lê *et al.*, 2004; Dobrev, 2005).

Total surface lipids

Lipids placed on the cutaneous surface come from sebaceous secretions and corneocytic structure remains, which are freed due to the turnover of the epidermal cells. Cosmetic treatment must be able to restore a suitable balance between oil and water secretions, especially when pronounced skin imbalances appear (alipic, greasy and/or acneic skins, hyperseborrhea, etc.) since healthiness and appearance of the skin depend on this balance as well as on a correct composition of the natural moist factor (NMF).

Different techniques enable lipids placed on the skin surface to be determined (Thune and Gustavsen, 1984; Clarys and Barel, 1995). Such techniques are used, for instance, to determine the efficacy of antiseborrheic or seboregulating products or treatments for greasy

skins or those with acneic tendency, etc., by comparing readings pre- and post-treatment (Piérard *et al.*, 2000) and can also back up diagnoses and monitoring of dermatological treatments (Rode *et al.*, 2000; Coderch *et al.*, 2002; Bornkessel *et al.*, 2005; Youn *et al.*, 2005). One such method is based on photometrically measuring the transparency degree of a translucent plastic strip of defined roughness and amplitude, to assess the amount of fat that adheres by contact with the skin surface (Courage-Khazaka Electronic, 1997). The results are expressed in μg of total fats/cm^2 of skin, since software transforms the transmittance into the previously mentioned units. Alipic skins have values below 50 and hyperseborrheic skins have values over 300 (Piérard-Franchimont *et al.*, 1999; Youn *et al.*, 2002).

Evaluating cutaneous pH complements the sebumetry and enables an indirect estimation of the state and quality of the skin's hydrolipidic mantle (Ehlers *et al.*, 2001).

Skin colour

Estimation of skin colour is important to diagnose skin type, since it is related to the phototype, which allows the specialist to better adjust the recommended cosmetic treatment (Andreassi *et al.*, 1990).

Determining skin colour variations also allows the evaluation of bronzing-agent efficacy with or without sun (Romero and del Pozo, 2001), quantify the degree of erythema and consequently the efficacy of skin congestion treatments and after-sun preparations (Rodrigues *et al.*, 2002) or to evaluate the degree of whitening induced by de-pigmentation agents (Zuidhoff and Van Rijsbergen, 2001; Gloor *et al.*, 2003; Petit and Piérard, 2003; Benchikhi *et al.*, 2004).

Moreover, the determination of skin colour is also used by dermatologists to monitor changes in freckles or other skin pigmentation disorders, thus it is obviously a very interesting work tool in the scope of photoprotection and cutaneous photobiology (Chardon and Crétois, 1991; Piérard and Piérard-Franchimont, 1993; Wee *et al.*, 1997; Piérard, 1998; Dornelles *et al.*, 2004).

Spectrophotometric techniques allow to determine a series of parameters related to skin colour; among these are the luminance (the luminous intensity of a surface in a given direction per unit of projected area), reflectance (relation between incident and reflected radiation) or the chromaticity parameters (related to the proportion of basic colours of the visible spectrum that define the colour of the studied sample) (Clarys *et al.*, 2000; Egea, 2005).

The results are shown as index numbers, pigmentation index also denominated melanin index, which can vary from 0 to 1000 (Edwards, 1995). The system can also be used to calculate "haemoglobin index" from the same experimental determinations; this index is of interest to quantify the cutaneous degree of erythema (Fullerton *et al.*, 2003) and consequently skin improvement after using after-sun cosmetics.

Other techniques used to evaluate cosmetic product efficacy

It is not possible to undertake a detailed study of this issue here, given the huge number of minimum or non-invasive techniques and procedures that, when suitably adapted, can be

used to estimate cosmetic efficacy. Therefore, only the most interesting, from the authors' viewpoint, will be described here.

Laser Doppler velocimetry techniques

The cutaneous microcirculation acts as a heat regulator and changes as a result of either skin disorders (cellulitis, aging, photoaging, solar erythema, etc.) or topical application of certain preparations (skin congestion treatments, lipolytics, products containing surfactants) (Duval *et al.*, 2003).

It is determined by deviations in Doppler frequency, which occur when a monochromatic laser light hits the skin and is dispersed due to red-cell movement. A detector catches the signal and the response is proportional to the number of erythrocytes according to their speed in the cutaneous microcirculation (Berardesca *et al.*, 2002; Wallen and Maibach, 2005).

Thermographic techniques

Skin temperature varies according to its basal or common levels depending on different processes; as an example, in cellulitic zones a reduction in cutaneous temperature takes place, whereas in cases of solar erythema (even subclinical), this parameter increases. Infrared thermography (changes in colouring on a plate with materials of thermotropic liquid crystal type) enables true thermal maps to be obtained, which are of considerable diagnostic value, and also establish criteria for cosmetic efficacy assessment (anticellulitic products, heavy-leg preparations, etc.). To achieve the latter aim, and in cases where study type requires greater precision, classical metallic thermal soundings can be used (Perin *et al.*, 2001).

Exfoliative cytology techniques

There are quantitative methods that are useful to characterize desquamation and removing of corneocytes process from their cellular turnover and degree of cohesion (Marks and Barton, 1983) as well as the possible changes in these processes brought about by dermatological or cosmetic preparations (antidandruff, dry skin products, etc.) (De Paepe *et al.*, 2001). Among these, exfoliative cytology techniques stand out (stripping studied by computerized image-analysis techniques) (Serup *et al.*, 1989; Piérard *et al.*, 1992; Gammal *et al.*, 1996; Piérard, 1996; Lagarde *et al.*, 2000; Gasser *et al.*, 2004).

Microphotographic and microscopy techniques

Sometimes, photographic images are the only alternative when studying cosmetic efficacy. This is the case, for example in trichology, when the aim is to study the efficacy of alopecia products. In principle a conventional digital camera is enough; however, it is recommendable to connect it to systems that optimize and guarantee both homogenous illumination and correction of the dimensions, distorted by curvature of the photographed plane, in order to correct the colour and diminish the presence of shadow, thus avoiding errors in the interpretation of results (Courage-Khazaka Electronic GMBH).

Different microscopy techniques can be used to obtain images (microphotographs), which are very useful in dermato-cosmetic diagnosis as well as to assess post-treatment results. Examples being scanning electronic microcopy (Lavker *et al.*, 1987; Wu *et al.*, 2001; Piérard *et al.*, 2004) and confocal microscopy (Sandoz *et al.*, 1996; Rhouzlane *et al.*, 2002; Sauermann *et al.*, 2002). They are commonly used in individuals to effectively assess different cosmetics: hair care, antiwrinkle, antiseborrheic, emollient and firming treatments, etc.

Computerized analysis image technique

Image analysis using silicone replica of certain zones of the skin enables their profilometric analysis (number, distribution, depth of wrinkles) (Zahouani and Vargiolu, 2000). This is an interesting tool not only for skin characterization (Lagarde *et al.*, 2005a), but also for efficient evaluation of different cosmetics (antiaging, antiwrinkles, exfoliation, etc.) (Corcuff *et al.*, 1985; Reece and Deeds, 1992; Smalls *et al.*, 2005).

Visualization is made using a video camera unit. The sample is placed in a previously fixed direction and illuminated to diminish the effect of shadows (that can lead to false interpretation of results). An opaque silicone replica can be used (Rosén *et al.*, 2005). Also, there are systems using transparent silicone moulds that interpret the characteristics of skin relief based on differences in transmittance between the different zones of the sample (Courage-Khazaka Electronic GMBH; Pena-Ferreira *et al.*, 2003). To take images directly is not recommendable due to the many factors that can distort them and make them difficult to interpret (deformations caused by greater or lesser pressure exerted by the video camera when sliding on the sampled skin zone, greater difficulty in shadow control) (Jacobi *et al.*, 2004; Sandoz *et al.*, 2004). Direct taking only has an orientative value, and sometimes it uses not only white light as the source of illumination of the sample skin zone, but also Wood light, which allows a better differentiation of the features, such as the characteristics of desquamation (Duval *et al.*, 2003). Suitable software will allow the calculation of roughness parameters, texture and anisotropy, in which changes are of interest in evaluating the efficacy of antiwrinkle, antiaging, exfoliation preparations, etc. (Lévêque, 1999; Casadó, 2000). The tribological techniques, based on evaluating the friction coefficient of the cutaneous surface complement the results obtained from silicone replica analysis (Sivamani *et al.*, 2003a, 2003b).

Echographic techniques

The thickness of the different skin layers is a useful parameter to know, when characterizing certain dermatological pathologies (psoriasis, eczema) and it is also of interest in the cosmetic field (study of dry and hyperkeratosic skins, cellulitic processes, etc.) after cosmetic application (Perin and Pittet, 1999; Schnebert *et al.*, 1999; Turck, 2002; Wallen and Maibach, 2005).

The technique involves evaluating reflection (echo) and transmission characteristics of an ultrasound radiation, when crossing layers of different density, which present different acoustic impedance. Illumination intensity of different zones using echography is proportional to the density of the crossed structures, thus, the technique detects the presence

and structure of blood vessels, cellulitic nodules, thickness and density of adipose panniculus, etc., and consequently changes in these dermal structures brought about by the action of cosmetic or dermatological treatments (Perin and Pittet, 1999; Schnebert *et al.*, 1999; Turck, 2002; Nouveau-Richard *et al.*, 2004; Sandby-Møller and Wulf, 2004; Lagarde *et al.*, 2005b).

CONCLUSIONS

Legislation in different countries indicates the need for a dossier showing the efficacy of commercial cosmetic preparations.

Researchers work should be carried out to validate simple, economic and rapid methods to carry out efficacy studies (Piérard, 2003). Biometrology, or more generally cutaneous bioengineering, seems to have the greatest future perspectives since it does not involve animal experimentation but rather uses non-invasive or minimally invasive techniques.

Given the lack of legislation on this issue, the researcher must always judge the necessary adaptation of work protocols, based on the promoter's requests (number of repetitions, number of volunteers, experimental conditions, statistical treatments to interpret results, etc.).

This is surely one of the fields with greatest future prospects in the research and development of cosmetic products.

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