Zacarías León González

Percutaneous Absorption of UV Filters Contained in Sunscreen Cosmetic Products

Development of Analytical Methods

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Percutaneous Absorption of UV Filters Contained in Sunscreen Cosmetic Products

Development of Analytical Methods

Doctoral Thesis accepted by
the University of Valencia, Spain

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2. León Z, Chisvert A, Balaguer A, Salvador A, "*Development of a fully automated sequential injection solid-phase extraction procedure coupled to liquid chromatography to determine free 2-hydroxy-4-methoxybenzophenone and 2-hydroxy-4-methoxy-benzophenone-5-sulphonic acid in human urine*", Anal Chim Acta (2010), 664:178–184. http://www.sciencedirect.com/science/article/pii/S000326701000228X

3. León Z, Chisvert A, Tarazona I, Salvador A, "*Solid-phase extraction liquid chromatography–tandem mass spectrometry analytical method for the determination of 2-hydroxy-4-methoxy-benzophenone and its metabolites in both human urine and semen*", Anal Bional Chem (2010), 398:831–843. http://link.springer.com/article/10.1007/s00216-010-3947-6

4. León Z, de Vlieger J, Chisvert A, Salvador A, Lingeman H, Irth H, Giera M, "*Identification of the Biotransformation Products of 2-Ethylhexyl 4-(N,N-Dimethylamino)benzoate*", Chromatographia (2010), 71:55–63. http://link.springer.com/article/10.1365/s10337-009-1386-3

5. León-González Z, Ferreiro-Vera C, Priego-Capote F, Luque de Castro MD, "*Targeting metabolomics analysis of the sunscreen agent 2-ethylhexyl 4-(N,N-dimethylamino)benzoate in human urine by automated on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry with liquid chromatography–time-of-flight/mass spectrometry confirmation*", J Chromatogr A (2011), 1218:3013–3021. http://www.sciencedirect.com/science/article/pii/S0021967311004080

6. León-González Z, Ferreiro-Vera C, Priego-Capote F, Luque de Castro MD, "*Bioaccumulation assessment of the sunscreen agent 2-ethylhexyl 4-(N,N-dimethylamino)benzoate in human semen by automated online SPE-LC–MS/MS*", Anal Bioanal Chem (2011), 401:1003–1011. http://link.springer.com/article/10.1007/s00216-011-5141-x

7. León-González Z, Chisvert A, Fernández I, Salvador A, "*A solid-phase extraction liquid chromatography-tandem mass spectrometry method for the percutaneous absorption assessment of 3-(4′-methyl-benzylidene)camphor* via *human urine analysis*", Anal Methods (2013), 5:367–375 (Reproduced by permission of The Royal Society of Chemistry). http://pubs.rsc.org/en/content/articlelanding/2013/ay/c2ay25490e

Apreta fort les dents,
apreta els punys.
Infla't d'aire els pulmons,
obre bé els ulls.
Fes treballar el cap,
controla el cor.
No tanques mai la boca,
crida ben fort.
Deixa't anar,
dóna't tot tú.

Ovidi Montllor

To my father,
because I know that wherever he is,
he's proud of me

To my mother,
because she is the reference of my life

Supervisors' Foreword

The use of high efficacy sunscreens is necessary to minimize the harmful effects that can be caused to the human body by an excessive exposure to sun radiation. Nowadays, dermatologists strongly recommend the use of sunscreen products not only when incidence of extreme sun exposure (i.e., beach, snow, etc.), but also in daily situations. However, these cosmetic formulations contain in their composition chemical compounds, commonly known as UV filters, which undergo some undesirable processes, such as percutaneous absorption and systemic effects.

The final aim of this Ph.D. thesis has been the development and validation of analytical methods based on both *in vitro* (Chap. 2) and non invasive *in vivo* (Chaps. 3–9) procedures which allow to gain more information about the adverse side effects that can be produced to the human body by the UV filters contained in sunscreen formulations. Hence, the described methodologies would help to contribute to the design of cosmetic formulations able to provide a high level of safety without any kind of risks to the users.

The *in vitro* methodology used to estimate percutaneous absorption processes of the most common water-soluble UV filters in sunscreen cosmetics (Chap. 2) was based on the use of diffusion cells.

In vivo procedures include the determination of UV filters in biological fluids from users of sunscreens that applied cosmetic formulations at different extent containing the target compounds in their composition. The target UV filters that were *in vivo* studied in this Ph.D. thesis have been benzophenone-3 (Chaps. 3–5), ethylhexyl dimethyl PABA (Chaps. 6–8), and methyl benzylidene camphor (Chap. 9).

The analytical techniques used along the study involved the use of LC-UV, LC-MS and GC-MS, coupled to sample preparation techniques (i.e., solid-phase extraction), thus providing the student a wide vision of analytical possibilities to apply in the future.

Furthermore, the metabolic pathway of a widely used UV filter was first elucidated by both *in vitro* and *in vivo* methodologies (Chaps. 6–8). Other important highlight from this thesis has been the determination of UV filters and its metabolites in human semen for the first time (Chaps. 5 and 8), thus opening

research lines concerning the establishment of relationships between the found amounts of these potential estrogenic compounds and the variation of specific parameters on semen quality.

Valencia, June 2013   Prof. Alberto Chisvert
                                                                 Prof. Amparo Salvador

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Part I
Introduction

Chapter 1
Sunscreen Cosmetics: Advantages and Drawbacks

1.1 The Protective Skin Barrier

The skin is the largest organ in the human body. Among its main functions, the skin is able to convey feelings, regulate body temperature, produce vitamin D and protect against external aggression. This protection is not based on the repulsion of noxious agents, but in the interaction with them through various defensive mechanisms which prevent the extension of possible skin lesions (Cohen and Rice 2008).

Regarding the histology of the skin, there are two main parts which are separated by a wavy basal membrane called papillary plexus. The outer part is called epidermis, while the innermost is dermis. In turn, the epidermal appendages, mainly the hair follicles, penetrate the epidermis and are embedded in the dermis (see Fig. 1.1).

The dermis is about 90 % of the thickness of the skin and acts as a cushion to support the epidermis. It consists mainly of collagen and elastin fibers, and contains nerves, blood and lymph vessels, and both sweat and sebaceous glands. Between the dermis and underlying tissues, there is a layer of adipocytes, also called hypodermis, where the fat is accumulated in the form of triglycerides.

Blood flow of the epidermis comes from the capillaries located in the papillary plexus. The epidermis consists of four distinct layers, which are called in order of increasing depth: corneum, granulosum, spinosum and basal or germinative layer. The outermost layer, the stratum corneum, consists mainly of keratinocytes, which are squamous cells strongly bonded together, and have no nucleus, then being biologically inactive. In the epidermis, there are also melanocytes, which produce melanin granulation under the stimulus of ultraviolet (UV) light. These granulations, once ejected, are incorporated into the surrounding epidermal cells that are then pigmented. Moreover, many Langerhans cells can be found in the epidermis, which have major importance in the immune reactions of the skin against foreign agents.
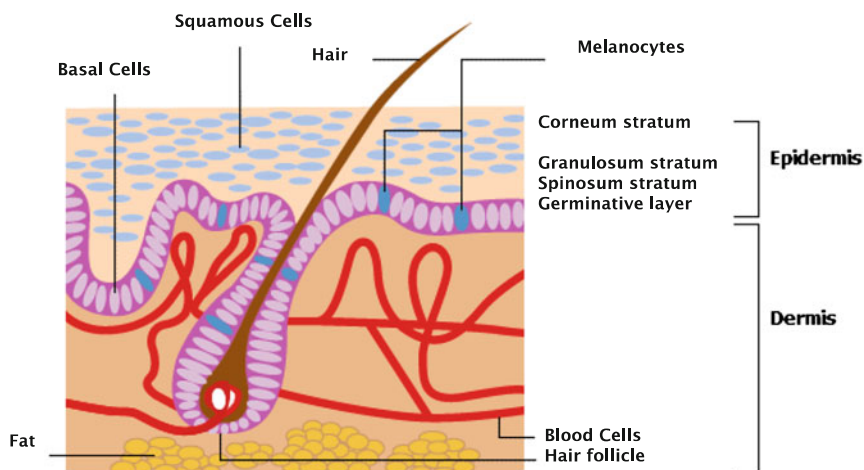
Fig. 1.1   Schematic drawing of a human skin section

1.2 Interaction Between Electromagnetic Radiation and Skin: Phototoxicology

Throughout life, the skin is exposed to different wavelengths of the electromagnetic spectrum that reach the Earth's surface, including UV, visible light and infrared (IR) radiation from the sun, artificial light and sources of heat. These radiations have different levels of penetration when they impact the human skin, as can be seen in Fig. 1.2.

It is interesting to compare the penetration ability of the UV-A (320–340 nm) and UV-B (290–320 nm) radiations through the skin. While the UV-A radiation gets through the dermis, the more energetic UV-B radiation only penetrates the
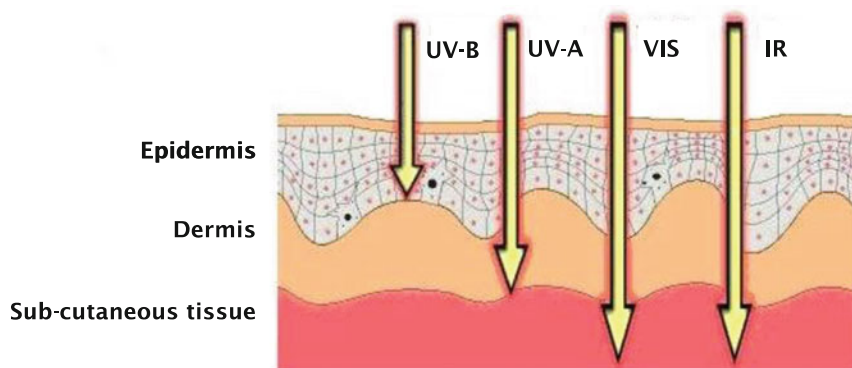
Fig. 1.2   Skin penetration of the different radiation achieving the Earth's surface

Fig. 1.3 Sunburn effects

epidermis. The visible and IR radiations have greater penetrating ability but, fortunately, they are less energetic.

Taking into account the effects induced by the UV radiation on the skin, it is important to consider the length of exposure, distinguishing between acute and chronic exposures, as well as the influence of environmental conditions, such as season, latitude, deterioration of the ozone layer, the exposed body region, skin pigmentation and the previous exposures (Herman et al. 1996).

The most obvious manifestation of acute exposure to UV radiation is sunburn (redness or sunburn, see Fig. 1.3). UV-B radiation has the greater capacity to cause sunburn in human skin.

Another typical sign of acute exposure to UV radiation is the dark pigmentation of the skin. This may be due to the increased production of melanin by melano-cytes or to the photo-oxidation of melanin. The tanning or increased pigmentation usually occurs after 3 days of exposure to UV light, while the photo-oxidation is observed immediately.

The pigment darkening that occurs immediately after exposure to UV-A and visible light does not increase the capacity of photo-protection. However, tan, which appears more readily after exposures to UV-B, enhances the protective effects of melanin on skin. Thus, acute exposure to UV-B radiation brings the thickening of the stratum corneum, which means, in turn, a greater protective effect against subsequent attacks caused by UV radiation.

Within this framework favourable, it is considered that the natural and environmental exposure to light is essential for the development of life. UV radiation is essential to promote the blood circulation (Barth et al. 1994) and the action of certain neurotransmitters in the brain that are responsible for good mood and feeling of well-being (Grant and Gruijl 2003; Lowry et al. 2009). In addition, the conversion of 7-dehydrocholesterol to provitamin D3, a necessary precursor for the endogenous formation of vitamin D, is enhanced (Chapuy et al. 1997). The toxic action of UV light emitted by artificial sources has also been used for decades to treat processes causing excessive proliferation of skin, as psoriasis and seborrheic dermatitis (Parrish and Jaenicke 1981).

In contrast, chronic exposure to solar radiation can stimulate some skin lesions that depend on the degree of basal pigmentation of the individual. Thus, freckles and skin spots, caused by inadequate distribution of pigments, wrinkles and actinic keratosis (pre-cancerous lesion) are direct consequences of prolonged exposure to UV light (Lim and Cooper 1999). Also, excessive exposure to the sun can lead to premature aging caused by the destruction of elastin and collagen fibers of the dermis, and skin cancer, which is the most common cancer malignancy in humans (Naylor and Farmer 1997). Even, it can be said that the main cause of skin cancer is sunlight. UV radiation can cause pyrimidine dimers in epidermal cells, thus triggering a series of mutations in genes. UV light has also immunosuppressive effects that can promote the persistence of some skin tumours. It should be noted that the incidence of skin cancer is higher in the tropics and in Caucasians and pale skin people. For this reason, there are many public health programs aimed at establishing measures for the sunscreen to reduce the risk of this cancer (ICNIRP 2007). Changes in social behaviour, including increased leisure activities outdoors (water sports, winter sports, etc.) require the consideration of prevention efforts on those risks (Hiom 2006).

The most effective photo-protective measure to suppress harmful effects of the UV radiation is to avoid sun exposure. However, this is not practical or desirable as indicated above. Therefore, it is recommended as far as possible, to minimize sun exposure during the hours when UV radiation is most harmful, to use suitable clothing and sunglasses, and to apply sunscreen cosmetics to obtain optimal protection (Nohynek and Schaefer 2001; Kullavanijaya and Lim 2005; Nash 2006; Gaspar and Fields 2007).

1.3 The Cosmetics for Sun Protection in the European Union

Cosmetic products for sun protection are classified in different categories by the different countries, which in turn depend on the relevant legislation. The three major regulatory systems of cosmetic products in the world are the Cosmetic Products Regulations of the European Union (EU), the rules of the food and drug administration (FDA) and Japanese legislation. Both the EU and Japan consider the sun protection products as cosmetics, while the United States considers them as over-the-counter products. Cosmetic products for sun protection include various chemicals in the composition, commonly known as UV filters, which act as active ingredients absorbing or reflecting solar radiation.

1.3.1   UV Filters. Classification and Properties

As indicated previously, cosmetics for sun protection are regulated in the EU by the Cosmetic Products Regulation (Regulation (EC) No 1223/2009), which replaces the previous Cosmetics Directive (Council Directive 76/768/ECC). Annex VI of this Regulation contains a list of chemicals that can be used as UV filters in cosmetic products, indicating their maximum allowed levels. UV filters can be defined as *substances which, contained in cosmetic sunscreen products, are specifically intended to filter certain radiation to protect skin from certain harmful effects of these rays* (Cosmetics Directive (76/768/ECC)). The allowed compounds are reviewed periodically by the Scientific Committee on Consumer Products (SCCP), which includes scientists from different member countries that study the experimental data obtained from the compounds of interest. After considering the relevant reports, the European Commission takes the appropriate actions. Currently, there are 26 substances that make up the Annex VI. Currently allowed UV filters and their authorized contents are shown in Table 1.1.

Note that *p*-amino benzoic acid (PABA) is the UV filter whose ban to be used as a cosmetic ingredient has been more recently applied (Directive 2008/123/EC). Nevertheless, no official analytical methods exist for the determination of these compounds in cosmetic products for sun protection (Salvador and Chisvert 2005).

UV filters can be divided into two groups, according to a classification based on chemical nature. On the one hand, the inorganic or physical UV filters, which act mainly reflecting or scattering the incident UV radiation and, on the other hand, the organic or chemical UV filters, which absorb UV light.

In general, physical UV filters are metal oxides. Although providing increased protection compared to chemical UV filters, they have a lower acceptance due to their low solubility in water that allows the formation of a protective film on the skin that is not pleasing to the user. Currently, only titanium dioxide is authorized as physical UV filter for Cosmetic Products Regulation in the EU, as shown in Table 1.1.

Meanwhile, the chemical UV filters are defined as organic compounds with a high molar absorptivity in the UV wavelength range. These compounds usually have one or more aromatic rings, sometimes conjugated with carbon–carbon double bonds and/or carbonyl groups. Cosmetic products containing these compounds normally have better acceptance than those formulated with physical UV filters due to their more convenient form of application. In turn, the chemical UV filters can be classified according to their chemical structures in different families, such as benzophenone derivatives (BZ3, BZ4, DHH), *p*-aminobenzoic acid derivatives (EDP, P25), salicylates (ES, HS), methoxycinnamates (EMC, BMI), camphor derivatives (3BC, MBC, BCS, CBM, TDS, PBC), triazine derivatives (ET, DBT, EMT), benzotriazole derivatives (DRT, MBT), benzimidazole derivatives (PBS, PDT) and others (BDM, OCR, P15). Some of these UV filters have a structure with ionizable functional groups (e.g., sulphonic) that confers water solubility.

Table 1.1 Updated list (April 2011) of the UV filters that can be used in sunscreen cosmetics according to the current legislation in the EU

Key[a]	INCI Nomenclature[b]	Maximum concentration[c]
3BC	3-benzylidene camphor	2
BCS	Benzylidene camphor sulphonic acid	6[d]
BDM	Butyl methoxydibenzoylmethane	5
BZ3	Benzophenone-3	10
BZ4	Benzophenone-4	5[d]
CBM	Camphor benzalkonium methosulphate	6
DBT	Diethylhexyl butamido triazone	10
DHH	Diethylamino hydroxybenzoil hexyl benzoate	10
DRT	Drometrizole trisiloxane	15
EDP	Ethylhexyl dimethyl PABA	8
EMC	Ethyhexyl methoxycinnamate	10
EMT	Bis-ethylhexyloxiphenol methoxyphenyl triazine	10
ES	Ethylhexyl salicylate	5
ET	Ethylhexyl triazone	5
HS	Homosalate	10
IMC	Isoamyl *p*-methoxycinnamate	10
MBC	4-methylbenzilidene camphor	4
MBT	Methylene bis-benzotriazolyl tetramethylbutylphenol	10
OCR	Octocrylene	10[d]
P15	Polysilicone-15	10
P25	PEG-25 PABA	10
PBC	Poliacrilamidomethyl benzilidene camphor	6
PBS	Phenylbenzimidazole sulphonic acid	8[d]
PDT	Phenyl dibenzimidazole tetrasulphonatoe disodium	10[d]
TDS	Tereftalidene dicamphor sulphonic acid	10[d]
TiO_2	Titanium dioxide	25

[a] Key used along this Ph.D. Thesis
[b] International Nomenclature of Cosmetic Ingredients
[c] Expressed as weight percentage in the final product (*m/m*)
[d] Expressed as acid

UV filters can also be classified into UV-A or UV-B, depending on which UV radiation that is attenuated. Generally, all absorb UV-B radiation, except BZ3, BZ4 and DHH that also absorb partially UV-A, and TDS and BDM, which only absorb UV-A radiation.

Given the potential of these compounds to protect the skin, the trend of the cosmetic industry is the inclusion of UV filters in the composition of daily-used cosmetic products, apart from their use in specific sunscreen products.

However, UV filters can produce undesirable side effects on the skin. Thus, cases of contact dermatitis and photo-allergies have been reported (Berne and Ros 1998; Alanko et al. 2001; Darvay et al. 2001; Maier and Korting 2005). Moreover, although cosmetic products for sun protection are designed to be applied externally and remain on the surface layers of the skin (Benson 2000; Nohynek and Schaefer 2001),

some UV filters can penetrate into the body through the skin (Arancibia et al. 1981; El Dareer et al. 1986; Hagedorn-Leweke and Lippold 1995; Marginean-Lazar et al. 1996). A number of effects that can be attributed to these processes of percutaneous absorption are the appearance of endocrine disruption effects, oestrogenic activity, the generation of free radicals and the induced toxicity in keratinocytes (Schlumpf et al. 2001; Heneweer et al. 2005; Sayre et al. 2005; Damiani et al. 2006; Kunz and Fent 2006; Soeborg et al. 2006). By contrast, other studies soften the extent that these side-effects can cause to humans (Janjua et al. 2004).

1.4 Disposition of the UV Filters in the Human Body

The toxicity of a substance, defined as the response to cause harmful effects to the body, depends on the concentration that reaches to the organ or tissue, which in turn depends on both the dose that the substance is administered and the kinetics of accumulation (Rozman and Klaasen 2008).

The disposition of the substance goes from absorption, ingestion or inhalation, until their excretion, taking into account the processes of distribution and biotransformation. Thus, the toxicokinetics and disposition of xenobiotics are closely related.

Considering the case of percutaneous absorption, if the absorbed amount or velocity of absorption is high, the xenobiotic can achieve a high enough concentration to cause toxicity in a particular place. Likewise, the more slowly the chemical is excreted outside the body, the higher is its concentration and, therefore, its toxicity in the corresponding tissue.

Furthermore, the distribution of the chemical cause that it reaches other tissues at lower concentration levels, then decreasing the toxicity.

In the particular case of the UV filters percutaneous absorption, their elimination from the general circulation of the body passes through biotransformation processes, the reservoir in the different parts of the body and excretion.

1.4.1 Percutaneous Absorption

The percutaneous absorption of the UV filters is defined as the process by which these compounds pass through the skin and are incorporated into the bloodstream. To be absorbed through the skin, UV filters must cross by diffusion the stratum corneum, which is the main barrier of the process. Then, they must come into contact with the deeper layers of the epidermis, the dermis and, finally, incorporated into the general circulation to reach the subcutaneous fatty tissue (see Fig. 1.4). During this way, cutaneous metabolism processes may occur, especially based on hydrolysis reactions catalyzed by enzymes present in the pilosebaceous system (Howes et al. 1996).
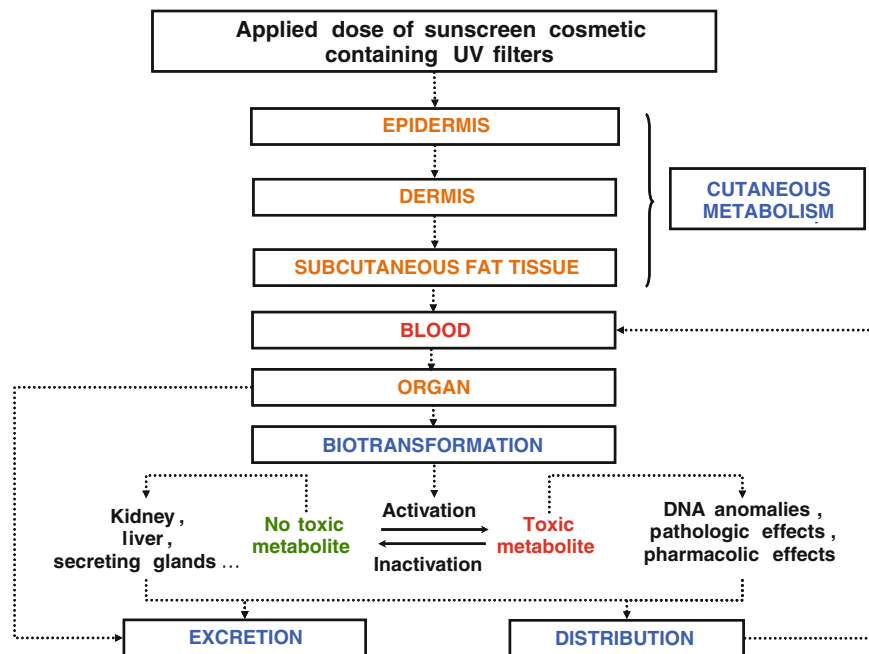
Fig. 1.4 Schematic representation of the body disposition and toxicity associated with percutaneous absorption of UV filters

Percutaneous absorption of UV filters depends on different aspects. First, the diffusion capacity of the UV filter to pass through the stratum corneum, which is the mechanism of conveyance and which is an inverse function of the molecular weight or volume. Second, the lipophilic nature of the UV filter, which affects their ability to disintegrate in the epidermal lipids and can be estimated using the partition coefficient octanol/water (K_{ow}). Third, the morphology of the stratum corneum, since the thickness of this skin barrier differs greatly depending on the area of the body. Finally, the nature of the dermatological carrier which the cosmetic preparation is formulated (Walters and Roberts 2002). In the latter case, it is important to discuss some details to understand the importance of this aspect.

Generally, the UV filters are not directly applied to the skin as pure chemical compounds, but they are usually incorporated via a suitable carrier, known as vehicle, which is defined as the set of accompanying ingredients to the UV filter in the formulation and represents the existence of a physically structured matrix (Smith et al. 2002). As indicated above, the vehicle type or the nature of the employed excipients can markedly affect the percutaneous absorption of a specific UV filter. Thus, formulations prepared with a particular UV filter at the same concentration but using different vehicles can cause different levels of percutaneous absorption for the target compound. Normally, the highest kinetic percutaneous absorption of UV filters is obtained with alcohol-based lotions and lipid ointments.

The sunscreen cosmetic formulations differ from pharmaceutical preparations for topical use in purpose, since the sunscreens should ideally remain on the skin surface without causing percutaneous absorption processes to carry out their protective function. Thus, during formulations development, the stability and compatibility of UV filters and excipients are taken into account, apart from the cosmetic acceptance of the vehicle itself. Therefore, the sunscreen cosmetic formulations can be defined as a situation of continuous and dynamic equilibrium, where the constituents interact with each other and with the skin, once it has been applied.

In this context, other important factors that determine the extent of the cutaneous absorption are the exposure conditions, mainly the UV filter concentration, the area of exposed skin surface and the application frequency of the cosmetic product.

1.4.2 Distribution

Once in the blood, the UV filters, like any other xenobiotic, can be distributed and/or move around the human body (see Fig. 1.4). Distribution to the various organs or tissues relies mainly on the blood flow and the rate of diffusion from the capillary bed into the corresponding cells. The final distribution depends largely on the affinity of the UV filter to the different tissues.

1.4.3 Biotransformation

The UV filters, similar to some endogenous substances, can undergo biotransformation processes, consisting of the metabolic conversion of parent compounds in other more soluble compounds (see Fig. 1.4). In general terms, the properties of lipophilicity, which promote the percutaneous absorption of the UV filters through the skin, are replaced by hydrophilic properties that facilitate their excretion, mainly via the urine or feces.

The chemical modification induced by the biotransformation can vary the biological effects of compound. In most cases, the biotransformation of the xenobiotic toxicity decreases. However, the formation of active metabolites can sometimes occur and then, they can exert more toxic effects than the own parent compounds (Koda et al. 2005; Jeon et al. 2008) and may cause various diseases and abnormalities.

The biotransformation of xenobiotics is competence of a small number of enzymes that have broad substrate specificity. Some of these enzymes are synthesized in response to the xenobiotic through enzyme induction process, but in most cases they are constitutive enzymes, and thus their synthesis is carried out in the absence of an external stimulus discernible. Moreover, the structure or amino

Table 1.2 General biotransformation mechanisms of xenobiotics and major subcellular localizations

Reaction	Enzyme	Subcellular localizations
Phase I		
Hydrolysis	Esterase	Microsomes, cytosol
	Peptidase	Lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo and nitro reductase	Microsomes, cytosol
	Carbonyl reductase	Microsomes, cytosol
	Disulfide reductase	Cytosol
	Sulfoxide reductase	Cytosol
	Quinone reductase	Microsomes, cytosol
	Dehalogen reductase	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	H Prostaglandin synthase	Microsomes
	P450 Cytochrome	Microsomes
Phase II		
Glucuronide conjugation	Uridine diphosphate-glucuronosyltransferase	Microsomes
Sulphate conjugation	Sulfotransferase	Cytosol
Glutathione conjugation	Glutathione S-transferase	Cytosol, microsomes
Aminoacids conjugation	Aminoacyl-tRNA synthetase	Mitochondria, microsomes
Acylation	N-acyltransferase	Mitochondria, cytosol
Methylation	Phenol-O-methyltransferase	Cytosol, microsomes

acid sequence of a biotransformer enzyme can display interindividual variations that can cause differences in the rate of biotransformation of the xenobiotic (Parkinson and Olgivie 2008).

The reactions catalyzed by enzymes that biotransform xenobiotics are divided into two groups and they are commonly known as phase I and phase II reactions, as can be seen in Table 1.2.

Phase I reactions are limited to hydrolysis and oxidation–reduction reactions. These reactions display or introduce a functional group, mainly hydroxyl, amino, carboxyl and sulphide, and typically result in a small increase in the hydrophilicity of the xenobiotic.

Phase II biotransformation covers glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis) and conjugation with amino acids such as glycine, glutamic acid and taurine. With the exception of methylation and acylation, the rest of these reactions increase

significantly the hydrophilicity of the xenobiotic, which greatly enhances its excretion (Parkinson and Olgivie 2008).

Xenobiotics biotransformation enzymes are widely distributed throughout the body and are located in various subcellular compartments. In humans, the most abundant source of enzymes that catalyze the biotransformation reactions is the liver. These enzymes are also found in skin, lung, nasal mucosa, kidney, eye and gastrointestinal tract as well as in various tissues. At a subcellular level, biotransformation enzymes are mainly located in the endoplasmic reticulum (microsomes) and the soluble fraction of the cytoplasm (cytosol), and they appear in smaller amounts in the mitochondria, nucleus and lysosomes.

Cofactors for phase II reactions react with the functional groups that are present in the xenobiotic or have been introduced in the phase I biotransformation. Glucuronidation, sulfation, acylation and methylation reactions require activated or high-energy cofactors, such as ATP (adenosine triphosphate), while the amino acid or glutathione conjugation is carried out with previously activated xenobiotics.

Most phase II biotransformation enzymes are mainly located in the cytosol. In general, phase II reactions are much faster than phase I reactions, and therefore, the removal rate of excretion of xenobiotics that depends on a phase I reaction followed by a phase II conjugation, will generally be defined by the first reaction.

1.4.4 Excretion

UV filters having a high lipophilicity, and thus a high coefficient K_{ow}, can efficiently be absorbed into the bloodstream and may undergo biotransformation process, while more polar compounds may be directly removed from the body by various means, including usually the urinary via (Okereke et al. 1993). However, apart from urine, it seems that all corporal secretions are able to excrete chemical substances administered externally to the human body. Thus, UV filters have been also found in faeces (Volkel et al. 2006), milk (Hany and Nagel 1995) and, as will be seen in this Thesis, in semen (Chaps. 5, 8).

1.5 Methodology for the Study of Processes Derived from the Percutaneous Absorption of UV Filters Contained in Sunscreen Cosmetics

As discussed above, there is a clear evidence to show that the human body can absorb through the skin some of the organic UV filters contained in sunscreen cosmetic formulations. These observations have prompted the researchers to study the disposition of these organic compounds in humans.

Despite the many complex interactions that can occur during transport of the UV filter from the outermost surface of the skin to the circulatory system or excretion, various systems have been proposed to study the series of processes that includes the UV filter body disposition. In this context, it should be noted the importance to develop analytical methods to obtain sensitive, selective and accurate estimations of the percutaneous absorption levels of UV filters by using *in vitro* systems or by means of the non-invasive determination of UV filters and/or their metabolites in biological fluids.

Researches found in the literature that employ *in vitro* and *in vivo* systems to consider processes derived from the body disposition of UV filters contained in sunscreen cosmetic products are detailed in **Part II** and **Part III** of this Ph.D. Thesis, respectively. Likewise, the main objectives that address these investigations and the instrumental techniques employed are also discussed.

1.5.1 In Vitro Methodology

The percutaneous absorption may be studied using *in vitro* methods, by means of membranes that simulate artificial skin or excised skin itself from animals or humans. This is possible because the excised skin maintains the barrier properties of the stratum corneum.

Among the advantages of using these alternatives, it must be highlighted not only the fact that the human skin can be used, but also that no procedures on live animals are carried out, allowing reduction and substitution of animal tests to estimate the percutaneous absorption of chemicals. The failure to obtain pharmacokinetic parameters and the possible difficulty of getting enough necessary human skin are the main drawbacks. Moreover, artificial membrane systems or animal skin have only limited use in predicting human absorption due to the large differences in skin permeability properties between human skin and animal skin or artificial membrane (Howes et al. 1996).

The primary methodology to estimate the *in vitro* percutaneous absorption of the UV filters is based on the use of diffusion cells. Furthermore, the distribution of UV filters can also be estimated from *in vitro* form using the tape-stripping technique on excised skin, which due to its non-invasive nature can also be applied directly on human volunteers and animals.

1.5.1.1 Diffusion Cells

The required number of physical and working conditions to carry out a general *in vitro* procedure based on the use of diffusion cells to assess the percutaneous absorption of UV filters contained in sunscreen products is described below.

Figure 1.5 shows the outline of the diffusion cells used in this Ph.D. thesis, which are based on Franz-type cells (Franz 1975) and designed by professors Dr.
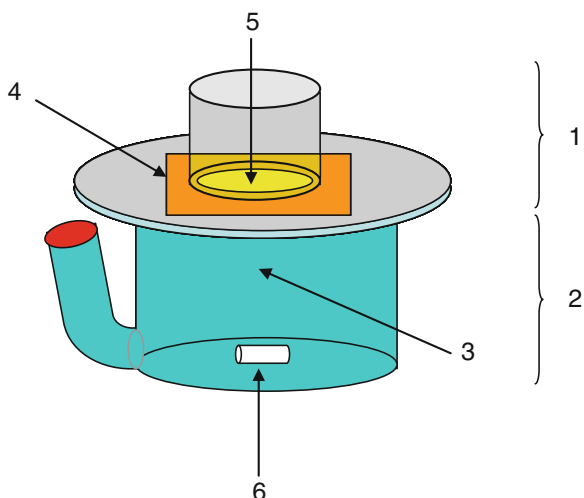
M. Herráez and Dr. O. Díez, from the Department of Pharmaceutical Technology
of the University of Valencia. They have been part of our research group for
several projects.

The used diffusion cells consisted mainly of glass and two compartments, called
donor and acceptor compartments. The receptor compartment contained a receiver
fluid and a magnetic stirrer. A piece of skin and the donor compartment were
subsequently placed over the receptor compartment with care, sealing the entire
cell with forceps and placing a plug in the hole on the receptor compartment that
allowed the sampling. Next, the sunscreen cosmetic product containing the UV
filter of interest was added on the skin surface available.

As previously discussed, to correctly predict the percutaneous absorption in
humans, it is recommended the use of split human skin samples, which can be
obtained from the surgeries remains or directly from corpses, strictly applying
ethic work practices. In order to minimize variability in the permeability properties
of the skin between the various anatomical parts, it is recommended to take the
skin from a single specific zone. Then, depending on the study, the skin can be
used either as a whole or only considering the epidermal membrane. In the last
case, the subcutaneous fat can be removed by applying a heat source to promote
the separation of the epidermal layer from the rest of the skin using tweezers (see
Fig. 1.6).

Typically, the skin portion is subjected to a thermostated water bath at 60 °C
for one minute. This separation process is carried out with extreme caution in order
to obtain the thin layer of epidermis without breaks. Next, the epidermal layer is
usually placed on absorbent paper to get greater rigidity when preparing the dif-
fusion cell.

To prepare the receptor fluid in the procedures followed in this Thesis, a
solution of phosphate salts was used under salinity and pH experimental conditions

Fig. 1.6 Process of
separation of the epidermis
layer
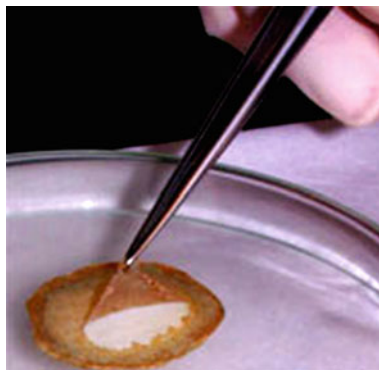
close to those found in body fluids. It must also be taken into account that the
receptor fluid must not alter the barrier properties of the skin. In this regard, the
physicochemical properties of the UV filter and the requirements for the final
analysis must be also considered

The cell must provide adequate clamping of the skin and enable a good tem-
perature control of the receptor compartment, which should be maintained con-
stant and close to normal skin temperature. Optimal homogenisation and easy
sampling of the receptor fluid should be also allowed.

The study of percutaneous absorption can be made by applying the cosmetic
formulation containing, besides the UV filter, excipients which may have their
own intrinsic effects on the skin. Hence, the choice of vehicle is one of the key
considerations in the study. Typically, the concentration of UV filter is selected
according to the authorized conditions of use. After the application of the cosmetic
product, the target UV filter remains in contact with the skin in the upper donor
compartment for a period of time. The average length of the experiments ranges
from 24 to 48 h. The absorbed amount of UV filter is collected in the receptor fluid
that is usually sampled at the end of the experiment or at intermediate times.

Although the state of the human skin before the initial preparation can be
assessed at a glance, it is recommended to check the integrity of the skin barrier
used before (Howes et al. 1996) or after (Balaguer et al. 2006) the application of
the sunscreen cosmetic product by conducting tests with coloured markers. Thus,
the viability of the assay can be confirmed (see Fig. 1.7), thus avoiding the con-
sideration of samples that are defective or skin that is damaged during handling
and, therefore, is abnormally permeable.

Finally, both the receptor fluid and skin itself can be analysed to verify the
results through the determination of the amount remaining on the surface, using
appropriate methods.

Fig. 1.7 Image obtained in our laboratory after a test of skin integrity with a marker (Balaguer et al. 2006) on a diffusion cell which proved to be discarded (*left*) and one that proved to be valid (*right*) after the corresponding analytical determinations of the marker in the receptor fluid

1.5.1.2 Tape-Stripping Technique

It is based on the application of a cosmetic formulation containing the UV filter on a limited surface of stratum corneum during a certain time, normally set to 30 min (see Fig. 1.8). Then, after removing the rest of the formulation and successive washings, the estimation is made from the amount recovered from the stratum corneum by application of various adhesive tapes at different times (Weigmann et al. 2001). The determination of absorbed UV filter is carried out using a suitable analytical method.

The methodology to predict *in vivo* topical bioavailability is largely based on the correlation between the application of the UV filter at a short-term and its permeability in the steady state. Thus, given a limited exposure period, the fraction of the dose which penetrates into the stratum corneum from the rest that cannot be removed by simple washing will be equal to the fraction which has reached the circulatory system.

Fig. 1.8 Application of tape-stripping technique on a volunteer

The procedure is non-invasive, direct and low cost although the skin preparation, regarding anatomical selection, shaving and cleaning is critical for the UV filter recovery.

1.5.2 In Vivo Methodology

The methodologies described in the literature to show the process of percutaneous absorption of UV filters using *in vivo* systems are based mainly on the application of a cosmetic formulation containing a UV filter of interest on the individual skin under certain conditions, in terms of applied amount of product, studied skin area and dose repetition. Subsequently, an analytical determination is performed to quantify the content of the UV filters and/or its metabolites in biological fluids and tissues at established time intervals after the product application.

The predominant biological matrix in these studies is urine, followed by plasma or serum, and quite far from biological tissues and breast milk, which have been only analyzed occasionally, and semen, whose analysis has been first proposed by our research group (see Chaps. 5, 8). Exceptionally, the tape-stripping technique have also been applied to human volunteers, especially to evaluate the effect of the vehicle on the percutaneous absorption of the UV filter.

1.6 Effect of the UV Filters in the Male Reproductive System

As the processes of percutaneous absorption of UV filters contained in sunscreen cosmetic products are evident, it is necessary to take a series of measures to establish adequate security for the user. First, the development of new products that protect and minimize the harmful effects of the sun through the use of UV filters that are non-toxic and maintain a minimum percutaneous absorption kinetics is essential. On the other hand, however, as a result of these processes, the expression of estrogen activity and the appearance of effects of endocrine dysfunction (Schlumpf et al. 2001), both associated with the use of such cosmetic products, have increased alarm on possible implications that UV filters can cause on the human reproductive system.

1.6.1 Effects of Toxics on the Reproductive System

The endocrine function of germ cells consists mainly in the perpetuation of the species. The genes located on the chromosomes of these cells and the transmitted genetic information modulate cell differentiation and organogenesis.

Contact with chemicals that disrupt the endocrine function has been linked to lower fertility in birds, fish, shellfish and mammals, with the loss of attributes of masculinity and the feminization of fish, birds and gastropods (Vos et al. 2000). In general, endocrine disorder mechanisms caused by chemicals except heavy metals, is based on competition with receptors or inhibition of the synthesis of steroids.

In humans, it is estimated that one in five couples are infertile unwillingly, that more than one-third of the embryos die early and that about 15 % of diagnosed pregnancies are aborted spontaneously. Of foetuses that survive and reach the birth, about 3 % has developmental defects (not always anatomical), from which more than twice of that number is detected during growth. Even under normal conditions, the reproductive system is not working fully. Therefore, if the presence of xenobiotics is added to these problems, it is not surprising that the interference of various processes or phenomena of reproduction increases markedly (Thomas and Thomas 2008).

1.6.2 Evaluation of Reproductive Capacity

There are several tests to assess endocrine function in humans (Thomas and Thomas 2001). The fact that there are chemicals capable of altering the reproductive system is an added difficulty when attempting to evaluate the harmful effects of toxic products in general. Apart from taking into account the considerable structural diversity of xenobiotics, the areas of the body where they may act and their very different mechanisms of action must be considered.

For men, the two basic methods to check if a chemical can be harmful to the generation of sperm are the testicular morphology evaluation and the functional assessment of spermatogenesis (Sharpe 1998). The finding of impaired spermatogenesis/testicular morphology, the degeneration of germ cells depending on their stage of development, and the insufficient delivery of normal sperm are included in these methods. At the molecular level, hormonally active androgens, primarily dihydrotestosterone and testosterone, stimulate anabolic and reproductive functions, which are mediated by its interaction with the nuclear receptors of steroid-androgen, also known as androgen receptors (AR). The action of the AR is very specific, despite the homology between them and other steroid receptors. The androgen target cells contain enzymes that can activate, deactivate and change the specificity of AR. However, different xenobiotics have been described to inhibit the binding of androgens to their receptors (Donovan et al. 1980) and to act as potent AR antagonists that can affect the reproduction of man (Kelce et al. 1995).

The evaluation of the processes of reproduction in mammals is much more complex in women than in men. Among females, those processes are ovulation, development of sexual receptivity, the transport of gametes and zygote, fertilization and implantation of the conception. All these phenomena may be disturbed by the action of external chemical agents.

It should be noted that estrogen, that are steroid hormones, have influence on growth, differentiation and function of several reproductive organs, such as mammary gland, uterus, vagina, ovary, as well as in some organs of male reproductive (testes, prostate). Estrogens can be located outside or inside the cells, but certain tissues can retain them with high avidity and specificity by action of intranuclear binding proteins known as estrogen receptor (ER).

Serum levels of estrogens, such as estradiol, and estrogenic effects on target tissues constitute a normal sign of follicular function. Using cell culture techniques, the chemicals ability to inhibit cell proliferation and estrogens production can be detected selectively (Zeleznik et al. 1979). The ratio of estrogen and progesterone in the nucleus and cytoplasm can have important applications in toxicology. Estradiol receptors and progesterone are particularly important because certain chemicals compete for these receptors and may alter its molecular conformation (Thomas 1975).

1.6.3 Risk Factors for Human Fertility

Most humans are exposed to a vast number of chemicals that can be dangerous to their reproductive capacity. Through laboratory studies, it is known that numerous chemicals are harmful to reproduction. Although the data obtained using laboratory animals may lose validity when extrapolated to the human species, it has also been shown that these chemicals exert harmful effects on human reproductive function.

It has been suggested that men are more vulnerable to environmental and occupational toxic than other mammals (Overstreet et al. 1988). The dangers and risks to reproduction have led to the formulation of policies for protection because certain professional occupations are related to semen quality. Thus, prolonged sitting, working with high heat sources, or exposure to X-rays can be causes of poor sperm quality.

In the early 1990, Carlsen and colleagues conducted an analysis of sperm density on average about 15,000 men over 50 years and it was revealed an alarming gradual decrease in semen quality (Carlsen et al. 1992). Although the application of mathematical models to describe the same set of data could allow the obtaining of other conclusions (Bromwich et al. 1994), there is a consistent evidence to suspect that changing lifestyles and increased exposure to estrogenic agents and other endocrine disruptors may correlate with the increased incidence of reproductive health problems of men, including testicular carcinomas, poor semen quality and other male reproductive system disorders (Skakkebaek et al. 2006).

Meanwhile, epidemiology has increased its importance in establishing cause-effect relationships, since it is inherently related to the risk control. The design of epidemiological studies may involve the use of both retrospective and prospective data, as well as statistical aspects such as the level of significance and effect size. Therefore, the importance of developing analytical methods to determine selectively and sensitively potential toxic compounds in biofluids related to human reproductive ability is unquestionable and can relate, for example, the concentration of a particular analyte to the ease binding with different hormone receptors.

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Part II
In Vitro Methodology

II.1 Background

In a query of scientific databases (Web of Knowledge, SciFinder®) in April 2011, around 50 publications that used *in vitro* methodologies to study the process of percutaneous absorption of the filters UV contained in sunscreen cosmetics have been found.

Table II.1 describes the highlights of these publications, including UV filter of interest, the technique used in the estimation of percutaneous absorption, the skin type, the analytical technique, and the most relevant comments.

In the search, PABA was also included as UV filter, despite its recent ban for use in sunscreen cosmetics under current legislation in the European Union (Directive 2008/123/EC). The published articles arising from this Ph.D. thesis and others carried out by our research group are also detailed. Figure II.1 shows the number of publications related to this type of study to date (April 2011).

Regarding the types of skin, about 42 % of the articles makes use of human skin. The remaining percentage corresponds to studies with pig skin (23 %), synthetic membranes (19 %), mouse skin (9 %), and rat skin (7 %). On the issue of the availability of human skin, it is true that *in vitro* models using skin from other species may be of interest if a fully validation is carried out. However, as discussed in Chap. 1, the use of human skin as the main *in vitro* model to study cutaneous permeation is recommended to complement the growing number of *in vivo* studies (see Part III). Figure II.2 shows the number of publications related to these studies based on the different UV filters of interest.

To date, BZ3 and EMC are the most studied UV filters by *in vitro* methodologies. Next, in descending order of publications number, BDM and BZ4 are followed by EDP, OCR, and TiO_2.

Table II.1 Published articles concerning the estimation of the *in vitro* percutaneous absorption of the UV filters by diffusion cells (DC) and tape-stripping (TS) techniques

Reference	UV filter	Technique	Type of skin	Analytical technique[a]	Comments
Blank et al. 1982	PABA, EDP	DC	Human	Radioactive techniques	Influence of the vehicle volatility
Kenney et al. 1995	EDP	DC (24 h)	Human and pig		Study of the *in vitro* cutaneous metabolism
Jiang et al. 1996	BZ3, BDM, EMC, ES, EDP	DC	Human	LC-UV/VIS	
Marginean-Laza et al. 1996	EMC, BDM	TS	Human	LC-UV/VIS	Influence of the vehicle
Treffel y Gabard 1996	EMC, BZ3, ES	DC	Human		Influence of the vehicle
Walters et al. 1997	ES	DC (48 h)	Human	Radioactive techniques	Influence of the vehicle. Percutaneous absorption of 0.6 %, approximately
Jiang et al. 1997	ES	DC	Human and synthetic	IR	Influence of the vehicle
Brinon et al. 1998	BZ4	DC	Rat	LC-UV/VIS	Influence of the vehicle
Jiang et al. 1998	BZ3	DC	Human and synthetic	LC-UV/VIS	Influence of the vehicle
Brinon et al. 1999	BZ4, EMC	DC	Pig	LC-UV/VIS	Influence of the vehicle
Aghazarian et al. 1999	3BC	DC	Rat and synthetic	LC-UV/VIS	Influence of the vehicle
Gupta et al. 1999	BZ3, EMC	DC, TS	Pig		Influence of the vehicle
Bench-Kieffer et al. 2000	BZ4, EMC	DC	Human and pig	Radioactive techniques	Interlaboratory data correlation
Potard et al. 2000	EMC, BZ4, BZ3, ET, OCR	TS (30 min and 16 h)	Human	LC-UV/VIS	Comparison of the cutaneous distribution and absorption between UV filters
Fernández et al. 2000a	BZ3	DC	Pig	LC-UV/VIS	Influence of the vehicle
Fernández et al. 2000b	BZ3	DC (8 h)	Pig	LC-UV/VIS	Influence of the vehicle evidence of absorption

(continued)

Table II.1 (continued)

Reference	UV filter	Technique	Type of skin	Analytical technique[e]	Comments
Brand et al. 2002	EMC, ES, HS BZ3, BDM, OCR, TiO$_2$	DC (24 h)	Mouse	Radioactive techniques	Synergy studies of percutaneous absorption between an herbicide and UV filters
Wissing and Muller	BZ3	DC	Synthetic membrane	UV/VIS	Influence of the vehicle: nanoparticles
Benech-Kieffer et al. 2003	TDS	DC (24 h)	Human	Radioactive techniques	The average absorption was around 0.16 % of the applied dose
Brand et al. 2003	TiO$_2$	DC	Mouse	Radioactive techniques	There is no effect on the herbicides absorption
Chatelain et al. 2003	EMC, BZ3, ES, HS, BDM	DC	Human	Radioactive techniques	Influence of the vehicle
Pont et al. 2003	EMC, ES, HS BZ3, OCR, EDP	DC (24 h)	Mouse	Radioactive techniques	Synergy studies of percutaneous absorption between an herbicide and UV filters
Yener et al. 2003	EMC	DC (6 h)	Artificial (cellulose acetate) and rat	UV/VIS LC-UV/VIS	Influence of the vehicle. Absorption reduction of 13–80 % with lipid microspheres
Gu et al. 2004	BZ3	DC	Pig and artificial membrane		Synergy effects with an insect repellent
Pont et al. 2004	EMC, OCR, BZ3, HS, ES, EDP, BZ4	DC (24 h)	Mouse and human	Radioactive techniques	Except OCR, the rest of UV filters enhance the absorption of an herbicide
Luppi et al. 2004	BZ3	DC, TS	Pig	LC-UV/VIS	Influence of the vehicle: nanoparticles
Jimenez et al. 2004	EMC			LC-UV/VIS	Influence of the vehicle: nanocapsulaes
Montenegro et al. 2004	EMC, BDM	DC (22 h)	Human and artificial membrane	LC-UV/VIS	Influence of the vehicle

(continued)

Table II.1 (continued)

Reference	UV filter	Technique	Type of skin	Analytical technique[c]	Comments
Gu et al. 2005	BZ3	DC	Pig	LC-UV/VIS	Synergy effects depending on the type of vehicle and the applied amount
Simeoni et al. 2006	BZ3	DC	Human	LC-UV/VIS	Influence of the vehicle: use of cyclodextrins
Balaguer et al. 2006b[a]	PDT	DC	Human	LC-F	Evidence of percutaneous absorption
Wang et al. 2006	BZ3	DC	Pig and artificial membrane	LC-UV/VIS	Synergy effects with an insect repellent
Brand et al. 2007	EMC, TiO$_2$	DC (24 h)	Rat	Radioactive techniques	Absorption enhancement of an herbicide
Mavon et al. 2007	MBT, TiO$_2$	DC	Human	LC-UV/VIS UV/VIS	Percutaneous absorption of MBT lower than 1 %. Absence of absorption for TiO$_2$
Wang and Gu 2007	BZ3	DC (6 h)	Human	LC-UV/VIS	Enhancement of an insect repellent absorption
Iannucelli et al. 2007	BDM	DC	Artificial membrane		Influence of the vehicle: lipid microparticles
Scalia et al. 2007	MBC	DC	Artificial membrane		Influence of the vehicle (microparticles: absorption reduction)
León et al. 2008[b]	BZ4, TDS, PDT, PBS	DC	Human	LC-UV/VIS	Evidence of percutaneous absorption of the UV filters
Klinubol et al. 2008	EMC, MBC, BDM	DC (24 h)	Mouse		Evidence of percutaneous absorption of BDM, EMC
Gu et al. 2008	BZ3	DC (6 h)	Synthetic membrane	LC-UV/VIS	Absence of absorption enhancement for ibuprofen
Durand et al. 2009	EMC, MBT, EMT, DHH, EDP, TiO$_2$	DC (24 h)	Human	LC-UV/VIS UV/VIS	Low penetration only for EMC and MBT. Absence of penetration for TiO$_2$
Puglia et al. 2009	EMC				Influence of the vehicle: nanoparticles

(continued)

Table II.1 (continued)

Reference	UV filter	Technique	Type of skin	Analytical technique[c]	Comments
Weiss-Angeli et al. 2010	EMC	DC			Influence of the vehicle: nanocapsules
Gulbake et al. 2010	BZ3	DC			Influence of the vehicle: nanoparticles
Vettor et al. 2010	EMC	DC			Influence of the vehicle: nanoparticles
Siqueira et al. 2011	BZ3	DC	Pig	Microscopic techniques, photon correlation spectroscopy	Influence of the vehicle(nanocapsules): absorption reduction

[a] Published articles of our research group
[b] Published articles included in this Ph.D. thesis
[c] The used keys are specified in the Annex I
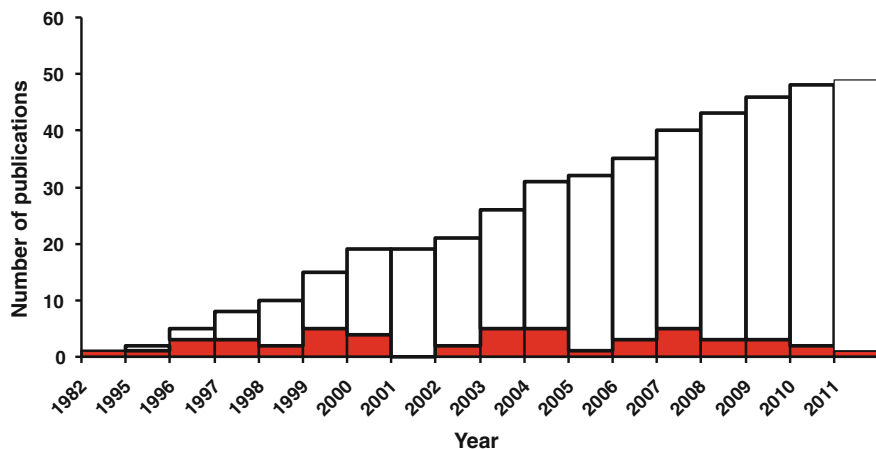
Fig. II.1 Temporal evolution of the number of scientific articles published concerning the estimation of percutaneous absorption of UV filters using *in vitro* techniques (■) and cumulative number of articles (□)
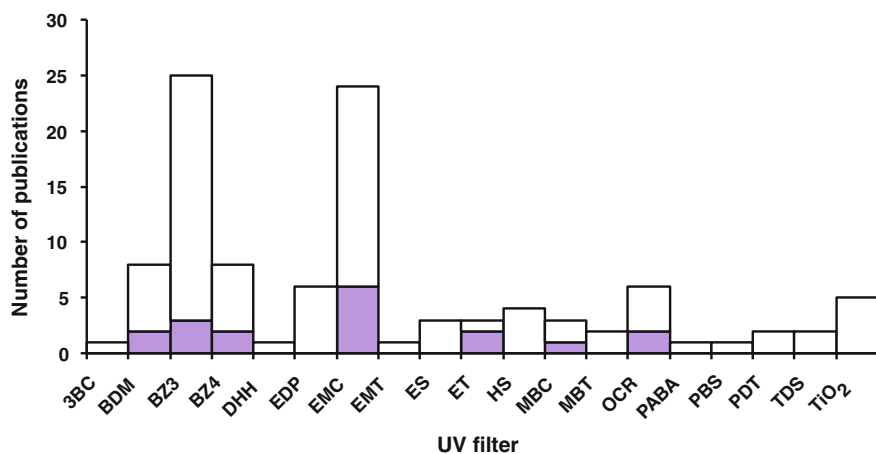
Fig. II.2 Publications related to the determination of UV filters to estimate the percutaneous absorption by the application of *in vitro* methods. Diffusion cells (□); *in vitro* tape-stripping technique (■)

II.2 Main Objectives of the *In Vitro* Studies

Experiments based on the use of *in vitro* methods allow the study of different aspects of the UV filters percutaneous absorption. Thus, studies have been conducted to estimate the level of percutaneous absorption for comparison among different UV filters (Jiang et al. 1996), to determine the distribution in the different layers of the skin (Potard et al. 1999; Potard et al. 2000) and to elucidate the

biotransformation reactions on the skin (Kenney et al. 1995). In fact, studies focused on *in vivo* percutaneous absorption are not able to distinguish between the processes of cutaneous and systemic metabolism, while *in vitro* tests do allow directly the assessment of cutaneous metabolism (see Chap. 1, Sect. 1.4).

However, the main objective of these methodologies is to evaluate the influence of the vehicle that contains the UV filter of interest on the percutaneous absorption processes (Blank et al. 1982; Chatelain et al. 2003; Siqueira et al. 2011). In this context, the possibility that synergy effects on absorption through the skin between UV filters and other compounds such as herbicides (Brand et al. 2002; Brand et al. 2003; Pont et al. 2003; Pont et al. 2004; Brand et al. 2007), insect repellants (Gu et al. 2004; Wang et al. 2006; Gu and Wang 2007), and drugs like ibuprofen (Gu et al. 2008) has also been considered.

II.3 Analytical Techniques

To determine the UV filters in the receptor fluid from diffusion cells and/or in the adhesive strips employed with the tape-stripping technique, liquid chromatography ultraviolet/visible spectrometry (LC-UV/VIS) has been extensively used (see Fig. II.3). The use of radiolabeled UV filters allowed the quantitation of analytes by radioactive techniques. Finally, UV/VIS spectrophotometry and other spectrometric techniques such as infrared spectrometry, molecular fluorescence, etc., have been also used in this research field

The concentration of these compounds in receptor fluid or in adhesive strips, using the methodology of diffusion cells or tape-stripping techniques, respectively, ranges between ng mL^{-1} and μg mL^{-1} levels, depending on the time elapsed
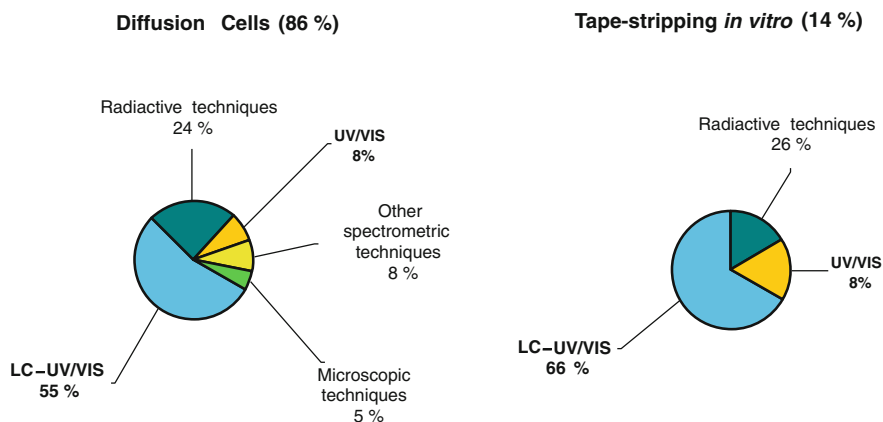
Fig. II.3 Percentage representation of the publications related to the determination of UV filters to estimate the percutaneous absorption by the application of *in vitro* methods based on the used analytical technique. Annex I specifies the employed keys

since the application of the sunscreen formulation. Therefore, the development of selective and accurate analytical methods to achieve these limits of quantification is required. In general, no particularly complex sample treatment steps are involved, and then simple dilutions or extractions of analytes from the adhesive strips by suitable extraction solvents are usually carried out.

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Chapter 2
Development of a Chromatographic Method to Estimate the *In Vitro* Percutaneous Absorption of Hydrosoluble UV Filters

2.1 Introduction

In recent years, new sunscreen cosmetic products increasingly more comfortable and easier to apply have been developed, based on an aqueous matrix and the use of hydrosoluble UV filters.

The most used hydrosoluble UV filters approved by the European legislation currently are, using the International Nomenclature of Cosmetic Ingredients, phenylbenzimidazole sulfonic acid (PBS), disodium phenyl tetrasulfonate dibencimidazol (PDT), benzophenone-4 (BZ4) and tereftaliden dicamphor sulfonic acid (TDS). The chemical structures of these compounds are shown in Fig. 2.1.

In the literature, studies that lead to a controversial situation about the side effects of BZ4 and PBS can be found. According to Hughes and Stones, the results obtained by analyzing skin allergy tests show that BZ4 produces significantly more positive reactions in the absence of photo-stimulation that any other UV filter (Hughes and Stone 2007). It was also found that UV filters are the substances that produce positive responses to photo-allergic reactions on the skin more frequently in comparison to the rest of the cosmetic ingredients and, in particular, BZ4 is the responsible of 2 % of the cases that involve patients with clinical diagnosis of photo-allergic contact dermatitis. In the case of PBS, the percentage is 1 % (Rodriguez et al. 2006). However, according to another study, BZ4 did not cause any allergic or photo-allergic reaction (Schauder and Ippen 1997). In this review, a case of allergic reaction and seven cases of photo-allergic reactions were related to the use of PBS, among a population of 402 patients. Accordingly, Darvay and colleagues claim that, despite the marked increase in the use of cosmetic products containing these compounds, contact allergic and photo-allergic reactions to UV filters are considered rare (Darvay et al. 2001).

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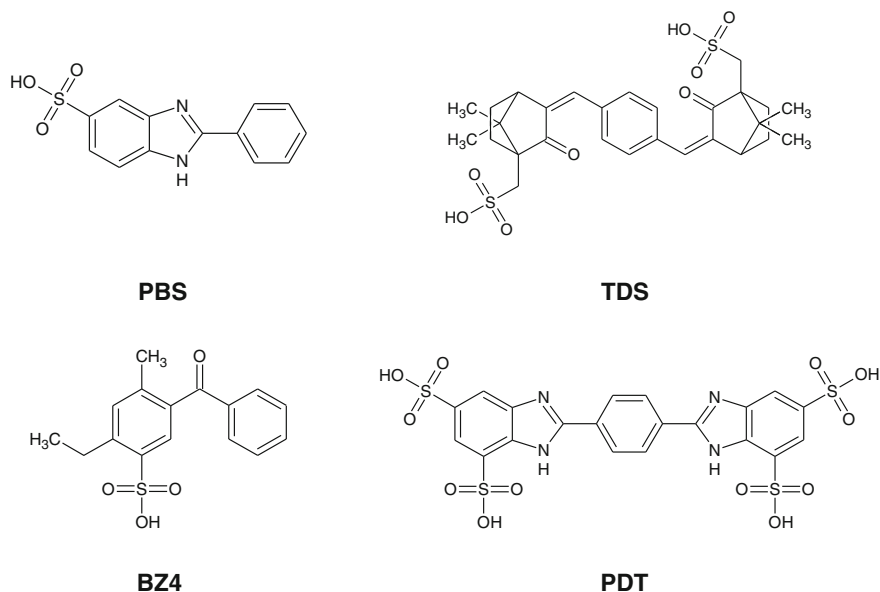
Fig. 2.1 Chemical structures of the most used hydrosoluble UV filters in sunscreen cosmetic products

Other of the side effects of PBS is the formation of free radicals caused by exposure to artificial sunlight (Inbaraj et al. 2002). Its inhibition was achieved through the interaction of the UV filter and derivatives of cyclodextrins, such that the decomposition of PBS induced by UV radiation was reduced (Scalia et al. 2004).

No publications related to the dermatologically harmful effects in the cases of PDT and TDS have been found. On the contrary, several articles show that TDS in contact with skin does not involve any risk to human health (Dean et al. 1992; Seite et al. 1998; Bernerd et al. 2000; Benech-Kieffer et al. 2003; Guenther et al. 2006). Furthermore, the protective properties of TDS regarding cytotoxicity and genotoxicity from solar UV radiation have been proved (Fourtanier et al. 1992; Fourtanier 1996; Marrot et al. 1998; Stege et al. 2000; Seite et al. 2000; Moyal 2004).

The UV/VIS absorption spectra of the target hydrosoluble UV filters shown in Fig. 2.2 indicate their protective ability to electromagnetic radiation on UV-A (320–340 nm) and UV-B (290–320 nm) areas.
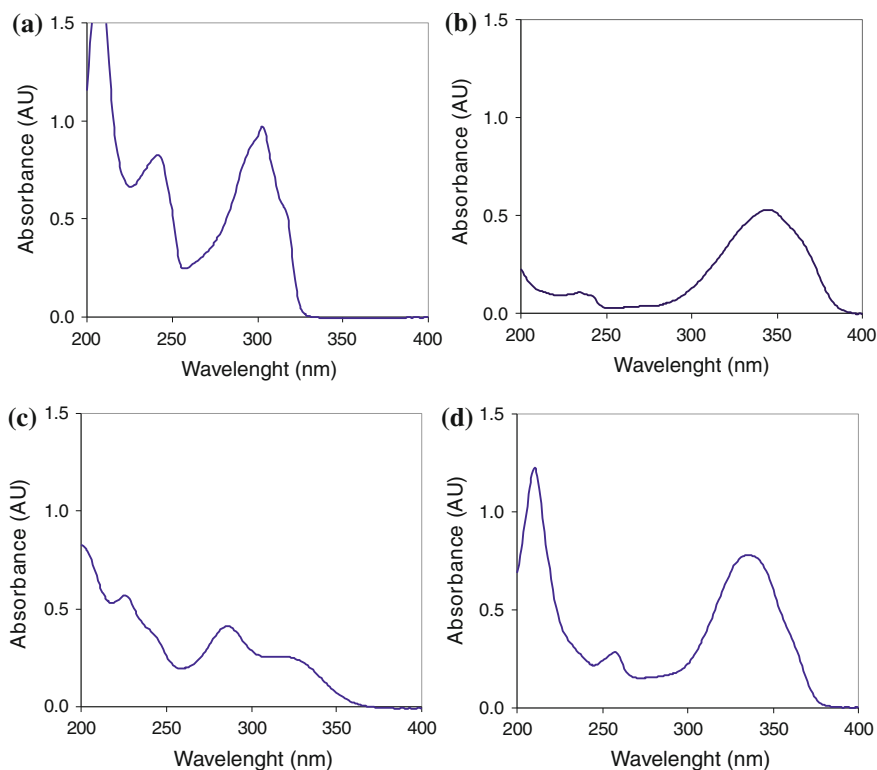
Fig. 2.2 UV/VIS absorption spectra obtained from 5 µg mL^{-1} aqueous solutions of PBS (**a**), TDS (**b**), BZ4 (**c**), and PDT (**d**)

2.1.1 Aim of the Study

As noted in Chap. 1, the fact that some of the organic UV filters penetrate through the skin is real. Considering this phenomenon, the need to perform skin permeation studies of these compounds becomes necessary. Hence, the development of analytical methods to determine UV filters with appropriate parameters of sensitivity, selectivity and accuracy and using preferably *in vitro* or non-invasive *in vivo* methodologies is required.

In this sense, the aim of this work is the development of an analytical method based on liquid chromatography-ultraviolet/visible spectrometry (LC-UV/VIS) to estimate simultaneously the *in vitro* percutaneous absorption of the most used hydrosoluble UV filters in sunscreen cosmetic products. The methodology is based on the application of a cosmetic product containing the UV filters of interest on human epidermis placed in a diffusion cell and the determination of the UV filters in the solution used as receptor fluid. The addition of an ion-pairing reagent to the mobile phase provides a good signal/noise ratio, which allows the determination of

the low content of the analytes in the receptor fluid from the first hours after the application of the cosmetic product.

Given that the *in vitro* percutaneous absorption procedures are complex and require a large number of experiments, the use of analytical methods to simultaneously study several components and evaluate the possibility of synergy effects can be very useful.

2.1.2 Background and Current Status of the Issue

In connection with the hydrosoluble UV filters considered in this study, analytical studies regarding percutaneous absorption by using different types of *in vitro* methodologies have been found in the literature (Part II, Fig. 2.2). Thus, diffusion cells have been used in the cases of BZ4 (Brinon et al. 1999; Potard et al. 1999; Benech-Kieffer et al. 2000) and TDS (Benech-Kieffer et al. 2003), as well as PDT, conducted by our research group (Balaguer et al. 2006b). Furthermore, *in vivo* tape-stripping techniques have also been used with BZ4 (Couteau et al. 2001; Potard et al. 1999).

On the other hand, studies to determine the target UV filters in blood and excretion products (urine or feces) from volunteers who had previously been applied cosmetics containing these sunscreen compounds have been conducted, as in the cases of TDS (Benech-Kieffer et al. 2003), PDT (Balaguer et al. 2006a) and PBS (Vidal et al. 2003) being the last two works from our research group. All these studies were carried out using analytical techniques such as LC-UV/VIS, molecular fluorescence spectrometry and radioactive techniques.

In previous articles published by our research group, the chromatographic separation and simultaneous determination of PBS, PDT, BZ4 and TDS in sunscreen cosmetics was achieved by using a LC-UV/VIS method (Salvador and Chisvert 2005b). However, the limits of detection of this method are not appropriate to determine the target UV filters in the receptor fluid from the *in vitro* diffusion cells, because UV filters are at low levels in the first hours after topical application of the cosmetic product.

It should be noted that the ion interaction chromatography is a technique that allows the separation of ions using conventional LC-UV/VIS instrumentation, unlike ion chromatography. The mobile phase is usually a hydro-organic solution containing a suitable ion-pairing reagent. In comparison to ion chromatography, this type of ion interaction methods provides advantages as lower equipment and columns cost, and the possibility of use in laboratories which only have conventional LC-UV/VIS systems. Furthermore, resolution and sensitivity of both techniques are comparable, given the appropriate choice of ion- pairing reagent.

Since the compounds of interest are acids that can undergo ionization acquiring anion form, a salt formed by a bulky cation (tetrabutylammonium, TBA) has been proposed as ion-pairing reagent. Thereby, ion pairs with the analytes can be formed, thus increasing the retention because of the greater affinity to the C18

stationary phase. However, this hypothesis of the mechanism of retention is not unique, and various alternatives have been proposed to explain how retention occurs (Gennaro and Angelino 1997).

The mobile phase composition should be optimized considering the different variables that control retention, such as content of organic modifier, concentration and nature of the ion-pairing reagent and pH. However, the choice of the experimental conditions to develop a method by ion interaction does not follow a general rule. In this sense, the dependence of the separation of analytes on so many different experimental conditions provides versatility to solve technical problems regarding retention and resolution.

The ability to obtain a good signal/noise ratio and therefore, a lower limit of detection, is another important consequence of the ion-pairing effect.

According to literature sources consulted to conduct this study, there are no published articles related to the estimation of *in vitro* percutaneous absorption of the target UV filters simultaneously and, moreover, published methods to estimate simultaneously the penetration through the skin of other UV filters are very limited (Potard et al. 1999).

2.2 Experimental

2.2.1 Reagents and Samples

Phenylbenzimidazole sulfonic acid (PBS), also called 2-phenylbenzimidazole-5-sulfonic acid, 99 % from Guinama (Valencia), disodium phenyl dibencimidazol tetrasulfonate (PDT) or acid 2-2′-bis-(1, 4-phenylene)-1H-benzimidazole-4,6-disulfonic, >99 % from Haarmann and Reimer (Parets del Valles), benzophenone-4 (BZ4) or 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, 99.9 % from Roig Farma (Terrassa) and tereftaliden dicamphor sulfonic acid (TDS) or triethanolamine salt of 3,3′-(1,4-phenylendimethyliden-bis-(7,7-dimethyl-2-oxo-bicyclo[2.2.1]heptane-1-methanesulfonic, 99 % supplied by L'Oreal (Madrid) were used as standards.

The solvents used for the preparation of the mobile phase were HPLC grade ethanol (EtOH) from Scharlab (Barcelona) and deionized water obtained from a NANOpure II water purification system (R $\geq$ 18 mΩ cm) from Barnstead (Boston).

To adjust the pH of the mobile phase, glacial acetic acid for analysis (AcOH) from Panreac (Barcelona) and an 25 % ammonium hydroxide solution (d = 0.91 g mL^{-1}) from Scharlab (Barcelona) were used. The ion-pairing reagent was tetra-n-butylammonium fluoride (TBA) >99 % from Acros Organics (Geel).

To prepare the solution used as receptor fluid, sodium monohydrogen monohydrate, anhydrous potassium dihydrogenphosphate, sodium hydroxide and sodium chloride, all from Scharlab (Barcelona), were employed.

To check the integrity of the skin, the phenol red (PR) marker >99 % from Scharlab (Barcelona) was used.

2.2.2 Equipment and Material

The LC-UV/VIS system consisted of a PU-2089 Plus® chromatograph connected to a MD-2010 Plus® UV/VIS detector, both from Jasco (Madrid). The chromatographic separation was performed using a Kromasil® 100 C18 analytical column (5 μm particle size, 125 mm long, 4 mm internal diameter) from Scharlab (Barcelona). A injection loop (20 μL) from Rheodyne (Wertheim-Mondfeld) was used.

Franz-type diffusion cells designed by Prof. Herráez and Prof. O. Díez, from the Department of Pharmaceutical Technology of the University of Valencia (Diez-Sales et al. 1991) were used.

A MicropH 2001pHmeter from Crison (Alella) and a Precisterm® thermostated water bath from JP Selecta (Barcelona) were also used.

2.2.3 Analytical Method for the Simultaneous Determination of Hydrosoluble UV Filters in the Receptor Fluid from Diffusion Cells

2.2.3.1 Preparation of Epidermis

All skin permeation studies were performed using abdominal skin samples obtained from plastic surgery of Caucasian women aged between 30 and 40 held at the University Hospital (Valencia). Previously, the patients signed an informed consent.

Abdominal skin samples were stored in a freezer at −40 °C for a maximum period of one month after conducting the removal of connective tissue and fat excess. After thawing, the skin was entered in a thermostated bath at 60 °C for 1 min and then, after holding the skin on a silicon support with pins, the epidermis was separated from the rest of the skin with tweezers. This separation process was carried out with extreme caution so as not to break the thin layer of epidermis. Finally, the epidermis was immersed in the receptor fluid for a few minutes to obtain a more effective conditioning.

In such assemblies, it is usual to place the epidermis on absorbent paper to prepare the diffusion cell in order to obtain greater rigidity. However, in this case, absorbent paper was not used because of the possible retention of UV filters on the paper, since it was observed in a previous study that the sulfonic groups present in the structure of these compounds enhance the retention over this support (Balaguer

et al. 2006b). Thus, the only barrier between the sunscreen containing the UV filters and the receptor fluid was the epidermis.

2.2.3.2 Diffusion Cell and Sampling

The receptor fluid consisted of 20 mL of an anhydrous potassium dihydrogen phosphate (10.4 g L^{-1}) solution and 80 mL of a sodium monohydrogen phosphate monohydrate (11.9 g L^{-1}) solution. Additionally, to get experimental conditions in the receptor fluid closer to those present in body fluids, pH and saline content were adjusted to 7.4 with sodium hydroxide and to 0.44 % with sodium chloride, respectively.

The diffusion cell consisted of both donor and receptor compartments. The 6 mL receptor compartment contained inside the receptor fluid and a magnetic stirrer. On this compartment, a portion of epidermis obtained as indicated above was placed completely stretched over the meniscus formed by the receptor fluid. Next, the donor compartment was carefully placed on the receptor, and the cell assembly was sealed with clamps. Figure 2.3 shows three diffusion cells completely assembled and ready to perform studies of *in vitro* percutaneous absorption.

Next, the skin permeability was studied by adding 100 μL of a cosmetic product containing the target UV filters on the available epidermis portion which had an area of 0.79 cm^2. To finish the installation, a cap was placed in the hole on the receptor compartment that allows sampling and donor compartment was covered with a piece of Parafilm® to prevent the sunscreen cosmetic product from evaporation. The diffusion cell was placed in a thermostatted water bath at 37 °C with magnetic stirring (see Fig. 2.4) and preventing from sunlight.

The assembly was maintained for 48 h. Two hours after the sunscreen cosmetic addition, 200 μL were obtained from the receptor compartment using a syringe. This volume was immediately replaced in the diffusion cell with fresh receptor

Fig. 2.3 Diffusion cells prepared in our laboratory to carry out *in vitro* percutaneous absorption studies
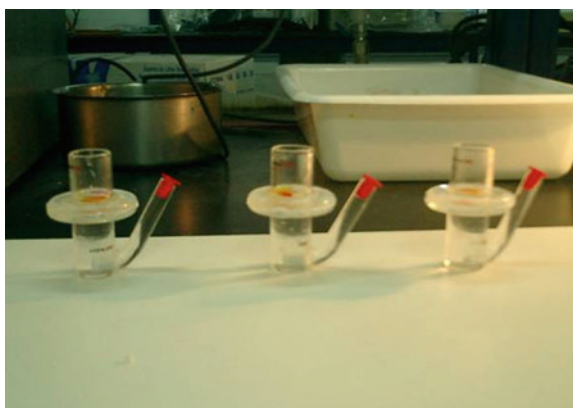
fluid so that the volume of receptor fluid in the receptor compartment was kept
constant throughout the experiment. The sampling process was repeated at 7 and
48 h. The obtained receptor fluid samples were stored at 4 °C.

2.2.3.3 Checking the Integrity of the Skin

To evaluate the integrity of the epidermis during the experiment and ensure that
the passage of the analytes through the skin was essentially due to percutaneous
absorption processes, thus avoiding the consideration of damaged epidermis, a PR
marker test was performed. Hence, after the last sampling, 1 mL of receptor fluid
solution containing 0.05 % PR was added to the donor compartment. The
assembly was maintained for 1 h under the same experimental conditions used to
study the *in vitro* percutaneous absorption and then, 200 μL from the receptor fluid
was obtained. Using an analytical method based on a solid-phase extraction (SPE)
on line coupled to a UV/VIS spectrophotometric detector using a sequential
injection that was previously developed by our research group (Balaguer et al.
2006b), PR was determined in the receptor fluid.

2.2.3.4 LC-UV/VIS Analysis

To construct the calibrate, BZ4, TDS, PDT and PBS standard solutions were
prepared in a concentration range between 0.1 and 10 μg mL^{-1}, using as solvent
the same solution that was used as a receptor fluid, and stored at 4 °C. 20 μL of
these solutions and the obtained sample solutions (in triplicate) were injected in
the chromatographic system.

   The chromatographic separation was carried out using a mobile phase con-
sisting of an ammonium acetate [pH 4, containing TBA (50 mM)] solution and
EtOH. An isocratic elution 65:35 (*v/v*) using a flow rate of 1 mL min^{-1} at 50 °C
was selected. The detection was performed at the wavelength corresponding to the

absorption maximum of each analyte, i.e. 304, 344, 288 and 344 nm for PBS, PDT, BZ4 and TDS, respectively.

According to the obtained volume of receptor fluid in each sample, data were adequately corrected and interpolated into the calibrate representing the area of each chromatographic peak versus the standard concentration.

2.3 Results and Discussion

2.3.1 Study of the Chromatographic Variables

To obtain the best resolution of the chromatographic peaks using the shortest analysis time possible, a series of experiments were conducted to select the experimental variables associated with the mobile phase (organic modifier content, pH, ion-pairing reagent concentration) and to evaluate the temperature effect over the analytical separation.

To select the chromatographic variables, a standard solution containing all four analytes (50 µg mL^{-1}) was used and the analytical signals obtained by measuring at the wavelength corresponding to the absorption maximum of each analyte were evaluated.

2.3.1.1 Mobile Phase

In preliminary studies, the percentage of organic modifier in the mobile phase consisting of a mixture of aqueous AcOH (1 %) containing 20 mM TBA and EtOH was studied. The best result concerning resolution and analysis time was obtained by using a mobile phase with a percentage of 35 % EtOH.

Another of the conclusions drawn from these preliminary studies was that the use of aqueous AcOH (1 %) buffered with ammonium hydroxide to pH 7.7 reduces the analysis time considerably in comparison to the use of aqueous AcOH (1 %) at pH 2.6. Thus, before performing a more comprehensive study of pH, an aqueous ammonium acetate (1 %) phase adjusted to pH 7.7 was used to perform the study of the other chromatographic variables.

2.3.1.2 Ion-Pairing Reagent Concentration and pH of the Mobile Phase

To select the concentration of TBA in the mobile phase, the analytes were analysed using different mobile phase solutions consisting of aqueous TBA (20–100 mM) in ammonium acetate (1 %) pH 7.7:EtOH 65:35 (*v/v*). The best results were obtained when using TBA concentrations between 40 and 60 mM as shown in Fig. 2.5.

Fig. 2.5 Results obtained by
LC-UV/VIS analysis to select
the concentration of TBA.
Mobile phases composed of
aqueous solutions of TBA
(20–100 mM) in ammonium
acetate (1 %) pH 7.7:EtOH,
65:35 (*v/v*) were used.
Experiments were performed
at room temperature. PBS,
(*blacklozenge*); PDT,
(*blacksquare*); BZ4,
(*blacktriangle*); TDS, (✗)
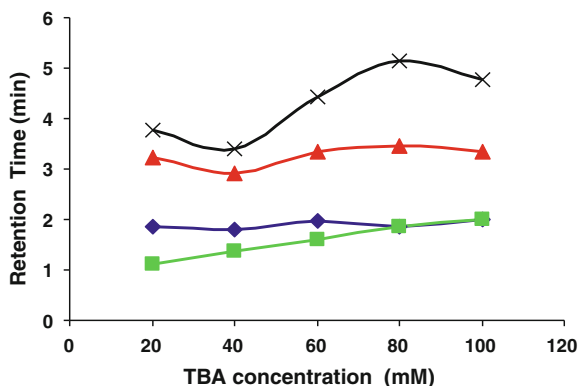
While concentrations of TBA lower than 40 mM caused the elution of PDT
next to the elution front, values higher than 60 mM caused the overlap of PDT and
PBS. For the other two analytes, the increase in the concentration of TBA did not
involve any significant variations in the case of BZ4, but increased the elution time
for TDS.

In general, it can be conclude that, on the one hand, a high amount of TBA in
the mobile phase did not allow the obtaining of a proper separation of analytes and,
on the other hand, a low concentration of TBA did not allow the separation of the
first eluted chromatographic peak from the elution front, although the analysis time
was reduced considerably. Therefore, a concentration of 50 mM TBA was
selected. Under these conditions, it was possible to achieve full resolution of all
the peaks in less than 5 min, as shown in Fig. 2.6.
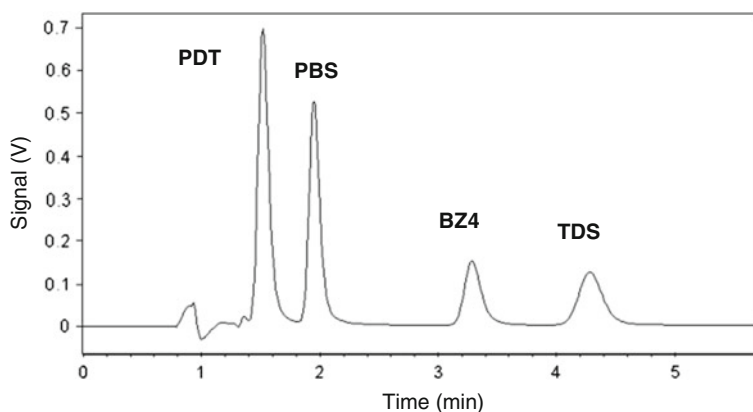
Fig. 2.6 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS
(50 μg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in
ammonium acetate (1 %) pH 7.7:EtOH, 65:35 (*v/v*). Experiments performed at room temperature.
Detection carried out at 316 nm

Fig. 2.7 Results obtained by LC-UV/VIS analysis to select the pH of the mobile phase. Mobile phases composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 % and different pH values):EtOH, 65:35 (*v/v*). Experiments were performed at room temperature. PBS, (*black lozenge*); PDT, (*black square*); BZ4, (*black triangle*); TDS, (✗)
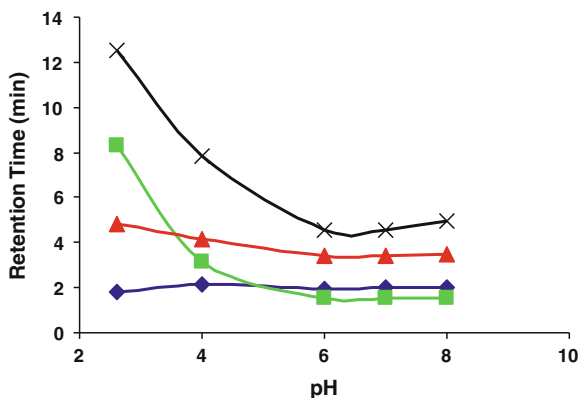
However, as the chromatographic peak corresponding to PDT still eluted close to the front of elution where other polar ingredients from the matrix could co-elute, other variables were explored to achieve a better separation from the front.

To study the pH effect of the aqueous phase in the chromatographic separation (see Fig. 2.7), the analytes were analysed using different mobile phases composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 %) pH (from 2.5 to 8.5):EtOH, 65:35 (*v/v*).

Values of pH above 4 caused the elution of PBS at 2 min steadily and the strong alteration of the PDT elution. Thus, at pH values of 6 and above, there was a reversal in the elution of PDT and PBS. Under these conditions, PDT showed a lower affinity to the stationary phase and increasingly approached to the front of elution. Increasing pH values allowed the suddenly elution of BZ4 and TDS, although their retention times remained constant from pH values higher than 5. For both analytes, no overlap occurred at any time under these conditions.

Furthermore, the analysys time was substantially lengthened at pH 2.6 and the definition of the peaks was worse, especially for PDT. Under these conditions, the retention time of PDT was even higher than that of BZ4.

Figure 2.8 shows a chromatogram obtained using a mobile phase composed by an aqueous solution containing 50 mM TBA in ammonium acetate at pH 4

As can be seen, the four chromatographic peaks eluted with good separation and resolution and additionally, the separation of the first eluted peak from the front of elution was achieved.

2.3.1.3 Effect of Temperature

The temperature can reduce the retention time of the analyte and therefore the analysis time, but also can affect the chromatographic resolution. Using a mobile phase consisting of an aqueous solution of TBA (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*) at 50 °C or lower, the first chromatographic peak
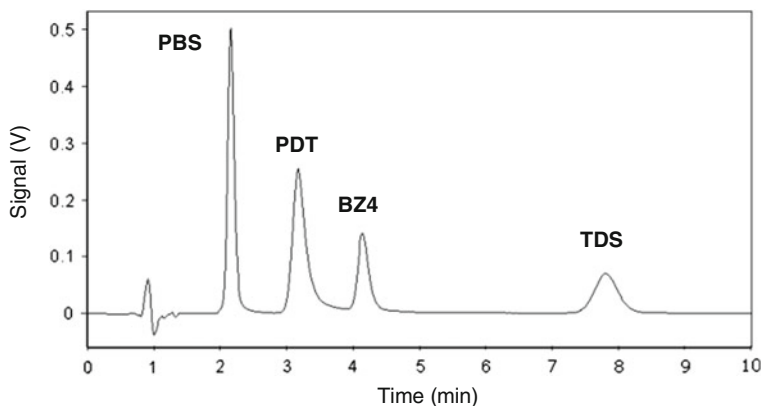
Fig. 2.8 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS (50 μg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*). Experiments performed at room temperature. Detection carried out at 316 nm

Fig. 2.9 Results obtained by LC-UV/VIS analysis to select the temperature. Mobile phase composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*) at different temperature values. PBS, (*blacklozenge*); PDT, (*blacksquare*); BZ4, (*blacktriangle*); TDS, (×)
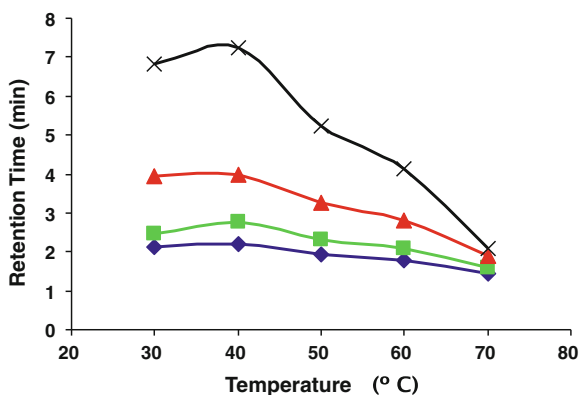
(PBS) eluted at 2 min. When increasing temperature, its retention time decreased (see Fig. 2.9).

The chromatographic profile showed some variations, especially with TDS. Basically, the retention time was reduced from 8 min to less than 6 min, when the temperature reached 50 °C. In contrast to that, values higher than 50 °C caused the elution of the first chromatographic peak closer to the elution front and a very noticeable loss of resolution, resulting in an overlap of the peaks.

Taken into consideration all of this, 50 °C was finally selected. Thus, the analysis time was reduced to less than 6 min, which led to a further improvement to the above advantages achieved of high resolution and good chromatographic separation from the front of elution. Figure 2.10 shows the separation of the target
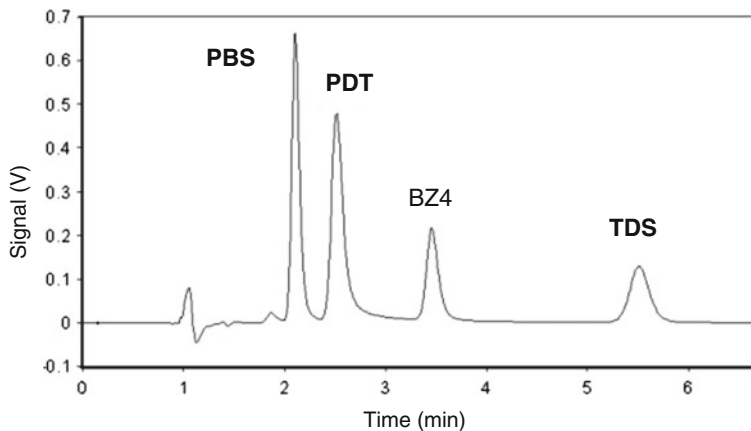
Fig. 2.10 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS (50 μg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*). Experiments performed at 50 °C. Detection was carried out at 316 nm

UV filters conducted under the selected chromatographic conditions. The resulting retention times (expressed in min) were: 1.9 to PBS, 2.3 to PDT, and 3.2 to 5.1 for TDS BZ4.

2.3.2 Validation of the Proposed Analytical Method: Study of the Interferences and Accuracy

Firstly, a study on the matrix effects caused by skin and/or the cosmetic product in the determination of the analytes was conducted. For this, a series of standard solutions of the four analytes (0.1–2 μg mL^{-1}) were prepared using, on the one hand, a solution of anhydrous potassium dihydrogen phosphate and sodium monohydrogen phosphate monohydrate used as receptor fluid and, on the other hand, receptor fluid obtained at 24 and 48 h from a skin permeation experiment based on the application of a cosmetic lotion containing no analyte.

The receptor fluid analysis obtained at both times showed the absence of peaks after the front of elution (1.8 min). Furthermore, when comparing by Student's *t*-tests (Annex III.4) the intercepts and slopes between the two calibrates, they were found statistically comparable for all analytes (see Table 2.1). In this way, the absence of proportional and/or constant interferences from the skin or the cosmetic sunscreen was showed.

Table 2.1 Statistical comparison of the linearity parameters obtained by conventional calibration and standard addition calibration

Parameter	t_{cal} [c]			
	PBS	PDT	BZ4	TDS
a (μV s)[a]	1.86	2.10	1.63	0.55
b (mL μg^{-1})[b]	0.33	1.15	2.17	0.79

[a] Intercept
[b] Slope
[c] Statistical $t_{\text{tab}(0.05,(N1-2)+(N2-2)=6)} = 2.45$

2.3.2.1 Analytical Parameters

Calibrates exhibited excellent linearity in the different concentration ranges for PBS, PDT, BZ4 and TDS with regression coefficients greater than 0.995 in all cases, establishing the working range between 0.1 and 10 μg mL^{-1} for all analytes. Table 2.2 shows the values corresponding to the limits of detection (LOD) and the slopes of the calibrates, which is the parameter used to estimate the sensitivity of the method, for each target UV filters.

The instrumental repeatability was studied by fivefold injecting a solution containing the analytes (10 μg mL^{-1}) prepared with receptor fluid. The relative standard deviations (RSD) in area values (N = 5) obtained for each analyte are also shown in Table 2.2.

2.3.3 Application of the Analytical Method

After preparing three diffusion cells according to the protocol described in Sect. 2.2.3, a volume of 100 μL of a sunscreen cosmetic lotion containing 3 % of the target hydrosoluble UV filters (Annex II.1) was added to the available epidermis. The cosmetic lotion was prepared in the laboratory using a protocol adapted from the manufacture of cosmetic products (Jordán and Jordán 1991).

As indicated above, the integrity of the epidermis was evaluated by an assay with PR marker, accordingly to a previous work (Balaguer et al. 2006b). As no PR values greater than 0.1 % of the total amount of PR applied to the donor compartment to none of the cells were detected in the receptor fluid, it was concluded that the skin remained intact during the experiment and then, the percutaneous absorption study was considered valid. The proposed chromatographic method was carried out to determine PBS, PDT, BZ4 and TDS in the receptor fluid.

Table 2.3 shows the concentrations found in the receptor fluid of the three cells for each analyte, considering representative sampling times at 2, 7 and 48 h after starting the experiment. The study was not extended beyond 48 h because the used *in vitro* system does not guarantee the integrity of the epidermis after this period of time. From the obtained concentrations, it is possible to estimate the penetration

Table 2.2 Analytical parameters of the proposed chromatographic method

Parameters	Analyte			
	PBS	PDT	BZ4	TDS
a (μV s)[a]	$(3 \pm 2) \times 10^{-5}$	$(-3 \pm 2) \times 10^{-5}$	$(-2 \pm 2) \times 10^{-5}$	$(-19 \pm 9) \times 10^{-5}$
b (mL μg^{-1})[b]	$(185 \pm 1) \times 10^{-5}$	$(134 \pm 1) \times 10^{-5}$	$(83 \pm 1) \times 10^{-5}$	$(93 \pm 2) \times 10^{-5}$
R^{2c}	0.99997	0.99995	0.99996	0.998
Repeatability (%)[d]	1.4	5.8	3.2	3.7
LOD[e] (ng mL^{-1})	16	44	36	65

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Expresed as peak area RSD ($N = 5$)
[e] Estimated as $3.s_{y/x}/b$, where b is the slope and $s_{y/x}$ the standard deviation of the residuals of the calibration curve

Table 2.3 Estimation of the *in vitro* percutaneous absorption of the target hydrosoluble UV filters obtained by applying the described method to receptor fluid samples

Diffusion cell	Analyte	Concentration (μg mL^{-1})		
		2 h	7 h	48 h
1	PBS	0.31 ± 0.03	0.34 ± 0.02	4.59 ± 0.03
	PDT	0.29 ± 0.01	0.35 ± 0.03	4.19 ± 0.02
	BZ4	0.34 ± 0.02	0.46 ± 0.07	5.52 ± 0.08
	TDS	0.70 ± 0.03	0.75 ± 0.05	2.74 ± 0.07
2	PBS	0.15 ± 0.01	0.20 ± 0.01	1.75 ± 0.03
	PDT	0.18 ± 0.01	0.21 ± 0.02	1.29 ± 0.03
	BZ4	0.21 ± 0.02	0.31 ± 0.04	2.41 ± 0.01
	TDS	0.54 ± 0.03	0.56 ± 0.03	1.07 ± 0.03
3	PBS	N.D.[a]	0.16 ± 0.01	5.06 ± 0.06
	PDT	N.D.[a]	0.16 ± 0.02	5.8 ± 0.9
	BZ4	N.D.[a]	0.22 ± 0.01	6.21 ± 0.04
	TDS	N.D.[a]	0.13 ± 0.03	1.98 ± 0.02
Average	PBS	0.2 ± 0.1	0.2 ± 0.1	4 ± 2
	PDT	0.2 ± 0.1	0.2 ± 0.1	4 ± 2
	BZ4	0.2 ± 0.1	0.3 ± 0.1	5 ± 2
	TDS	0.4 ± 0.1	0.5 ± 0.3	2 ± 1

[a] ND not detected

through the skin, so that the percentage of percutaneous absorption after 48 h from the application of the lotion on the skin was 0.8 ± 0.4, 0.8 ± 0.5, 0.9 ± 0.4 and 0.4 ± 0.2 % for PBS, PDT, BZ4 and TDS, respectively.

The accuracy of the results can be estimated by considering the standard deviation of each cell to a specific time (see Table 2.3). In this case, the obtained results can be considered accurate, since the average of the RSD values obtained in the determinations reaches 5.8 %. However, it is not so easy to draw the same conclusion when considering the standard deviation of the three cells to a specific time, because the RSD values increase significantly, probably due to the own peculiarities of the skin portion used in each cell.

It should be emphasised that the delicate step of separation of the epidermis from the dermis can cause the obtaining of epidermis portions seemingly similar but having different biomechanical parameters, such as elastic and viscoelastic deformation, porosity, thick, etc., which would explain the variability of the results obtained. Only the application of the described analytical method to the study of a large number of diffusion cells using a wide variety of skin types will enable the average of these inherent differences and provide more specific data.

2.4 Conclusions

In this Chapter, a liquid chromatography analytical method based on ion inter-action with UV/VIS to estimate the *in vitro* percutaneous absorption of the most used hydrosoluble UV filters in sunscreen cosmetic products has been proposed.

The addition of an ion-pairing reagent to the mobile phase allowed the simultaneous determination of the analytes in the fluid receptor from diffusion cells with a low detection limit, which allows the quantification of the target compounds from the first hours after the application of the cosmetic product on the epidermis.

Although the estimation of the obtained percutaneous absorption may not be conclusive, a general idea of how the analyte passes through the skin surface is achieved. No more than 1 % of the total applied amount of each UV filter was determined in the receptor fluid after 48 h in any case. It is not possible to ensure that more percutaneous absorption occurs after this time due to the used *in vitro* system does not guarantee the integrity of the epidermis (possible occurrence of decomposition processes). It would be reasonable to assume that the absorption of the tested compounds occurs over a time less than 48 h, although a more com-prehensive biokinetic study would be necessary to confirm this. Moreover, it should be noted that the actual *in vivo* situation would be even more adverse, since users of such cosmetic products rarely stay with the product applied after 24 h, tending to remove the product in the shower, when covering with clothes, etc.

This method may be useful in future studies to develop more exhaustive bi-okinetic studies regarding percutaneous absorption of these UV filters, thus con-sidering different times and employing a greater number of diffusion cells that minimize variability in the used skin portions. In this context, the study of synergy effects of the UV filters would be also possible by using this analytical method.

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Part III
In Vivo Methodology

III.1 Background

In a query of scientific databases (Web of Knowledge, SciFinder®) in April 2011, around 60 publications that used *in vivo* methodologies to study the process of percutaneous absorption of the UV filters have been found, mainly through the determination of the found content in biological fluids after topical application of the sunscreen cosmetics and *in vivo* tape-stripping techniques.

Table III.1 describes the highlights of these publications including UV filter of interest, application of the cosmetic formulation, sample collection, sample treatment, analytical technique, and the most relevant comments.

In the search, PABA was also included as UV filter, despite its recent ban for use in sunscreen cosmetics under current legislation in the European Union (Directive 2008/123/EC). The published articles arising from this Ph.D. thesis and others carried out by our research group are also detailed.

Regarding the subject under study, about 73 % of the articles are based on the application of the cosmetic formulation on human volunteers. The remaining percentage corresponds to studies with rat (20 %), pigs (5 %), and mice (2 %).

It should be noted that the methodologies based on *in vivo* animal trials are being gradually banned within the EU (Directive 2003/15/EC). Thus, according to this Directive, September 2004 was set as the deadline for the testing of finished cosmetic products on animals. Also, March 2009 was set as the deadline for the testing of cosmetic ingredients that are included in cosmetic products, which have been evaluated with animal testing. In addition, from that date, the marketing in the EU was banned to both products and ingredients that have been tested in this way.

This prohibition was applied to all effects that impact on human health, except for repeated dose toxicity assays, reproductive toxicity, and toxicokinetics. In these particular cases, the prohibition shall apply gradually while accepting and adopting alternative methods by the European legislation. However, a maximum period of 10 years was set up after the entry into force of the Directive 2003/15/EC (i.e., March 2013), regardless of the availability of alternative methods.

Table III.1 Published articles showing evidences of the *in vivo* percutaneous absorption of UV filters

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Arancibia et al. 1981	PABA	Oral and topical (six male volunteers)	Urine excretion study for 48 h	–	–	Topical application provides higher metabolism (acetyled conjugate content 70–90 %) than oral (50–80 %)
El Dareer et al. 1986	BZ3	Oral, intravenous and topical (rats)	Urine excretion study for 72 h	–	Radioactivity measures	Nine new radiactive compounds are detected, two of them identified as BZ3 and its glucuronide conjugate
Abdel-Naby et al. 1992	BZ3	Oral (rats)	Biological fluids and tissues	Hydrolysis (HCl)	LC-UV/VIS	Content of BZ3 in: Blood: maximum after 1 h / Urine: low after 12 h, present as glucuronide conjugate
Daston et al. 1993	BZ3	Topical (mice)	Biological fluids and tissues for 13 weeks	–	Weight of organs and sperm concentration	No potential reproductive toxicity for BZ3 in mice
Okereke et al. 1993	BZ3	Oral (rats). Metabolism study	Urine excretion study for 96 h. Plasma sampling for 20 h	Enzymatic (ß-glucuronidase) or hydrolytic (HCl)	LC-UV/VIS	Identification of DHB, DHMB and THB as phase I metabolites of BZ3. Urine is the main way of excretion
Okereke et al. 1994	BZ3	Topical (rats)	Plasma sampling for 48 h	Hydrolysis; LLE; Evaporation; Reconstitution	LC-UV/VIS	Maximum concentration 35 µg L^{-1} (2.5 h)
Hany and Nagel. 1995	HS, IMC, BZ3, MBC, EDP, EMC	Topical (6 female volunteers)	Breastmilk	SPE GPC	GC-MS	Determination of BZ3 and EMC in 5 samples (between 16 and 417 ng g^{-1} of fat)
Kadry et al. 1995	BZ3	Oral (rats). Pharmacokinetics study	Biological fluids and tissues for 6 h	–	LC-UV/VIS	Prevalence of glucuronide conjugation. Content of BZ3 in: plasma: maximum after 3 h ($\sim$ 25 µg L^{-1}) liver > kidney > testicles, after 6 h
Okereke et al. 1995	BZ3	Topical and repeated (rats)	Plasma (for 16 days)	–	LC-UV/VIS	Security evaluation: toxicity absence

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Hagedorn-Leweke and Lippold 1995	BZ3, MBC, IMC, EDP	Topical (volunteers)	–	–	–	Correlation method for maximim fluxes and relevant properties
Treffel and Gabbard 1996	ES, EMC, BZ3	Topical (volunteers)	Tape-stripping	–	–	Vehicle influence
Jiang et al. 1996	BZ3, EMC, BDM, EDP, ES	–	Plasma	LLE; Centrifuge	LC-UV/VIS	Not applied to real samples
Hayden et al. 1997	BZ3, EMC, ES, OCR	Topical (9 volunteers)	Urinary excretion study for 48 h	Enzymatic treatment (β-glucuronidase)	LC-UV/VIS	Evidence of percutaneous absorption (1–2 %) for BZ3 and urinary excretion
Marginean-Lazar et al. 1997	EMC, BDM, MBC	Topical (volunteers)	Tape-stripping	–	UV/VIS	Influence of radiation photochemical behavior
Felix et al. 1998	BZ3	Topical (1 volunteer)	Urinary excretion study	SPME and SPE	GC-MS	Determination of BZ3 and DHB in urine (~ 260 and 200 ng mL^{-1})
Lademann et al. 1999	TiO^2	Topical (volunteers)	Tape-stripping	–	Espectroscopy	Microparticles. Low absorption
Fernández et al. 2000b	BZ3	Topical (6 volunteers)	Tape-stripping (30 min)	–	LC-UV/VIS	Influence of vehicle, evidence of absorption
Cambon et al. 2001	EMC	Topical (volunteers)	Tape-stripping	–	LC-UV/VIS	Influence of UV radiation
Couteau et al. 2001	BZ4, P25	Topical (21 female volunteers)	Tape-stripping (7 h)	–	LC-UV/VIS	Better remanence for BZ4
Laugel et al. 2001	EMC	Topical (volunteers)	Tape-stripping	–	ATR-IR	*In vivo* tool
Gonzalez et al. 2002	BZ3	Topical (11 volunteers)	Urinary excretion study (0–48 h)	Hydrolytic treatment (HCl); SPE (C8)	LC-UV/VIS	Total excreted amount: 0.4 %

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Chatelain et al. 2003	EMC, BZ3,ES, HS, BDM	Topical (volunteers)	Tape-stripping (30 min)	–	–	Influence of vehicle, evidence of absorption
Vidal et al. 2003a	PBS	Topical (3 volunteers)	Urinary excretion study (0–12 h)	on line SPE	SI-SPE-F	Total excreted amount: 10–30 μg
Benech-Kieffer et al. 2003	TDS	Topical (volunteers)	Urinary excretion study	–	Measurements of radioactivity	Evidence of low percutaneous absorption (< 0.1 %) for TDS
Gottbrath et al. 2003	TiO$_2$	Topical (volunteers)	Tape-stripping	–	TEM	Influence of vehicle, evidence of absorption
Sarveiya et al. 2004	BZ3, EMC ES, HS	Topical (volunteers)	Urinary excretion study (0–48 h). Plasma sampling (0–24 h) Tape-stripping	Urine: enzymatic treatment (ß-glucuronidase); Plasma: extraction (ACN)centrifuge	LC-UV/VIS	Evidence of percutaneous absorption (1 %) for BZ3
Jacobi et al. 2004	BDM, MBC	Topical (volunteers)	Tape-Stripping	–	Espectroscopy	Presence of competitive processes with skin penetration
Janjua et al. 2004	BZ3, MBC, EMC	Topical and repeated (15 male and 17 female volunteers)	Urinary excretion study and plasma sampling (0–96 h). Study of hormone levels for 2 weeks	–	–	Evidence of percutaneous absorption for BZ3, MBC, OMC. Absence of relationship between hormone levels and differences in exposure to UV filters
Ye et al. 2005	BZ3	Topical (volunteers)	Urinary excretion study. 30 samples collected between 2000 and 2004	Enzymatic treatment (ß-glucuronidase/ sulfatase)	on line SPE-Isotopic dilution-LC-MS/MS	Evidence of percutaneous absorption for BZ3 (detection of the 85 % as glucuronide conjugate)
Olvera-Martínez et al. 2005	EMC	Topical (6 volunteers)	Tape-Stripping (1 h)	–	UV/VIS	Vehicle influence (nanoencapsulation)
Kasichayanula et al. 2005	BZ3	Topical (pigs)	Urinary excretion study and plasma sampling (0–48 h)	LLE (MeOH:ACN); Centrifuge	LC-UV/VIS	Found concentrations: THB < BZ3 < DHB DHMB, not detected

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Balaguer et al. 2006b[a]	PDT	Topical (5 volunteers)	Urinary excretion study (0–48 h)	on line SPE	SI-SPE-F	Total excreted mass: 3.5 µg at 8 h 300 µg at 30 h 35 µg at 35 h 45 µg at 50 h
Volkel et al. 2006	MBC	Oral (rats). Characterization of metabolites. Toxicokinetics	Urinary excretion study. Plasmasampling	Extraction (MeOH). Centrifuge. Extraction (ACN)	LC-MS/MS	Identificationof MB-6HC y CBC in urine and faeces. Slow excretion of metabolites in urine
Schauer et al. 2006	MBC	Topical (3 male and 3 female volunteers).	Urinary excretion study (0–96 h)	Enzymatic treatment (ß-glucuronidase); LLE (MeOH)	LC-MS/MS	Evidence of low percutaneous absorption (<0.1 %) of MBC
Soeborg et al. 2006	3BC	Topical (rats)	Plasma (for 65 days)	LLE (heptane)	LC-MS/MS	Found concentrations: 16–89 ng mL^{-1}
Gonzalez et al. 2006	BZ3	Topical and repeated for 5 days (25 volunteers)	Urinary excretion study (0–10 days)	–	LC-UV/VIS	Evidence of percutaneous absorption and accumulation BZ3 (3.7 % recovered)
Kasichayanula et al. 2007	BZ3	Topical (pigs)	Urinary excretion study. Plasmasampling (0–48 h) Tape-stripping	–	LC-UV/VIS	Evidence BZ3 percutaneous absorption. Synergy effects with an insecticide
Vidal et al. 2007[a]	BZ3	Topical (1 volunteer)	Urinary excretion study. (0–12 h)	SDME	LC-UV/VIS	Evidence of percutaneous absorption BZ3
Iannuccelli et al. 2007	BDM	–	Tape-stripping	–	–	Vehicle influence: lipid microparticles
Mavon et al. 2007	MBP, TiO^2	Topical (volunteers)	Tape-stripping	–	LC-UV/VIS TEM	Minimal percutaneous absorption for TiO^2
Wang et al. 2007	PABA	Topical (volunteers)	Urinary excretion study (0–48 h)	LLE (EtOH and Ethyl Acetate); SPE (C18)	LC-ECD	Evidence of percutaneous absorption for PABA. Biotransformation pathway

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Scalia et al. 2007	MBC	–	Tape-stripping	–	–	Vehicle influence (lipid microparticles): lower absorption
Klinubol et al. 2008	EMC, MBC, BDM	Topical (5 volunteers)	Suction technique	–	–	Evidence of percutaneous absorption for BDM and EMC
Golmohammadzadeh et al. 2008	EMC	Topical (6 volunteers)	Tape-stripping	–	LC-UV/VIS	Vehicle influence (liposomes)
Balaguer et al. 2008a	P25	Topical (3 volunteers)	Urinary excretion study (0–90 h)	SPE (silica)	LC-F	Evidence of percutaneous absorption (~0,015 %) for P25
Janjua et al. 2008	BZ3, MBC, EMC	Topical and repeated (15 male and 17 female volunteers)	Urinary excretion study and plasma sampling (0–96 h)	Plasma: Precipitate (ACN) Urine: Lyophilized and suspended	LC-UV/VIS	Maximum average concentration in plasma and urine: BZ3: ~200 and ~60 ng mL^{-1} MBC: ~17 and ~4 ng mL^{-1} EMC: ~12 and ~5 ng mL^{-1}
Ye et al. 2008a	BZ3	Topical (female volunteers)	Breastmilk	Enzymatic treatment (β-glucuronidase)	SPE-LC-MS/MS on line	Unconjugated species prevails. Found concentrations up to 1.28 ng mL^{-1}
Gonzalez et al. 2008	BZ3	Topical (25 volunteers) Repeated doses (5 days)	Urinary excretion study (0–10 days)	Enzymatic treatment (β-glucuronidase-arylsulfatase); SPE (C8)	LC-UV/VIS	Valid procedure for routine analysis. No conclusions on the obtained results
Jeon et al. 2008	BZ3	Oral (rats)	Plasma (0–24 h)	LLE; Hydrolysis; Extraction; Evaporation; Reconstitution (MSTFA)	GC-MS	The concentration of BZ3 metabolites decreases more slowly with time than BZ3
Schlumpf et al. 2008	MBC, 3BC(rats)BZ3, 3BC MBC, EMC HS, OCR EDP(female)	Topical (female volunteers, rats)	Breastmilk. Organs of growing rats	Milk: Extraction, Reconstitution, GPC Rat organs: Extraction, Reconstitution	GC-MS	Evidence of percutaneous absorption (~80 % of all samples). Estrogenic effects on reproductive organs of growing rats after exposure to MBC and 3BC

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
Kawaguchi et al. 2008	BZ3	Topical (6 volunteers)	Urinary excretion study	Enzymatic treatment (β-glucuronidase); SBSE	TD-GC-MS	Found concentrations up to 1.2 ng mL^{-1}
Ye et al. 2008b	BZ3	Topical (25 volunteers)	Serum	Enzymatic treatment (β-glucuronidase)	on line SPE-LC-MS/MS	Absence in serum
Filipe et al. 2009	TiO$_2$	Topical (volunteers)	Cryosections of biopsied skin at 2 h	–	–	Vehicle influence (nanoparticles): absorption minimizes
March et al. 2009a[a]	EDP	Topical (volunteers)	Urinary excretion study after 2 h	MALLE	GC-MS	Evidence of percutaneous absorption
Kawaguchi et al. 2009	BZ3	Topical (10 volunteers)	Urinary excretion study	Enzymatic treatment (β-glucuronidase-sulfatase) HF-LPME	GC-MS	Evidence of percutaneous absorption of BZ3. Concentrations: 0.43–5.17 μg L^{-1}
Kunisue et al. 2010	BZ3	Topical (23 American and 22 Japanese volunteers)	Urinary excretion study	Enzymatic treatment (β-glucuronidase)	LC-MS/MS	Evidence of percutaneous absorption
Gulbake et al. 2010	BZ3	Topical	Morphological study of the skin	–	Microscopic techniques	Vehicle influence (nanoparticles)
Adachi et al. 2010	TiO$_2$ (ultrafine)	Topical (rats)	Morphological study of the skin	–	Microscopy RX espectrometry	Absence of percutaneous absorption
Sadrieh et al. 2010	TiO$_2$	Topical (pigs)	Morphological study of the skin and other organs	–	ICP-MS	Vehicle influence (nanoparticles)
Fediuk et al. 2010	BZ3	Topical (rats).Toxicological cell tests	Plasma and other organs sampling. Tape-stripping	Homogenization (ACN) SPE or LLE	LC-UV/VIS	Evidence of accumulation in blood, liver, brain. No neurotoxicological deficiencies
León et al. 2010a[b]	BZ3, BZ4	Topical (2 male and 1 female volunteers)	Urinary excretion study	SPE (C18 and DEA)	SI-SPE-LC-UV/VIS	Evidence of percutaneous absorption of BZ3 and BZ4

(continued)

Table III.1 (continued)

Reference	UV filter	Application	Sampling	Sample treatment	Analytical technique[c]	Comments
León et al. 2010b[b]	EDP	In vitro metabolism study	Not applied to real samples	SPE (Plexa)	LC-MS	Identification of DMP and MMP as in vitro phase I metabolites of EDP by GC-MS analysis
León et al. 2010c[b]	BZ3	Topical (1male volunteer)	Urinary excretion study and semen sampling (0–48 h)	Enzymatic treatment (ß-glucuronidase) SPE (C18)	LC-MS/MS	Evidence of percutaneous absorption and potential bioaccumulation of BZ3
León et al. 2011a[b]	EDP	Topical (2 male and 2 female volunteers)	Urinary excretion study for 8 days	Enzymatic treatment (ß-glucuronidase)	SPE-LC-MS/MS on line	Identification of DMP and MMP glucuronides conjugates as in vivo phase II metabolites of EDP by LC-TOF-MS analysis
León et al. 2011b[b]	EDP	Topical (2 male volunteers)	Semen sampling for 8 days	Enzymatic treatment (ß-glucuronidase)	SPE–LC–MS/MS on line	Evidence of percutaneous absorption and potential bioaccumulation of EDP
Scalia et al. 2011	BDM	Topical (volunteers)	Tape-stripping	LLE	LC-UV/VIS	Vehicle influence (charged microparticles with a complex of cyclodextrins and BDM); reduction of absorption
León et al. 2011c[b]	MBC	Topical (1 volunteer)	Urinary excretion study for 4 days	SPE (C18)	LC-MS/MS	Evidence of percutaneous absorption. CBC glucuronide as predominant metabolite

[a] Published articles by the research group where this PhD thesis was carried out
[b] Published articles that are part of this PhD thesis
[c] The employed keys are specified in Annex I
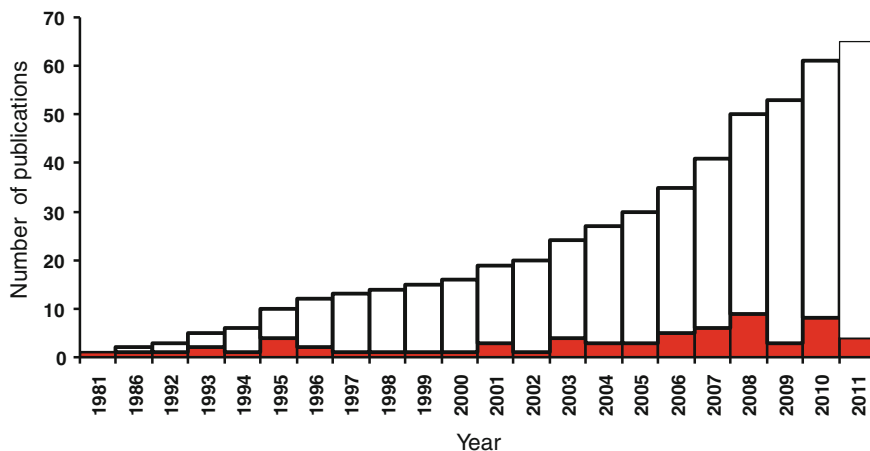
Fig. III.1 Evolution of scientific articles regarding the estimation of the percutaneous absorption of UV filters using *in vivo* techniques (■) and cumulative number of articles (□)

Figure III.1 shows the number of publications related to this research area versus time to date (April 2011). A significant increase in the number of articles published in the last 5 years can be seen. Therefore, data are a clear evidence of the social concern about the safety of daily used cosmetic products containing UV filters in their composition.

III.2 Main Objectives of the *In Vivo* Studies

Although the determination of UV filters in biological matrices do not provide reliable information about the level of percutaneous absorption in comparison to in vitro methodologies, the process of absorption through the skin is evidenced, as part of the global process that constitutes the body disposition of the UV filters in the organism. In addition, research on the evaluation of toxicity and systemic biotransformation processes is also enhanced.

In this context, it should be highlighted the great variability observed in the published estimations when comparing these processes among different individuals. Therefore, the development of analytical methods to determine UV filters in biological fluids should encourage their use as tools for application to a large number of individuals in order to reach more general conclusions.

Regarding toxicological findings, a low toxicity level associated to BZ3 after topical applications of cosmetic formulations in rats and mice has been found (Daston et al. 1993; Okereke et al. 1995; Fediuk et al. 2010). Moreover, the *in vitro* estrogenic activity showed by various UV filters (Schlumpf et al. 2001) has been also confirmed to MBC and 3BC under *in vivo* conditions (Schlumpf et al. 2008).

Meanwhile, the metabolic pathway of some UV filters such as BZ3, MBC and, as indicated in Chap. 6 of this Ph.D. Thesis, EDP has been clarified and confirmed by characterization of metabolites. Actually, the aforementioned compounds are the only UV filters that have been determined along with all its metabolites in biological matrices to date. The processes related to the biotransformation reactions are described in more detail in Sect. 1.4.3.

In the case of BZ3, the characterization of the metabolites has been carried out by analysis of urine (El Dareer et al. 1986; Okereke et al. 1993; Felix et al. 1998; Kasichayanula et al. 2005; Ye et al. 2005; León et al. 2010c, see Chap. 4), plasma (Okereke et al. 1993; Kasichayanula et al. 2005; Jeon et al. 2008), milk (Ye et al. 2008) and semen (León et al. 2010c, see Chap. 5) from humans, rats and pigs after both oral and topical application. In the case of MBC, the analyses of urine (Volkel et al. 2006; Schauer et al. 2006; León et al. 2011c, see Chap. 9) and plasma (Volkel et al. 2006) from humans and rats after both oral and topical application have been described. Finally, in the case of EDP, *in vitro* analyses (León et al. 2010b, see Chap. 6) and determination in urine (León et al. 2011a, see Chap. 7) and semen (León et al. 2011b, see Chap. 8) from humans after topical application have been carried out.

Furthermore, the effects of the vehicle where the UV filter is formulated have been evaluated in parallel to the studies of percutaneous absorption in order to optimize the final cosmetic product by minimizing these processes. In general, mainly using tape-stripping technique on human volunteers, an increase in UV filter accumulation on the skin surface has been observed by encapsulating the active ingredients alone or complexed with cyclodextrins (Scalia et al. 2011), in lipid microparticles (Iannucelli et al. 2007; Scalia et al. 2007), in solid lipid nanoparticles (Gulbake et al. 2010; Sadrieh et al. 2010), or in polymeric lipid nanoparticles (Olvera-Martinez et al. 2005; Filipe et al. 2009). These proposals do not involve a loss of efficiency for solar protection of the cosmetic formulation, but rather keep longer by minimizing the percutaneous absorption processes.

Finally, an *in vivo* assay with pigs that showed the appearance of synergy effects of percutaneous absorption of BZ3 and *N,N*-diethyl-m-toluamide (DEET), a known repellent insect (Kasichayanula et al. 2007) has been also published.

III.3 Biological Matrices

To date, BZ3 is the UV filter whose percutaneous absorption has been more extensively studied using *in vivo* methodologies. BZ3 has been determined in biological tissues (liver, kidney, spleen, and testes) and biofluids (urine, plasma, breast milk, and even semen, being the first UV filter that has been found in such biological matrix, as described in Chap. 5). The concentrations of this compound in biological fluids range from a few ng mL^{-1} to several tens of μg mL^{-1}, depending on the nature of the matrix and the time elapsed from the application,
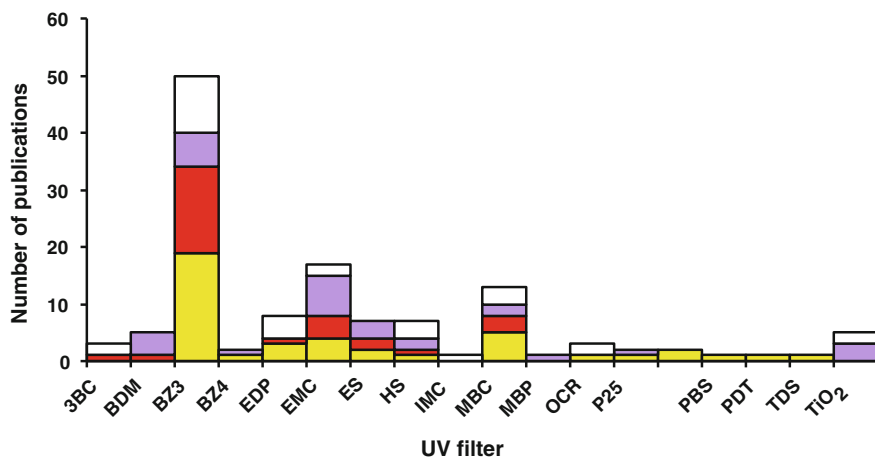
Fig. III.2 Number of publications related to the determination of UV filters by applying *in vivo* methodologies to evidence the percutaneous absorption. Urine analysis ((■)), plasma or serum analysis ((■)), other fluids or tissues ((□)) and *in vivo* Tape-stripping technique ((■))

resulting in estimated absorption rates between 0.1 and 3.7 % of the total amount of the applied UV filter.

As shown in Fig. III.2, in decreasing order of publications number, EMC (5 and 300 ng mL^{-1} in urine, plasma and milk) MBC (4 and 300 ng mL^{-1} in urine and plasma), followed by EDP, ES, and HS have been also studied.

The prevalent biological matrix to the *in vivo* study of the processes resulting from percutaneous absorption of UV filters is urine, followed by plasma or serum, and very distant from biological tissues, breast milk, and semen, which only have been analyzed occasionally. Exceptionally, tape-stripping methods applied to human volunteers have been also used often to evaluate the effect of the vehicle where the UV filter is formulated, as discussed above.

III.4 Analytical Techniques

Regarding the analytical techniques used for the determination of UV filters in biological matrices, liquid chromatography-ultraviolet/visible spectrometry (LC-UV/VIS) is the one that is most often used to date (see Fig. III.3). Liquid chromatographic (LC) and gas chromatographic (GC) techniques coupled to mass spectrometry (MS) have also been widely used. Lastly, the UV/VIS absorption spectrometry, the infrared absorption spectrometry, and the fluorescence spectrometry have been used to a lower extent.

Not coincidentally, chromatographic techniques have been largely used to determine UV filters in such complex biological matrices. Furthermore, treatment
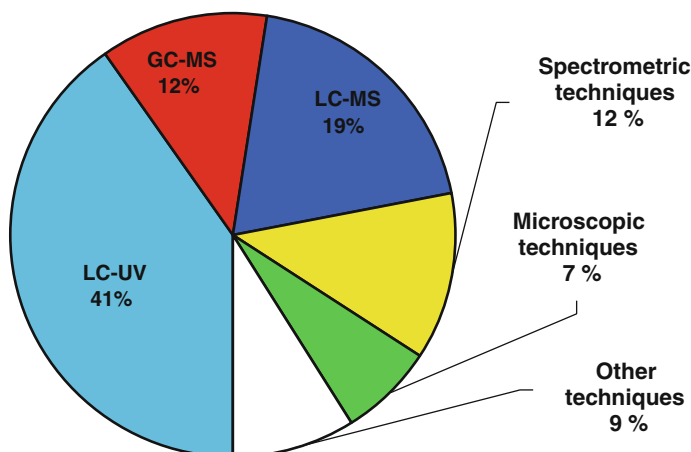
Fig. III.3 Percentage representation of the publications related to the determination of UV filters to evidence percutaneous absorption by application of *in vivo* methodologies classified according to the analytical technique used. Annex I specifies the keys used

of these samples has become an indispensable step to remove those components that produce matrix interferences in the development of new analytical methodologies.

Thus, acid and enzymatic hydrolysis have been used in the sample treatment to denature proteins present in the medium or deconjugate phase II biotransformation conjugates (see Sect. 1.4.3), respectively. Both hydrolytic treatments agree to facilitate the determination of the free or unconjugated species (Okereke et al. 1993).

In addition, processes allowing both sample clean-up and concentration of the analytes are also common, such as liquid-liquid extraction (LLE) (Scalia et al. 2011) which involves subsequent stages of evaporation and reconstitution, solid-phase extraction (SPE) (Vidal et al. 2003), and solid-phase microextraction (SPME) (Felix et al. 1998).

Less commonly, extraction techniques based on stir-bar sorptive extraction (SBSE) (Kawaguchi et al. 2008), membrane-assisted liquid-liquid extraction (MALLE) (March et al. 2009c), hollow fiber liquid phase microextraction (HF-LPME) (Kawaguchi et al. 2009), and single-drop microextraction (SDME) (Vidal et al. 2007) have also been used. Finally, lyophilization of urine samples, which are analyzed directly after resuspension in a suitable solvent (Janjua et al. 2008), has been described.

Furthermore, the coupling of chromatographic techniques to sensitive detection systems (i.e., MS) allows the development of selective analytical methods that are able to achieve low limits of detection and to determine UV filters accurately in biological matrices (León et al. 2010c).

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III.1 Benzophenone-3

Note: Chapter 3–5 comes under "Benzophenone-3"

III.1.1   *Definition*

Benzophenone-3 (BZ3), also known as 2-hydroxy-4-methoxy-benzophenone, is a very common UV filter used in sunscreen cosmetics alone or in combination with other UV filters to protect against the harmful effects of the UV radiation from the sun.

As indicated above, the levels of this compound in sunscreen formulations are currently regulated by different laws worldwide. Hence, BZ3 can be used up to 10, 6, and 5 % (m/m) in the final product, according to the current legislation in the EU, the USA, and Japan, respectively (Chisvert and Salvador 2007). In addition, the European Union legislation requires the inclusion of a warning on the label to show the presence of BZ3 in the cosmetic product, due to the high incidence of allergy caused by this compound.

According to the available data related to safety considerations (Colipa 2006), the use of BZ3 is considered safe for topical application to human skin under the concentrations and conditions of use specified in cosmetic products. The chemical structure and the UV/VIS absorption spectrum of BZ3 are shown in Fig. III.4.

III.1.2   *Undesired Side Effects*

Some studies questioned the supposed safety of using BZ3. There are published data showing evident positive allergic reactions to consider BZ3 as the most
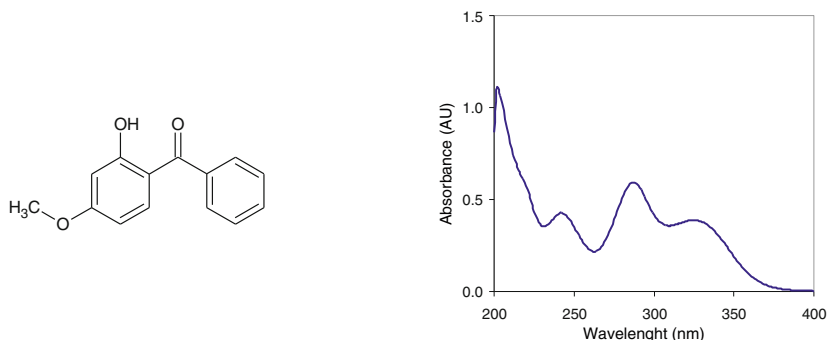
Fig. III.4 Chemical structure of BZ3 and UV/VIS spectrum of a BZ3 solution (5 μg mL^{-1}) prepared in EtOH

common allergic (Berne and Ros 1998; Landers et al. 2003) and photo-contact allergic agent (Darvay et al. 2001) that are currently still used.

Regarding both *in vitro* and *in vivo* percutaneous absorption studies, it was shown that BZ3 is absorbed through the skin to a certain extent (Gabard and Treffel 1996; Giokas et al. 2007).

Recent research related to hormonal activity concluded that daily exposure to cosmetic formulations containing BZ3 could produce estrogenic effects in humans (Schlumpf et al. 2001; Schreurs et al. 2002; Ma et al. 2003; Henewer et al. 2005; Matsumoto et al. 2005), suggesting the need to further study the endocrine activity of this substance. Furthermore, a certain type of endocrine disrupting which has not been described for classical estrogenic markers was shown for BZ3 (Schlecht et al. 2004; Coronado et al. 2008). By contrast, other studies have suggested that the *in vivo* genotoxicity of BZ3 is reduced (Janjua et al. 2004; Robison et al. 1994; Okereke et al. 1995; Hayden et al. 2005).

One of the direct consequences arising from the percutaneous absorption of BZ3 is the appearance of a number of biotransformation reactions in the body causing changes in physical properties of the compound. These processes are performed by a limited number of enzymes as discussed in Chap. 1. Toxicokinetic studies with rats (Okereke et al. 1993) and humans (Sarveiya et al. 2004) indicate that BZ3 rapidly biotransformed to three phase I metabolites, called 2,4-dihydroxybenzophenone (DHB), 2, 2′-dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB), as shown in Fig. III.5.

Considering phase II enzymatic reactions, BZ3 and its phase I metabolites have been also identified in their glucuronide and sulfatase conjugated forms (Kadry et al. 1995).

Due to the fact that the concentration of these metabolites in blood decreases much more slowly with time compared to the parent compound, it is possible that the metabolites of BZ3 may have more long-term adverse effects than BZ3 (Jeon et al. 2008). Different *in vitro* studies have identified DHB as a metabolite of BZ3 with estrogenic character and antiandrogenic activity much higher than that of
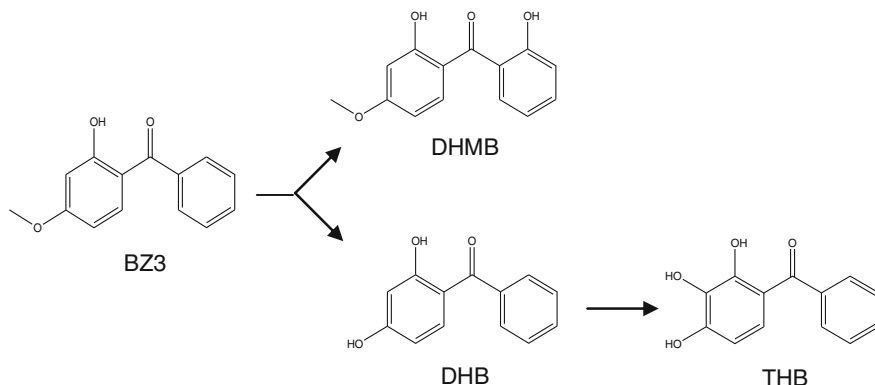
Fig. III.5 Proposed metabolism pathway of BZ3 (Okereke et al. 1993)

BZ3 has been attributed to this compound (Koda et al. 2005; Jeon et al. 2008; Molina Molina et al. 2008). Furthermore, estrogenic activity of BZ3 and DHMB was confirmed (Ogawa et al. 2006) and additionally, it was shown that DHB and THB exhibit higher estrogenic activity than other potent and well established endocrine disruptors, such as bisphenol A (Kawamura et al. 2003).

Clear evidences showing that various environmental estrogens, including the most potent xenoestrogen (i.e., 17ß-estradiol), significantly had influenced on the mature sperm function in mice, causing variations in their fertilizing ability (Adeoya-Osiguwa et al. 2003). This finding has led to the development of studies with other potential estrogenic agents. For BZ3, controversial results have been found when studying specific parameters of reproductive toxicity. First, the potential of BZ3 to cause alterations in breeding male mice was evaluated and the results indicated that, when applied topically, BZ3 lacked reproductive toxicity under the conditions of study (Daston et al. 1993). However, consistent conclusions drawn from a toxicity report (National Toxicology Program, 1992) showed that BZ3 produced in general the same effects whether the administration to rats was topically and orally, and these findings included decreasing in sperm density and elongation of the estrogen cycle. Therefore, taking into account the potential estrogenic activity of BZ3 and especially of its metabolites, further research on the toxicity of this xenobiotic on the male reproductive system is required. Thus, the development of analytical methods to determine BZ3 and other UV filters in biological fluids related to the reproductive system can be interesting to find a relationship between the found concentrations and the caused toxicological effects.

III.1.3 Benzophenone-4

Furthermore, benzophenone-4 (BZ4), also called 2-hydroxy-4-methoxybenz-ophenone-5-sulfonic acid, is defined as the soluble version of BZ3 because of the
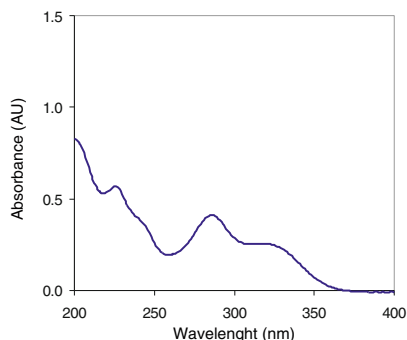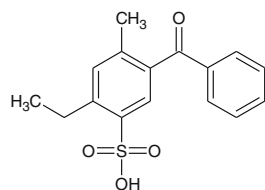
Fig. III.6 Chemical structure of BZ4 and UV/VIS absorption spectrum of a BZ4 solution (5 μg mL^{-1}) prepared in EtOH

sulfonic group present in its chemical structure which confers water solubility. Hence, the incorporation into aqueous sunscreen cosmetic formulations such as spray lotions is enhanced. The chemical structure and the UV/VIS absorption spectrum of BZ4 are shown in Fig. III.6.

The maximum content of BZ4 in the finished cosmetic product authorized by the European Union is 5 % (m/m), while in the USA and Japan, BZ4 can be used up to 10 % (m/m) (Chisvert and Salvador 2007).

Several studies have evaluated the frequency of the incidence of both allergic reactions as photo-allergic contact caused by some UV filters (Schauder and Ippen 1997; Fischer and Bergstrom 1991). Among the cases of photo-positive allergies, the most frequent sensitizers corresponded to the family of benzophenones (Bisland and Ferguson 1993) and, in particular, Hugues and Stone (2007) found a high allergen incidence caused by BZ4 that was significantly higher than that of any other studied UV filter.

As evidence for percutaneous absorption processes of BZ4, *in vitro* methodology based on the use of diffusion cells with skin of rat (Brinon et al. 1998), pig (Brinon et al. 1999), human (Benech-Kieffer et al. 2000; Pont et al. 2004; Leon et al. 2008 (see Chap. 2)) and mouse (Pont et al. 2004) has been employed. Similarly, tape-stripping technique has been also used *in vitro* (Potard et al. 2000) and *in vivo* (Couteau et al. 2001) with human skin, leading to the unique found reference that stated *in vivo* percutaneous absorption for BZ4.

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III.2 Ethylhexyl Dimethyl PABA

Note: Chapter 6–8 comes under "Ethylhexyl Dimethyl PABA"

III.2.1 *Definition*

The organic UV filter called ethylhexyl dimethyl PABA (EDP) according to the International Nomenclature Cosmetic Ingredient, or 2-ethylhexyl-4- (N, N-dimethylamino) benzoate, absorbs electromagnetic radiation mainly in the UVB range (290–320 nm). The UV/VIS absorption spectrum and the chemical structure of EDP are shown in Fig. III.7.

According to both the European and American laws, EDP can be used up to a maximum concentration of 8 % (m/m) in the final cosmetic product, while Japanese law allows its use up to 10 % (m/m) (Chisvert and Salvador 2007).

The efficiency of EDP to protect against the formation of erythema and edema induced by UV radiation (Reeve et al. 1991) and to avoid the formation of photocarcinomas (Kerr 1998) has been showed. According to an *in vitro* study regarding the influence of the UV filters concentration on Sun Protection Factor (SPF), EDP was considered one of the most effective UV filter authorized (Couteau et al. 2007), thus being one of the most popular sunscreen agents (Roelandts 1998).
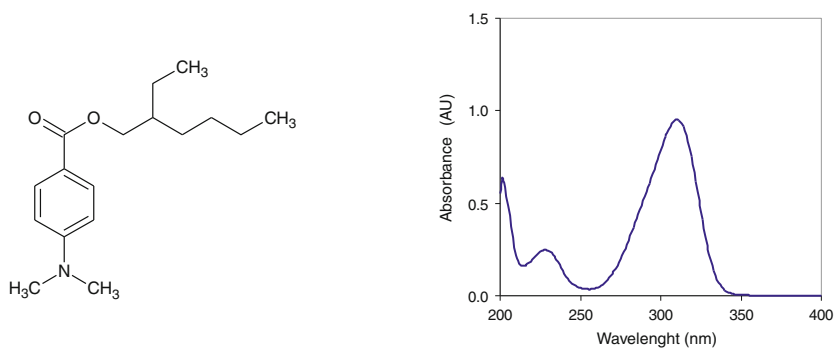
Fig. III.7 Chemical structure of EDP and UV/VIS absorption spectrum of a 5 μg mL^{-1} solution of EDP in EtOH

III.2.2 Undesired Side Effects

Despite these almost ideal features showed by EDP (Klein 1997), there are clear evidences that warn about the safety of this compound.

An undesired side effect of this substance is systemic percutaneous absorption (Blank et al. 1982; Hagedorn-Leweke and Lippold 1995; Kenney et al. 1995; Pont et al. 2003; Pont et al. 2004; Hayden et al. 2005). Nevertheless, according to the consulted literature, the mechanism of biotransformation of EDP was unknown.

In endocrine activity studies carried out by *in vitro* assays using different breast cancer cell lines, certain estrogenicity was found for EDP (Schlumpf et al. 2001; Gomez et al. 2005; Cargouet et al. 2006) but it was not agonist to androgen receptors (Ma et al. 2003). Furthermore, different *in vitro* approaches assessing its potential genotoxicity showed evidence of photo-mutagenicity, so that EDP selectively damaged DNA when irradiated with UV light, thus causing breakage of the double helix strands and other injuries which could contribute to the increase of the appearance of carcinomas related to sunlight (Knowland et al. 1993; McHugh and Knowland 1997; Gulston and Knowland 1999; Xu et al. 2001).

On the other hand, the potential of EDP and its potential metabolites to cause toxicity in the human reproductive system has not been evaluated yet. In this context, in addition to the relevant studies to evaluate sperm quality through the required histological parameters (i.e., mobility, vitality and sperm morphology, etc.), the determination of EDP and/or its possible metabolites in human semen may be of great interest to establish relationships between the concentration of these substances in such biofluid and the potential adverse effects that they may cause to the male reproductive system.

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III.3 Methyl Benzilidene Camphor

Note: Chapter 9 comes under "Methyl Benzylidene Camphor"

III.3.1   Definition

The organic UV filter named methyl benzylidene camphor (MBC) according to the International Nomenclature Cosmetic Ingredient, or 3-(4′-methylbenzylidene) camphor, absorbs electromagnetic radiation in the UVB range and exhibits a maximum absorption at 300 nm. The UV/VIS absorption spectrum and the chemical structure of MBC are shown in Fig. III.8.
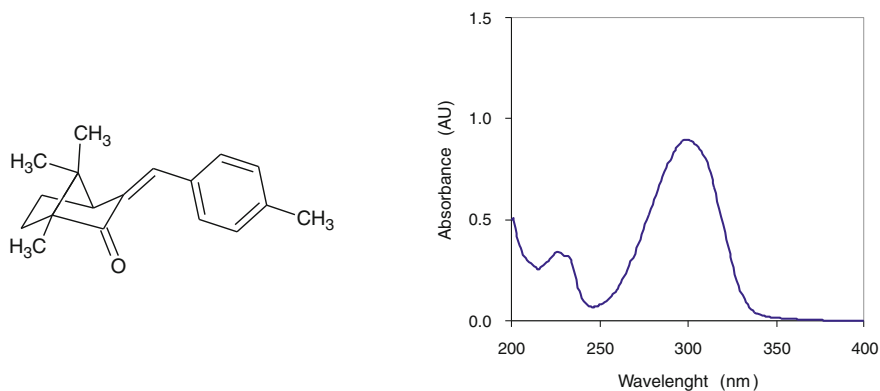
Fig. III.8 Chemical structure of MBC and UV/VIS absorption spectrum of a 5 μg mL^{-1} solution of MBC prepared in EtOH

The European legislation, which is the only that allows the use of MBC, restricts its content to a maximum concentration of 4 % (m/m) in the final cosmetic product. Meanwhile, both the American and the Japanese laws do not allow the use of MBC as a cosmetic ingredient (Chisvert and Salvador 2007).

III.3.2   Undesired Side Effects

Since 2000, potential safety issues related to MBC have been warned. In response, the Scientific Committee for Consumer Products Non-Food Products (SCCNFP) stated in 2001 that the organic UV filters used in sunscreen cosmetic products had no estrogenic effects that could affect human health (SCCNFP/0483/01).

However, based on the evaluation of new data, SCCNFP considered in 2004 that using MBC in this type of cosmetic products supposed a concern and then, additional information was urgently needed. After the study of these data, SCCNFP argued that a safe use of MBC with a maximum concentration up to 4 % in sunscreen cosmetic products could not be established (SCCNFP/1042/06).

In 2008, the last report related to MBC by SCCNFP was elaborated and the new safety assessment allowed MBC to be considered as a safe substance for topical use up to a concentration of 4 % in finished cosmetic products, without taking into account other routes of exposure such as inhalation or orally (SCCNFP/1184/08).

In this context, various studies related to percutaneous absorption of MBC have been conducted in humans after topical application of cosmetic products containing this UV filter. MBC was detected in urine and plasma mean concentrations around 20 ng mL^{-1} have been quantified to both women and men (Janjua et al. 2004; Janjua et al. 2008).

Based on this evidence, toxicokinetics and biotransformation processes of MBC have been characterized after its oral administration in rats by techniques such as proton nuclear magnetic resonance (^{1}H-NMR) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Volkel et al. 2006; Schauer et al. 2006). Thus, 3-(4′-carboxybenziliden)-camphor (CBC) and four isomers of 3-(4′-carboxybenziliden)-hydroxycanfor (CBC-OH), with predominance of the species 3-(4′-carboxybenziliden)-6-hydroxycanfor, were identified as phase I metabolites of MBC (see Fig. III.9).

In addition, the estrogenic capacity of MBC was evaluated by both *in vitro* and *in vivo* methodologies. Thus, several *in vitro* studies have shown that MBC increases cellular proliferation in MCF-7 breast cancer cell lines and that an antagonist estrogen blocks its proliferative effects (Schlumpf et al. 2001). The antagonist ability of MBC to androgen and progesterone receptors by employing specific and sensitive cell lines has also been observed at low concentrations (Schreurs et al. 2005). Therefore, in view of these results, it must be taken into account that daily exposure to cosmetic products containing MBC can bring the appearance of estrogenic effects in humans (Heneweer et al. 2005). However, evidence of *in vitro* estrogenicity in estrogen receptor binding and transactivation
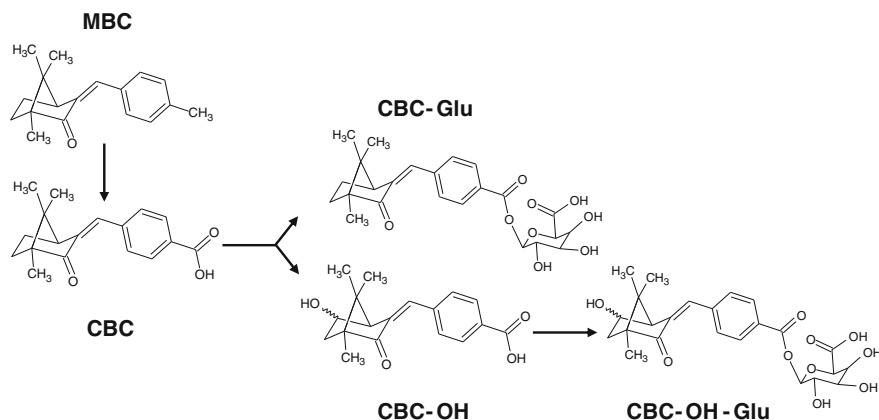
Fig. III.9 *In vivo* biotransformation of MBC in CBC, CBC-OH and their glucuronide conjugates (Schauer et al. 2006)

assays in yeast can sometimes be misleading, thus suggesting that MBC can be metabolized resulting in estrogen derivatives in MCF-7 cells and rats but not in yeast cells (Tinwell et al. 2002). Consequently, both reproductive and developing *in vivo* tests based on the application of MBC are required to anticipate the assessment of endocrine toxicity of this UV filter.

Regarding *in vivo* methods, recent studies have suggested that the uterine weight of young rats increases depending on the dosage of MBC administered orally and topically. Thus, it has been shown that exposure to MBC in developing rats affects the regulation of target estrogen gene (Mueller et al. 2003; Durrer et al. 2005; Schlumpf et al. 2008), interferes in a specific way with sexual gene expression in the brain (Maerkel et al. 2005; Maerkel et al. 2007; Carou et al. 2008; Carou et al. 2009a; Carou et al. 2009b), influences the development of organs and the reproductive functions in males (Durrer et al. 2007; Hofkamp et al. 2008) and even promotes the growth of the prostate (Schlumpf et al. 2008).

Nevertheless, controversial situations can be also found. While some researchers claim that MBC does not adversely affect the thyroid gland and the sex ratio of frogs at environmental concentrations (Kunz et al. 2004), others show MBC as a environmental substance especially harmful to frogs at concentrations of the order of micromolar (Klann et al. 2005). Thus, due to the widespread use of sunscreen cosmetic products, damage that MBC can cause to such invertebrates should not be overlooked (Schmitt et al. 2008).

An interesting study that involve the monitoring of MBC in relation to other endocrine disruptors in breast milk with three mother-infant pairs allowed a significant correlation between the use of the UV filter and its concentration in breast milk (Schlumpf et al. 2008).

The high risk associated with the use of cosmetic formulations containing this potential endocrine disruption compound should call to further research in order to

assess in a comprehensive manner possible long-term effects that can occur in both humans and environment.

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Chapter 3
Determination of the Unconjugated Forms of Benzophenone-3 and Benzophenone-4 in Urine by Solid-Phase Extraction Coupled to a Liquid Chromatographic System with UV/Vis Detection by Using Automated Sequential Injection Analysis

3.1 Introduction

3.1.1 Aim of the Study

The evidence of percutaneous absorption processes associated with benzophenone-3 (BZ3) and benzophenone-4 (BZ4) justifies the need to develop selective analytical methods to determine these UV filters in biological fluids, as they can become powerful tools for obtaining essential information regarding estimation of the excreted levels by *in vivo* methodology.

The aim of this paper is to develop and validate an analytical method for the simultaneous determination BZ3 and BZ4, which are the only authorized UV filters of the benzophenones family worldwide accepted (Chisvert and Salvador 2007), in urine from users of cosmetic products containing these compounds.

The methodology is based on a sequential injection (SI) (Ruzicka and Marshall 1990) that enables *on line* coupling a solid-phase extraction (SPE) procedure to liquid chromatography-UV/VIS spectrometry (LC-UV/VIS). Thus, the proposed experimental configuration enhances automated operation, thus minimizing errors in sample handling.

3.1.2 Background and Current Status of the Issue

Despite the undesired side effects of BZ3 and BZ4 (see Part III.1), only a few studies focused on the determination of these compounds in urine (Giokas et al. 2007), which is the main excretion pathway for BZ3 according to a

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pharmacokinetic study after oral administration in rats (Okereke et al. 1993), have been published.

Regarding the analytical techniques, BZ3 has been determined in human urine by gas chromatography-mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS) and LC-UV/VIS, after treatment of the urine sample with *on line* SPE (Ye et al. 2005), solid-phase microextraction (SPME) (Felix et al. 1998) single-drop microextraction (SDME) (Vidal et al. 2007), stir bar sorptive extraction (SBSE) (Kawaguchi et al. 2008) and hollow fiber assisted liquid-phase microextraction (HF-LPME) (Kawaguchi et al. 2009). Furthermore, the consulted scientific literature shows that BZ4 has not been determined previously in urine.

Some research has focused on the determination of the total amount of BZ3 excreted by urine after repeated whole-body applications of a cosmetic product containing the UV filter in humans. Urinary excretions of BZ3 were set in the range of 0.4 and 3.7 % of the total applied amount (Gonzalez et al. 2002, 2006; Sarveiya et al. 2004). Other studies have determined mean maximum concentration intervals in urine, which varied from 7 to 260 ng mL^{-1} (Felix et al. 1998; Janjua et al. 2004, 2008; Vidal et al. 2007).

There are precedents in the development of automated SPE procedures that are based on the principle of sequential injection analysis prior to analysis by LC, such as the determination of caffeine in urine, coffee and other beverages (Theodoridis et al. 2004). However, according to a review on sequential injection chromatography (Chocholous et al. 2007), there are few publications based on such SI-SPE-LC-UV/VIS systems.

Moreover, SI-SPE coupled to fluorimetric detector methods have also been developed by our research group to determine other UV filters, such as PBS (Vidal et al. 2003) and PDT (Balaguer et al. 2006a) in urine.

No publications related to the percutaneous absorption processes of BZ4 by means of its determination in biological fluids or tissues were found in the databases consulted during the development of this study, to the best of our knowledge.

3.2 Experimental

3.2.1 Reagents and Samples

Benzophenone-3 (BZ3), also called 2-hydroxy-4-methoxybenzophenone, 98 % from Sigma-Aldrich (Barcelona) and benzophenone-4 (BZ4), also called 2-hydroxy-4-methoxybenzophenone-5 sulfonic acid, 99.9 % from Roig Farma (Terrassa) were used as standards.

Sodium monohydrogen phosphate from Merck (Darmstadt), sodium hydroxide and 30 % ammonium hydroxide (d = 0.91 g mL^{-1}) from Probus (Badalona) and glacial acetic acid for analysis (AcOH) from Panreac (Barcelona) were also used.

The solvents used were LC grade absolute ethanol (EtOH) and 37 % analysis grade hydrochloric acid (d = 1.19 g mL^{-1}) from Scharlab (Barcelona). Deionized water obtained from a NANOpure II water purification system (R $\geq$ 18 mΩ cm) from Barnstead (Boston).

Urine samples used to develop and validate the analytical method were obtained from volunteers who had not applied any cosmetic product containing BZ3 and BZ4, and stored at 4 °C until analysis.

3.2.2 Instruments and Material

A L-7100$^{®}$ liquid chromatograph equipped with a L-7420$^{®}$ UV/VIS detector, both from Hitachi (Tokyo) were used. The chromatographic separation was carried out using a LiChrospher$^{®}$ RP-18 analytical column (5 μm particle size, 125 mm long, 4 mm internal diameter) from Merck (Darmstadt). The UV/VIS detection was performed at the wavelength of 288 nm. Data acquisition was controlled by the D-7000 HSM$^{®}$ software from Hitachi (Tokyo).

To configure the SI system, a 2031$^{®}$ automatic burette equipped with a 2.5 mL syringe, a 2030$^{®}$ eight channels automatic valve, all from Crison (Alella) and a storage loop made with Teflon$^{®}$ tube of 0.8 mm internal diameter from VICI (Houston) that could hold up to 3 mL were used.

Studies were conducted using SPE cartridges (100 mg, 10 mm long, 5 mm internal diameter) with different sorbents, such as octadecyl (C18), diethylami-nopropyl (DEA), trimetilaminopropilo (SAX) aminopropyl (NH$_2$) and ethylene-diamine-N-propyl (PSA), all from Varian (Barcelona).

The SPE cartridges were adapted to the SI-SPE-LC-UV/VIS analytical platform by using a six channels injection valve from Omnifit (Cambridge) and Teflon$^{®}$ tube. The automation of the extraction step was carried out using a sequence analysis software designed for this system.

A 8453$^{®}$ UV–VIS spectrophotometer from Hewlett Packard (Palo Alto), a MicropH 2001$^{®}$ pH meter from Crison (Alella), a Precisterm thermostated water bath from JP Selecta (Barcelona) and filter plates # 4 from Schott (Stafford) were also employed.

3.2.3 Analytical Method for the Determination of Benzophenone-3 and Benzophenone-4 in Their Unconjugated Forms in Urine

BZ3 and BZ4 stock standard solutions (100 μg mL^{-1}) were daily prepared in deionized water, adding a few drops of a 10 % sodium hydroxide solution to promote the dissolution of BZ3.

Urine samples were filtered through the filter plates and adjusted to pH 6 using a solution of 1 M hydrochloric acid or a solution of 1 M ammonium hydroxide, depending on the initial pH of the urine.

To correct the error caused by the matrix effects (see Sect. 3.3.2), the standard addition calibration was used. The calibrate solutions were prepared fortifying five aliquots (4950 μL) of urine containing 0, 10, 20, 30 and 40 μL of the aqueous standard solution (100 μg mL^{-1}), to which 50, 40, 30, 20 or 10 μL of deionized water was added, respectively, to reach the same content of deionized water in all calibrate solutions. Each urine solution contained a final volume of 5 mL and was analyzed according to the protocol specified in Table 3.1, using the SI-SPE-LC-UV/VIS system schematized in Fig. 3.1. It should be noted that chromatographic monitoring to determine BZ4 began at the end of stage 7 of the SI-SPE procedure (see Table 3.1), being the analytical column conditioned with 20 % EtOH. For BZ3, monitoring began at the end of stage 10, being the analytical column conditioned with 55 % EtOH.

Calibration curves were obtained by representing the peak areas for each analyte versus the added concentration. Figure 3.2a shows a chromatogram obtained by applying the SI-SPE-LC-UV/VIS method to a urine sample fortified with BZ4 and BZ3 at 600 ng mL^{-1}. To compare, Fig. 3.2b shows the chromatogram obtained from the analysis of a urine sample from the same volunteer that did not contain BZ4 or BZ3. As can be observed, no chromatographic peaks associated to potential interferents from the sample matrix eluted at the retention times of BZ4 or BZ3.

3.3 Results and Discussion

First, preliminary studies were conducted to select the conditions of retention and elution of the *off line* SPE procedure. For this purpose, aqueous standard solutions of BZ4 and BZ3 were prepared (1 μg mL^{-1}) and loaded in separate into the SPE cartridges. Absorbance measurements were performed using an UV/VIS spectrophotometer.

After selecting the SPE conditions, the SPE cartridges were *on line* connected to the LC-UV/VIS system by using a sequential injection system and chromatographic variables were studied to obtain the best performance. Then, the validation of the method to determine the analytes in urine was proceeded and finally, the proposed method was applied to the analysis of urine samples from volunteers who had applied a cosmetic sunscreen containing BZ3 and BZ4.

Table 3.1 SI-SPE-LC-UV/VIS system steps to determine BZ4 and BZ3 in their unconjugated forms in urine

Step	Operation to perform		
1. DEA cartridge solvation	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (EtOH)		
	Propulsion of 2.5 mL to waste		
2. DEA cartridge conditioning	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (deionized water)		
	Propulsion of 2.5 mL to waste		
3. C18 cartridge solvation	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (EtOH)		
	Propulsion of 2.5 mL to waste		
4. C18 cartridge conditioning	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (deionized water)		
	Propulsion of 2.5 mL to waste		
5. DEA cartridge urine sample loading	Aspiration of 0.5 mL (carrier)		
	Aspiration of 1 mL (urine solution)		
	Propulsion of 1.5 mL to waste		
6. DEA cartridge washing	Aspiration of 0.5 mL (carrier)		
	Aspiration of 1 mL (0.1 M HCl solution)		
	Propulsion of 1.5 mL to waste		
7. BZ4 elution from DEA cartridge	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (0.25 M HCl solution)		
	Propulsion of 2.5 mL to injection loop		
8. LC-UV/VIS injection	Elution program[a]		
	T (min)	1 % Ammonium acetate (pH 6) (%)	EtOH (%)
	0	80	20
	10	45	55
9. C18 cartridge urine sample loading	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (urine solution)		
	Propulsion of 2.5 mL to waste		
10. C18 cartridge washing	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (40:60, (v/v) EtOH:water solution)		
	Propulsion of 2.5 mL to waste		
11. BZ3 elution from C18 cartridge	Aspiration of 0.5 mL (carrier)		
	Aspiration of 2 mL (75:25, (v/v) EtOH:water solution)		
	Propulsion of 2.5 mL to injection loop		
12. LC-UV/VIS injection	Elution program[a]		
	t (min)	1 % Ammonium acetate (pH 6) (%)	EtOH (%)
	10	45	55
	20	45	55

[a] Flow 1 mL min^{-1}; T = 60 °C
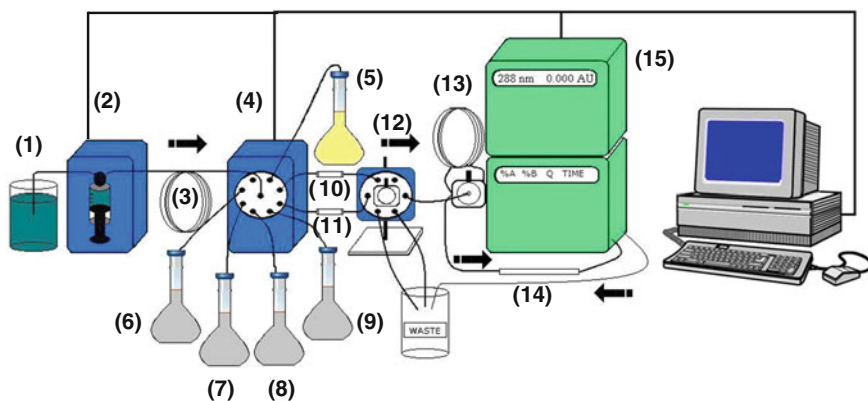
Fig. 3.1 SI-SPE-LC-UV/VIS system for the simultaneous determination of BZ3 and BZ4 in their unconjugated forms in human urine. (*1*) Carrier, deionized water, (*2*) burette with 2.5 mL syringe, (*3*) 3 mL holding loop, (*4*) eight-channel automatic valve, (*5*) urine sample solutions; (*6*) 0.1 M HCl solution, (*7*) 0.25 M HCl solution, (*8*) water:EtOH 60:40 (*v/v*) solution, (*9*) water:EtOH 25:75 (*v/v*) solution, (*10*) C18 SPE cartridge, (*11*) DEA SPE cartridge, (*12*) six-channel valve, (*13*) 2 mL injection loop, (*14*) analytical column, (*15*) LC-UV/VIS system

3.3.1 Study of the Experimental Variables

3.3.1.1 Solid-Phase Extraction: Retention and Elution Steps

For BZ4, different modified silica SPE cartridges (i.e., C18, NH_2, SAX, DEA and PSA) were studied. EtOH was the elution solvent used for C18 and NH_2 cartridges, while 1 M hydrochloric acid was employed for the rest SPE cartridges. The extraction efficiency values were 15, 23, 77, 91 and 8 % for C18, NH_2, SAX, DEA and PSA cartridges, respectively, and then, DEA cartridges were selected to provide the best retention of BZ4.

For BZ3, C18 and DEA cartridges were tested. When eluting the DEA with 1 M hydrochloric acid, no signal was observed, thus showing that the retention of BZ3 on this sorbent was negligible. By contrast, when eluting the C18 with EtOH, the extraction efficiency was close to 100 %. Therefore, C18 cartridges were selected for the SPE process of BZ3.

Initially, a mixed DEA and C18 cartridge was tested to study the simultaneous retention of BZ3 and BZ4 and the sequential and selective elution of both analytes using different conditions of elution. However, as BZ4 was also partially retained in the C18 sorbent, a loss of sensitivity would be produced in the determinaion of this analyte. The same problem was observed when the cartridges were connected in series. Then, to improve the sensitivity of the analytical method, the SPE cartridges were disposed in parallel.

A 2 mL injection loop was used to ensure that all of the eluted fraction from the SPE cartridges was efectively transferred to the LC-UV/VIS system. The main
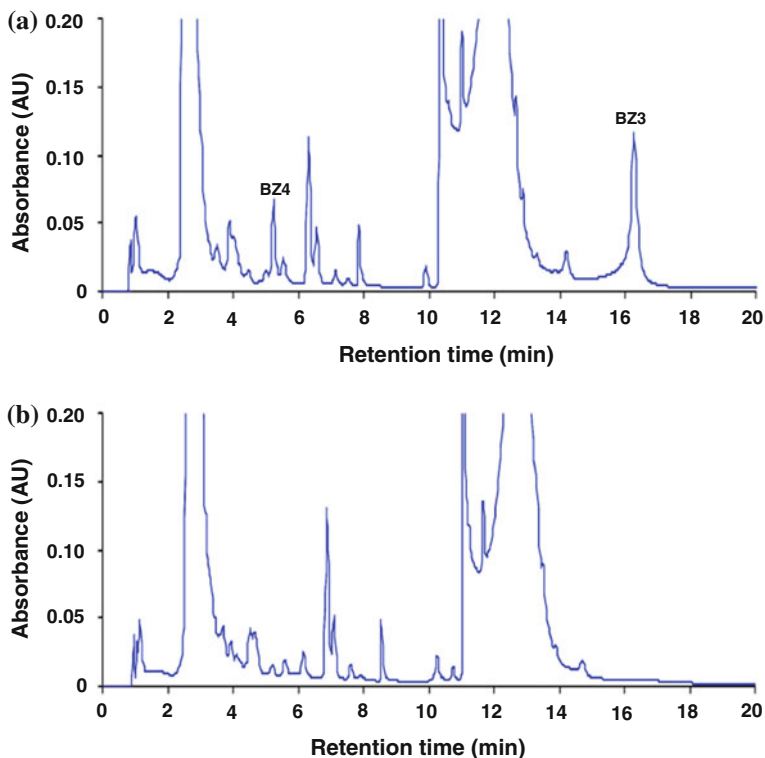
Fig. 3.2 Chromatogram obtained by applying the proposed method to a urine sample from a volunteer who had applied a cosmetic product containing BZ3 and BZ4, fortified with 600 ng mL^{-1} BZ4 and BZ3 (**a**), a urine sample that did not contain the analytes from the same volunteer before applying the cosmetic product (**b**)

drawback that the use of this injection loop could cause was the posible enhancement of dispersion effects, thus producing relatively wide chromatographic peaks. However, the width of the obtained peaks were comparable to those obtained with conventional 20 μL injection loops, probably due to the analytes were compacted on the head of the column.

The efficiency of the elution solutions was also checked. The conducted studies showed that quantitative elution of BZ3 and BZ4 occurred when eluting the C18 cartridge with 2 mL of a EtOH:deionized water (75:25, *v/v*) solution and the DEA cartridge with 2 mL of a 0.25 M hydrochloric acid solution, respectively.

3.3.1.2 Solid-Phase Extraction: Washing Step

To study the best conditions to determine BZ3 and BZ4 in urine without the presence of interference that may co-eluting with the analytes, a selective washing step of the SPE cartridge was required prior to the elution step.

Thus, two urine samples containing no analytes were separately fortified with 1 $\mu g\ mL^{-1}$ of BZ3 and 1 $\mu g\ mL^{-1}$ of BZ4. After the *on line* conditioning of the SPE cartridge (see Sect. 3.2.3), 2 mL of the urine solution containing BZ3 was loaded in the C18 cartridge and 1 mL of the urine solution containing BZ4 was loaded in the DEA cartridge. Different washing conditions were checked previous to the analyte elution performed under the aforementioned selected conditions.

For BZ3, 2 mL of different solutions of EtOH:deionized water (i.e., 0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, *v/v*) were used as washing solutions of the C18 cartridge, using the system shown in Fig. 3.3.

As can be seen in Fig. 3.4, a reduction in the peak area of BZ3 was obtained when the content of EtOH in the washing solution was above 50 %, while the signal of BZ3 remained constant when the content of EtOH was 50 % or less. Therefore, a volume of 2 mL of a deionized water:EtOH (60:40, *v/v*) solution was selected as the washing solution of the C18 cartridges thereby preventing a significant loss of BZ3.

It should be noted that, due to the polar characteristics of BZ4, the partially retained BZ4 in the C18 cartridges would be eluted in this washing step. Then, the elution of BZ3 from the C18 cartridge was carried out (2 mL of a EtOH:deionized water (75:25, *v/v*) solution) and the chromatographic separation was monitored by the LC-UV/VIS system using a mobile phase consisting of a provisional 70 % of
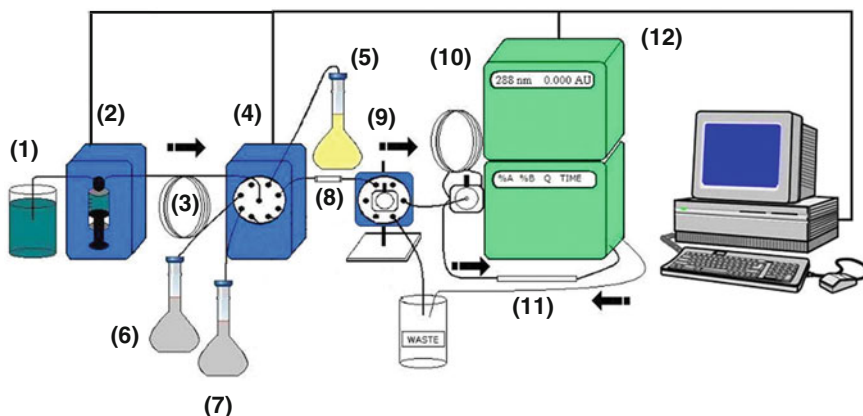
Fig. 3.3 SI-SPE-LC-UV/VIS system to study the washing step of the C18 cartridges. (*1*) carrier, deionized water, (*2*) burette with 2.5 mL syringe, (*3*) 3 mL holding loop, (*4*) eight-channel automatic valve, (*5*) urine sample solutions fortified with BZ3 (1 $\mu g\ mL^{-1}$), (*6*) water:EtOH 60:40 (*v/v*) solution, (*7*) water:EtOH 25:75 (*v/v*) solution, (*8*) C18 SPE cartridge, (*9*) six-channel valve, (*10*) 2 mL injection loop, (*11*) analytical column; (*12*) LC-UV/VIS system

Fig. 3.4 Effect of the EtOH content on the washing step of C18 cartridges for the SI-SPE process of BZ3. The *dashed line* fits the experimental values
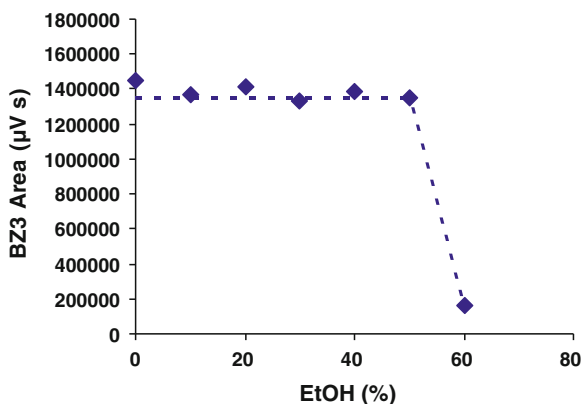
EtOH under isocratic conditions. Figure 3.5 shows the chromatograms obtained when loading on the C18 cartridges a urine solution not containing BZ3 and a urine solution containing BZ3 (1 µg mL^{-1}). The lack of resolution of the chromatographic peak is evident. Further detailed studies were carried out to improve the chromatographic resolution of BZ3 (Sect. 3.3.1.3).

Similarly, the system showed in Fig. 3.6 was used to study the washing step of the DEA cartridge using different volumes of a 0.1 M hydrochloric acid solution, taking into account that more concentrated hydrochloric acid solutions caused the partial elution of BZ4. In each case, after the washing step, the elution of BZ4 from the DEA cartridge (2 mL of a 0.25 M hydrochloric acid solution) was proceeded and chromatographic separation was monitored by the LC-UV/VIS system using a temporary mobile phase consisting of 30 % of EtOH under isocratic conditions.

As can be seen in Fig. 3.7, the washing step with 0.25 mL of 0.1 M hydrochloric acid allowed to obtain a greater area for BZ4.
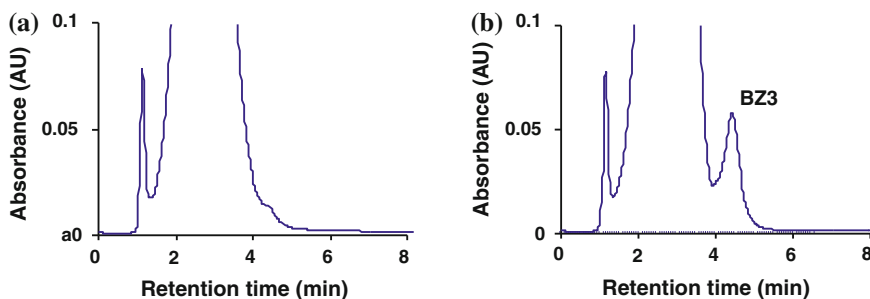
Fig. 3.5 Chromatograms obtained by analyzing a BZ3-free urine solution (**a**), a BZ3-containing urine solution (1 µg mL^{-1}) (**b**) under the selected extraction conditions (see Sect. 3.3.1.2). Mobile phase composition: 1 % ammonium acetate (pH 6):EtOH, 30:70 (*v/v*), at a flow rate of 1 mL min^{-1} and at room temperature
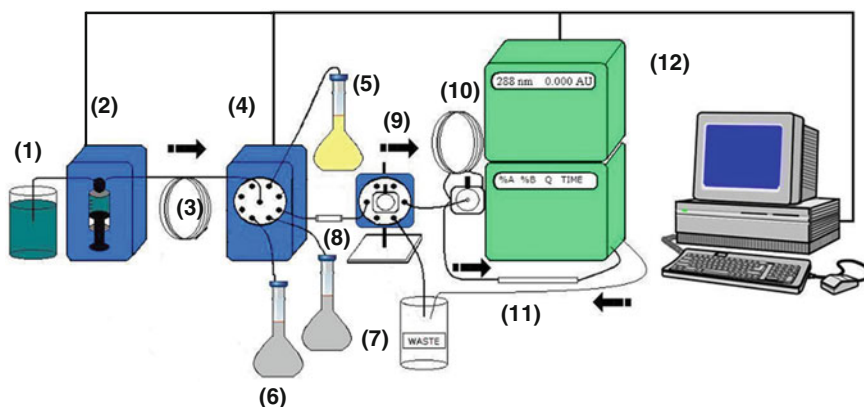
Fig. 3.6 SI-SPE-LC-UV/VIS system to study the washing step of the DEA cartridges. (*1*) carrier, deionized water, (*2*) burette with 2.5 mL syringe, (*3*) 3 mL holding loop, (*4*) eight-channel automatic valve, (*5*) urine sample solutions fortified with BZ4 (1 μg mL^{-1}), (*6*) 0.1 M HCl solution, (*7*) 0.25 M HCl solution, (*8*) DEA SPE cartridge, (*9*) six-channel valve, (*10*) 2 mL injection loop, (*11*) analytical column, (*12*) LC-UV/VIS system

Fig. 3.7 Effect of volume of solution of 0.1 M hydrochloric acid in the washing step of the process cartridges for DEA SI-BZ4 SPE
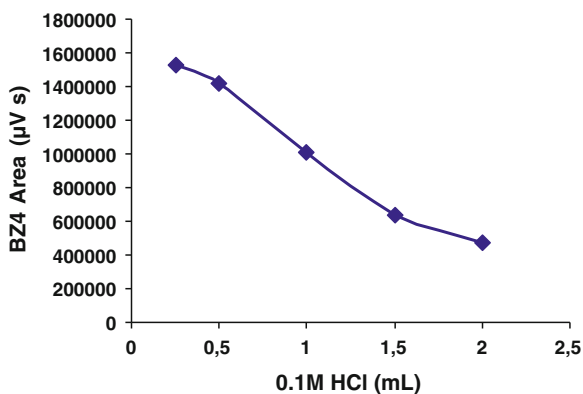
However, when washing with 1 mL of 0.1 M hydrochloric acid, the chromatographic profile of BZ4 was proved to be cleaner and therefore free of interferences (see Fig. 3.8), despite its lower signal resulting from losses during the washing stage. Washing volumes greater than 1 mL did not improve the chromatographic profile and additionally caused considerable losses for BZ4, thus losing sensitivity (see Fig. 3.7). For this reason, a volume of 1 mL of 0.1 M hydrochloric acid was selected as the washing condition for BZ4, to obtain the maximum signal with the lowest presence of interferences.

Moreover, it was found that the cartridges could be reused up to 30 times without losing in their extraction efficiency.
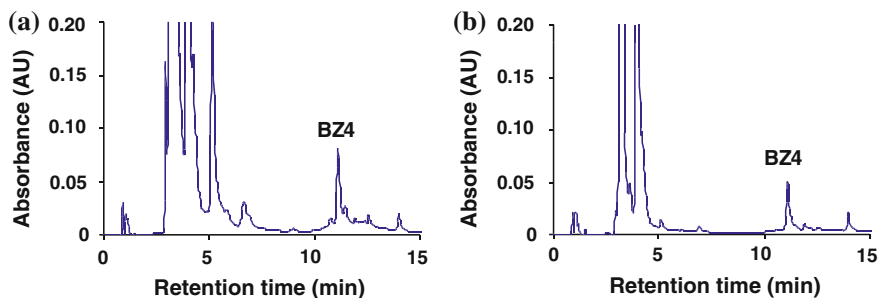
Fig. 3.8 Chromatograms obtained by analyzing solutions 1 μg mL^{-1} of BZ4 in urine, using 0.5 mL (**a**) or 1 mL (**b**) of 0.1 M hydrochloric acid as washing solution under the selected extraction conditions (see Sect. 3.3.1.2). Mobile phase composition: 1 % ammonium acetate (pH 6):EtOH, 70:30 (*v/v*), at a flow rate of 1 mL min^{-1} and at room temperature

3.3.1.3 Chromatographic Conditions

To obtain the optimal resolution of the chromatographic peaks in the shortest analysis time, the composition of the mobile phase was studied. Hence, urine samples containing no analytes and fortified with 1 μg mL^{-1} of BZ3 and BZ4, were analyzed with an isocratic elution program of 1 % ammonium acetate (pH 6):EtOH, 70:30 (*v/v*) at a flow rate of 1 mL min^{-1}.

As expected when using a reverse phase chromatographic mode, increasing the proportion of EtOH in the mobile phase caused the reduction of the retention time of BZ3 and therefore, the approximation of the chromatographic peak to the front of elution. Thus, when using mobile phases with a high content of organic phase (above 55 %), the separation of BZ3 from the rest of interferents was not obtained. However, when using a 55 % of EtOH, the separation was possible and, moreover, in the shortest possible time (6.3 min).

For BZ4, increasing the content of EtOH in the mobile phase leads to an analogous situation to that obtained with BZ3. When the percentage of EtOH was increased from 30 to 40 %, the retention time of BZ4 decreased from 11.4 to 3.7 min. However, a content of 20 % or lower of organic modifier was not sufficient to elute BZ4 from the analytical column. Therefore, studies were conducted to test a gradient elution program instead of applying isocratic conditions. The gradient was increased from 20 % of EtOH up to 55 %, thus matching the EtOH composition that was selected for the subsequent chromatographic separation of BZ3. In this sense, the more polar interferents retained in the DEA cartridge were eluted first and the organic composition of the mobile phase was increased gradually to achieve the elution of BZ4. Thus, the chromatographic elution of BZ4 was achieved at 5.2 min.

In summary, the selected elution program started with a 20 % of EtOH in the mobile phase that was increased to 55 % for the chromatographic separation of

BZ4. Then, this percentage of EtOH remained constant (55 %) for the subsequent chromatographic separation of BZ3.

3.3.1.4 Effect of pH in Urine

To study the effect of pH in urine on the retention process of the analytes by SPE, five aliquots of the same urine sample containing no analyte were fortified with 1 μg mL^{-1} of BZ3 and BZ4, and the pH was adjusted to 2, 4, 6, 7 and 8 using 1 M hydrochloric acid or 2 M ammonium hydroxide solutions as appropriate. Urine samples were analyzed by applying the SI-SPE-LC-UV/VIS methodology described above (see Sect. 3.2.3).

In Fig. 3.9, it can be seen that at pH values above 6, the signal of BZ4 increases while the signal of BZ3 decreases. This result are in accordance to the pK$_a$ values of the analytes (*BZ3 pK$_a$:7.5; BZ4 pK$_{a2}$ (hydroxyl group): 6.3, calculated using the Advanced Chemistry Development (ACD/Labs) V8.14 software*) and the retention nature of each SPE cartridge (reverse phase and non-polar interactions in the case of C18 and anion exchange cartridges in the case of DEA cartridge). Under these conditions (pH > 6) the ionized species of BZ3, which cannot be retained in the C18 cartridges, and the double charged species of BZ4, which are retained by the DEA sorbent to a greater extent than the mono charged species, are prevalent in the solution. In this context, it was expected the constant response of both analytes when the pH was in the range from 4 to 6, when neutral species of BZ3 and mono ionized species of BZ4 are predominant.

Therefore, to achieve a compromise situation taking into account the optimal pH conditions for the determination of both analytes simultaneously, urine samples were adjusted to pH 6.

Fig. 3.9 Influence of pH to determine BZ3 (*diamond*) and BZ4 (*square*) in urine samples
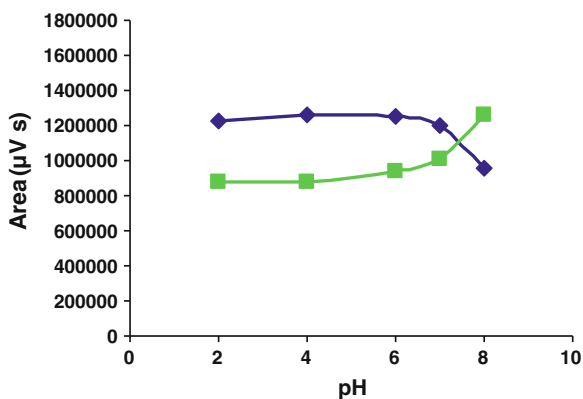
3.3.1.5 Effect of Temperature

When analyzing urine samples from different volunteers who had applied a cosmetic product containing BZ3 and BZ4, new peaks that interfered the determination of BZ4 were observed. For the determination of BZ3, no interferences were seen under the working conditions described above. Therefore, to determine BZ4 free from interferences, the effect of temperature was considered. Thus, it was found that maintaining the analytical column at 60 °C afforded a good separation of BZ4 from the abovementioned interfering compounds (see Fig. 3.10).

3.3.1.6 Stability of the Urine Samples

To study the stability of the urine samples from excretion until analysis, two aliquots of a urine sample containing no analytes were fortified with BZ3 and BZ4 at 150 ng mL^{-1}. One aliquot was maintained at room temperature and the other aliquot was stored at 4 °C. Contents of BZ4 and BZ3 were determined in both
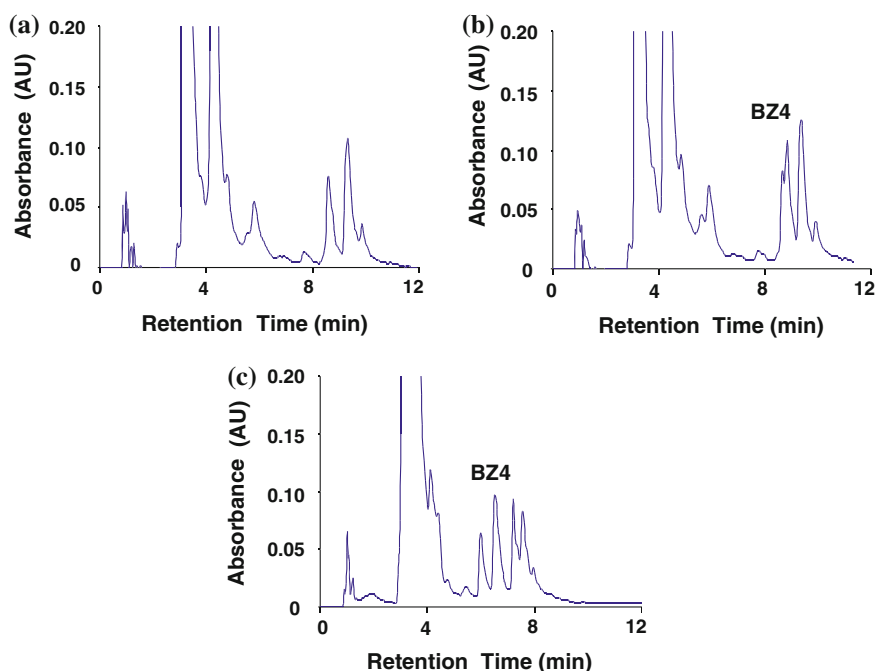
Fig. 3.10 Chromatograms obtained by analyzing urine samples from a volunteer who had applied a cosmetic product containing BZ3 and BZ4. Unfortified urine sample analyzed at room temperature (**a**), BZ4 fortified (1 μg mL^{-1}) urine sample analyzed at room temperature (**b**), BZ4 fortified (1 μg mL^{-1}) urine sample analyzed at 60 °C (**c**). The extraction and chromatography conditions are indicated in Sect. 3.2.3

samples using the methodology proposed in the following six days. Figure 3.11 shows the values obtained for BZ3 and BZ4 in each urine sample with its standard deviation values that were calculated as the corresponding standard deviations of the extrapolated values in the standard addition calibration curve (Miller and Miller 2005).

The results suggest that urine samples stored at 4 °C can be analyzed six days after the collection of the urinary excretion without causing problems related to the degradation of the analytes. However, if the urine sample is maintained at room temperature, the analysis must be carried out during the following two days after the urine collection to prevent from stability problems.

3.3.2 Validation of the Analytical Method: Study of the Interferences

The presence of the so-called matrix effects associated with the determination of BZ4 and BZ3 in urine samples by the SI-SPE-LC-UV/VIS method was studied. Hence, the differences between the responses obtained from a series of standard solutions of BZ4 and BZ3 (0.2-1 μg mL^{-1}) using, on one hand, a urine sample containing no analytes (*urine calibrate*) and, on the other hand, an aqueous solution of sodium monohydrogenphosphate (1 %) adjusted to pH 6 (*aqueous calibrate*), were compared (see Table 3.2).

The fact that the intercepts were statistically comparable to zero in all cases indicates that all other components of the matrix do not coelute with the analytes. In this case, the value of the tabulated t statistics (t_{tab}) for a significance level of 5 % and $(N_1 - 2) + (N_2 - 2) = 6$ degrees of freedom (where N is the number of points of each calibration line) is 2.45, which is higher than the values calculated t statistics for BZ3 and BZ4, $(t_{tal})_{BZ3} = 0.14$ and $(t_{tal})_{BZ4} = 1.21$, respectively (see
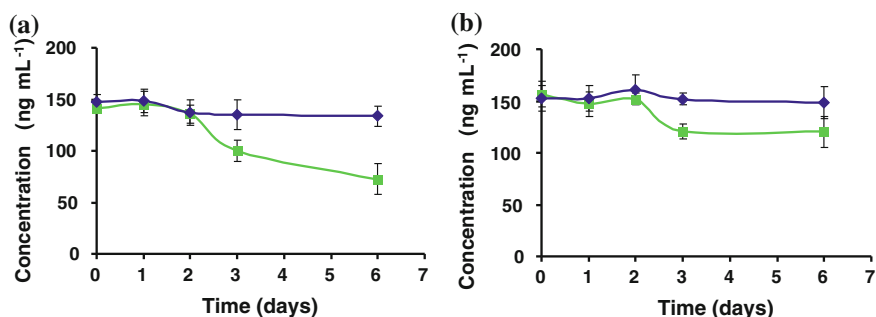
Fig. 3.11 Found BZ3 (**a**) and BZ4 (**b**) contents by applying the proposed method to urine samples stored at 4 °C (*diamond*) and at room temperature (*square*). Error bars show the standard deviation of the results

Table 3.2 Comparison of aqueous and urine calibrates for BZ3 and BZ4

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^f
		Urine[d]	Aqueous[d]		
BZ3	**a** (μV s)[a]	$(-7 \pm 100).10^2$	$(-6 \pm 10).10^3$		
	b (μV s mL μg^{-1})[b]	$(970 \pm 20).10^3$	$(1630 \pm 30).10^3$	60	23.0
	R^{2c}	0.998	0.999		
BZ4	**a** (μV s)[a]	$(10 \pm 10).10^3$	$(-5 \pm 10).10^3$		
	b (μV s mL μg^{-1})[b]	$(600 \pm 20).10^3$	$(1240 \pm 20).10^3$	48	23.8
	R^{2c}	0.996	0.999		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, N = 5
[e] Recovery coefficient estimated as the ratio between the slopes obtained using both calibrates
[f] Estatistical $t_{tab(0.05, (N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

Annex III.4). As a result, it can be concluded that no constant interferences were present in the SI-SPE-LC-UV/VIS methodology.

However, when comparing the aqueous and urine calibrates, statistically different slopes were obtained for a significance level of 5 % to both BZ4 and BZ3, thus showing the presence of the so-called matrix effects (see Table 3.2). Therefore, standard addition calibration was used to correct the error caused by the proportional interferences.

3.3.2.1 Accuracy

The accuracy of the method was evaluated using the SI-SPE-LC-UV/VIS system to the analysis of nine urine samples fortified with known amounts of BZ3 and BZ4. The urine samples were from volunteers who had not applied any cosmetic product containing these UV filters. Table 3.3 shows the results obtained for each sample and its corresponding standard deviation, that was calculated as the error of the extrapolated value of x in the standard addition calibration curve (Miller and Miller 2005).

The results obtained for BZ3 and BZ4 applying the SI-SPE-LC-UV/VIS methodology were compared with the fortified amounts by a linear regression model (see Annex III.5). The lines obtained by representing the found amounts (y) versus the fortified amounts (x), all expressed in ng mL^{-1}, were:

$$y = (1.18 \pm 0.09)\,x - (0.02 \pm 0.01)\,\left(R^2 = 0.96, N = 9\right) \text{ for BZ3}$$

$$y = (1.03 \pm 0.08)\,x - (0.00 \pm 0.01)\,\left(R^2 = 0.96, N = 9\right) \text{ for BZ4}$$

Considering that the critical value of tabulated t statistics (t_{tab}) for a significance level of 5 % and N $-$ 2 = 7 degrees of freedom (where N is the number of urine

Table 3.3 Determination of BZ3 and BZ4 in fortified urine samples

Sample[a]	BZ3 concentration (ng mL^{-1})		BZ4 concentration (ng mL^{-1})	
	Fortified	Obtained	Fortified	Obtained
1	144	140 ± 20	166	162 ± 3
2	154	150 ± 20	133	137 ± 7
3	123	113 ± 8	105	102 ± 12
4	192	190 ± 20	208	220 ± 12
5	212	230 ± 30	115	125 ± 15
6	172	180 ± 30	223	222 ± 8
7	75	71 ± 10	166	167 ± 2
8	154	165 ± 8	215	234 ± 13
9	183	200 ± 30	156	156 ± 12

[a] Urine samples from different volunteers

samples) is 2.36 and that the calculated t statistics (t_{cal}) for the slope and intercept are lower than the t_{tab} value in all cases (2.14 and 1.77 for BZ3, 0.41 and 0.12 for BZ4, respectively), it can be concluded that there is no significant differences between the results obtained by applying the SI-SPE-LC-UV/VIS methodology and the actual values, thus showing the accuracy of the proposed method.

3.3.2.2 Other Analytical Parameters

The limits of detection (LOD) and quantification (LOQ), estimated according to the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) are shown in Table 3.4 for BZ3 and BZ4 to each analyzed urine samples. LOQ values vary from 68 to 112 ng mL^{-1} for BZ3 and from 34 to 41 ng mL^{-1} for BZ4, depending on the sample.

A linear response up to concentrations of 20 µg mL^{-1} was observed to both analytes. The precision of the SI-SPE-LC-UV/VIS method, expressed as relative standard deviation (RSD) of the average concentration values obtained for the analyzed samples was 13 and 6 % for BZ3 and BZ4, respectively, showing an acceptable level of precision.

3.3.3 Application of the Analytical Method

The proposed analytical method was applied to the determination of BZ3 and BZ4 in urine samples from three volunteers, two men and one woman, who had applied 12.5 g of a sunscreen cosmetic product prepared in the laboratory containing 8 % of BZ3 and 4 % of BZ4 (see Annex II.2). The applied doses are in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of

Table 3.4 Limits of detection (LOD) and quantitation (LOQ) of the developed methodology to determine BZ4 and BZ3 in urine

Sample[a]	BZ3		BZ4	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	35	107	12	36
2	36	109	13	38
3	33	98	13	40
4	36	109	13	41
5	32	97	14	35
6	35	106	12	34
7	23	68	12	37
8	37	112	14	41
9	35	105	13	40

[a] Urine samples from different volunteers
[b] Estimated in the urine sample, taking into account the dilution factor during the treatment of the sample, as $3.3.s_{Blank}/b$, where b is the slope of the standard addition calibration curve and s_{Blank} is the standard deviation value of the responses obtained by measuring the analytical background noise ($N = 5$). Values expressed as ng mL^{-1}
[c] Estimated in the urine sample as $10.s_{Blank}/b$ (see annotation b)

Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007). The cosmetic product was prepared following an adapted protocol (Jordán and Jordán 1991).

Urine samples were collected immediately before (blank sample) and after the application of the cosmetic product, until the amounts of the excreted analytes were no longer detectable. In all cases, the volunteers were instructed to collect the total volume of urine excretion in sterile commercially available containers. To determine the total amount of analytes in each urinary excretion, the total volume of excreted urine was measured, although only a fraction was stored (at 4 °C) to perform urine analysis. Urine samples were analyzed applying the SI-SPE-LC-UV/VIS method described above. The results, expressed as the total excreted amount of BZ4 and BZ3 depending on the time elapsed from the application of the sunscreen cosmetic product are shown in Fig. 3.12.

The results indicate that the excretion of BZ3 and BZ4 tends to increase after the application of the cosmetic product to reach a maximum value, and then, the excreted amounts decrease to values below the detection limit. The excretion time depends on the volunteer and the analyte. Thus, BZ3 was no longer detected 25–30 h after the application of the cosmetic product, while BZ4 was still detected in urine samples collected 50 h after the application.

The total excreted amount of BZ3 was in the range of 55–180 μg (corresponding to 0.006–0.018 % of the total amount applied BZ3), while volunteers excreted between 190 and 540 μg BZ4 (corresponding to 0.04–0.1 % of the total amount applied BZ4). These results show that the process of absorption/excretion

(a)

(b)
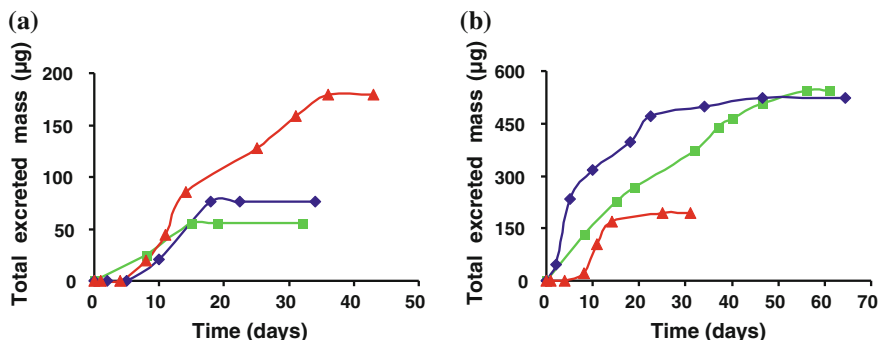
Fig. 3.12 Total excreted amount of BZ3 (**a**) and BZ4 (**b**) by three volunteers that had applied a sunscreen containing BZ3 (8 %) and BZ4 (4 %). Volunteer 1, (*diamond*); volunteer 2, (*square*); volunteer 3, (*triangle*)

of the analytes in the human body depends greatly on the characteristics of each individual.

By comparing the estimated excretion levels of BZ4 and BZ3, it can be concluded that BZ4 was detected in urine samples before and more extensively than BZ3, probably due to the fact that BZ4 is a hydrosoluble UV filter. In fact, the excretion of BZ3 from the human body involves a series of biotransformation reactions, as discussed in Part III.1.

3.4 Conclusions

The proposed method for the simultaneous determination of BZ3 and BZ4 in human urine involves the use of a solid-phase extraction process automated with a sequential injection system in combination with a liquid chromatography system with UV/VIS detection. The combination of sequential injection system and liquid chromatography provides robustness, ease of sample handling, precision, reproducibility and economic profitability (Chocholous et al. 2007).

The proposed analytical method was validated and the obtained analytical parameters showed the potential application of the methodology to carry out *in vivo* pharmacokinetic and pharmacodynamic studies. Finally, the proposed method has been applied successfully to the analysis of urine samples collected from volunteers who had applied a cosmetic product containing both UV filters.

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Chapter 4
Determination of Benzophenone-3 and Their Metabolites in Urine by Solid-Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

4.1 Introduction

4.1.1 Aim of the Study

This chapter focuses on the development and validation of an analytical method based on a solid-phase extraction (SPE) procedure prior to liquid chromatography with tandem mass spectrometric (LC–MS/MS) that allows the determination of BZ3 and its three metabolites (DHB, DHMB and THB) in human urine.

To determine the content of the analytes in both their unconjugated and conjugated by glucuronidation forms, an enzymatic hydrolysis of the conjugates with a ß-glucuronidase solution was carried out.

4.1.2 Background and Current Status of the Issue

Different methodologies that allow the *in vivo* percutaneous absorption monitoring of BZ3 by means of its determination in biological fluids have been described. However, some of these analytical methods did not consider the contribution of the metabolites of BZ3 (Ye et al. 2005; Vidal et al. 2007; Kawaguchi et al. 2008, 2009) and those who did consider this contribution, had significantly higher limits of detection (Sarveiya et al. 2004; Kasichayanula et al. 2005) or did not take into account all the metabolites (Felix et al. 1998).

Moreover, analytical methods based on both liquid and gas chromatography and mass spectrometry (LC–MS, GC–MS) have been widely applied to determine BZ3 and their metabolites in aqueous samples (Giokas et al. 2007; Diaz-Cruz et al.

2008; Tarazona et al. 2010). However, the higher complexity of the urine matrices in comparison to aqueous samples makes difficult the application of these analytical methods to determine BZ3 and its metabolites simultaneously with low limits of detection.

4.2 Experimental

4.2.1 Reagents and Samples

Benzophenone-3 (BZ3), also called 2-hydroxy-4-methoxybenzophenone, 98 %, 2,4-dihydroxybenzophenone (DHB) 99 %, 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) 98 %, and 2,3,4-trihydroxy-benzophenone (THB) 99 % from Sigma-Aldrich (Steinheim) were used as analytical standards. 2,2′-dihydroxy-4,4′-dimethoxybenzophenone (DHDMB) 98 % from Sigma-Aldrich was used as internal standard.

The structures of these compounds are shown in Figs. III.1.1 and III.1.2, and some properties of interest are specified in Table 4.1.

The solvents used were absolute ethanol (EtOH), LC grade methanol (MeOH), LC grade ultrapure acetone, 37 % analysis grade hydrochloric acid (d = 1.19 g mL^{-1}), and analysis grade sodium hydroxide, all from Scharlab (Barcelona). Deionized water obtained from a NANOpure II water purification system (R ≥ 18 mΩ cm) from Barnstead (Boston).

Formic acid (FA) from Fluka Chemie (Steinheim), and ß-glucuronidase solution from Helix pomatia (type HP-2) with 116,300 U mL^{-1} activity and sulfatase activity ≤7,500 U mL^{-1} from Sigma- Aldrich (Steinheim) were also used.

Urine samples used during the development and validation of the method were obtained from different male volunteers who had not applied any cosmetic product containing BZ3.

Table 4.1 Relevant information about the considered compounds

Compound	Molecular weight[b]	pKa[c]
2-Hydroxy-4-methoxybenzophenone (BZ3)	228.2	7.56 ± 0.35
2,4-Dihydroxy-benzophenone (DHB)	214.2	7.53 ± 0.35
2,2′-Dihydroxy-4-methoxybenzophenone (DHMB)	244.2	6.99 ± 0.35
2,3,4-Trihydroxybenzophenone (THB)	230.2	7.51 ± 0.40
2,2′-Dihydroxy-4,4′-dimethoxybenzophenone (DHDMB)[a]	274.3	6.80 ± 0.35

[a] Used as internal standard
[b] Expressed in g mol^{-1}
[c] Calculated using *Advanced Chemistry Development (ACD/Labs) V8.14 software*

4.2.2 Instruments and Material

The LC–MS/MS equipment consisted of a LC-10AD® liquid chromatograph from Shimadzu (Duisburg) coupled to a Micromass Quattro® triple quadrupole mass spectrometer from Waters (Beverly) equipped with an electrospray ionization source. A high purity generator from CLAN Technology (Sevilla) provided nitrogen used as both source of ionization and nebulizer gas. The MS/MS spectra were acquired using collision-induced dissociation (CID) of selected precursor ions using Premier® helium from Air Products (Barcelona) as the collision gas.

A Mediterranea® SEA 18 analytical column (3 μm particle size, 50 mm long, 2.1 mm internal diameter) with a Ultraguard® SEA (10 × 3.2 mm) precolumn, from Teknokroma (Barcelona) was used.

C18 solid-phase extraction cartridges (100 mg, 1 cm long, 5 mm internal diameter) and a SPE vacuum system from Varian (Barcelona) were also used.

A MicropH 2001® pH meter from Crison (Alella), a ZX3® vibrating shaker from Velp (Usmate), a EBA 21® centrifuge from Zentrifugen Hettich (Tuttlingen) and a Precisterm® thermostated water bath and an incubation chamber, both from JP Selecta (Barcelona) were employed.

4.2.3 Analytical Method for the Determination of Benzophenone-3 and its Metabolites in Urine

4.2.3.1 Preparation of Solutions

Target standard (BZ3, DHB, DHMB and THB) and internal standard (DHDMB) solutions were prepared separately at a concentration level of 200 μg mL^{-1} in EtOH, and stored at 4 °C. Working solutions (5 μg mL^{-1}) were prepared daily in deionized water from the respective standard solutions.

To correct the error caused by the matrix effects (see Sect. 4.3.2), the standard addition calibration was employed. The calibrate solutions were prepared by fortifying five aliquots (5 mL) of urine containing 0, 20, 40, 60 and 80 μL of the multicomponent solution (5 μg mL^{-1}), to which 100, 80, 60, 40 and 20 μL of deionized water was added, respectively, to fix the same deionized water content in all of the calibrate solutions. Furthermore, 100 μL of the DHDMB (5 μg mL^{-1}) solution was also added to each calibrate solution.

4.2.3.2 Enzymatic Hydrolysis

To determine the total content of the analytes, i.e., both their unconjugated and glucuronide conjugated forms, 50 μL of the solution of ß-glucuronidase was added to the urine samples. Alternatively, urine samples were also treated with deionized water to determine only the content of unconjugated analyte.

The urine solutions were stirred and incubated at 37 °C for 12 h. Finally, 30 μL of FA were added to each solution urine. Thus, the optimal pH was adjusted (pH 3) to the subsequent SPE procedure of the analytes. The final volume of the urine sample solutions was 5,230 μL.

4.2.3.3 Solid-Phase Extraction

The C18 solid-phase extraction cartridges were conditioned with 2 mL of EtOH followed by 2 mL of deionized water. Next, 5 mL of the urine solution was loaded into the cartridge at a flow rate of 0.5 mL min^{-1}, washed with 8 × 1 mL of deionized water and dried under vacuum for 10 min. Analytes were eluted with 3 × 0.35 mL of acetone. Then, the eluted fractions were evaporated to dryness under a stream of air and reconstituted with 60 μL of a mixture of deionized water (0.1 % FA):MeOH (0.1 % FA), 1:1 (v/v). Finally, reconstituted samples were injected into the LC–MS/MS system.

4.2.3.4 LC–MS/MS Analysis

The mobile phase consisted of deionized water and MeOH, both containing 0.1 % of FA. The used elution gradient is shown in Table 4.2, at a flow rate of 0.2 mL min^{-1} and at room temperature. The injection volume was 20 μL.

The analytes were determined by selected reaction monitoring (SRM) in positive electrospray ionization mode (ESI$^+$). The conditions of the mass spectrometer and triple quadrupole spray chamber were as follows: temperature of gas ionization source, 120 °C; gas flow ionization source, 600 L h^{-1}; nebulizer gas temperature, 350 °C, nebulizer gas flow, 33 L h^{-1}, capillary voltage, 3,500 V. Two SRM transitions to quantify and to confirm the compounds of interest were selected. Relevant MS detection information is specified in Table 4.3.

Calibration curves were obtained by representing the area ratio of each analyte and internal standard (DHDMB) versus the added concentration of analyte. Figure 4.1 shows the SRM chromatograms of the target compounds obtained by applying the SPE-LC–MS/MS procedure to a urine sample containing no analytes and a urine sample fortified at 120 ng mL^{-1}.

Time (min)	0.1 % FA in deionized water (%)	0.1 % FA in MeOH (%)
0	60	40
1	60	40
9.5	5	95
14.5	5	95

Table 4.2 Elution gradient used in the LC–MS/MS analysis to determine BZ3 and their metabolites in urine

Table 4.3 SRM parameters in positive electrospray ionisation mode used to determine BZ3 and their metabolites in urine

Compound	Parameter			
	First quad voltage (V)	Collision energy (V)	SRM transition to quantify	SRM transition to confirm
THB	30	10	231.3 → 105.1	231.3 → 152.5
DHB	25	15	215.4 → 137.2	215.4 → 141.2
DHMB	30	15	245.2 → 121.1	245.2 → 151.3
DHDMB[a]	20	15	275.3 → 151.2	275.3 → 105.1
BZ3	30	20	229.3 → 151.3	229.3 → 105.1

[a] Internal standard

4.3 Results and Discussion

4.3.1 Study of Experimental Variables

4.3.1.1 LC–MS/MS Analysis

The MS/MS spectra of the compounds of interest were obtained by collision induced dissociation (CID) experiments from direct infusion of standard solutions (500 µg mL^{-1}) prepared in EtOH, operating with electrospray ionization source in both positive (ESI$^+$) and negative (ESI$^-$) modes. The most intense MS/MS transitions were obtained in all cases in positive ionization mode through the corresponding protonated molecular ions [(M+H)$^+$]. Figure 4.2 shows the mechanism of MS/MS fragmentation and the proposed molecular structures of each fragment.

4.3.1.2 Solid-Phase Extraction Procedure

The C18 cartridges used in SPE showed suitable capacity to simultaneously retain both types of analytes, more lipophilic (BZ3) and more polar (DHB, DHMB and THB). At pH values below 5, which correspond to 2 units below their pK_a values (see Table 4.1), the prevalent species of the analytes are in their protonated forms (i.e., uncharged forms). As neutral compounds, they tend to be retained by hydrophobic interactions and then, they can be retained more easily in the C18 cartridges. Therefore, the pH of the urine solutions was adjusted to 3 by adding 30 µL of AF before being subjected to the SPE procedure.

The SPE conditions for the extraction and concentration processes of the target compounds were studied. To study the loading capacity, different aliquots of aqueous standard solutions (1,000 ng mL^{-1}) were loaded into two C18 cartridges assembled in series. Based on previous experiments that showed that compounds of interest were quantitatively eluted with 1 mL of acetone, SPE cartridges were disassembled, dried and eluted with 3 × 0.35 mL of acetone.
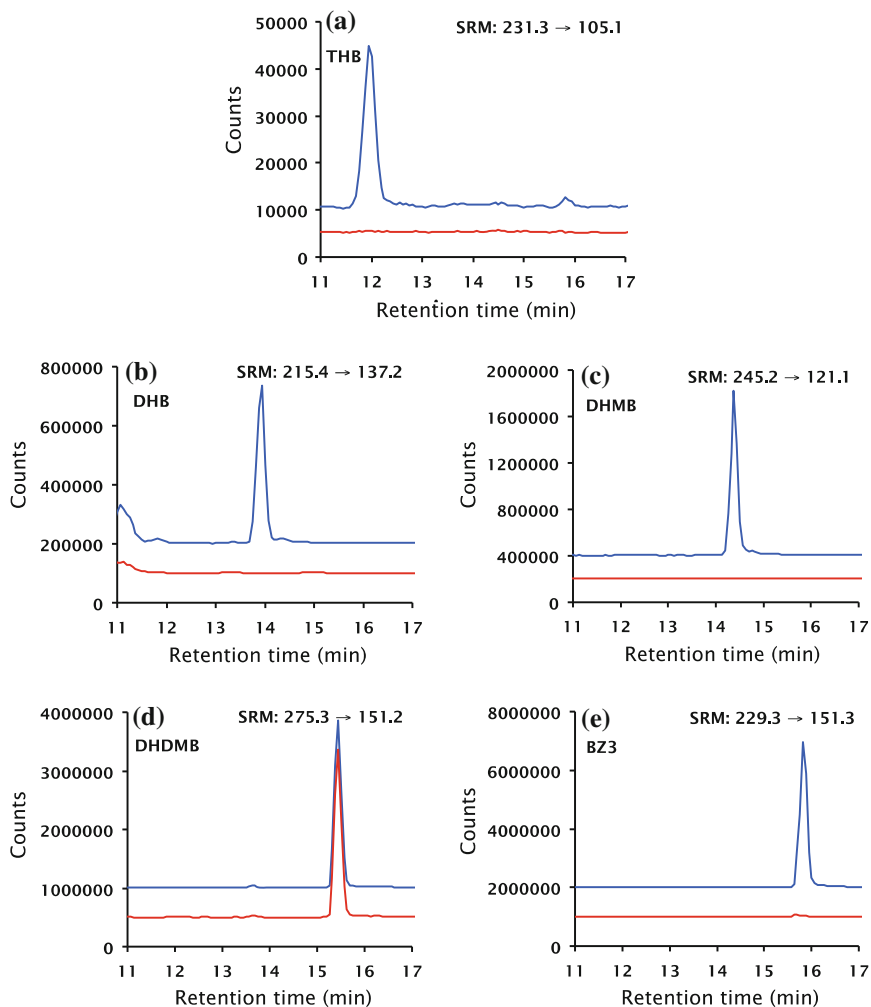
Fig. 4.1 SRM chromatograms of each target compound obtained by analyzing a urine sample containing no compounds (Red line) and the same urine sample fortified at a concentration of 120 ng mL^{-1} (Blue line). The internal standard (DHDMB) was included in both types of samples. Experimental conditions are described in Sect. 4.2.3

Then, the eluted fractions were evaporated to dryness and reconstituted with 60 μL of a deionized water (0.1 % FA):MeOH (0.1 % AF), 1:1 (*v*/*v*) solution. The quantitative retention of all the target compounds was produced in the first cartridge when 5 mL of standard solution was loaded (R > 95 %). The extraction efficiency was estimated by comparison with external calibration, and the obtained values were in the range of 100 % for BZ3, DHMB and DHB, and about 40 % for THB. The lower extraction efficiency was obtained with the more polar compound (THB), which agrees with the extraction process nature of the C18 cartridges.
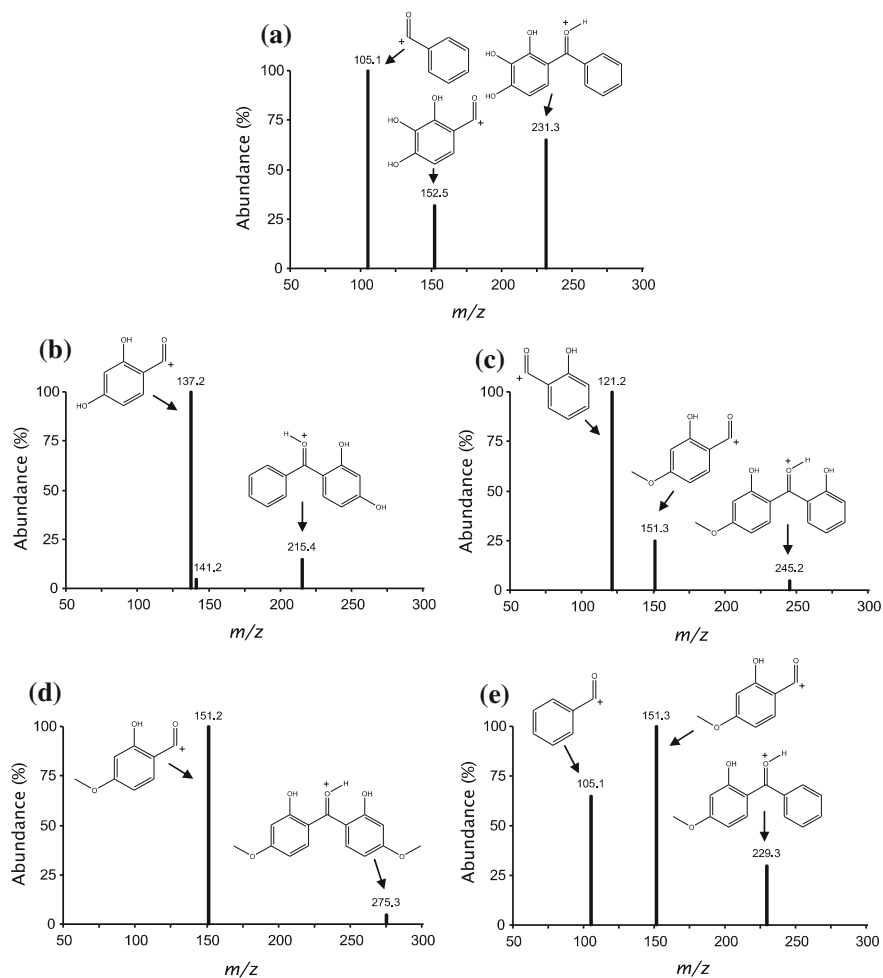
Fig. 4.2 MS/MS spectra obtained by CID experiments including proposed fragmentation mechanisms for THB (**a**), DHB (**b**), DHMB (**c**), DHDMB (internal standard) (**d**) and BZ3 (**e**). All compounds were analyzed in positive electrospray ionization mode (ESI⁺)

4.3.2 Validation of the Analytical Method: Study of the Interferences

The matrix effects, which may occur in both the extraction process and the detection, were evaluated. The differences between the responses obtained for a series of standard solutions (20–100 ng mL^{-1}) using, on one hand, deionized water (*aqueous calibrate*) and, on the other hand, urine from male volunteers who had not applied any cosmetic product containing BZ3 (*urine calibrate*) were

compared. Moreover, all the aliquots were fortified with DHDMB solution (100 ng mL^{-1}) and pH was adjusted by adding 30 µL of FA (pH 3). Then, the described SPE–LC–MS/MS process was carried out (see Table 4.4).

Statistically different slopes were obtained when compared both calibrates for all analytes. Considering the experimental results, the standard addition calibration was used to correct the error caused by the matrix interferences and, thus, to assess levels of the target compounds in the urine samples.

4.3.2.1 Accuracy

Accuracy was evaluated applying the analytical method to urine samples from male volunteers who had not applied any cosmetic product containing BZ3 fortified with known amounts of BZ3, DHB, DHMB and THB. The obtained values are shown in Table 4.5. Standard deviations were obtained as the standard deviation of the extrapolated value in the standard addition line (Miller and Miller 2005).

A Student's t test was used to confirm the absence of significant differences between the found concentration values and the fortified concentration values of urine samples (see Annex III.3), thus showing the accuracy of the methodology.

4.3.2.2 Other Analytical Parameters

The calibration graphs (N = 5) were linear for BZ3, DHB, DHMB and THB over a range of concentrations from 20 to 100 ng mL^{-1} (working range) with a

Table 4.4 Comparison of the aqueous and urine calibrates for BZ3, DHB, DHMB and THB

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^{f}
		Urine[d]	Aqueous[d]		
BZ3	**a**[a]	0.02 ± 0.02	0.00 ± 0.03		
	b (mL µg^{-1})[b]	14.1 ± 0.5	17.2 ± 0.6	82	4.5
	R^{2c}	0.997	0.997		
DHB	**a**[a]	0.001 ± 0.001	−0.01 ± 0.01		
	b (mL µg^{-1})[b]	1.08 ± 0.02	6.1 ± 0.3	18	36.8
	R^{2c}	0.998	0.997		
DHMB	**a**[a]	0.010 ± 0.003	0.000 ± 0.007		
	b (mL µg^{-1})[b]	2.60 ± 0.04	9.2 ± 0.2	28	64.2
	R^{2c}	0.9992	0.9990		
THB	**a**[a]	0.0002 ± 0.0001	0.001 ± 0.001		
	b (mL µg^{-1})[b]	0.078 ± 0.002	0.56 ± 0.02	14	51.8
	R^{2c}	0.998	0.997		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number pf points, $N = 5$
[e] Recovery coefficient estimated as ratio between the slopes obtained using both calibrates
[f] Statistical $t_{tab(0.05,(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

regression coefficient greater than 0.995 in all cases. The slope of the calibration curve by the standard addition calibration was the parameter used to estimate the sensitivity of the method.

The limits of detection (LOD) and quantification (LOQ) of the analytes were estimated based on the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) (see Table 4.6).

It should be emphasized the low LOQ values obtained, ranging from 0.083 to 0.312 ng mL^{-1} in urine samples, depending on the compound and sample.

The instrumental precision was determined by repeated injections (N = 5) of a urine solution fortified at 100 ng mL^{-1}. The repeatability of the analytical method was evaluated by applying the SPE-LC–MS/MS procedure to the extraction of analytes from five aliquots of the same urine sample fortified at 100 ng mL^{-1}, as can be seen in Table 4.7.

4.3.3   Application of the Proposed Method

A male volunteer applied 13 g of a sunscreen cosmetic product containing 10 % BZ3 (see Annex II.3). The applied dose is in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007). The cosmetic product was prepared following an adapted protocol (Jordán and Jordán 1991).

Urine samples were collected before and after the application of the sunscreen cream. The volunteer was instructed to accurately collect the total volume of each

Table 4.5 Determination of BZ3, DHB, DHMB and THB in fortified urine samples

Analyte	Parameter	Urine samples				
		1	2	3	4	5
BZ3	μ^a	20	52	100	152	200
	$C \pm s^b$	23 ± 2	51 ± 1	105 ± 4	159 ± 6	208 ± 10
	t_{cal}^c	2.91	2.17	2.85	2.34	1.78
DHB	μ^a	20	52	100	152	200
	$C \pm s^b$	21 ± 1	52 ± 3	106 ± 5	158 ± 7	197 ± 6
	t_{cal}^c	2.57	0.04	2.57	2.02	0.91
DHMB	μ^a	19	50	96	146	192
	$C \pm s^b$	20 ± 2	50 ± 2	99 ± 6	149 ± 10	200 ± 6
	t_{cal}^c	1.19	0.24	0.97	0.67	2.78
THB	μ^a	21	54	104	158	208
	$C \pm s^b$	24 ± 2	61 ± 4	112 ± 7	170 ± 9	211 ± 9
	t_{cal}^c	2.36	2.91	2.77	3.04	0.70

[a] Fortified concentration (ng mL^{-1})

[b] Found concentration (ng mL^{-1}) by standard addition calibration

[c] Statistical $t_{tab\ (0.05, N-2=3)} = 3.18$

Table 4.6 Limits of detection (LOD) and quantitation (LOQ) of the developed methodology to determine BZ3 and its metabolites in urine

Sample[a]	BZ3		DHB		DHMB		THB	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	0.065	0.196	0.035	0.106	0.049	0.148	0.044	0.131
2	0.028	0.083	0.065	0.194	0.044	0.133	0.086	0.258
3	0.065	0.196	0.080	0.241	0.097	0.292	0.096	0.287
4	0.069	0.208	0.076	0.227	0.100	0.300	0.104	0.312
5	0.097	0.291	0.066	0.197	0.049	0.148	0.086	0.257

[a] Urine samples from different volunteers

[b] Estimated in the urine sample, taking into account the dilution factor during the treatment of the sample as 3.3 s_a/b, where b is the slope of the standard addition calibration curve, and s_a the standard deviation of the intercept in the calibration curve. Values expressed as ng mL^{-1}

[c] Estimated in the urine sample as 10 s_a/b (see annotation b)

Table 4.7 Precision parameters for the determination of BZ3, DHB, and THB DHMB in urine samples

Analyte	Parameter	
	Instrumental precision (%)[a]	Repeatability (%)[a]
BZ3	1.8	7.2
DHB	2.1	9.2
DHMB	1.5	7.4
THB	2.6	8.1

[a] Expressed as relative standard deviation (RSD), N = 5

urine excretion in sterile commercially available containers. The total volume of urine was measured to determine the excreted amount of analytes and stored at −20 °C until analysis. The samples were analyzed using the SPE–LC–MS/MS analytical methodology described above.
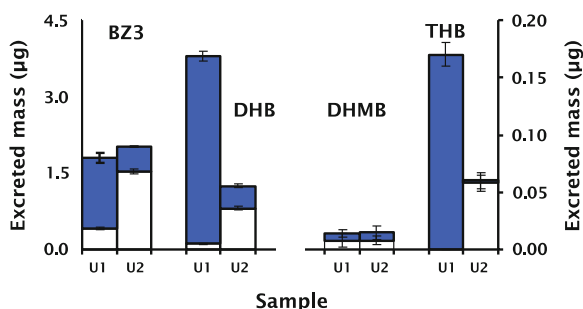
Fig. 4.3 Found amounts of BZ3, DHB, DHMB and THB in two urine samples collected at the times specified in Sect. 4.3.3, from a male volunteer after topical application of a sunscreen cosmetic containing BZ3 (10 %). *Error bars* show the standard deviation of the results. The results are expressed in terms of absolute mass considering the dilution factor corresponding to the total volume collected in each case. Unconjugated content, (*square*); glucuronide conjugated contents (*filled square*)

The urine sample obtained 20 h after the sunscreen application (U1) was divided into two fractions. Each fraction was treated with deionized water or with ß-glucuronidase solution to determine the unconjugated content of analytes or the total content of analytes, respectively. A similar procedure was followed with the urine sample 2 (U2), collected 30 h after the sunscreen application.

As can be seen in Fig. 4.3, the contribution of the glucuronide conjugate content to the total excreted amount by urine of BZ3 and its metabolites depends greatly on the analyte and the sample. Although the described analytical method should be applied to a larger number of urine samples from different people and collected at different times to get a more consistent idea of the toxicokinetics of BZ3 and its metabolites, some interesting conclusions can be drawn from this study.

Hence, DHB and BZ3 prevail in the two urine samples that were analyzed in comparison to the found contents of DHMB and THB. It should be also noted that the respective contents of DHMB and BZ3 are similar in both urine samples and the conjugated content of DHB and THB is more relevant in U1 than in U2.

4.4 Conclusions

An analytical method based on the combination of SPE procedure and the subsequent LC–MS/MS analysis for the sensitive determination of BZ3, DHB, DHMB and THB in human urine has been developed. Standard addition calibration was employed to correct the error caused by the proportional matrix effect observed. Full validation of the analytical method was conducted and statistically accurate results were obtained by analyzing urine samples previously fortified. The methodology was applied successfully to the analysis of urine samples collected from a volunteer who had applied a sunscreen cosmetic product containing BZ3.

The analytical methodology can be applied to assess the *in vivo* pharmacokinetics of BZ3, and especially of its metabolites, which may have more long-term side effects than the parent compound (Jeon et al. 2008).

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Chapter 5
Determination of Benzophenone-3 and its Metabolites in Human Semen by Solid-Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

5.1 Introduction

5.1.1 Aim of the Study

The contribution of analytical tools to develop further research on the process of bioaccumulation and toxicity in the male reproductive system of BZ3 and its metabolites is necessary considering the systemic properties that exhibit this type of compounds.

The process of bioaccumulation can be defined, generally, as a phenomenon originating from the incorporation of xenobiotics to the human body (by breathing, ingestion or percutaneous absorption) and linked to retention and elimination mechanisms, such as metabolism and excretion.

This paper focuses on the development and validation of an analytical method based on a solid-phase extraction (SPE) procedure prior to liquid chromatography tandem mass spectrometry (LC-MS/MS) for the determination of BZ3 and its three metabolites (DHB, DHMB and THB) in human semen.

To determine the content of the analytes in their unconjugated and glucuronide conjugated forms, samples were treated with a ß-glucuronidase solution to carry out the enzymatic hydrolysis of the conjugates.

5.1.2 Background and Current Status of the Issue

BZ3 has been determined in biological fluids different from urine, such as plasma (Sarveiya et al. 2004; Jeon et al. 2008; Kasichayanula et al. 2005), serum (Jiang

et al. 1996; Ye et al. 2008b) and breast milk (Hany and Nagel 1995; Ye et al. 2008a). Okereke and colleagues further argue that contents of BZ3 and its metabolites were found in different tissues and organs (liver, kidneys, heart and testes) from rats that had been treated with this UV filter (Okereke et al. 1993). According to the revised literature, no publications regarding analytical methods to determine BZ3 and/or its metabolites in semen have been published.

5.2 Experimental

5.2.1 Reagents and Samples

Benzophenone-3 (BZ3), also called 2-hydroxy-4-methoxybenzophenone, 98 %, 2,4-dihydroxybenzophenone (DHB) 99 %, 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) 98 %, and 2,3,4-trihydroxy-benzophenone (THB) 99 % from Sigma-Aldrich (Steinheim) were used as analytical standards. The structures of these compounds are shown in Figs. III.1.1 and III.1.2. 2,2′-dihydroxy-4,4′-dimethoxy-benzophenone (DHDMB) 98 % from Sigma-Aldrich was used as internal standard.

The solvents used were absolute ethanol (EtOH), LC grade methanol (MeOH), LC grade ultrapure acetone, 37 % analysis grade hydrochloric acid $(d = 1.19 \text{ g mL}^{-1})$, and analysis grade sodium hydroxide, all from Scharlab (Barcelona). Deionized water obtained from a NANOpure II water purification system (R $\geq$ 18 mΩ cm) from Barnstead (Boston).

Formic acid (FA) from Fluka Chemie (Steinheim), and ß-glucuronidase solution from Helix pomatia (type HP-2) with 116 300 U mL^{-1} activity and sulfatase activity $\leq$7,500 U mL^{-1} from Sigma-Aldrich (Steinheim) were also used.

The semen samples used to develop and validate the analytical method were obtained from healthy male volunteers who had not applied any cosmetic product containing BZ3, according to an official protocol of the World Health Organization for the examination and processing of human semen.[1] They were stored at −20 °C.

5.2.2 Instruments and Material

The LC-MS/MS equipment consisted of a LC-10AD® liquid chromatograph from Shimadzu (Duisburg) coupled to a Micromass Quattro® triple quadrupole mass spectrometer from Waters (Beverly) equipped with an electrospray ionization

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[1] WHO Laboratory Manual for the Examination and Processing of Human Semen (2010), 5th edn. World Health Organization.

source. A CLAN Technology high purity generator (Sevilla) provided nitrogen used as both source of ionization and nebulizer gas. The MS/MS spectra were acquired using collision-induced dissociation (CID) of selected precursor ions using Premier® helium from Air Products (Barcelona) as the collision gas.

A Mediterranea® SEA 18 analytical column (3 μm particle size, 50 mm long, 2.1 mm internal diameter) with an Ultraguard® SEA (10 × 3.2 mm) precolumn, from Teknokroma (Barcelona) was used. C18 solid-phase extraction cartridges (100 mg, 1 cm long, 5 mm internal diameter) and a SPE vacuum system from Varian (Barcelona) were also used.

A MicropH 2001® pH meter from Crison (Alella), a ZX3® vibrating shaker from Velp (Usmate), a EBA 21® centrifuge from Zentrifugen Hettich (Tuttlingen) and a Precisterm® thermostated water bath and an incubation chamber, both from JP Selecta (Barcelona) were employed.

5.2.3 Analytical Method for the Determination of Benzophenone-3 and its Metabolites in Semen

5.2.3.1 Preparation of Solutions

Target standard (BZ3, DHB, DHMB and THB) and internal standard (DHDMB) solutions were prepared separately at a concentration level of 200 μg mL^{-1} in EtOH, and stored at 4 °C. Working solutions (1 μg mL^{-1}) were prepared daily in deionized water from the respective stock standard solutions.

2 mL of semen sample were placed in a 15 mL conical tube and acidified with 300 μL of a 1 M hydrochloric acid solution to denature the proteins present in the matrix. Then, the mixture was stirred and centrifuged (6,000 g) over 3 min at room temperature.

To correct the error caused by the matrix effects (see Sect. 5.3.2), the standard addition calibration was employed. The calibrate solutions were prepared by fortifying five aliquots (340 μL) of semen supernatant with 0, 20, 40, 60 and 80 μL of the multicomponent solution (1 μg mL^{-1}), to which 100, 80, 60, 40 and 20 μL of deionized water was added, respectively, to fix the same content of deionized water in all the calibrate solutions. Furthermore, 100 μL of the DHDMB (1 μg mL^{-1}) solution and 60 μL of a 1 M sodium hydroxide solution were also added to each calibrate solution. Thus, the pH was adjusted (6.5) to proceed with the enzymatic hydrolysis step.

5.2.3.2 Enzymatic Hydrolysis

To determine the total content of the analytes, i.e., both their unconjugated and glucuronide conjugated forms, 50 μL of ß-glucuronidase aqueous diluted (1:50,

v/v) solution was added to the semen samples. Alternatively, semen samples were also treated with deionized water to determine only the content of unconjugated analyte.

The semen solutions were stirred and incubated at 37 °C for 12 h. Finally, 30 μL of FA were added after the hydrolytic incubation. Thus, the optimal pH was adjusted (pH 3) to the subsequent SPE procedure of the analytes. The final volume of the semen sample solutions was 870 μL.

5.2.3.3 Solid-Phase Extraction

The C18 solid-phase extraction cartridges were conditioned with 2 mL of EtOH followed by 2 mL of deionized water. Next, 0.7 mL of the semen solution was loaded into the cartridge at a flow rate of 0.5 mL min^{-1}, washed with 3×0.7 mL of deionized water and dried under vacuum for 10 min. Analytes were eluted with 3×0.35 mL of acetone. Then, the eluted fractions were evaporated to dryness under a stream of air and reconstituted with 60 μL of a mixture of deionized water (0.1 % FA):MeOH (0.1 % FA), 1:1 (*v/v*). Finally, reconstituted samples were injected into the LC-MS/MS system.

5.2.3.4 LC-MS/MS Analysis

The mobile phase consisted of deionized water and MeOH, both containing 0.1 % of FA. The used elution gradient is shown in Table 5.1, at a flow rate of 0.2 mL min^{-1} and at room temperature. The injection volume was 20 μL.

The analytes were determined by selected reaction monitoring (SRM) in positive electrospray ionization mode (ESI$^+$). The conditions of the mass spectrometer and triple quadrupole spray chamber were as follows: temperature of gas ionization source, 120 °C; gas flow ionization source, 600 L h^{-1}; nebulizer gas temperature, 350 °C, nebulizer gas flow, 33 L h^{-1}, capillary voltage, 3,500 V. Two SRM transitions to quantify and to confirm the compounds of interest were selected. Relevant MS detection information is specified in Table 5.2.

Calibration curves were obtained by representing the ratio area of each analyte and internal standard (DHDMB) versus the added concentration of analyte. Figure 5.1 shows the SRM chromatograms of the target compounds obtained by

Time (min)	0.1 % FA in deionized water (%)	0.1 % FA in MeOH (%)
0	60	40
1	60	40
9.5	5	95
14.5	5	95

Table 5.1 Elution gradient used in the LC-MS/MS analysis to determine BZ3 and its metabolites in human semen

Table 5.2 SRM parameters in positive electrospray ionisation mode to determine BZ3 and its metabolites in human semen

Compound	Parameter			
	First Quad voltage (V)	Collision energy (V)	SRM transition to quantify	SRM transition to confirm
THB	30	10	231.3 → 105.1	231.3 → 152.5
DHB	25	15	215.4 → 137.2	215.4 → 141.2
DHMB	30	15	245.2 → 121.1	245.2 → 151.3
DHDMB[a]	20	15	275.3 → 151.2	275.3 → 105.1
BZ3	30	20	229.3 → 151.3	229.3 → 105.1

[a] Internal standard

applying the SPE-LC-MS/MS procedure to a semen sample containing no analytes and a semen sample fortified at 120 ng mL^{-1}.

5.3 Results and Discussion

5.3.1 Study of the Experimental Variables

Most of the conditions employed in the analytical methodology were adapted from those described in Chap. 4. In this regard, C18 SPE cartridges were selected as they were the most suitable for BZ3 and its metabolites in the case of urine samples, after the pH adjustment of the enzymatically hydrolyzed semen solutions by adding 20 μL of FA (pH 3). Likewise, LC-MS/MS conditions were similar to those used in the case of urine samples analysis.

5.3.2 Validation of the Analytical Method: Study of the Interferences

The matrix effects, which may occur in both the extraction process and the detection, were evaluated. The differences between the responses obtained for a series of standard solutions (20–100 ng mL^{-1}) using, on one hand, deionized water (*aqueous calibrate*) and, on the other hand, semen from male volunteers who had not applied any cosmetic product containing BZ3 (*semen calibrate*) were compared.

The slopes and intercepts of both calibrates were statistically compared using a Student's *t*-test (see Annex III.4). Statistically different slopes were obtained when compared both calibrates for all analytes, thus showing the occurrence of the so-called matrix effects (see Table 5.3). Therefore, the standard addition calibration was used to correct this undesired effect.
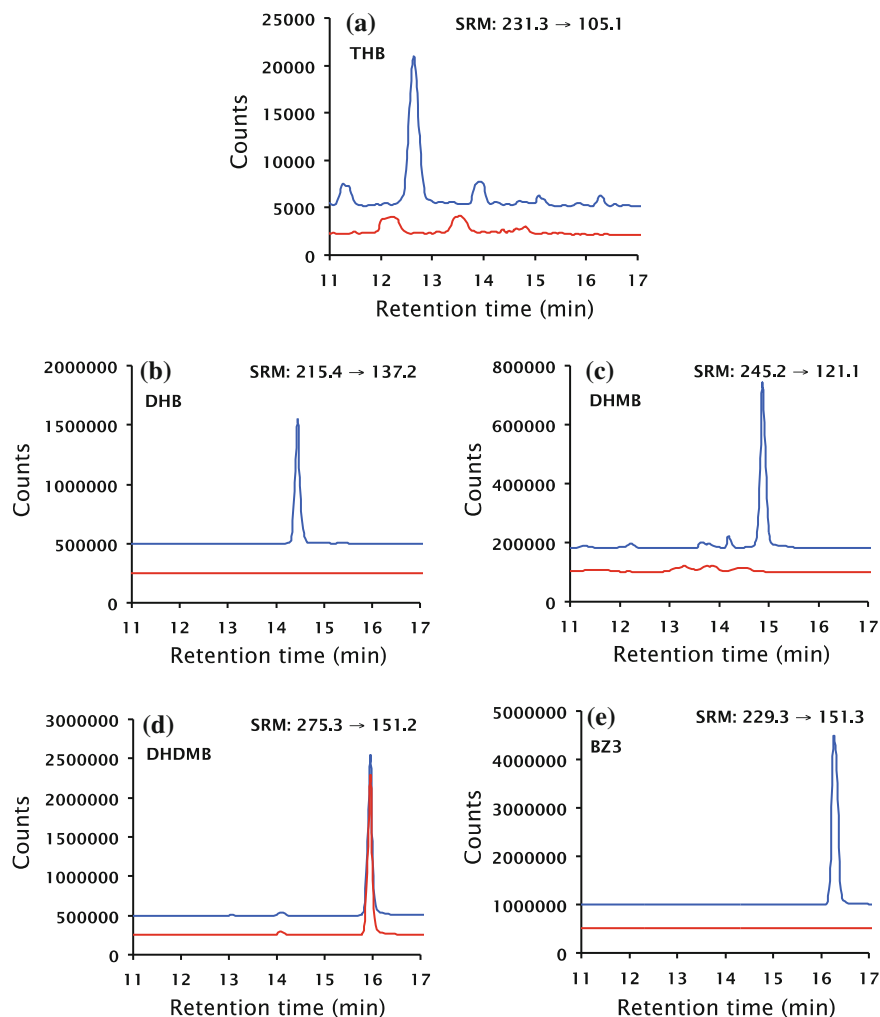
Fig. 5.1 SRM chromatograms of each target compound obtained by analyzing a semen sample containing no compounds (*Red line*) and the same semen sample fortified at 120 ng mL^{-1} (*Blue line*). The internal standard (DHDMB) was included in both types of samples. Experimental conditions are described in Sect. 5.2.3

5.3.2.1 Accuracy

Accuracy was evaluated applying the analytical method to semen samples from male volunteers who had not applied any cosmetic product containing BZ3. Semen samples were fortified with known amounts of BZ3, DHB, DHMB and THB. The obtained values are shown in Table 5.4. Standard deviations were obtained as the standard deviation of the extrapolated value in the standard addition line (Miller and Miller 2005).

Table 5.3 Comparison of calibrates prepared in deionized water and semen for BZ3, DHB, DHMB and THB

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^{f}
		Semen[d]	Aqueous[d]		
BZ3	a[a]	0.13 ± 0.02	-0.01 ± 0.03		
	b (mL µg^{-1})[b]	16.9 ± 0.2	19.6 ± 0.4	86	8.7
	R^{2} [c]	0.9994	0.998		
DHB	a[a]	0.04 ± 0.01	0.02 ± 0.02		
	b (mL µg^{-1})[b]	6.6 ± 0.2	10.1 ± 0.3	65	12.2
	R^{2} [c]	0.998	0.9993		
DHMB	a[a]	0.00 ± 0.01	0.13 ± 0.03		
	b (mL µg^{-1})[b]	6.5 ± 0.2	10.8 ± 0.4	60	13.9
	R^{2} [c]	0.998	0.998		
THB	a[a]	-0.0008 ± 0.0003	-0.001 ± 0.001		
	b (mL µg^{-1})[b]	0.115 ± 0.004	0.47 ± 0.02	24	34.5
	R^{2c}	0.997	0.995		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the slopes obtained using both calibrates
[f] Statistical $t_{tab(0.05(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

Table 5.4 Determination of BZ3, DHB, DHMB and THB in fortified semen samples

Analyte	Parameter	Semen samples				
		1	2	3	4	5
BZ3	μ[a]	21	41	62	73	104
	$C \pm s$[b]	18 ± 3	38 ± 4	57 ± 5	68 ± 6	95 ± 7
	t_{cal}[c]	2.06	1.87	2.47	1.57	2.54
DHB	μ[a]	19	39	58	67	96
	$C \pm s$[b]	17 ± 2	35 ± 3	53 ± 4	65 ± 5	90 ± 5
	t_{cal}[c]	2.60	2.44	2.27	1.33	2.50
DHMB	μ[a]	18	37	55	64	92
	$C \pm s$[b]	16 ± 2	35 ± 4	53 ± 4	61 ± 3	87 ± 4
	t_{cal}[c]	3.11	1.09	1.17	2.19	2.29
THB	μ[a]	19	37	56	65	93
	$C \pm s$[b]	21 ± 3	31 ± 6	55 ± 5	61 ± 4	87 ± 7
	t_{cal}[c]	1.81	2.19	0.48	2.12	1.96

[a] Added concentration (ng mL^{-1})
[b] Found concentration (ng mL^{-1}) by standard addition calibration
[c] Statistical $t_{tab(0.05,N-2=3)} = 3.18$

Table 5.5 Limits of detection (LOD) and limits of quantitation (LOQ) of the developed methodology to determine BZ3 and its metabolites in semen

Sample[a]	BZ3		DHB		DHMB		THB	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	1	4	1	4	1	4	1	4
2	2	6	2	6	2	7	2	7
3	2	8	2	7	2	5	1	2
4	3	9	2	6	1	4	2	6
5	3	9	3	7	2	5	3	8

[a] Semen samples from different volunteers
[b] Estimated in the semen sample, taking into account the dilution factor during the treatment of the sample as **3.3 s_a/b**, where **b** is the slope of the standard addition calibration curve and s_a is the standard deviation of the intercept of the calibrate. Values expressed as ng mL^{-1}
[c] Estimated in the urine sample as **10 s_a/b** (see annotation b)

A Student's *t*-test was used to confirm the absence of significant differences between the found concentration values and the fortified concentration values of semen samples (see Annex III.3), thus showing the accuracy methodology.

5.3.2.2 Other Analytical Parameters

The calibration graphs (N = 5) were linear for BZ3, DHB, DHMB and THB over a range of concentrations from 20 to 100 ng mL^{-1} (working range) with a regression coefficient greater than 0.995 in all cases.

The limits of detection (LOD) and quantification (LOQ) of the analytes were estimated based on the International Conference on Harmonization on validation of analytical procedures.[2] Notably low LOQ values were obtained (see Table 5.5), ranging from 2 to 9 ng mL^{-1}, depending on both compound and sample.

The instrumental precision was determined by repeated injections (N = 5) of a semen sample fortified at 100 ng mL^{-1}. The repeatability of the methodology was evaluated by applying the described SPE-LC-MS/MS to five aliquots from the same semen sample fortified at 100 ng mL^{-1} (see Table 5.6).

5.3.3 Application of the Analytical Method

In order to evaluate the applicability of the proposed analytical method, precise instructions were given to a male volunteer to apply once 13 g of a sunscreen cosmetic product containing 10 % BZ3 (see Annex II.3).

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[2] ICH validation of analytical procedures methodology: text and methodology Q2(R1), ICH harmonised tripartite guidelines, Adopted November (2005).

Table 5.6 Precision parameters for the determination of BZ3, DHB, DHMB and THB in human semen samples

Analyte	Parameter	
	Instrumental precision (%)[a]	Repeatability (%)[a]
BZ3	1.0	2.2
DHB	1.3	5.2
DHMB	0.8	4.8
THB	3.0	6.4

[a] Expressed as relative standard deviation (RSD), N = 5
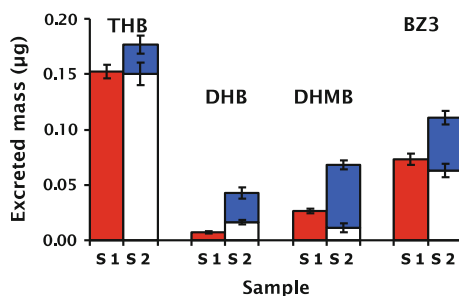
Fig. 5.2 Found amounts of BZ3, DHB, THB and DHMB in two semen samples (S1 and S2) collected at the times specified in Sect. 5.3.3, from a male volunteer after topical application of a cosmetic product containing BZ3 (10 %). *Error bars* show the standard deviation of the results. The results are expressed in terms of absolute mass considering the dilution factor corresponding to the total volume collected. Unconjugated content (*Empty square*); conjugated content (*Blue square*); total content (*Red square*)

The applied dose is in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007). The cosmetic product was prepared following an adapted protocol (Jordán and Jordán 1991). Semen samples were collected before and after the application of the sunscreen cream. The volunteer was instructed to accurately collect the total volume of each semen excretion in periods of 24 h in sterile commercially available containers. The collected volume of semen was measured to determine the excreted amount of analytes and stored at −20 °C until analysis. The semen samples were analyzed using the SPE-LC-MS/MS analytical methodology described above.

The semen sample obtained during the first 24 h after the sunscreen application (S1) was treated with ß-glucuronidase solution to determine the total content of analytes. Due to the lack of sufficient sample, the determination of the unconjugated content of analytes was not carried out.

The semen sample obtained during the second 24 h after the sunscreen application (S2) was divided into two fractions. Each fraction was treated either with

deionized water or ß-glucuronidase solution to determine the unconjugated content of analytes or the total content of analytes, respectively.

Figure 5.2 shows that the contribution of the glucuronide conjugated content to the total amount of analyte depends greatly on the target analyte.

BZ3 and THB were longer excreted than DHB and DHMB, although the excreted amounts increased with time for all analytes, being the found content higher in S2 than in S1. However, no general conclusions can be drawn from the observed results because absorption/excretion bioprocesses in the human body depend largely on the characteristics of each person.

5.4 Conclusions

For the first time, an analytical method capable to determine BZ3 and its metabolites in human semen has been developed. The methodology may be useful to obtain a more conclusive toxicological evaluation of the negative effects that these estrogenic compounds may cause on the male reproductive system.

The described method is based on the combination of a SPE procedure and LC-MS/MS analysis to determine selectively and sensitively BZ3, DHB, DHMB and THB in semen samples from users of sunscreen cosmetic products containing BZ3.

After performing a full validation of the methodology, the standard addition calibration was employed to correct the error caused by the matrix effect. Thus, statistically accurate results were obtained by analyzing semen samples previously fortified.

The described analytical method has been applied successfully to the analysis of semen samples collected from a volunteer who had applied a sunscreen cosmetic product containing BZ3. Moreover, the methodology can be applied to establish relationships between the presence of estrogenic metabolites of this widely used UV filter and the variation of specific parameters of reproductive toxicity.

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Chapter 6
Elucidation of the *In Vitro* Biotransformation Products of Ethylhexyl Dimethyl PABA by Chromatographic Techniques Coupled to Mass Spectrometry

6.1 Introduction

6.1.1 Aim of the Study

Given the side effects associated with the use of EDP (Part III.2), this study aims to provide additional information to clarify the metabolic pathway of EDP by *in vitro* experiments and develop an analytical tool to determine the so widely used UV filter along with its possible metabolites in urine.

To study the metabolism of EDP, different phase I and phase II metabolic reactions (see Sect. 1.4.3) were considered using enzymatic systems present in microsomes and cytosol, and different biochemical cofactors. Subsequently, the solutions resulting from these *in vitro* incubations were analyzed by gas and liquid chromatographic techniques coupled to mass spectrometry (GC–MS, LC–MS).

Given that EDP is a derivative of p-aminobenzoic acid (PABA), whose metabolism is well established (see Fig. 6.1), this substance was chosen as "positive control" in the study of the biotransformation reactions of EDP (Brown et al. 1974).

Furthermore, it should be noted that the high incidence of dermatologic side effects observed for PABA (Chignell et al. 1980; Sutherland and Griffin 1984; Dromgoole and Mailbach 1990; Shaw et al. 1992; Aliwell et al. 1993; Allen et al. 1996; Mackie and Mackie 1999) was the reason why this sunscreen agent, widely used in the past, is not currently approved for use as a UV filter in cosmetic products in the European Union (Regulation (EC) No 1223/2009). Therefore, it becomes necessary to combine different scientific disciplines to further study the systemic effects that may be caused by possible metabolites of EDP.
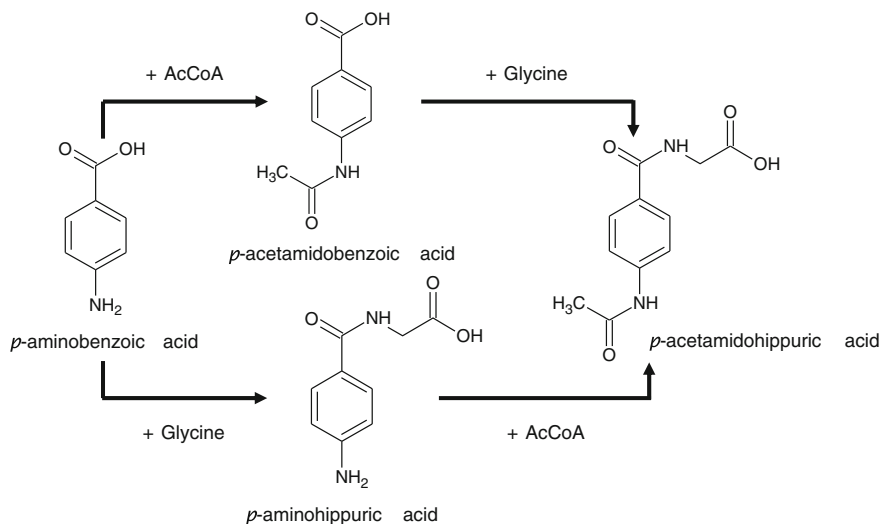
Fig. 6.1 Proposed biotransformation pathway of PABA (Brown et al. 1974)

Furthermore, to determine both the parent compound (EDP) and its possible metabolites in human urine, an analytical method based on solid phase extraction (SPE) prior to LC–MS analysis is developed and validated.

6.1.2 Background and Current Status of the Issue

Because the route of urinary excretion is the main elimination mechanism of some UV filters and their metabolites (Okereke et al. 1993), our research group has published several articles focused on the determination of UV filters in human urine (Vidal et al. 2003; Vidal et al. 2007; Balaguer et al. 2008).

Moreover, some analytical methods have been developed to determine EDP in human plasma (Jiang et al. 1996) and breast milk (Hany and Nagel 1995). However, there is no evidence in the scientific literature consulted to conduct this study on the determination of EDP in human urine or on the determination of EDP and its possible metabolites in any biological fluid. In fact, possible metabolites of this UV filter are unknown.

6.2 Experimental

6.2.1 Reagents and Samples

Ethylhexyl dimethyl PABA (EDP), also called 2-ethylhexyl 4-(N,N-dimethyl-amino) benzoate, 98 % from Aldrich (Milwaukee), 4-(N,N-dimethylamino) benzoic acid (DMP) 98 % and 4-acetamidobenzoic acid, both from Aldrich (Barcelona), 4-(N-methylamino) benzoic acid (MMP) 97 % from Sigma-Aldrich (Schnelldorf) and p-aminobenzoic acid (PABA) 99.7 % from Guinama (Valencia) were used as standards. Trihexylamine (THA) from Eastman (Rochester) and caffeine (CAF) from Janssen Chimica (Geel) were used as internal standards for LC–MS procedures and GC–MS, respectively.

The solvents used were absolute LC grade methanol (MeOH) and LC grade acetonitrile (ACN), both from Biosolve BV (Leenderweg) and GC grade dichloromethane (DCM) from Sigma-Aldrich (St. Louis). Deionized water was obtained from a Milli-Q® water purification system (R $\geq$ 18 mΩ cm) from Millipore (Amsterdam).

Formic acid (FA) from BV Biosolve (Leenderweg), magnesium chloride hexahydrate $\geq$99 % from Fluka Chemie (Steinheim), potassium monohydrogen phosphate and 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl) acetamide (MSTFA), both from Merck (Darmstadt), glacial acetic acid (AcOH) and 30 % ammonium hydroxide (d = 0.910 g mL^{-1}) both of Riedel-de Haën (Seelze) and sodium chloride, potassium chloride, calcium chloride, magnesium sulfate and 37 % analysis grade hydrochloric acid (d = 1.19 g mL^{-1}), all from Sigma-Aldrich (Schnelldorf), were also used.

Likewise, ß-nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt (NADPH), uridine 5′-diphosphoglucuronic acid trisodium salt (UDP-GA) 98–100 %, adenosine 5′-triphosphate magnesium salt (ATP) $\geq$95 % and acetyl coenzyme A sodium salt (AcCoA) $\geq$93 %, all from Sigma-Aldrich (Schnelldorf), were used.

Urine samples used to develop and validate the proposed analytical method were obtained from volunteers who had not applied any cosmetic product containing EDP.

6.2.2 Instruments and Material

The GC–MS equipment consisted of an HP 6890® gas chromatograph and a HP 5973® mass spectrometer, both from Agilent (Waldbronn). Factor A Four® VF-5MS analytical column was used (0.25 μm particle size, 30 m length, 0.25 mm internal diameter) from Varian (Darmstadt), using a constant flow rate of 1 mL min^{-1} of Helium 99.9990 % from Praxiar (Oevel). An ATAS-2® programmable injector from Optic (Cambridge) was used in splitless mode (1 min),

with a injection volume of 1 μL. The mass spectrometer operated in electron impact ionisation mode, with an ionization energy of 70 eV.

The LC–MS equipment consisted of a LC-2010A® high pressure pump from Shimadzu (Duisburg) and a 1100® ion trap detector equipped with an electrospray ionization source from Agilent (Waldbronn). A Phenyl XTerra® (3.5 μm particle size, 100 mm long, 2.1 mm internal diameter) analytical column from Waters (Milford) coupled to a precolumn (5 mm long, 4 mm internal diameter) from Restek GmbH (Bad Homburg) was used. Detection was initially carried out in full scan mode from 50 to 500 m/z and was on line coupled to a 2010A® UV/VIS detector from Shimadzu (Duisburg). 99.9990 % nitrogen from Praxiar (Oevel)) was used as both nebulizer and drying gas. The MS/MS spectra were obtained by collision induced dissociation (CID) of the selected precursor ions using 99.9990 % helium from Praxiar (Oevel) as collision gas.

Plexa Bond Elut® SPE cartridges (30 mg, 10 mm length, 5 mm internal diameter) from Varian (Middelburg) and a SPE vacuum system from Waters (Milford) were employed.

A HI 2212® pH meter from Hanna Instruments BV (IJsselstein) and 595–110 mm filter paper from Schleicher and Schuell (Dasel) were also used.

6.2.3 Experimental Procedures

Microsomes (13 mg mL^{-1} average protein concentration) and cytosol (38 mg mL^{-1} average protein concentration) solutions from rat liver were prepared according to the protocol described by Rooseboom et al. (2001) and stored at −80 °C until use. In addition, human liver microsomes from 50 donors (Lot No. 0710619) from Xenotech (Lenexa) containing an average protein concentration of 20 mg mL^{-1} were employed.

Separate standard solutions of EDP (2 mM, substrate solution), PABA (2 mM) and CAF (20 μg mL^{-1}) were prepared in ACN. An aqueous solution of potassium monohydrogen phosphate (100 mM) containing magnesium chloride (5 mM) at pH 7.4 (phosphate solution) was also prepared and stored at 4 °C.

ATP, AcCoA and UDP-GA cofactors solutions were prepared at 10 mM in phosphate solution. Analogously, a 20 mM NADPH solution was also prepared.

Separate standard solutions of THA (25 μg mL^{-1}) and EDP, DMP and MMP (25 μg mL^{-1}), both in MeOH, were also prepared.

Meanwhile, the urine samples used in the development of the analytical method were filtered through filter paper.

6.2.3.1 Study of the *in vitro* Phase I Metabolism of Ethylhexyl Dimethyl PABA

To investigate the *in vitro* phase I metabolism of EDP, 50 μL of rat liver microsomes were mixed with 395 μL of phosphate solution and 50 μL of NADPH (20 mM). Then, 5 μL of the EDP substrate solution was added. The mixture was incubated at 37 °C for 1 h. Control incubations were also performed in the absence of substrate solution or rat liver microsomes.

After 1 h, the reaction was stopped by adding 10 μL of FA, thus causing enzyme denaturation and resulting in a pH 3. The obtained mixture was stirred and loaded into a SPE cartridge that was conditioned with 2 mL of MeOH followed by 2 mL of deionized water at a flow rate of 0.5 mL min^{-1}. Then, the SPE cartridge was washed with 0.5 mL of deionized water and dried under vacuum for 10 min. 2×0.5 mL of MeOH:DCM (1:1, *v/v*) was used to elute. The eluted fraction was evaporated to dryness under a nitrogen stream at room temperature and reconstituted with 150 μL of ACN. A volume of 100 μL was transferred to a vial, to which 20 μL of MSTFA and 20 μL of CAF (internal standard solution, 20 μg mL^{-1}) were added. Finally, the solution was injected into the GC–MS system.

For GC–MS analysis the following temperature program was used: 0–1 min, maintained at 50 °C, linear gradient to 150 °C at 30 °C min^{-1}, linear gradient up to 200 °C at 5 °C min^{-1} and then linear gradient to 300 °C at 10 °C min^{-1}, hold for 5 min. Transfer line temperature was set at 270 °C and injector temperature at 280 °C. Detection was carried out in full scan mode from 50 to 500 m/z.

6.2.3.2 Study of the *in vitro* Phase II Metabolism of Ethylhexyl Dimethyl PABA

To investigate the *in vitro* phase II metabolism of EDP, different cofactors were added to the incubation solution.

To study the acetylation process, 50 μL of rat liver cytosol were mixed with 295 μL of phosphate solution, 50 μL of NADPH (20 mM), 50 μL of ATP (10 mM) and 50 μL of AcCoA (10 mM). Then, 5 μL of the EDP substrate solution was added.

For glucuronidation, 50 μL of either human or rat liver microsomes were mixed with 345 μL of phosphate solution, 50 μL of NADPH (20 mM) and 50 μL of UDP-GA (10 mM). Then, 5 μL of the EDP substrate solution was added.

In both cases, solutions were incubated at 37 °C for 1 h. Control incubations were performed in the absence of EDP substrate solution or enzyme solutions.

To compare the biotransformation processes of EDP and PABA, additional incubations were carried out using conditions similar to those described above but using a PABA standard solution (2 mM) instead of the EDP standard solution (2 mM).

Time (min)	Aqueous solution of MeOH (0.5 %, *v/v*) containing 0.1 % FA (%)	MeOH containing 0.1 % FA (%)
0	100	0
8	100	0
16	0	100
24	0	100

Table 6.1 Elution gradient used in the LC–MS analysis to elucidate the biotransformation process of EDP

After 1 h, the reaction was stopped by adding 1 mL of ice cold MeOH, resulting in the denaturation of enzymes. The resulting mixture was stirred and centrifuged at 5,000 g for 15 min at room temperature. The supernatant was evaporated to dryness under a stream of nitrogen at room temperature, reconstituted with 150 μL of MeOH in deionized water (0.5 %, *v/v*):MeOH (1:1, *v/v*) with 0.1 % FA, and analyzed by LC–MS.

For LC–MS analysis, the elution gradient outlined in Table 6.1 was used, at a flow rate of 0.5 mL min^{-1} and at 22 °C. The injection volume was 50 μL.

The search for possible phase II metabolites of EDP, selected ion monitoring (SIM) was performed in positive electrospray ionization mode (ESI$^+$), through the corresponding protonated molecular ions [(M + H)$^+$]. The mass spectrometer operated with the following parameters: capillary voltage, 40 eV; nebulizer gas pressure, 30 psi; drying gas flow, 8 mL min^{-1}; capillary temperature, 350 °C.

6.2.3.3 Determination of Ethylhexyl Dimethyl PABA and its Phase I Metabolites in Urine

Once the identification of the phase I metabolites of EDP (see Sect. 6.3.2) was confimed, an analytical method to determine EDP, DMP and MMP in urine using the standard addition calibration to correct the error caused by the matrix effect was used (see Sect. 6.3.4.1). Thus, the calibrate solutions were prepared by fortifying five aliquots (9,640 μL) of urine samples with 0, 20, 40, 60 and 80 μL of EDP, MMP and DMP (25 μg mL^{-1}) solution, to which 100, 80, 60, 40 and 20 μL of MeOH, respectively, was added to fix the same content of MeOH in each solutions. Then, 200 μL of THA (25 μg mL^{-1}) solution and 60 μL of FA were added to each calibrate solution, resulting in a pH 3. The resulting final volume of each solution was 10 mL.

Then, 5 mL from each urine solution was loaded the SPE cartridges, that were conditioned with 2 mL of MeOH followed by 2 mL of deionized water at a flow rate of 0.5 mL min^{-1}. SPE cartridges were washed with 5 mL of deionized water and dried under vacuum for 10 min. Then, analytes were eluted with 3 × 0.4 mL of MeOH:DCM (1:1, *v/v*). The eluted fractions were evaporated to dryness under a nitrogen stream at room temperature, reconstituted with 400 μL of MeOH in deionized water (0.5 %, *v/v*):MeOH (1:1, *v/v*) with 0.1 % FA, and analyzed by LC–MS.

Table 6.2 Elution gradient used in the LC–MS analysis for the determination of EDP and its metabolites in urine

Time (min)	Aqueous solution of MeOH (0.5 %, *v/v*) containing 0.1 % FA (%)	MeOH containing 0.1 % FA (%)
0	100	0
1	100	0
16	0	100
24	0	100

Table 6.3 SIM parameters in positive electrospray ionisation mode to determine EDP and its metabolites in urine

Compound	Parameter	
	Amplitude (V)	Monitored ion (m/z)
MMP	0.60	152
DMP	0.65	166
THA[a]	0.75	270
EDP	0.75	278

[a] Internal standard

For LC–MS analysis, the elution gradient outlined in Table 6.2 was employed, at a flow rate of 0.5 mL min^{-1} and at 22 °C. The injection volume was 30 μL.

The analytes were determined by SIM in ESI$^+$ mode. The SIM selected parameters are specified in Table 6.3. The mass spectrometer operated with the following parameters: capillary voltage, 40 eV; nebulizer gas pressure, 30 psi; drying gas flow, 8 mL min^{-1}; capillary temperature, 350 °C.

Figure 6.2 shows a SIM chromatogram obtained from the analysis of a urine sample containing no analyte that was fortified with EDP, DMP and MMP (250 ng mL^{-1}).

Calibration curves for each analyte were obtained by representing the ratios between the analyte and internal standard (THA) peak areas versus the added concentration of analyte.

6.3 Results and Discussion

First, the *in vitro* phase I metabolism of EDP was studied by means of microsomal incubations. Subsequently, the *in vitro* phase II metabolism of EDP was also studied and a comparison between the biotransformation processes of EDP and PABA was established. Finally, an analytical method based on SPE and LC–MS for the determination of the EDP and its phase I metabolites in human urine was developed and validated.
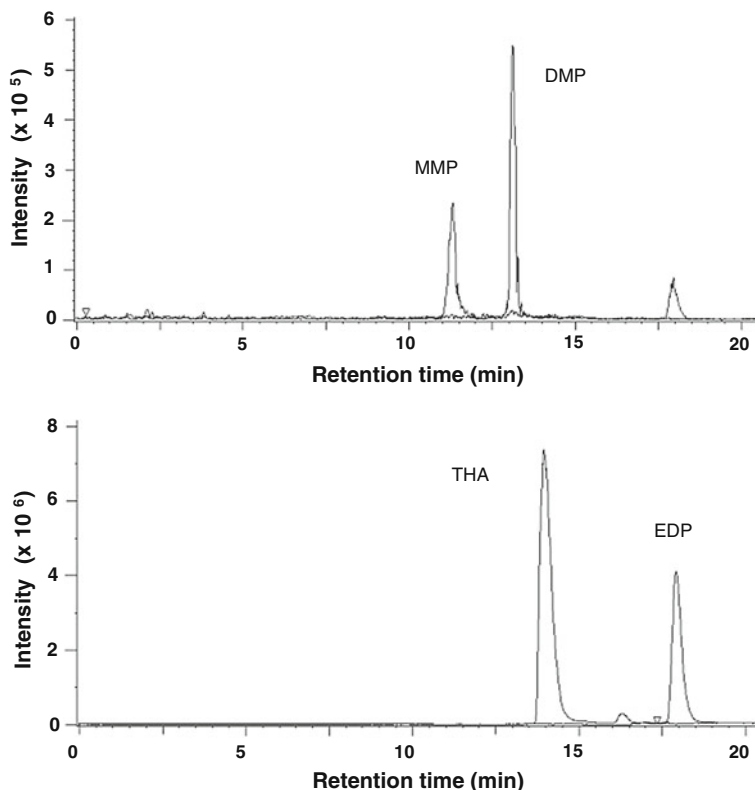
Fig. 6.2 SIM chromatograms obtained from the analysis of a fortified urine sample (25 ng mL^{-1}) using the proposed SPE-LC–MS methodology for MMP (m/z = 152), DMP (m/z = 166), EDP (m/z = 278) and THA (m/z = 270)

6.3.1 Study of the Experimental Variables

Plexa$^®$ Bond Elut cartridges showed good ability to adequately retain both types of analytes, both EDP (more lipophilic) as well as MMP and DMP. SPE procedure was optimized to achieve an efficient clean-up of the microsomal incubations prior to the subsequent GC–MS analysis and obtain a proper extraction and concentration of the analytes in urine by LC–MS analysis.

6.3.2 Identification of the Phase I Metabolites Ethylhexyl Dimethyl PABA

Figure 6.3 shows the chromatograms obtained from the application of the analytical methodology described in Sect. 6.2.3.1 to a incubated microsomal solution in the presence and absence of EDP substrate solution.

As can be seen, MMP form DMP and analyzing the incubated mixture in the presence of substrate solution EDP.

After performing appropriate derivatization reactions with MSTFA, identification of MMP and DMP was verified by comparison of the relative retention times of internal standard (CAF) and mass spectra data from reference standard solutions (see Table 6.4). The hydrolysis of the ester group of EDP allowed the trimethylsilylation of the free carboxyl group of DMP and MMP.

To confirm the origin of MMP, that is, whether it was directly formed from EDP or from the intermediate DMP, an additional incubation was carried out employing DMP as a substrate. MMP was identified, but the signal intensity was significantly lower than that in the case of the EDP incubation. So, from tests carried out, it was not clarified if MMP was formed by demethylation of DMP or from hydrolysis of the 2-ethylhexyl group and subsequent demethylation of EDP.
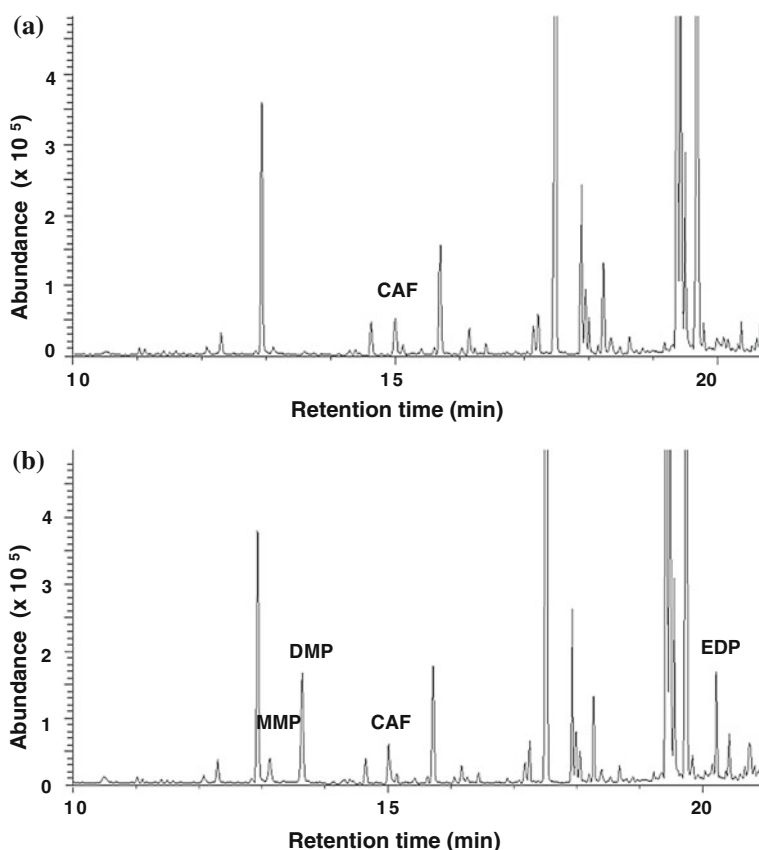
Fig. 6.3 Chromatograms obtained by total ion monitoring from GC–MS analysis of the control incubation in absence of the EDP substrate solution (**a**) and the incubation in presence of the EDP substrate solution (**b**). Experimental conditions are described in Sect. 6.2.3.1

Table 6.4 Relevant data obtained from GC–MS analysis for EDP and its phase I metabolites

Compound	Relative retention time	m/z[c] (%)
CAF[a]	1.00	194 (100), 104 (90), 67 (80)
MMP-TMS[b]	0.87	134 (100), 164 (50), 208 (40)
DMP-TMS[b]	0.91	148 (100), 178 (50), 237 (40)
EDP	1.35	165 (100), 277 (40), 148 (30)

[a] Internal standard
[b] Trimethylsilylated analogue
[c] Mass charge ratio. Relative abundance is expressed in percentage of the base peak

The possibility that the degradation of EDP under the incubation conditions may be the origin of the presence of DMP and MMP in the incubation mixture was discarded by carrying out control experiments in the absence of the microsomal solution. After performing these experiments, neither DMP nor MMP were identified, so it was confirmed that the formation of both compounds was due to the *in vitro* metabolism of EDP.

Thus, spectral data obtained from the GC–MS analysis by full scan mode allow the *in vitro* biotransformation of EDP illustrated in Fig. 6.4 to be proposed.

Furthermore, the extraction efficiency associated with the SPE-GC–MS process for DMP and EDP was found to be about 80 and 60 %, respectively, by comparison with external standards (4 µg mL^{-1}).

6.3.3 Study of the In Vitro Phase II Metabolism of Ethylhexyl Dimethyl PABA

The *in vitro* phase II metabolism of EDP was studied by means of the incubation of different solutions with cytosol or microsomes and corresponding cofactors. The reaction products were analyzed by LC–MS (see Sect. 6.2.3.2 for further details).

Among all the reactions included in the phase II metabolism, acetylation and glucuronidation were considered including the respective AcCoA and UDP-GA. In these cases, the phase I metabolism of EDP is a necessary requirement for the subsequent conjugation with these cofactors, since acetylation and glucuronidation can only occur after the hydrolysis of the ester group.

The acetylation reaction was activated by adding ATP to the cytosolic solution. Considering that the amino group of DMP is dimethylated, acetylation was only possible for MMP by means of the reaction of the monomethylated amino group with AcCoA. However, the expected metabolite was found.

As previously discussed, the comparison between the biotransformation of EDP and PABA may be useful to understand the metabolism of EDP and to check the effectiveness of the biotransformation reactions performed. Thus, when using PABA as substrate under the same incubation conditions as those used with MMP, the acetylated PABA metabolite was detected (see Fig. 6.5).
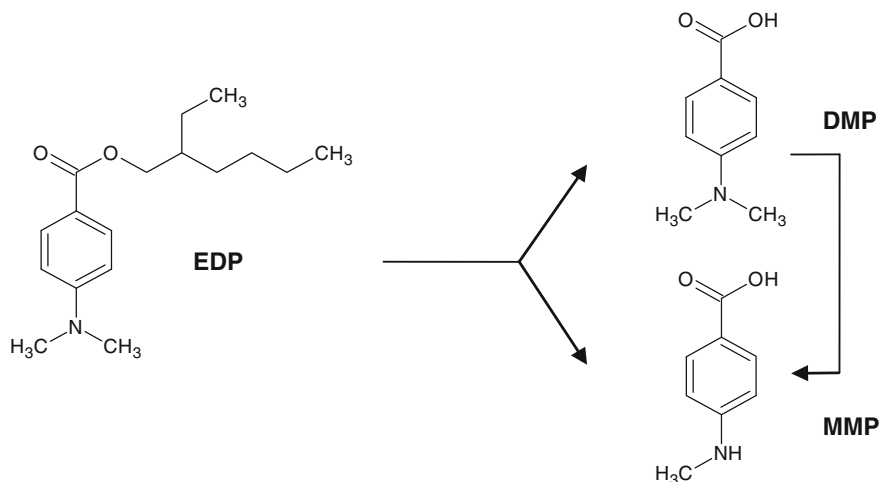
Fig. 6.4 *In vitro* metabolic pathway proposed for EDP

This result showed that the chosen reaction conditions for the acetylation study were correct. Figure 6.6 shows the MS/MS spectrum of the acetylated conjugated of PABA, whose identification was confirmed by comparison of the MS/MS spectra with reference standard solutions. In fact, PABA is considered a typical substrate of the arylamine N-acetyltransferase, an enzyme present in the cytosol that enables the acetylation reaction (Gusson et al. 2006).

The *in vitro* conjugation with glucuronic acid requires UDP-GA cofactor and UDP-glucuronyltransferase (UGT) enzyme, which is present in the microsomes. Glucuronidation usually occur in nucleophylic heteroatoms, such as O, N or S, which are rich in electrons. Therefore, in this case, the reaction may occur with either the carboxyl group formed during phase I biotransformation of EDP for DMP and MMP, or the secondary amino group for MMP.

To assess the reaction of glucuronidation for DMP, MMP and PABA, eight series of experiments were performed using EDP, DMP, MMP and PABA substrate solutions, and both human and rat liver microsomes. These experiments included the possibility of both ester hydrolysis and glucuronidation by incubations with EDP, because both reactions are catalyzed by enzymes present in the microsomes. However, no corresponding glucuronide conjugates were detected in either case (see Fig. 6.7).

The results from the *in vitro* phase II metabolism of EDP indicate that DMP and MMP are not involved in the process of acetylation or glucuronidation. Accordingly, *in vitro* biotransformation of EDP seems to be limited to phase I metabolism, although other types of conjugations (sulfations, etc.) should be considered to obtain a more general idea of the phase II metabolism of EDP.

The use of PABA as control substance in the biotransformation reactions of EDP helped to confirm that the experimental conditions used were adequate to
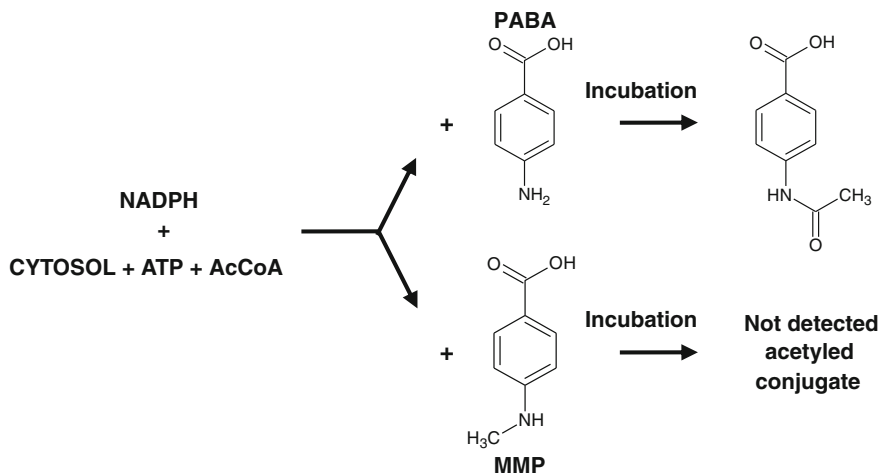
Fig. 6.5 Schematic description of the acetylation processes conducted with MMP and PABA

carry out the study. Thus, when using the same incubation conditions in the case of PABA, the results established in the scientific literature were obtained (Gusson et al. 2006) and then, the formation of the acetylated product and the absence of the corresponding glucuronide conjugate were observed. A possible explanation about the no formation of the acetylated adduct for MMP could be the steric impedance presented by the monomethylated amino group.

6.3.4 Validation of the Analytical Method to Determine Ethylhexyl Dimethyl PABA and its Phase I Metabolites in Urine

To study the robustness of the analytical method, pH effect on the urine sample and SPE load capacity were evaluated.

Thus, four aliquots of urine containing no analyte were fortified with MMP, DMP and EDP (200 ng mL^{-1}). The pH was adjusted to 3, 4, 6 and 9 by adding FA, AcOH, deionized water and ammonium hydroxide, respectively.

The results obtained by applying the analytical method to the various urine samples are described in Fig. 6.8. As can be seen, the response of EDP was not pH dependent. Moreover, by increasing the pH value, the obtained responses for MMP decreasing until being zero at pH 9. In the case of DMP, responses were constant at pH 3, 4 and 6, but they were null at pH 9.

The results are consistent to the pK$_a$ values of the analytes (EDP pK$_a$, 2.5; pK$_{a1}$ of DMP and MMP, ~2.5; pK$_{a2}$ of DMP and MMP, ~5) calculated using the *Advanced Chemistry Development (ACD/Labs) V8.14 software*. Therefore, the

Fig. 6.6 MS/MS spectrum of the acetylated conjugate species of PABA. The parent ion $(M+H)^+$ (180.2 (m/z)) was fragmented by applying a collision induced dissociation of 0.55 V. The isolated window was 1 m/z
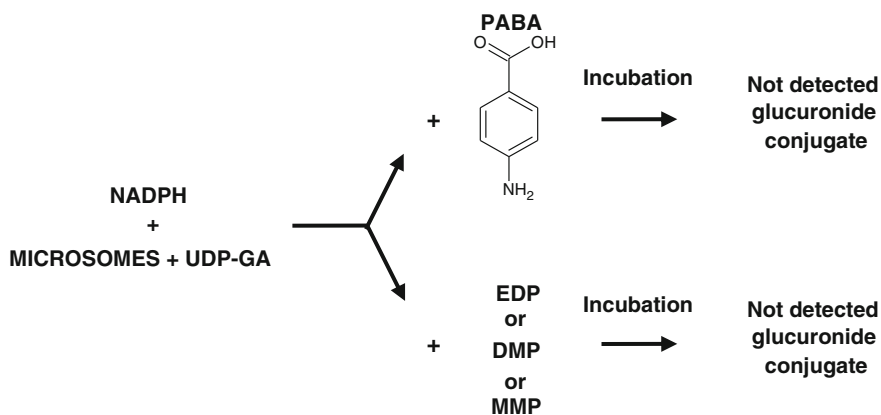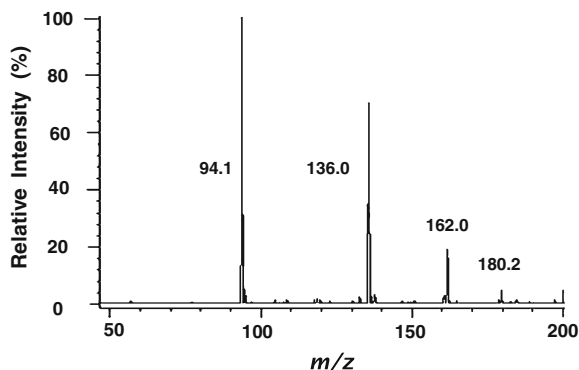
Fig. 6.7 Schematic description of glucuronidation processes performed with MMP, DMP, EDP and PABA

extraction conditions of MMP, DMP and EDP were suitable at pH values when their predominant species in solution are neutral.

On the other hand, the eluted fractions from SPE experiments carried out with increasing loaded sample volumes were analyzed. The cartridges allowed the loading of at least 5 mL of urine sample solution (150 ng mL^{-1}, 750 ng) without analytes breakthrough. Furthermore, the efficacy of the eluent solution was also evaluated and 1 mL of MeOH: DCM (1:1, *v/v*) was found to be enough to quantitatively elute the analytes.

6.3.4.1 Study of the Interferences

The matrix effects associated to the determination of EDP and its metabolites in urine were studied by applying the described SPE-LC–MS methodology and by
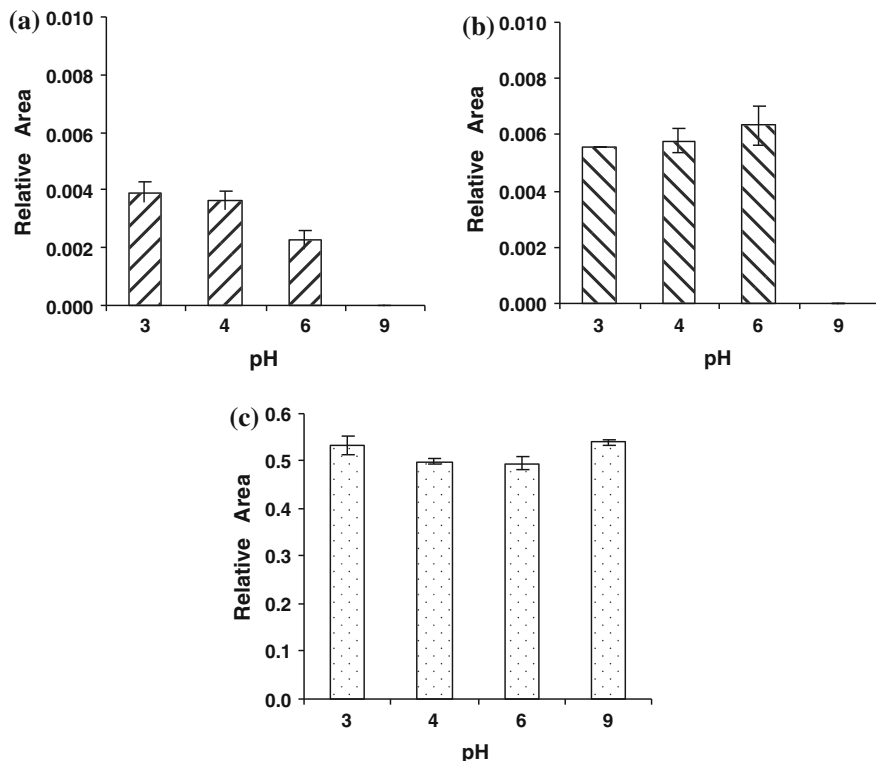
Fig. 6.8 Effect of pH on the extraction of MMP (**a**), DMP (**b**) and EDP (**c**) from a fortified human urine sample (200 ng mL^{-1}). Error bars show the standard deviation of the results (N = 3)

comparison of the responses obtained from a set of solutions (50–250 ng mL^{-1}) prepared, on one hand, in artificial urine (Tanaka and Hayashi 1986) (artificial urine calibrate) and, on the other hand, in a urine sample containing no analyte (real urine calibrate). Internal standard was added (THA, 500 ng mL^{-1}) to the solutions of both urine calibrates and the pH was adjusted by adding 60 μL of FA (pH 3). Calibrate curves were constructed by representing the peak area ratio of each analyte and THA versus the added concentration of analyte.

The parameters of the obtained calibrates are shown in Table 6.5. As can be seen, the intercept of both calibrates were statistically comparable to zero in all cases, showing the absence of constant interference from urine.

Moreover, when comparing both calibrates for DMP and MMP, statistically different slopes were obtained ($P_0 = 0.05$), thus showing the presence of matrix effects. However, in the case of EDP, no significant difference was obtained. Hence, to correct the error caused by the effect matrix which affects the determination of MMP and DMP, standard addition calibration was used.

Table 6.5 Comparison of calibrates prepared with artificial and real urine for MMP, DMP and EDP

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^{f}
		Real urine[d]	Artificial urine[d]		
MMP	a[a]	$(1 \pm 7) \times 10^{-5}$	$-3 \pm 6) \times 10^{-4}$		
	b (mL µg^{-1})[b]	0.0175 ± 0.0004	0.106 ± 0.003	17	61.7
	R^{2c}	0.998	0.997		
DMP	a[a]	$(2 \pm 1) \times 10^{-4}$	$(-1 \pm 6) \times 10^{-5}$		
	b (mL µg^{-1})[b]	0.0403 ± 0.0009	0.155 ± 0.003	26	65.2
	R^{2c}	0.998	0.998		
EDP	a[a]	$(-9 \pm 4) \times 10^{-3}$	$(-1 \pm 1) \times 10^{-2}$		
	b (mL µg^{-1})[b]	2.00 ± 0.02	2.02 ± 0.06	99	0.5
	R^{2c}	0.999	0.996		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the slopes obtained using both calibrates
[f] Statistical $t_{tab(0.05,(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

6.3.4.2 Accuracy

Accuracy was evaluated by applying the developed method to the analysis of urine samples from different volunteers who had not applied any cosmetic product containing EDP fortified with known amounts of MMP, DMP and EDP.

Table 6.6 shows the results obtained in the study of accuracy. Standard deviation values were obtained as the corresponding standard deviations of the extrapolated values of the standard addition calibration curve (see Miller and Miller 2005). To verify that the added concentration values were statistically comparable to those experimentally found, a Student's t-test was used (see Annex III.3). No significant difference were obtained between the fortified and found values, thus showing the accuracy of the developed analytical method.

6.3.4.3 Other Analytical Parameters

Limits of detection (LOD) and quantification (LOQ) were determined based on the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) (see Table 6.7).

The calibration lines (N = 5) were found to be linear for EDP, DMP and MMP on a range of concentrations from 50 to 250 ng mL^{-1} with regression coefficients greater than 0.995 in all cases. The slope of the standard addition calibration curve was the parameter used to estimate the sensitivity of the method. The instrumental precision was determined by repeated analysis (N = 5) of a fortified urine sample (100 ng mL^{-1}). The method precision (repeatability) was assessed by extracting

Table 6.6 Determination of MMP, DMP and EDP in fortified urine samples

Analyte	Parameter	Urine samples				
		1	2	3	4	5
MMP	μ^a	34	90	112	140	169
	$C \pm s^b$	26 ± 7	88 ± 6	104 ± 8	132 ± 10	167 ± 6
	t_{cal}^c	2.30	0.59	2.24	1.60	0.49
DMP	μ^a	32	84	105	132	158
	$C \pm s^b$	36 ± 7	81 ± 10	108 ± 9	123 ± 9	153 ± 10
	t_{cal}^c	1.59	0.69	0.50	2.22	1.27
EDP	μ^a	29	78	98	122	146
	$C \pm s^b$	34 ± 5	83 ± 6	107 ± 8	128 ± 5	148 ± 6
	t_{cal}^c	1.90	1.88	2.74	2.41	0.62

[a] Fortified concentration (ng mL^{-1})
[b] Found concentration (ng mL^{-1}) by standard addition calibration
[c] Statistical $t_{tab(0.05,N-2=3)} = 3.18$

Table 6.7 Limits of detection (LOD) and quantitation (LOQ) of the developed methodology to determine EDP, DMP and MMP in urine

Sample[a]	MMP		DMP		EDP	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	2	5	1	4	1	4
2	2	5	2	6	1	4
3	2	5	2	5	2	5
4	3	8	1	4	1	4
5	1	3	1	4	1	2

[a] Urine samples from different volunteers
[b] Estimated in the urine sample, taking into account the dilution factor during the treatment of the sample, as $3.3 \cdot s_a/b$, where b is the slope of the standard addition calibration curve and s_a is the standard deviation value of the intercept in the calibrate. Values expressed as ng mL^{-1}
[c] Estimated in the urine sample as $10 \cdot s_a/b$ (see annotation b)

Table 6.8 Parameters of the analytical method validation for the determination of MMP, DMP and EDP in urine samples

Parameter	Analyte		
	MMP	DMP	EDP
Working range (ng mL^{-1})	50–250	50–250	50–250
Slope (mL µg^{-1})	0.012–0.047	0.022–0.045	1.278–2.004
Instrumental precision (%)[a]	3.2	2.5	2.4
Method precision (%)[a]	6.2	3.4	7.4

[a] Expressed as relative standard deviation (RSD), N = 5

the analytes from five different aliquots of the same urine sample containing no analyte that was fortified (100 ng mL^{-1}) and analysed in the same batch. These results are shown in Table 6.8.

6.4   Conclusions

The study of the *in vitro* biotransformation of EDP, one of the most commonly used UV filters in sunscreen cosmetic products, was carried out by means of different incubations with enzymatic solutions. Hence, the *in vitro* biotransformation pathway of EDP was established, and two phase I metabolites, MMP and DMP, were identified by GC–MS and subsequently, confirmed by LC–MS.

Phase II metabolism of EDP was also investigated via glucuronidation with UDP-GA and acetylation with AcCoA. In both cases, PABA was used as reference compound due to PABA metabolism is well described. Although the acetylated conjugate of PABA was identified, neither acetylated or glucuronide conjugates of the phase I metabolites of EDP were detected. Based on these *in vitro* results, EDP mainly undergoes phase I biotransformation reactions.

Furthermore, a sensitive analytical method based on the combination of SPE and LC–MS to determine EDP and its phase I metabolites in urine at ng mL^{-1} levels has been developed. The standard addition calibration was used to correct the error caused by the proportional matrix effect. Full validation of the analytical method was conducted, thus obtaining statistically accurate results.

The high sensitivity of the developed method allows the implementation of *in vivo* studies considering the urinary excretion of both EDP and its phase I metabolites. Hence, it is possible to estimate the absorption, accumulation and excretion processes of EDP. In general, increasing knowledge with regards of the toxicology of this widely used substance can be obtained from these estimations.

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Chapter 7
Elucidation of the *In Vivo* Biotransformation Products of Ethylhexyl Dimethyl PABA by Liquid Chromatographic Techniques with Mass Spectrometry Detectors

7.1 Introduction

7.1.1 Aim of the Study

As noted in Chap. 6, the study of the *in vitro* biotransformation of ethylhexyl dimethyl PABA (EDP) allowed the identification of two phase I metabolites by gas and liquid chromatography mass spectrometry (GC-MS, LC-MS) analysis, named acid 4-(*N,N*-dimethylamino) benzoic acid (DMP) and 4-(*N*-methylamino) benzoic acid (MMP).

Since urinary excretion is the main mechanism of elimination for some UV filters and their metabolites (Okereke et al. 1993), the development of analytical methods to determine both types of compounds in urine has become a necessary tool to gain more information about the toxicology of these substances. The need to carry out further toxicological studies increases in this case, because the phase I metabolites EDP are structurally very similar to the *p*-aminobenzoic acid (PABA), a UV filter widely used in the past, which is not authorized for use as an ingredient in cosmetic products in the European Union[1] due to its high incidence of dermatological effects, including damage to DNA (Shaw et al. 1992).

To complement the in vitro studies conducted in Chap. 6, the aim of this study is double. First, the development and validation of an analytical method based on automated solid phase extraction (SPE) followed by LC-MS/MS analysis to determine EDP and its phase I metabolites in urine. Thus, additionally to confirm the *in vitro* results of the previously conducted methodology, the study would provide greater insight into the percutaneous absorption process of the UV filter in

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[1] Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products Text with EEA relevance.
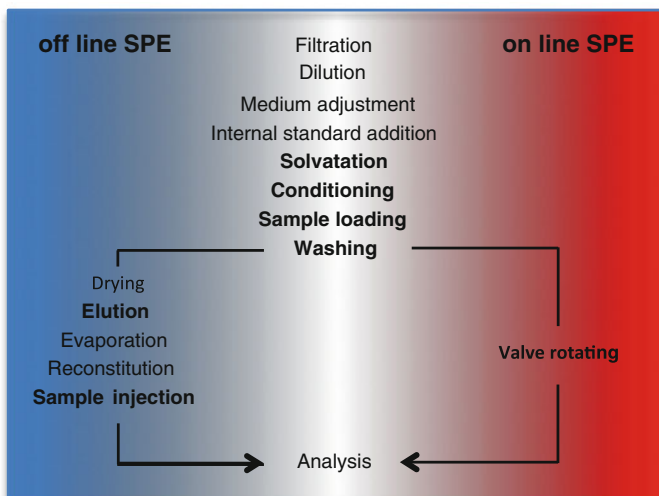
Fig. 7.1 Comparison of the SPE steps between the *off line* and *on line* modes. The steps in bold correspond to those which are basic in each procedure

humans. The second objective is the identification and characterization of potential *in vivo* phase II metabolites of EDP, by means of mass spectrometry analyzers such as triple quadrupole (QqQ) and time of flight (TOF).

In automated or *on line* SPE, unlike *off line* SPE, the elution is enhanced by connecting the SPE cartridge to the analytical column directly after the washing step, so that sample handling is reduced and time consuming steps are removed (see Fig. 7.1), thus minimizing the sources of error in the analytical procedure.

Another advantage offered by the *on line* mode is that, as soon as the analyte is eluted from the SPE cartridge, valve can be returned to the initial position, and matrix compounds more intensively retained in the cartridge that the analyte itself will never reach the analytical column, thus avoiding potential ion suppression problems on the ionization source. Furthermore, as all of the analyte that has been loaded into the SPE cartridge will elute to the analytical column, the sensitivity increases significantly compared to the *off line* mode.

7.1.2 Background and Current Status of the Issue

Only few articles regarding the determination of EDP in urine have neen published. Besides the described analytical method in Chap. 6, which allows the determination of this UV filter and its phase I metabolites by conventional or *off line* SPE combined with LC-MS analysis (León et al. 2010b), EDP has been determined by GC-MS analysis after a concentration step using microporous

membranes assisted liquid–liquid extraction (March et al. 2009a, b), which provides operational simplicity and low consumption of organic solvents.

7.2   Experimental

7.2.1   Reagents and Samples

Ethylhexyl dimethyl PABA (EDP), also called 2-ethylhexyl 4-(*N,N*-dimethyl-amino) benzoate, 98 % from Aldrich (Milwaukee) and 4-(*N,N*-dimethylamino) benzoic acid (DMP) 98 % and 4-(*N*-methylamino) benzoic acid (MMP) 97 %, both from Sigma-Aldrich (Schnelldorf) were used as analytical standards. Trihexylamine (THA) from Sigma-Aldrich was used as internal standard.

The solvents used were LC grade methanol (MeOH) and LC grade acetonitrile (ACN), both from Scharlab (Barcelona). Deionized water was obtained from a Milli-Q water purification system (R $\geq$ 18 mΩ cm) from Millipore (Billerica). LC-MS/MS grade ACN and MeOH were also employed.

Other reagents used were ammonium formate and ammonium acetate from Sigma (St. Louis), citric acid from Panreac (Barcelona) and formic acid (FA) and 32 % (*m/v*) ammonium hydroxide (d = 0.910 g mL^{-1}) from Scharlab (Barcelona). ß-glucuronidase enzyme from *Helix pomatia* (type H-1) with activity $\geq$300,000 U g^{-1} and sulfatase activity $\geq$10,000 U g^{-1} from Sigma-Aldrich was also used.

Urine samples used to develop and validate the analytical method were obtained from volunteers who had not applied any cosmetic product containing EDP.

7.2.2   Instruments and Material

An automated SPE-LC-MS/MS system consisted of a Prospekt-2® SPE platform from Spark Holland (Emmen) coupled to an 1,200 Series LC System® from Agilent Technologies (Palo Alto) that was connected to a 6460 LC/MS® Triple Quad mass spectrometry detector equipped with a Jet Stream® electrospray ionization source, also from Agilent, were used.

The automated Prospekt-2® SPE platform consisted of an automatic cartridge exchanger (ACE) and a high pressure dispenser (HPD) syringe to distribute the SPE solvents. ACE and HPD were connected to a Midas® autosampler from Spark Holland equipped with a 100 μL loop. 0.25 mm internal diameter PEEK (polyether ether ketone) tube from VICI (Houston) was employed for all connections between valves. The automation of the extraction step was performed using the Sparklink V. 2.10 software.

The following HySphere® SPE cartridges from Spark Holland were studied: CN-SE (cyanopropyl phase based on silica, particle size 7 μm), C2-SE (acetate phase based on silica, particle size 7 μm), C8 EC-SE (octyl phase based on silica, particle size 10 μm), C18-HD (high density phase of octadecyl chains based on silica, particle size 7 μm), GP Resin (polydivinylbenzene polymer phase, particle size 5–15 μm), Resin SH (polystyrene-divinylbenzene phase with hydrophobic modifiers, particle size 20–50 μm), MM anion (anion exchanger with mixed mode based on polydivinylbenzene, particle size 10 μm) and MM cation (cation exchanger with mixed mode based on polydivinylbenzene, particle size 10 μm). In all cases, the dimensions of the cartridges were 10 mm long and 2 mm internal diameter.

The MS/MS spectra were acquired using collision-induced dissociation (CID) of selected precursor ions using 99.999 % nitrogen from Air Products (Córdoba) as collision gas. Data acquisition, control of the SPE analysis sequences and development of qualitative and quantitative analyses were carried out with Agilent MassHunter Workstation software.

Moreover, a 1,200 Series LC System® from Agilent connected to a 6540 UHD Accurate-TOF Mass LC/MS® mass spectrometric detector equipped with a Jet Stream® electrospray ionization source from Agilent was also used. The acquisition of data (2.5 Hz) in both profile and centroid modes was controlled by Agilent MassHunter Workstation software.

In both LC-MS systems, a C18 Mediterranean Sea® analytical column (3 μm particle size, 150 mm long, 4.6 mm internal diameter) from Teknokroma (Barcelona) was used. Moreover, a high purity generator from CLAN Technology (Sevilla) provided nitrogen gas used in the ionization source.

Finally, a 2001 MicropH® pH meter from Crison (Alella), a thermostated water bath from Selecta (Barcelona) and a 450 digital sonicator (20 kHz, 450 W) from Branson Ultrasonics Corporation (Danbury) with adjustable amplitude and duty cycle, equipped with a cylindrical probe of titanium alloy and 12.7 mm in diameter were also used.

7.2.3 Analytical Method to Determine Ethylhexyl Dimethyl PABA and its Metabolites in Urine

7.2.3.1 Preparation of Solutions

Separate analytes standard (EDP, DMP, MMP) and internal standard (THA) solutions in MeOH (200 μg mL^{-1}) were prepared and stored at 4 °C. From these stock solutions, a solution of THA (2 μg mL^{-1}) in deionized water and a solution of EDP, DMP and MMP (2 μg mL^{-1}) in the urine sample to be analyzed were prepared.

To correct the error caused by the matrix effects (see Sect. 7.3.2), the standard addition calibration was employed. The calibrate standard solutions were prepared by fortifying five aliquots of urine sample (650 μL) containing 0, 20, 40, 60 and 80 μL of the analytes solution (2 μg mL^{-1}) to which 100, 80, 60, 40 or 20 μL of urine was added, respectively, to fix the same final volume in all the calibrate solutions. Furthermore, a volume of 50 μL of THA (2 μg mL^{-1}) was added to each calibrate solution.

7.2.3.2 Enzymatic Treatment

0.01 g of enzyme was added to a 5 mL of 1 M ammonium acetate (pH 5), solution which was stored at 4 °C.

To determine the total content of the analytes (i.e., both unconjugated and conjugated forms) 100 μL of the ß-glucuronidase solution was added to each calibrate solutions. Next, these solutions were subjected to an enzymatic hydrolysis treatment assisted by ultrasound to deconjugate the possible glucuronide adducts of the phase I metabolites of EDP. Thus, the calibrate solutions were immersed in a thermostated water bath (37 °C) together with the probe tip, which was located at the same distance from each solution (5 cm) and at a fixed distance from the bath background (5 cm). Hydrolysis of conjugated metabolites of EDP was conducted essentially under the optimal conditions obtained previously by Alvarez-Sánchez and colleagues (Álvarez-Sánchez et al. 2009). Thus, ultrasonic radiation was applied for 50 min at 35 % duty cycle and 50 % amplitude (converted applied power, 400 W).

7.2.3.3 Automated Solid-Phase Extraction

The autosampler added 100 μL of a 1 M citric acid/citrate buffer solution, adjusted to pH 3 with ammonium hydroxide to each urine sample solution. Homogenization by mixing was performed systematically by the autosampler for 2 min before the automated extraction was carried out. The final volume of each of the solutions was 1 mL of urine.

Selected SPE cartridges (C18 HD) were automatically solvated with 4 mL of MeOH at 5 mL min^{-1}, conditioned with 4 mL of deionized water at 5 mL min^{-1} and equilibrated with 3 mL of deionized water at 1 mL min^{-1} (Fig. 7.2a). The cartridges were then loaded with 100 μL of urine by propelling 3 mL of deionized water at 1 mL min^{-1} (Fig. 7.2b). Finally, the analytes were eluted by flowing the LC mobile phase through the SPE cartridges for 4 min (Fig. 7.2c). Thus, the eluate from each SPE cartridge was chromatographically separated in the analytical column prior to the detection with the mass spectrometer. Then, the cartridges were washed with 6 mL of MeOH at 5 mL min^{-1} and 6 mL of deionized water at 5 mL min^{-1} for the next extraction (Fig. 7.2d).

(a)

(b)

(c)

(d)
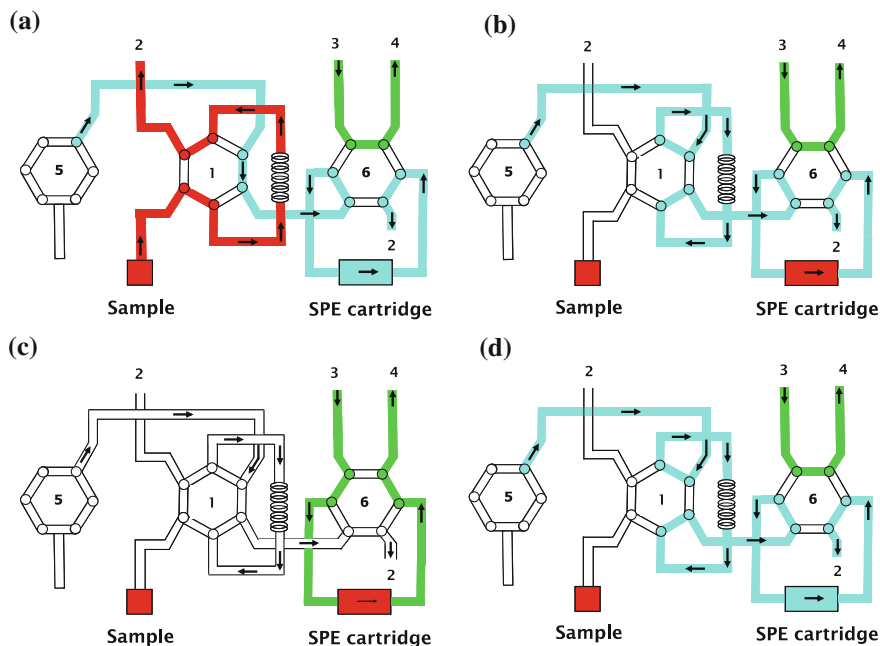
Fig. 7.2 Schematic illustration of the steps performed by the automated SPE system, including sample loop filling and cartridge solvation and conditioning (**a**); sample loading (**b**); analyte elution (**c**); cartridge post-extraction washing (**d**). Autosampler *1*; waste *2*; LC pump *3*; LC analytical column *4*; HPD *5*; ACE *6*

Table 7.1 Elution gradient used in the LC-MS/MS analysis to determine EDP and its metabolites in urine

Time (min)	0.2 % FA in deionized water (%)	0.2 % FA in MeOH:ACN (1:1, *v/v*) (%)
0	60	40
1	60	40
3	0	100
15	0	100

7.2.3.4 LC-MS/MS Analysis

The chromatographic separation of analytes was carried out using a mobile phase consisting of deionized water and MeOH:ACN (1:1, *v/v*), both containing 0.2 % FA. The elution gradient outlined in Table 7.1 was used at a flow rate of 0.8 mL min^{-1} and at 20 °C. The analytical column was equilibrated with a post-analysis time of 5 min.

The conditions of QqQ mass spectrometer analyzer and spray chamber were: temperature of gas ionization source, 350 °C; drying gas flow, 10 L min^{-1}; nebulizer gas pressure, 35 psi; nebulizer gas temperature, 380 °C; nebulizer gas flow, 10 L min^{-1}; capillary voltage, 4,450 V. The dwell time was set to 70 ms.

Table 7.2 SRM parameters in positive electrospray ionisation mode to determine EDP and its metabolites in urine

Compound	Parameter			
	First quad voltage (V)	Collision energy (eV)	SRM transition to quantify	SRM transition to confirm
MMP	90	15	151.8 → 136.8	151.8 → 119.9
DMP	90	25	165.8 → 149.8	165.8 → 133.9
EDP	100	15	277.8 → 165.8	277.8 → 150.8
THA[a]	120	20	270.1 → 185.8	270.1 → 102.0

[a] Internal standard

The analytes were determined by selected reaction monitoring (SRM) in positive electrospray ionization (ESI$^+$) mode. The selected confirmation and quantification SRM transitions are shown in Table 7.2.

Calibration curves were obtained by representing the peak area ratio of each analyte to the internal standard (THA) versus the added concentration of analyte.

Figure 7.3 shows the SRM chromatograms obtained for each analyte in urine samples containing no analyte that were either fortified (40 ng mL^{-1}; blue line) and unfortified (red line) subjected to the analytical procedure described above.

7.2.4 In Vivo Metabolism of Ethylhexyl Dimethyl PABA

To confirm the presence of phase I metabolites of EDP and elucidate its potential phase II metabolites, LC-ToF-MS analysis of urine samples from volunteers before and after applying a sunscreen cosmetic product containing EDP were performed.

Chromatographic separation of the target compounds was carried out using a mobile phase consisting of deionized water and MeOH:ACN (1:1, v/v), both containing 0.2 % FA. The same elution gradient described in Sect. 7.2.3.4 was used. The injected sample volume was 100 μL.

The working conditions of the TOF analyzer operating in positive electrospray ionization (ESI$^+$) mode were as follows: temperature of the source gas, 350 °C; drying gas flow, 10 L min^{-1}; nebulizer gas pressure, 35 psi; nebulizer gas temperature, 380 °C; nebulizer gas flow, 10 L min^{-1}; capillary voltage, 4,450 V; inlet cone potential, 65 V; octopole radiofrequency voltage, 750 V; focus voltage, 90 V. The mass range and the detection window was set to 100–1100 (m/z) and 100 ppm, respectively. A 20,000 resolution power was used to examine the isotopic content of the EDP metabolites. The analytes were identified by accuracy mass measurements.
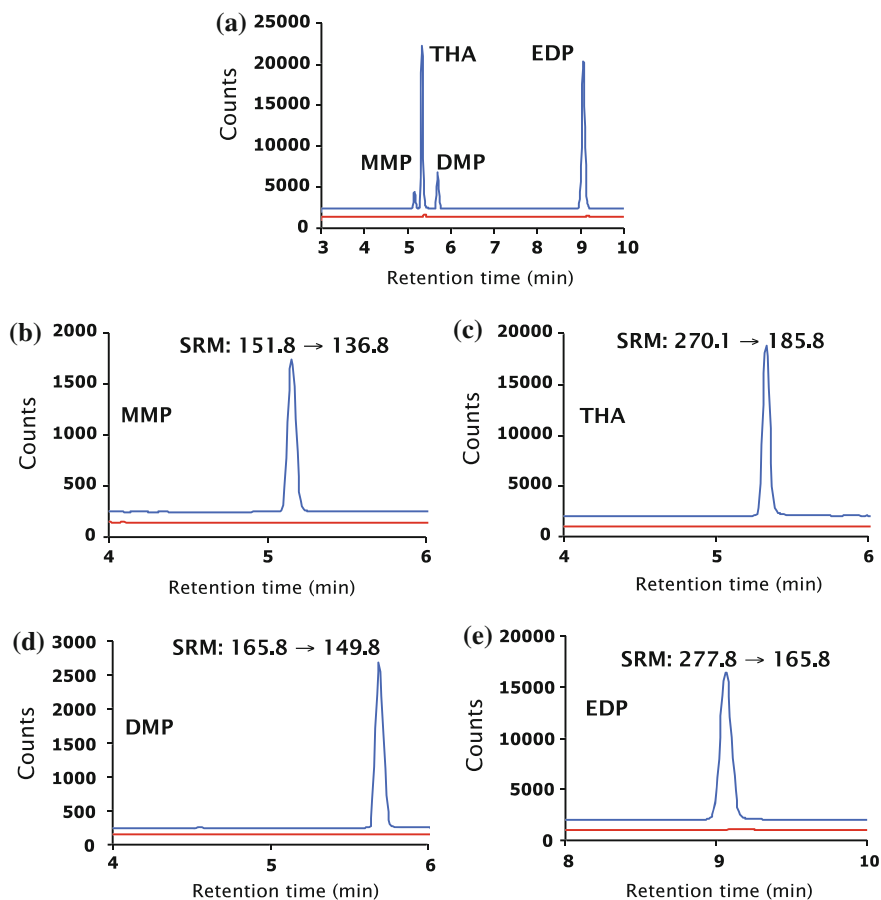
Fig. 7.3 Total ion chromatograms (TIC) in positive ionization mode for each compound of interest (**a**) and SRM chromatograms for MMP (**b**), THA (**c**), DMP (**d**) and EDP (**e**) obtained by analyzing a urine sample containing no compounds (*red line*) and the same urine sample fortified at a concentration of 40 ng mL^{-1} (*blue line*). Experimental conditions are described in Sect. 7.2.3

7.3 Results and Discussion

7.3.1 Study of the Experimental Variables

7.3.1.1 LC-MS/MS

Collision induced dissociation values were investigated for all analytes by direct injection of individual standard solutions (5 µg mL^{-1}) in MeOH using both positive and negative ESI modes. Based on the intensity values obtained with both ESI-MS/MS fragmentation, ESI$^+$ was selected to determine EDP, DMP and MMP.

The effect of the modifier addition to the mobile phase was evaluated in terms of sensitivity and signal peak shape. For this purpose, different concentrations of FA and two volatile salts, such as ammonium formate and ammonium acetate, were assayed. The best results were obtained by using a mobile phase consisting of deionized water and MeOH:ACN (1:1, *v/v*), both containing 0.2 % FA.

Under these conditions, tandem mass spectrometry parameters were selected. The optimum working conditions are indicated in Table 7.1.

7.3.1.2 Automated SPE Procedure

Different variables regarding the automated sample preparation were selected using a univariate approach to study the retention-elution step and the washing step.

First, the response of the analytes by using various SPE sorbents (i.e., CN-SE, C2-SE, SE EC-C8, C18 HD, GP Resin, Resin SH, MM MM anion and cation) was examined. To improve retention and considering the pKa values of the analytes (EDP pKa, 2.5; pK_{a2} of DMP and MMP, $\sim$5; pK_{a1} of DMP and MMP, $\sim$2.5), calculated using the *Advanced Chemistry Development (ACD/Labs) V8.14 software*, urine samples containing no analytes were fortified (1 μg mL^{-1}) and the behavior of EDP, DMP and MMP was studied under pH conditions 3 and 8. The prevalent species in solution of the three analytes are neutral at pH 3, but they are non-protonated for MMP and DMP and neutral for EDP at pH 8. The retention capacity of the sorbent was evaluated eluting with mobile phase for a time enough to ensure the complete elution of the retained analytes (100 % of MeOH:ACN (1:1, *v/v*) with 0.2 % FA, 10 min).

In general, a better response was obtained for the analytes at pH 3 with the C8 EC-SE, C18 HD, Resin GP and Resin SH cartridges, based on non-polar interactions, than at pH 8 with the MM anion and MM cation cartridges, based on ionic interactions. The worst responses were obtained with the CN-SE and C2-SE cartridges.

Therefore, as C18 HD cartridges provided the overall optimum performance for all the analytes, they were selected for the following experiments. Morever, pH of urine samples was adjusted to 3 prior to the application of automated SPE procedure.

An *in situ* acidification procedure was carried out, thus allowing the urine at pH 3 the shortest time possible before the analysis to avoid the occurrence of potential precipitation processes. To this end, the autosampler added different volumes of FA or a 1 M citric acid/citrate (pH 3) buffer solution to the urine samples immediately prior to the automated SPE procedure. Best and more reproducible results were obtained when adding 100 μL of a 1 M citric acid/citrate (pH 3) buffer solution. Thus, the SPE procedure was conducted effectively under these in situ acidification conditions.

To measure the retention capacity of the analytes in the C18 HD sorbents, breakthrough process was evaluated using a dual cartridge configuration in the

Table 7.3 Evaluation of the retention capacity of EDP and its metabolites in urine by the *C18 HD* cartridges

Concentration (ng mL^{-1})	Retention capacity (%)[a]		
	EDP	DMP	MMP
10	98.7 ± 0.2	99.2 ± 0.1	90.3 ± 0.4
100	99.8 ± 0.1	99.8 ± 0.1	93.0 ± 2.0
500	99.7 ± 0.1	99.7 ± 0.1	95.0 ± 1.0

[a] Analysis in triplicate

SPE automated platform.[2] In this way, two C18 HD cartridges were connected in series to retain in the second cartridge the analytes that have not been retained in the first cartridge during the loading step. Thus, if breakthrough actually happens in the first cartridge, the ranalytes will be retained in the second.

Breakthrough was studied in triplicate analyzing urine samples fortified at three levels of concentration. Retention efficiency was calculated as the ratio between the analyte response in the first cartridge and the combined response in both cartridges. The experiments conducted under the selected experimental conditions showed that no breakthrough was produced for any analyte at any concentration tested (see Table 7.3).

Different proportions of MeOH:deionized water as loading solvent were studied. Due to the more polar analyte (MMP) was eluted even with the lowest content of MeOH, deionized water was selected as loading solvent. Furthermore, the influence of the loading volume was examined in the range from 0.5 to 8 mL. A volume of 3 mL of deionized water was selected because larger volumes caused the partial elution of MMP. Similarly, different loading flows between 0.5 and 2 mL min^{-1} were examined and the best yields were obtained using a flow rate of 1 mL min^{-1}.

To eliminate potential polar interferents present in urine samples, deionized water and aqueous solutions of MeOH (2.5 and 5 % (*v/v*)) were evaluated as SPE washing solutions. Given that partial elution of the most polar analyte was produced when propelling high volumes of deionized water in the sample loading step, it was not surprising that the same situation was repeated even using low volumes of deionized water as washing solvent. Then, the washing step was removed in the following experiments.

The retained analytes were eluted by flowing the LC mobile phase through the SPE sorbent, by rotating the automated valve. The time during the mobile phase was propelled through the SPE cartridge to the analytical column, namely the elution time, must allow the quantitative elution of the analytes but avoiding the elution of interferences that may cause ion suppression in the ionization source and the obtaining of broad chromatographic peaks. A time of 4 min was selected as

[2] Prospeckt Technical Note 6 (1996) Strategy for Accelerated On-line Solid Phase Extraction Method Development; Serial Extraction. B. V. Spark Holland.

elution time and thereafter, the mobile phase was propelled again directly to the analytical column, by means of a new rotation of the automated valve.

To study the adsorption of the analytes on the automated platform connections was not necessary since the obtained yields using C18 HD cartridges were quantitative. As a result, the memory effect caused by the possible adsorption of hydrophobic analytes (EDP) in the connections (Rodil et al. 2008) were negligible mainly due to the systematic washing step performed after each SPE extraction.

It was also found that the C18 HD cartridges could be reused up to five times without causing a significant loss on efficiency extraction (<5 %).

7.3.1.3 Ultrasonic Assisted Enzymatic Hydrolysis

Considering that glucuronidation and sulfation are the most common phase II biotransformation reactions (Olgivie and Parkinson 2008), urine samples were subjected to enzymatic hydrolysis with glucuronidase/sulfatase activity to perform hydrolysis of conjugated metabolites of EDP. For this, the conditions set by Alvarez-Sánchez and colleagues (Álvarez-Sánchez et al. 2009) as described in Sect. 7.2.3.2 were basically used. However, the ultrasonic irradiation time was extended from 30 min to 50 min to improve the deconjugation efficiency from 60 to 90 %, approximately, with respect to the efficacy obtained by conventional hydrolysis treatment (12 h at 37 °C) of the conjugated metabolites of EDP (see Fig. 7.4). Taking into account this modification, both hydrolytic treatments may be considered comparable.

The notable reduction of time required for the deconjugation process in comparison to traditional methods based on long hours of enzymatic incubation, and the operational simplicity of the process justified the use of sonication.
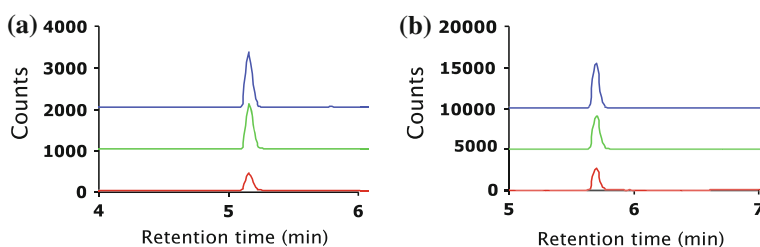
Fig. 7.4 SRM chromatograms for MMP (**a**) and DMP (**b**) obtained by analyzing a urine sample from a volunteer who had applied a cosmetic formulation containing EDP treated by conventional enizmatic hydrolysis overnight at 37 °C (*blue line*) and treated by ultrasonic assisted enizmatic hydrolysis for 50 min (*green line*) and 10 min (*red line*) at 37 °C. Experimental conditions are described in Sect. 7.2.3.2
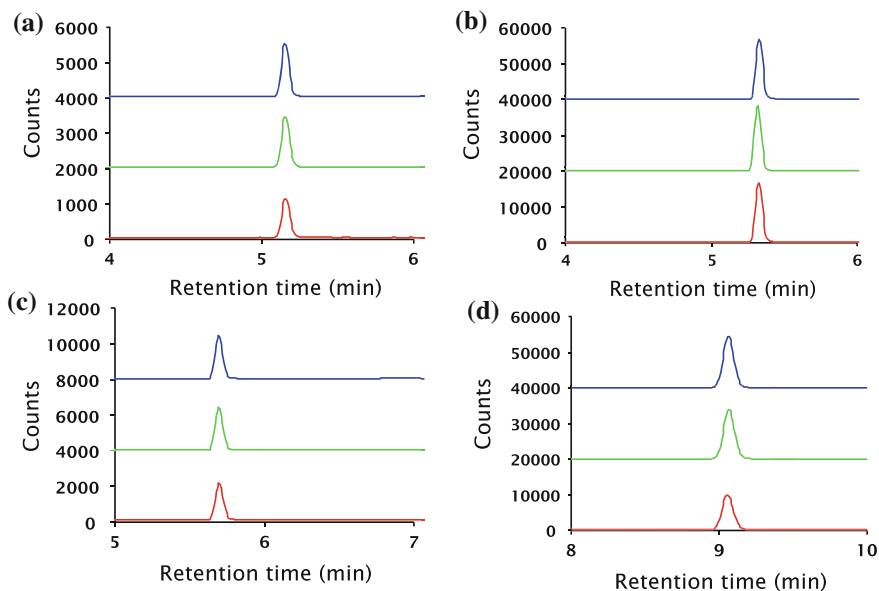
Fig. 7.5 SRM chromatograms for MMP (**a**), THA (**b**), DMP (**c**) and EDP (**d**) obtained by analyzing a urine sample fortified at 50 ng mL^{-1} at time zero (*blue line*), at 72 h and stored at 4 °C (*green line*) and at 80 h and stored at 4 °C (*red line*). Experimental conditions are described in Sect. 7.2.3

7.3.1.4 Study of Sample Stability

The stability of EDP and its metabolites in human urine samples were studied at 4 °C. As can be seen in Fig. 7.5, the reproducibility of the analysis at time zero (blue line) and at 72 h (green line) indicated the stability of the analytes at least during this period of time, under the study conditions. After this time, however, a slight instability of the analytes was exhibited in urine samples, particularly for EDP. Therefore, urine samples were kept stored at 4 °C and analyzed always within 72 h from collection.

Additionally, the analytes were stable during the time that urine samples remained in the autosampler. Only special care was required with the 1 M citric acid/citrate buffer solution, which had to be prepared fresh approximately every 4 h.

7.3.2 Validation of the Analytical Method: Study of the Interferences

The matrix effects associated to the automated determination of EDP and its metabolites in urine samples by SPE-LC-MS/MS was evaluated comparing the

Table 7.4 Comparison of the calibrates obtained from a single sample of urine or a pool of several urine samples for EDP, DMP and MMP

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^{f}
		Single[d]	Pool[d]		
MMP	a[a]	0.000 ± 0.002	0.01 ± 0.01		
	b (mL μg^{-1})[b]	0.00089 ± 0.00003	0.0038 ± 0.0001	23	44.2
	R^{2c}	0.996	0.995		
DMP	a[a]	0.000 ± 0.001	0.00 ± 0.01		
	b (mL μg^{-1})[b]	0.00164 ± 0.00002	0.0060 ± 0.0002	27	58.7
	R^{2c}	0.9993	0.997		
EDP	a[a]	0.06 ± 0.05	0.2 ± 0.2		
	b (mL μg^{-1})[b]	0.0241 ± 0.0009	0.126 ± 0.003	19	63.5
	R^{2c}	0.995	0.997		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the obtained slopes by both calibrates
[f] Statistical $t_{tab(0.05(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

obtained responses from two calibrates prepared in two urine samples containing no analyte. One urine sample was obtained from a single volunteer (*single urine calibrate*) and the other was prepared by mixing urine samples from several volunteers (*pool urine calibrate*).

The slopes of both calibrates were statistically compared using a Student's *t* test (see Appendix III.4) as shown in Table 7.4. Statistically different slopes were obtained ($P_0 = 0.05$) between the two calibrates for all analytes. Therefore, to correct the errors caused by matrix interferences and to measure accurately the analyte levels in urine samples, the standard addition calibration was used.

7.3.2.1 Accuracy

The accuracy was evaluated applying the proposed method to the analysis of urine samples containing no analytes from different volunteers who had not applied any cosmetic product containing EDP. Samples were fortified with known amounts of EDP, DMP and MMP.

Table 7.5 shows the results obtained for each sample. The standard deviation was calculated as the error of the extrapolated value in the standard addition calibration curve (Miller and Miller 2005).

The Student's *t*-test confirmed the absence of significant differences between the found concentrations by the described method and the fortified concentrations (see Annex III.3), thus showing the accuracy of the analytical method.

Table 7.5 Determination of MMP, DMP and EDP in fortified urine samples

Analyte	Parameter	Urine samples				
		1	2	3	4	5
MMP	μ^a	12	40	52	110	120
	$C \pm s^b$	11 ± 1	40 ± 2	56 ± 4	108 ± 8	115 ± 7
	t_{cal}^c	2.33	0.46	2.17	0.44	1.54
DMP	μ^a	13	36	50	107	116
	$C \pm s^b$	13 ± 1	33 ± 2	47 ± 4	111 ± 6	111 ± 8
	t_{cal}^c	0.05	2.71	1.99	1.49	1.24
EDP	μ^a	11	38	52	103	113
	$C \pm s^b$	10 ± 1	35 ± 3	51 ± 1	102 ± 4	107 ± 5
	t_{cal}^c	3.04	2.18	1.93	0.80	2.73

[a] Fortified concentration (ng mL^{-1})
[b] Found concentration (ng mL^{-1}) by standard addition calibration
[c] Statistical $t_{tab(0.05, N-2=3)} = 3.18$

7.3.2.2 Other Analytical Parameters

The calibrate curves (N = 5) exhibited excellent linearity for EDP, DMP and MMP with regression coefficients greater than 0.995 over the range of concentration between 20 and 100 ng mL^{-1} in all cases.

The limits of detection (LOD) and quantification (LOQ) were estimated based on the International Conference on Harmonization on validation of analytical procedures (see Table 7.6).[3] Notably low on column LOD values were obtained (i.e., from 0.1 to 1.1 ng). Nevertheless, in the case that lower LOD could be required, that would be possible by increasing the sample volume.

The intra-day variability (repeatability) was assessed by analysing five different urine solutions containing no analytes that were fortified (50 ng mL^{-1}) in the same batch. The inter-day variability (repeatability) was determined by analysing a single urine solution containing no analytes that was fortified (50 ng mL^{-1}) in five different batches. The intra- and inter-day variability, expressed as relative standard deviation (RSD) ranged from 1.5 to 8.7 % and from 3.8 to 11.2 %, respectively, depending on the analyte (see Table 7.7).

7.3.3 Elucidation of the In Vivo Phase II Metabolism of Ethylhexyl Dimethyl PABA

First, the described analytical methodology without including the enzymatic hydrolysis treatment was applied to a urine sample from a volunteer who had applied a sunscreen cosmetic product containing EDP. Under these conditions, the

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[3] ICH validation of analytical procedures methodology: text and methodology Q2(R1), ICH harmonised tripartite guidelines, Adopted November (2005).

Table 7.6 Limits of detection (LOD) and quantitation (LOQ) of the developed methodology to determine EDP, DMP and MMP in urine

Sample[a]	MMP		DMP		EDP	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	0.2	0.6	0.3	1.1	0.3	0.9
2	0.6	2.1	0.5	1.5	0.5	1.5
3	0.1	0.3	0.7	2.3	0.7	2.2
4	0.5	1.6	0.7	2.5	1.1	3.5
5	0.5	1.8	0.9	3.1	0.7	2.2

[a] Urine samples from different volunteers
[b] Estimated in the urine sample, taking into account the dilution factor during the pretreatment of the sample as $3.3 \cdot s_a / b$, where b is the slope of the standard addition calibration curve and s_a is the standard deviation value of the intercept calibrate. Valued on column, expresed as ng, being 100 µL the injection volume
[c] Estimated in the urine sample as $10 \cdot s_a / b$ (see annotation b)

Table 7.7 Precision parameters for the determination of MMP, DMP and EDP in urine samples

Analyte	Parameter	
	Repeatability (%)[a]	Reproducibility (%)[a]
MMP	8.7	11.2
DMP	4.7	5.6
EDP	1.5	3.8

[a] Expressed as RSD ($N = 5$)

in vitro phase I metabolites of EDP, DMP and MMP, were not detected. However, a chromatographic peak was observed in the SRM transition of DMP, but at 4.9 min instead of 5.7 min, that is the retention time of DMP. From this fact, the idea about the possibility of *in vivo* phase II biotransformation mechanisms to detoxify EDP was taken into account. The observed chromatographic peak exhibited a fragmentation mechanism similar to DMP and therefore, it could be assigned as a conjugated form.

Secondly, different QqQ monitoring modes were evaluated to identify the metabolites of EDP. Hence, the ion precursor (IP) scan mode was selected to monitor any compound having an ion precursor that, after fragmentation, generates product ions with a mass-to-charge ratio 165.8 and 151.8 *m/z*, corresponding to the protonated molecular masses of DMP and MMP, respectively. This approach showed that the glucuronide conjugate of DMP (DMP-Glu, 341.8 (*m/z*)) fragmented to give the product ion 165.8 (*m/z*), which was previously observed at 4.9 min (see Fig. 7.6a).

To carry out the confirmation of the presence of the glucuronide conjugates of EDP, other QqQ monitoring mode, named neutral loss (NL), was used to monitor any compound whose fragment lost after MS/MS activation was 176.0 (*m/z*), which corresponds to the characteristic loss of neutral fragment of a glucuronide
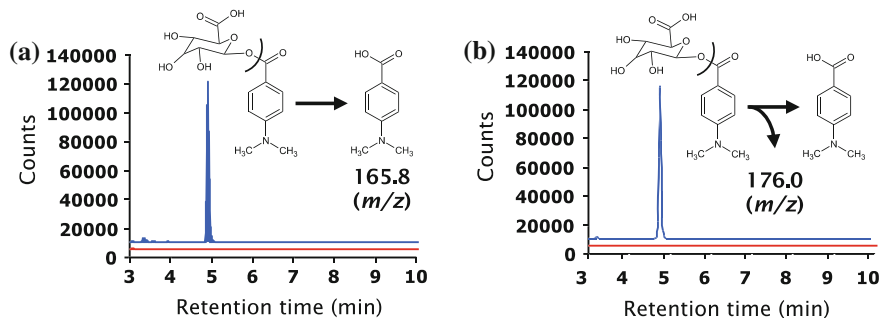
Fig. 7.6 Chromatograms obtained by precursor ion (**a**) and neutral loss (**b**) monitoring when analyzing a urine sample containing no analyte (*red line*) and a urine sample from a volunteer who had applied a cosmetic containing EDP (*blue line*). Experimental conditions are described in Sect. 7.2.3

conjugate (Levsen et al. 2005). The results shown in Fig. 7.6b confirm the contribution of the *in vivo* phase II metabolism in the detoxification of EDP.

Next, the same urine sample was analyzed with the proposed methodology, using the SRM monitoring mode and including the enzymatic hydrolysis treatment with the ß-glucuronidase/sulfatase solution. As can be seen in Fig. 7.7, both MMP and DMP were effectively detected. Comparing the urine sample treated (blue line) and not treated (red line) to the ultrasonic assisted enzymatic hydrolysis treatment, the signal of DMP-Glu at 4.9 min can be seen (red line). It should be also noted that, although the presence of DMP was expected in the urine sample accordingly to the observation of DMP-Glu, the presence of MMP was not expected.

To confirm the presence of the glucuronide conjugate of MMP (MMP-Glu), experiments with a high resolution mass spectrometry analyzer were carried out. For this purpose, urine samples were collected from the same volunteer at two different times (i.e., before applying a sunscreen cosmetic product containing EDP
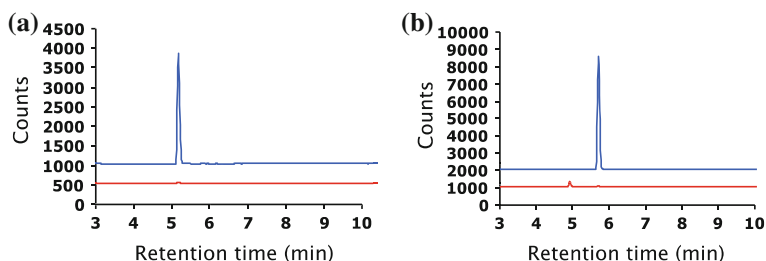
Fig. 7.7 SRM chromatograms for MMP (**a**) and DMP (**b**) obtained by analyzing a urine sample from a volunteer who had applied a cosmetic product containing EDP, after subjecting the urine to ultrasonic assisted enzymatic hydrolysis treatment (*blue line*) and without subjecting any hydrolytic treatment (*red line*). Experimental conditions are described in Sect. 7.2.3

(*blank sample*) and 24 h after the sunscreen application (*sample*)). Each urine sample was divided into two fractions to which 100 μL of a 1 M ammonium acetate (pH 5) solution or 100 μL of the ß-glucuronidase/sulfatase solution was added. Then, all these solutions were subjected to the ultrasound assisted enzymatic hydrolysis procedure and injected into the LC-TOF-MS system, without carrying out the SPE procedure.

Data processing consisted on the extraction of the molecular features of the two *blank sample* solutions (enzyme-treated or not). A common organic molecules isotopic model was used with a tolerance of 0.0025 peak area (*m/z*). After subtracting the list of compounds extracted from the analysis of the *blank sample* solutions (enzyme-treated or not) to the data files obtained by analyzing the two *sample* solutions (enzyme-treated or not), the identification of the resulting compounds was carried out by means of the generation of candidate formulas with a mass accuracy limit of 5 ppm. The contribution to the mass accuracy and the isotopic abundance and separation were 100, 60 and 50 ppm, respectively. Retention times, formulas, experimental and theoretical masses, and errors (expressed in ppm) of the urinary metabolites of EDP were obtained by accurate mass measurements (see Table 7.8).

The lower retention times of the glucuronide conjugates in comparison to the unconjugated metabolites agree with the higher hydrophilicity of the phase II metabolites and the reverse phase chromatographic mode employed. As shown in Fig. 7.8, DMP and MMP were detected in the sample treated with ß-glucuronidase, while MMP-Glu and DMP-Glu were identified in the enzymatically untreated sample.

A possible explanation of the observed facts is based on the hydrophilicity of the EDP metabolites. Probably, the most hydrophilic metabolite, MMP-Glu, was not retained in the C18 HD SPE cartridges during the automated SPE procedure of urine samples and then, it could not be detected with the triple quadrupole under these conditions. However, as the described method was aimed to determine the unconjugated forms of DMP and MMP, the poor SPE extraction of the conjugated metabolite MMP-Glu in the C18 HD cartridges was not an inconvenience, due to the ultrasonic assisted enzymatic hydrolysis treatment carried out that ensured the quantitative deconjugation of the glucuronide forms.

Table 7.8 Accurate mass measurements obtained by LC-TOF-MS analysis to identify EDP metabolites in human urine

Parameter	Compound			
	MMP-Glu	DMP-Glu	MMP	DMP
Retention time (min)	3.90	4.96	5.34	5.85
Molecular ion	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$
Real *m/z* value	328.1039	342.1199	152.0708	166.0869
Empirical formulae	$C_{14}H_{17}NO_8$	$C_{15}H_{19}NO_8$	$C_8H_9NO_2$	$C_9H_{11}NO_2$
Experimental *m/z* value	328.1027	342.1183	152.0706	166.0863
Error (ppm)	3.7	4.7	1.3	3.6
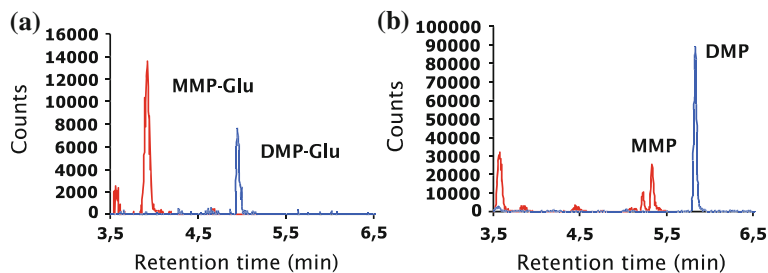
Fig. 7.8 Extracted ion chromatograms (EIC) for accurate mass measurements obtained from LC-TOF-MS analysis for identification of EDP metabolites in urine from a volunteer who had applied a cosmetic product containing EDP without performing any enzymatic treatment (**a**) or after treatment by ultrasound assisted enzymatic hydrolysis (**b**). Experimental conditions are described in Sect. 7.2.3 and the details of the measurements are shown in Table 7.7

Other phase II biotransformation reactions, such as acetylation and conjugation with glycine reactions, which are very well established for a structurally similar compound to EDP and its metabolites [i.e., *p*-aminobenzoic acid (PABA) (Gusson et al. 2006)] were also considered. However, no more phase II metabolites of EDP were identified with none of the mass analyzers employed. This *in vivo* information confirms the *in vitro* biotransformation mechanism of EDP previously studied by our research group with rat microsomes (León et al. 2010b) and described in Chap. 6. Based on the consulted literature, this is the first study that confirms the presence of EDP metabolites in human urine samples.

7.3.4 Application of the Analytical Method

The described analytical method was applied to the determination of EDP and its metabolites in urine from 4 volunteers, 2 men and 2 women, which had applied an amount of 13 g of a sunscreen cosmetic product prepared in the laboratory according to an adapted protocol (Jordán and Jordán 1991) containing 8 % of EDP (see Annex II.4). The applied doses are in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007).

Urine samples were collected immediately before and after the application of the sunscreen cosmetic product. For samples collected after application, precise instructions were given to the volunteers to collect the total volume of urine excretions into 24 h sterile commercially available containers. During the study, which lasted four days, the urine samples were stored at 4 °C until analysis, which always was within 72 h after collection. In addition, urine samples were collected on the eighth day after the application of the cosmetic product to verify the persistence of EDP and/or its metabolites.

   After measuring the total volume of urine collected daily by each volunteer, urine from men and from women were mixed separately, and stored at 4 °C. At the time of analysis, an aliquot of each urine samples mixture (male and female) was divided into two fractions.

   As noted in Sect. 7.2.3, the standard addition calibration was used by adding 100 μL of a 1 M ammonium acetate (pH 5) solution or 100 μL of a ß-glucuronidase (prepared in a 1 M ammonium acetate (pH 5) solution) solution to each urine fraction. In this way, the unconjugated analyte content and both conjugated and unconjugated content (total content of analyte) were determined.

   As can be seen in Table 7.9, EDP was not found in any of the urine samples. Furthermore, the contribution of the unconjugated content of the phase I metabolites, DMP and MMP, to the total content was very low, as DMP and MMP were mainly conjugated to the glucuronic acid (DMP-Glu and MMP-Glu). In fact, unconjugated DMP was only detected in the urine sample collected from the male volunteers 24 h after the application of the cosmetic product. It was also detected in the urine sample collected from the female volunteers the second day.

   The higher concentrations of MMP and DMP to both men and women were found in the urine samples collected 48 h after the application of the sunscreen cosmetic product. Subsequently, the contents of the EDP metabolites decreased gradually in urine. However, both compounds were still detected in urine samples collected 8 days after the application of the cosmetic.

   Based on the total contribution of DMP and MMP to the systemic disposition processes of EDP, which include percutaneous absorption, distribution,

Table 7.9 Excreted amounts of DMP and MMP by urine from volunteers that applied once a cosmetic product containing EDP

Days	Excreted mass DMP (μg)[c]		Excreted mass MMP (μg)[c]	
	Men	Women	Men	Women
0[a]	N.D.[d]	N.D.	N.D.	N.D.
0[b]	N.D.	N.D.	N.D.	N.D.
1[a]	17 ± 1	N.D.	N.D.	N.D.
1[b]	970 ± 15	1040 ± 15	440 ± 11	160 ± 4
2[a]	N.D.	46 ± 2	N.D.	N.D.
2[b]	1380 ± 50	1000 ± 14	680 ± 16	500 ± 20
3[a]	N.D.	N.D.	N.D.	N.D.
3[b]	690 ± 19	240 ± 5	330 ± 10	140 ± 5
4[a]	N.D.	N.D.	N.D.	N.D.
4[b]	140 ± 8	61 ± 5	129 ± 3	122 ± 3
8[a]	N.D.	N.D.	N.D.	N.D.
8[b]	51 ± 5	116 ± 7	N.D.	46 ± 4

[a] Not applying the enzymatic hydrolysis treatment
[b] Applying the enzymatic hydrolysis treatment
[c] The results are expressed in terms of absolute excreted mass considering the dilution factor corresponding to the total volume of the collected sample
[d] N.D., not detected

biotransformation and excretion, the estimated levels excreted by urine were approximately 0.5 % (males) and 0.4 % (women) of the total applied EDP, predominantly as glucuronide conjugates of DMP and MMP.

7.4 Conclusions

An analytical method based on automated SPE coupled to LC-MS/MS to determine EDP and its phase I metabolites (DMP and MMP) in human urine has been developed. The described method requires minimal human intervention and is noted for its high simplicity, selectivity and sensitivity. Ultrasonic assisted enzymatic hydrolysis was applied to deconjugate the glucuronide phase II metabolites of EDP.

The analytical procedure has been validated and statistically accurate results were obtained using the standard addition calibration to correct quantitatively the errors caused by the observed matrix effect.

The described method was applied to determine the analytes in urine from men and women volunteers who had applied a sunscreen cosmetic product containing EDP. Only DMP and MMP were found in urine, mainly in the form of glucuronide conjugates, which were characterized by LC-TOF-MS analysis based on accurate mass measurements and confirmed by LC-QqQ-MS analysis.

The results of the *in vivo* systemic disposition of EDP after a single application of a cosmetic product containing the UV filter revealed that around 0.5 % of the applied amount was excreted by urine.

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Chapter 8
Determination of Ethylhexyl Dimethyl PABA and its Metabolites in Semen by Automated Solid-Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

8.1 Introduction

8.1.1 Aim of the Study

Due to the hydrophobicity of EDP, this UV filter can undergo presumably bioaccumulation in humans and stay in the body longer than other hydrophilic xenobiotics, thus being metabolized and/or excreted. This hypothesis is evident from the results obtained in the *in vivo* study regarding the determination of the excreted levels in urine from volunteers that had applied a sunscreen cosmetic product containing EDP (see Chap. 7). In this study, the metabolites of EDP were detected in urine samples collected 8 days after a single application of the cosmetic product (León-González et al. 2011).

The development of long-term toxicological studies is particularly important because of the currently tendency of the cosmetic sector about the inclusión of UV filters not only in sunscreen cosmetic products, but also in other daily products, such as face creams, aftershave products, etc.

This study aims to develop and validate an analytical method to determine EDP and its metabolites in human semen using an automated methodology based on the application of a solid-phase extraction (SPE) procedure prior to liquid chromatography tandem mass spectrometry (LC–MS/MS) to evidence bioaccumulation and provide a basis for assessing the toxicity of this widely used UV filter.

8.1.2 Background and Current Status of the Issue

According to the literature consulted, there was no precedent for the determination of UV filters in human semen, except benzophenone-3, whose determination was carried out using the analytical method described in Chap. 5 of this PhD Thesis (León et al. 2010). The analytical method was based on a SPE procedure and subsequent LC–MS/MS analysis, and allowed simultaneously the determination of the parent compound and its metabolites at the ng mL^{-1} level.

8.2 Experimental

8.2.1 Reagents and Samples

Ethylhexyl dimethyl PABA (EDP), also called 2-ethylhexyl 4-(N,N-dimethyl-amino) benzoate, 98 % from Aldrich (Milwaukee) and 4-(N,N-dimethylamino) benzoic acid (DMP) 98 % and 4-(N-methylamino) benzoic acid (MMP) 97 %, both from Sigma-Aldrich (Schnelldorf) were used as analytical standards. Trihexylamine (THA) from Sigma-Aldrich was used as internal standard.

The solvents used were LC grade methanol (MeOH) and LC grade acetonitrile (ACN), both from Scharlab (Barcelona). Deionized water was obtained from a Milli-Q water purification system (R ≥ 18 mΩ cm) from Millipore (Billerica).

Other reagents used were 85 % (m/v) ortho-phosphoric acid (d = 1,710 g mL^{-1}) from Merck (Darmstadt), ammonium acetate from Sigma (St. Louis), citric acid from Panreac (Barcelona) and formic acid (FA) and 32 % (m/v) ammonium hydroxide (d = 0.910 g mL^{-1}) from Scharlab (Barcelona). ß-glucuronidase enzyme from *Helix pomatia* (type H-1) with activity ≥300,000 U g^{-1} and sulfatase activity ≥10,000 U g^{-1} from Sigma-Aldrich was also used.

Semen samples used to develop and validate the analytical method were obtained from healthy male volunteers who had not applied any cosmetic product containing EDP, according to an official protocol of the World Health Organization for the examination and processing of human semen (WHO Laboratory Manual 2010).

8.2.2 Instruments and Material

An automated SPE-LC–MS/MS system consisted of a Prospekt-2® SPE platform from Spark Holland (Emmen) coupled to an 1200 Series LC System® from Agilent Technologies (Palo Alto) that was connected to a 6460 LC/MS® Triple Quad mass spectrometry detector equipped with a Jet Stream® electrospray ionization source, also from Agilent, were used. The automated Prospekt-2® SPE

platform consisted of an automatic cartridge exchanger (ACE) and a high pressure dispenser (HPD) syringe to distribute the SPE solvents. ACE and HPD were connected to a Midas® autosampler from Spark Holland equipped with a 100 μL loop. 0.25 mm internal diameter PEEK (polyether ether ketone) tube from VICI (Houston) was employed for all connections between valves. The automation of the extraction step was performed using the Sparklink V. 2.10 software.

C18-HD (high density phase of octadecyl chains based on silica, particle size 7 μm) HySphere® SPE cartridges (10 mm long and 2 mm internal diameter) from Spark Holland were used.

A C18 Mediterranean Sea® analytical column (3 μm particle size, 150 mm long, 4.6 mm internal diameter) from Teknokroma (Barcelona) was used. Moreover, a high purity generator from CLAN Technology (Sevilla) provided nitrogen gas used in the ionization source. As collision gas, 99.999 % nitrogen from Air Products (Córdoba) was used.

Data acquisition, control of the SPE analysis sequences and development of qualitative and quantitative analyses were carried out with Agilent MassHunter Workstation software.

Finally, a 2001 MicropH® pH meter from Crison (Alella), Eppendorf® tubes (2.5 mL) from Nirco (Granada), a Sorvall Legend Micro 21R® centrifuge from Thermo Scientific (Barcelona), a thermostated water bath from Selecta (Barcelona) and a 450 digital sonicator (20 kHz, 450 W) from Branson Ultrasonics Corporation (Danbury) with adjustable amplitude and duty cycle, equipped with a cylindrical probe of titanium alloy and 12.7 mm in diameter were also used.

8.2.3 Analytical Method to Determine Ethylhexyl Dimethyl PABA and its Metabolites in Semen

8.2.3.1 Preparation of Solutions

2 mL of semen was mixed in an Eppendorf® tube with 0.3 mL of 1 M orthophosphoric acid to denature the proteins present in the biological matrix. Then, the mixture was centrifuged at 10,000 rpm for 10 min at 5 °C. After discarding the pellet, the pH of the supernatant was adjusted to 4.5 or 5 considered with diluted ammonium hydroxide if needed.

Separate analytes standard (EDP, DMP, MMP) and internal standard (THA) solutions in MeOH (200 μg mL^{-1}) were prepared and stored at 4 °C. From these stock solutions, a solution of THA (0.5 μg mL^{-1}) in deionized water and a solution of EDP, DMP and MMP (2 μg mL^{-1}) in the semen supernatant to be analyzed were prepared.

To correct the error caused by the matrix effects (see Sect. 8.3.2), the standard addition calibration was employed. The calibrate standard solutions were prepared by fortifying five aliquots of semen supernatant (270 μL) containing 0, 20, 40, 60

and 80 μL of the analytes solution (0.5 μg mL^{-1}) to which 100, 80, 60, 40 or 20 μL of semen supernatant was added, respectively, to fix the same final volume in all the calibrate solutions. Furthermore, a volume of 50 μL of THA (2 μg mL^{-1}) was added to each calibrate solution.

8.2.3.2 Ultrasonic Assisted Enzymatic Hydrolysis

0.01 g of enzyme was added to a 5 mL of 1 M ammonium acetate (pH 5), solution which was stored at 4 °C.

To determine the total content of the analytes (i.e., both unconjugated and conjugated forms), 100 μL of the ß-glucuronidase solution was added to each calibrate solutions. Next, these solutions were subjected to an enzymatic hydrolysis treatment assisted by ultrasound to deconjugate the possible glucuronide adducts of the phase I metabolites of EDP. Thus, the calibrate solutions were immersed in a thermostated water bath (37 °C) together with the probe tip, which was located at the same distance from each solution (5 cm) and at a fixed distance from the bath background (5 cm). Hydrolysis of conjugated metabolites of EDP was conducted essentially under the conditions previously obtained and described in Sect. 7.2.3.2. Thus, ultrasonic radiation was applied for 50 min at 35 % duty cycle and 50 % amplitude (converted applied power, 400 W).

8.2.3.3 Automated Solid-Phase Extraction

From the final volume of each semen sample (0.5 mL), an aliquot of 250 μL was mixed with 50 μL of a 1 M citric acid/citrate buffer solution, adjusted to pH 3 with ammonium hydroxide and the mixture was homogenized prior to performing the automated SPE procedure.

Selected SPE cartridges (C18 HD) were automatically solvated with 2 mL of MeOH at 5 mL min^{-1}, conditioned with 2 mL of deionized water at 5 mL min^{-1} and equilibrated with 1 mL of deionized water at 0.5 mL min^{-1}. The cartridges were then loaded with 100 μL of semen solution by propelling 2 mL of deionized water at 0.5 mL min^{-1}. Next, the SPE cartridge was washed with 1 mL of an aqueous methanol 5 % (v/v) solution at 0.5 mL min^{-1}. Finally, the analytes were eluted by flowing the LC mobile phase through the SPE cartridges for 4.5 min. The eluate from each SPE cartridge was chromatographically separated in the analytical column prior to the detection with the mass spectrometer. Then, the cartridges were washed with 2 mL of MeOH at 5 mL min^{-1} and 2 mL of deionized water at 5 mL min^{-1} for further extractions. The sequence of operations of the automated SPE procedure is summarized in Table 8.1.

Table 8.1 Main features of the automated SPE process

Process	Solution	Volume (mL)	Flow (mL min^{-1})	Comment
Placing new cartridge				C18 HD
Autosampler starting				Sample loading (100 μL)
Solvatation step	MeOH	2	5	
Conditioning step 1	Deionized water	2	5	
Conditioning step 2	Deionized water	1	0.5	
Sample injection	Deionized water	2	0.5	Sample injection (100 μL)
Washing step	Deionized water:MeOH (95:5, *v/v*)	1	0.5	
Elution step	LC mobile phase	3.6	0.8	Along 4.5 min
Post-extraction washing 1	MeOH	2	5	
Post-extraction washing 2	Deionized water	2	5	

8.2.3.4 LC–MS/MS Analysis

The chromatographic separation of analytes was carried out using a mobile phase consisting of deionized water and MeOH:ACN (1:1, *v/v*), both containing 0.2 % FA. The elution gradient outlined in Table 8.2 was used at a flow rate of 0.8 mL min^{-1} and at 20 °C. The analytical column was equilibrated with a post-analysis time of 5 min.

The conditions of the mass spectrometer analyzer and spray chamber were: temperature of gas ionization source, 350 °C; drying gas flow, 10 L min^{-1}; nebulizer gas pressure, 35 psi; nebulizer gas temperature, 380 °C; nebulizer gas flow, 10 L min^{-1}; capillary voltage, 4450 V. The dwell time was set to 70 ms.

The analytes were determined by selected reaction monitoring (SRM) in positive electrospray ionization (ESI$^+$) mode. The selected confirmation and quantification SRM transitions are shown in Table 8.3.

Calibration curves were obtained by representing the peak area ratio of each analyte to the internal standard (THA) versus the added concentration of analyte.

Table 8.2 Elution gradient of the LC–MS analysis to determine EDP and its metabolites in semen

Time (min)	0.2 % FA in deionized water (%)	0.2 % FA in MeOH:ACN (1:1, *v/v*) (%)
0	60	40
1	60	40
3	0	100
15	0	100

Table 8.3 SRM parameters in positive electrospray ionisation mode to determine EDP and its metabolites in semen

Compound	Parameter			
	First quad voltage (V)	Collision energy (eV)	SRM transition to quantify	SRM transition to confirm
MMP	90	15	$151.8 \rightarrow 136.8$	$151.8 \rightarrow 119.9$
DMP	90	25	$165.8 \rightarrow 149.8$	$165.8 \rightarrow 133.9$
EDP	100	15	$277.8 \rightarrow 165.8$	$277.8 \rightarrow 150.8$
THA[a]	120	20	$270.1 \rightarrow 185.8$	$270.1 \rightarrow 102.0$

[a] Internal standard

Figure 8.1 shows the SRM chromatograms obtained for each analyte in semen samples containing no analyte that were either fortified (40 ng mL^{-1}; blue line) and unfortified (red line) subjected to the analytical procedure described above.

8.3 Results and Discussion

8.3.1 Study of the Experimental Variables

Most of the conditions used in the automated analytical platform were adapted from those described in Chap. 7. Thus, C18 HD cartridges were selected for subsequent experiments, as they provided the best results for these analytes in the case of urine samples analysis.

As the mechanism of extraction was based on the interaction of analytes with the C18 HD sorbent, semen samples were fortified (1 μg mL^{-1}) and acidified to pH 3 with a 1 M citric acid/citrate buffer solution (pH 3) before the automated SPE procedure. The analytes were stable during the time that the samples remained in the autosampler prior to the analysis. No precipitation of the semen samples despite remaining at pH 3 was observed during the complete sequence of analysis. Acceptable variability was obtained [12.6 % for MMP, 5.7 % for DMP and 10.1 % for EDP, expressed as relative standard deviation (RSD)] for at least 4 h for all analytes.

The influence of the loading volume was examined in the range from 0.5 to 5 mL. A volume of 2 mL of deionized water was selected because larger volumes caused the partial elution of MMP and lower volumes did not allow the loading properly. Similarly, different loading flows between 0.25 and 1.5 mL min^{-1} were examined and the best yields were obtained using a flow rate of 0.5 mL min^{-1}.

To eliminate potential polar interferents present in semen samples, deionized water and aqueous solutions of MeOH (2.5 % and 5 % (v/v)) were evaluated as SPE washing solutions. 1 mL of an aqueous solution of MeOH (5 % (v/v)) was selected as washing condition because no losses were observed when the analyte responses were compared to those obtained in the absence of the washing step.
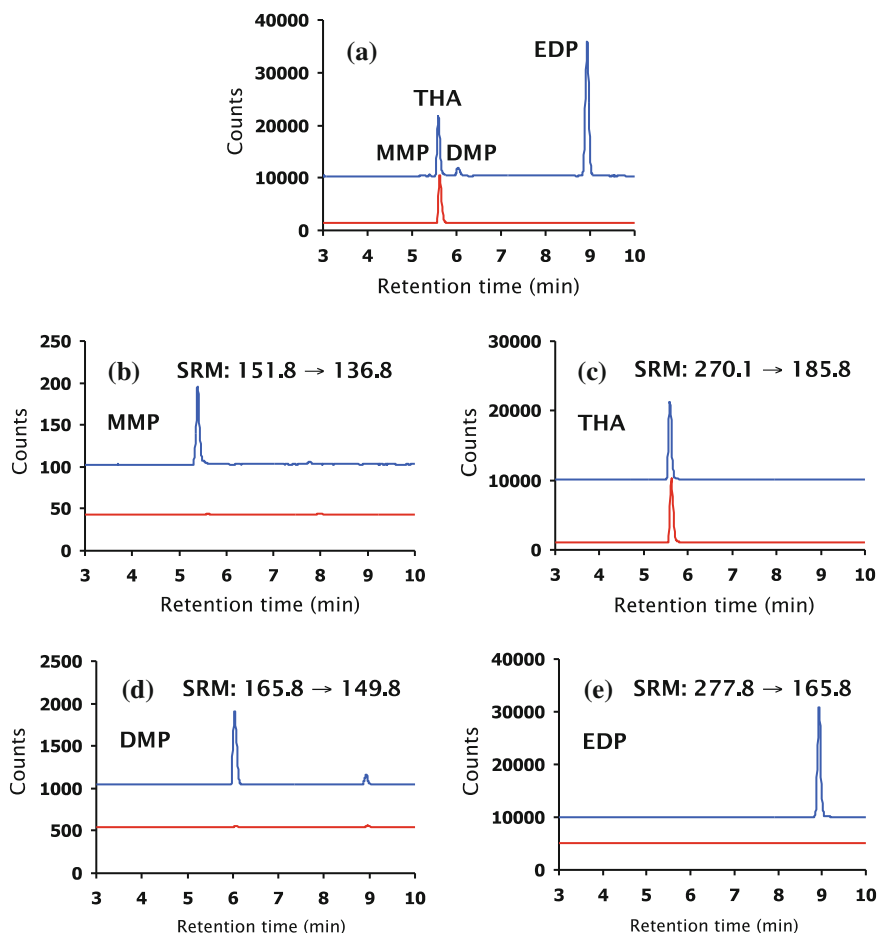
Fig. 8.1 Total ion chromatograms (TIC) in positive ionization mode for each compound of interest (**a**) and selected reaction monitoring (SRM) chromatograms for MMP (**b**), THA (**c**), DMP (**d**) and EDP (**e**) obtained by analyzing a semen sample containing no compounds (Red line) and the same semen sample fortified at a concentration of 50 ng mL^{-1} (Blue line). The internal standard (THA) was included in both types of samples. Experimental conditions are described in Sect. 8.2.3

Analogously, different loading flows were examined and the best yields were obtained using a flow rate of 0.5 mL min^{-1}, as higher flow rates caused the partial elution of the analytes.

The retained analytes were eluted by flowing the LC mobile phase through the SPE sorbent for 4.5 min. Longer elution times allowed the elution of interferents causing ion suppression in the ionization source, while shorter elution times did not provide the quantitative elution of the analytes.

To measure the retention capacity of the analytes in the C18 HD sorbents, breakthrough process was evaluated using a dual cartridge configuration in the

SPE automated platform (Prospekt Technical Note 1996). In this way, two C18 HD cartridges were connected in series to retain in the second cartridge the analytes that have not been retained in the first cartridge during the loading step.

Breakthrough was studied in triplicate analyzing urine samples fortified at three levels of concentration (20, 100 and 500 ng mL^{-1}). Retention efficiency was calculated as the ratio between the analyte response in the first cartridge and the combined response in both cartridges. The experiments conducted under the selected experimental conditions showed that no breakthrough was produced for any analyte at any concentration tested (extraction efficiencies above 95 % in all cases).

Moreover, the cartridges could be reused up to 3 times without causing a significant loss of extraction efficiency (<5 %).

8.3.2 Validation of the Analytical Method: Study of the Interferences

To estimate the influence of the matrix sample on the determination of EDP and its metabolites, the responses obtained from a set of standard solutions prepared in a semen sample from a single volunteer containing no analyte (*single semen calibrate*) were compared to those obtained from a set of standard solutions prepared in a pool of semen samples from 5 volunteers containing no analyte (*pooled semen calibrate*) (see Table 8.4).

Table 8.4 Comparison of EDP, DMP and MMP calibrates obtained from single and pooled semen samples

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}^{f}
		Single[d]	Pooled[d]		
MMP	a[a]	0.01 ± 0.07	−0.03 ± 0.05		0.2
	b (mL μg^{-1})[b]	53 ± 2	56 ± 1	95	1.2
	R^{2}[c]	0.995	0.998		
DMP	a[a]	0.000 ± 0.001	0.0002 ± 0.0006		0.5
	b (mL μg^{-1})[b]	1.97 ± 0.02	1.57 ± 0.02	125	14.9
	R^{2}[c]	0.9995	0.9996		
EDP	a[a]	0.0000 ± 0.0001	0.0000 ± 0.0001		0.9
	b (mL μg^{-1})[b]	0.164 ± 0.003	0.139 ± 0.004	118	5.9
	R^{2}[c]	0.998	0.997		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the slopes ontained using both calibrates
[f] Statistical $t_{tab(0.05,(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

The slopes and intercepts of both calibrates were statistically compared using a Student's *t* test (see Annex III.4). The intercepts were statistically comparable to zero (at a significance level of 5 %), showing the absence of constant errors. However, the slopes were statistically different at the same level of significance when comparing both calibrates in the cases of DMP and MMP. The t_{cal} statistical values were higher than the t_{tab} values, thus showing a matrix dependency. Therefore, standard addition calibration was used to correct the proportional errors caused by matrix interference.

8.3.2.1 Accuracy

The accuracy was evaluated by applying the SPE-LC–MS/MS method to the analysis of semen samples from different volunteers who had not applied any cosmetic product containing EDP that were fortified with known amounts of EDP, DMP and MMP.

The results obtained for each sample are shown in Table 8.5. The standard deviation was calculated as the error of the extrapolated value in the standard addition curve (Miller and Miller 2005).

The Student's *t*-test confirmed the absence of significant differences between the found concentrations and the fortified concentrations (see Annex III.3), thus showing the accuracy of the proposed methodology.

8.3.2.2 Other Analytical Parameters

Calibration curves (N = 5) exhibited excellent linearity for EDP, DMP and MMP with regression coefficients greater than 0.995 in all cases for a range of concentrations between 20 and 100 ng mL^{-1}.

Table 8.5 Determination of MMP, DMP and EDP in fortified semen samples

Analyte	Parameter	Semen samples				
		1	2	3	4	5
MMP	μ[a]	38	47	75	141	235
	C ± s[b]	39 ± 4	49 ± 3	78 ± 5	139 ± 6	228 ± 12
	t_{cal}[c]	0.79	1.40	1.09	0.87	1.31
DMP	μ[a]	39	49	78	146	243
	C ± s[b]	37 ± 2	52 ± 3	85 ± 6	151 ± 6	246 ± 13
	t_{cal}[c]	1.51	2.12	2.96	1.92	0.69
EDP	μ[a]	40	50	80	150	250
	C ± s[b]	43 ± 4	52 ± 5	81 ± 3	157 ± 8	247 ± 10
	t_{cal}[c]	2.17	1.07	0.51	1.85	0.73

[a] Fortified concentration (ng mL^{-1})
[b] Found concentration (ng mL^{-1}) by standard addition calibration
[c] Statistical $t_{tab(0.05,N-2=3)} = 3.18$

The limits of detection (LOD) and quantification (LOQ) were determined based on the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) (see Table 8.6).

The intra-day variability (repeatability) was assessed by analysing five different semen solutions containing no analytes that were fortified (50 ng mL^{-1}) in the same batch. The inter-day variability (repeatability) was determined by analysing a single semen solution containing no analytes that was fortified (50 ng mL^{-1}) in five different batches.

As can be seen in Table 8.7, the intra-day and inter-day variability, expressed as relative standard deviation (RSD), were in the range from 4.6 to 9.4 %, and from 8.1 to 12.7 %, respectively, depending on the analyte.

8.3.3 Application of the Analytical Method

The described analytical method was applied to the determination of EDP and its metabolites in semen from volunteers which had applied an amount of 13 g of a sunscreen cosmetic product prepared in the laboratory according to an adapted protocol (Jordán and Jordán 1991) containing 8 % of EDP (see Annex II.4). The applied doses are in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007).

In concrete, two different studies were carried out, based on single and repeated sunscreen applications (i.e., in the morning and evening for 4 days, making a total of 8 applications) of the cosmetic product. In the last case, the volunteer was allowed to have a shower per day just before the second daily application. The reason for conducting this study was to simulate a real situation of sunscreen

Table 8.6 Limits of detection (LOD) and limits of quantitation (LOQ) of the developed methodology to determine EDP, DMP and MMP in semen

Sample[a]	MMP		DMP		EDP	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	0.5	1.5	0.3	1.0	0.4	1.2
2	0.3	0.9	0.3	1.0	0.6	1.8
3	0.4	1.2	0.4	1.2	0.2	0.6
4	0.4	1.2	0.3	1.0	0.4	1.2
5	0.5	1.5	0.4	1.2	0.4	1.2

[a] Semen samples from different volunteers
[b] Estimated in the semen sample, taking into account the dilution factor during the treatment of the sample, as $3.3.s_a/b$, where b is the slope of the standard addition calibration curve and s_a is the standard deviation value of the intercept in the calibrate. On-column values, expressed as ng, being the injection volume 100 μL
[c] Estimated in the urine sample as $10.s_a/b$ (see annotation b)

Table 8.7 Precision parameters for the determination of MMP, DMP and EDP in human semen

Analyte	Parameter	
	Repeatability (%)[a]	Reproducibility (%)[a]
MMP	6.0	8.1
DMP	4.6	12.5
EDP	9.4	12.7

[a] Expressed as RSD ($N = 5$)

cosmetic product application, which usually involves the application of repeated doses during the sun bathing.

During 8 days from the first or the unique application of the cosmetic product, the whole secreted semen was collected for periods of 24 h into sterile commercially available containers. The total volume of semen daily collected was measured and stored at -20 °C. Considering the total volume of semen, the bioaccumulation process of EDP can be then estimated. Before the first or the unique application of the cosmetic product, semen samples were also collected to check for the absence of EDP and/or its metabolites.

Analogously to the results obtained in urine analysis, EDP was not observed in any of the semen samples that were analyzed, thus showing that EDP follows an extensive biotransformation process in the human body. However, there are clear differences when comparing the studies of semen and urine analysis. Firstly, neither DMP or MMP were detected in semen samples after a single application of the cosmetic product containing EDP. Considering that semen is not considered a proper excretion biofluid, it was not surprising to observe the absence of the metabolites of EDP in semen.

More interesting information could be drawn from study of repeated applications. Thus, the presence of the two metabolites of EDP were detected at different extension (see Fig. 8.2).

Due to lack of enough sample, semen samples were only treated with the ß-glucuronidase solution to determine the total content of the analytes and then, the contribution of both the unconjugated and conjugated content to the total content

Fig. 8.2 Found amounts of DMP (*blue colour square*) and MMP (*red colour square*) in semen from a volunteer after repeated application of a sunscreen cosmetic product containing EDP. The results are expressed in terms of absolute mass considering the total volume of sample collected
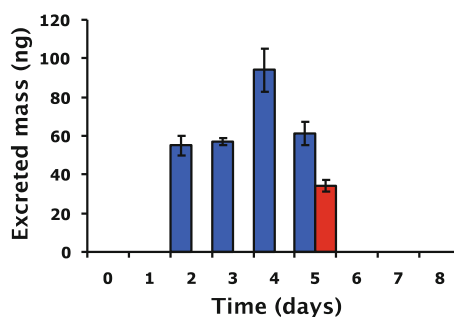
was not determined. However, according to the previous study in urine (see Chap. 7), a low contribution of the unconjugated species to the total content would be expected.

DMP was first detected in the semen sample collected during the period between 24 and 48 h since the start of the study, once the cosmetic product had been applied four times. DMP excretions were measured also in the third, fourth and fifth day after the first cosmetic application. The highest concentration of DMP in human semen was found in the sample collected on the fourth day.

MMP was only detected in the semen sample collected 5 days after the start of the study, corresponding to the samples collected during the 24 h after the eighth application of the cosmetic product. Subsequently, the content of the metabolites of EDP in semen decreased gradually.

Figure 8.3 shows a SRM chromatogram obtained after the application of the SPE-LC-MS/MS methodology to a semen sample collected the fifth day.

As a consequence of the dynamic equilibrium process of bioaccumulation, increasing the incorporation of topically applied EDP to the human body could allow the accumulation of the metabolites of this substance in semen, apart from urine, as an alternative and secondary route of excretion. Taking into account the different levels at which the metabolites of EDP are excreted by semen, more information regarding the mechanism of biotransformation of EDP in humans can be obtained (see Fig. 8.4).

The fact that MMP was only detected in semen after repeated applications of the cosmetic product containing EDP means that initially DMP is formed from EDP by hydrolysis of the 2-ethylhexyl catalyzed by cytochrome P450. Then, this metabolite can be conjugated with glucuronic acid, resulting in the predominant species in urine (DMP-Glu, see Chap. 7) and/or continue the biotransformation process to yield MMP, which may also be conjugated with glucuronic acid, depending on the quantity of EDP absorbed and the studied biofluid.
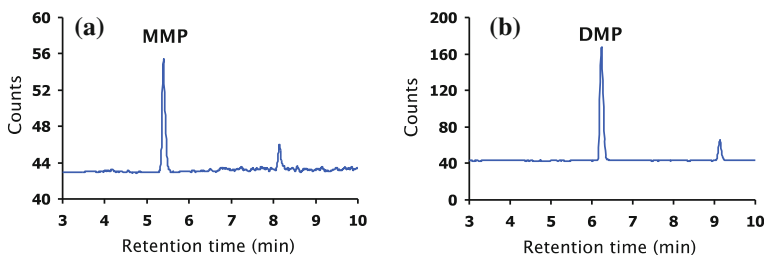
Fig. 8.3 SRM chromatograms for MMP (**a**) and DMP (**b**) obtained by analyzing a semen sample collected the fifth day from a volunteer who had repeatedly applied a cosmetic product containing EDP. Experimental conditions are described in Sect. 8.2.3
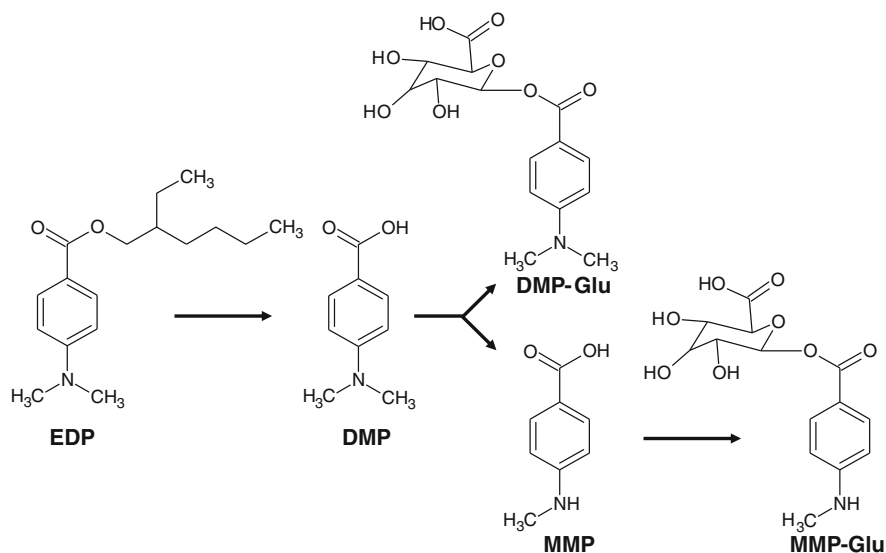
Fig. 8.4 *In vivo* biotransformation mechanism proposed for EDP, thus resulting in DMP, MMP and their respective glucuronide conjugates

8.4 Conclusions

A sensitive analytical method based on automated SPE-LC-MS/MS to determine EDP and its phase I metabolites (DMP and MMP) in human semen with previous ultrasonic assisted enzymatic hydrolysis treatment has been described. The standard addition calibration was used to correct errors caused by matrix interferences. The validation of the method was carried out, thus providing statistically accurate results in the analysis of fortified semen samples.

Furthermore, the proposed methodology has been applied successfully to the analysis of semen samples from volunteers who had applied repeatedly a cosmetic product containing EDP. DMP and MMP were found at different levels in the semen samples, showig evidences of bioaccumulation processes of EDP in humans.

In general, the described analytical methodology could be used to provide more information regarding the toxicology of this substance. Research in the area of male reproductive health should continue in this line, trying to elucidate the magnitude that the effects of EDP and its metabolites can cause in the male reproductive system.

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Chapter 9
Determination of Methyl Benzylidene Camphor and its Main Metabolite in Urine by Solid-Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

9.1 Introduction

9.1.1 Aim of the Study

The development and validation of an analytical method based on a solid-phase extraction (SPE) prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine methyl benzylidene camphor (MBC) and its major metabolite, i.e., carboxy benzylidene camphor (CBC), in urine from users of sunscreen cosmetic products containing MBC has been carried out.

Since CBC is not a commercially available standard, it was synthesized from MBC. The purified product was used as analytical standard in the development and validation of the analytical methodology.

Furthermore, the aim of the study also includes the detection of the other major phase I metabolite of MBC, i.e., CBC-OH, and the formation and excretion assessment of the phase II metabolites, mainly glucuronide conjugates, by appropriate enzymatic hydrolysis treatment of urine samples.

9.1.2 Background and Current Status of the Issue

Different methodologies that allow the determination of MBC in human plasma, urine and breast milk by liquid chromatography with UV/VIS detection (LC-UV/VIS) (Janjua et al. 2008), LC-MS/MS (Volkel et al. 2006; Schauer et al. 2006) and gas chromatography-mass spectrometry (GC-MS) (Hany and Nagel 1995; Schlumpf et al. 2008) have been found. However, some of these methods involve the use of long and laborious extraction procedures such as liquid-liquid extraction (LLE) (Schlumpf et al. 2008) and gel permeation chromatography (Hany and

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Nagel 1995), while others do not consider the contribution of metabolites to consider the overall body disposition process of MBC (Janjua et al. 2008). Furthermore, the methodologies described in the literature considering the contribution of metabolites of MBC do not allow, however, the determination of analytes using a single analytical method, and then different ionization sources are employed depending on the compound. Moreover, the analytical validation of these methods is not fully complete.

9.2 Experimental

9.2.1 Reagents and Samples

Methyl benzylidene camphor (MBC), also named 3-(4′-methylbenzylidene) camphor, 99.7 % from Guinama SL (Valencia) and carboxy benzylidene camphor (CBC), also named 3-(4′-carboxibenziliden) camphor, >95 %, which was synthesized as described in Sect. 9.2.3, were used as analytical standards. Benzophenone-d10 (BZ-d), 99 % D atom from Sigma-Aldrich (Schnelldorf) was used as deuterated internal standard. Some properties of interest of these compounds are shown in Table 9.1.

The solvents used were absolute ethanol (EtOH), LC grade methanol (MeOH), LC grade acetonitrile (ACN) and LC ultrapure grade acetone, all from Scharlab (Barcelona). Deionized water was obtained from a NANOpure II water purification system (R $\geq$ 18 mΩ cm) from Barnstead (Boston).

Formic acid (FA) from Fluka Chemie (Steinheim), analysis grade 37 % hydrochloric acid (d = 1.19 g mL^{-1}) and analysis grade sodium hydroxide, both from Scharlab (Barcelona), and ß-glucuronidase from Helix pomatia (type HP-2) with 116 300 U mL^{-1} activity, and sulfatase activity $\leq$7,500 U mL^{-1}, from Sigma-Aldrich (Steinheim), were also employed.

Urine samples used to develop and validate the method were obtained from different volunteers who had not applied any cosmetic product containing MBC and stored at 4 °C until analysis.

All reagents and solvents used in the synthesis of CBC (see Sect. 9.2.3) were of analytical or purest grade and obtained from Fluka Chemika (Buchs) or Strem

Table 9.1 Relevant information about the studied compounds

Compound	Molecular weight[b]	pKa[c]
Methyl benzylidene camphor (MBC)	254.4	–
Carboxy benzylidene camphor (CBC)	284.3	5.06 ± 0.10
Benzophenone-d10 (BZ-d)[a]	192.3	–

[a] Used as internal standard
[b] Expressed as g mol^{-1}
[c] Calculated using *Advanced Chemistry Development (ACD/Labs) V8.14 software*

Chemicals (Newburyport). Deuterochloroform ($CDCl_3$) 99.9 % D atom, and NMR grade trimethylsilane (TMS) both from Sigma-Aldrich (Steinheim) were also used.

9.2.2 Instruments and Material

A L-7100 liquid chromatograph® connected to a L-7420® UV/VIS detector both from Hitachi (Tokyo) was used. The chromatographic separation was carried out on a LiChrospher® RP-18 analytical column (5 μm particle size, 125 mm long, 4 mm internal diameter) from Merck (Darmstadt). The UV/VIS detection was performed at the wavelength of 300 nm. Data acquisition was controlled by the D-7000 HSM software from Hitachi (Tokyo).

The LC-MS/MS system consisted of an Acquity UltraPerformance LC® liquid chromatograph coupled to a TQD® triple quadrupole mass spectrometer from Waters (Barcelona). A CLAN Technology high purity generator (Sevilla) provided nitrogen that was used as both ionization source and nebulizer gas. The MS/MS spectra were acquired by collision-induced dissociation (CID) of the selected precursor ions using Premier® argon from Air Products (Barcelona) as collision gas. The acquisition of data, and qualitative and quantitative analyses were performed using the MassLynx software. An Acquity UPLC BEH C18® analytical column (1.7 μm particle size, 50 mm long, 2.1 mm internal diameter) with an Acquity BEH C18 Vanguard® precolumn (1.7 μm particle size, 5 mm length, 2.1 mm internal diameter) also from Waters were used.

An Avance 300® spectrometer from Bruker (Madrid) to obtain the nuclear magnetic resonance (NMR) spectra at 299.95 MHz ($CDCl_3$) for ^1H NMR and at 75.43 MHz ($CDCl_3$) for ^{13}C NMR was also employed.

C18 solid-phase extraction cartridges (100 mg, 1 cm long, 5 mm internal diameter) and a SPE vacuum system from Varian (Barcelona) were used.

A MicropH 2001 pH meter from Crison (Alella), a ZX3® vibrating agitator from VELP Scientifica (Usmate), Silica Gel 60 F-254® thin layer plates for thin-layer chromatography (TLC) from Merck (Barcelona), a R-210/R-215® rotavapor from Buchi (Flawil) and a Precisterm® thermostated water bath, an incubation chamber and a Digitheat® oven, all from JP Selecta (Barcelona) were also used.

9.2.3 Synthesis and Characterization of Carboxy Benzylidene Camphor

The synthesis CBC was conducted through an initial reaction of MBC bromination followed by the Kornblum reaction to obtain the aldehyde analogue and a final oxidation with the Jones reagent (see Fig. 9.1).
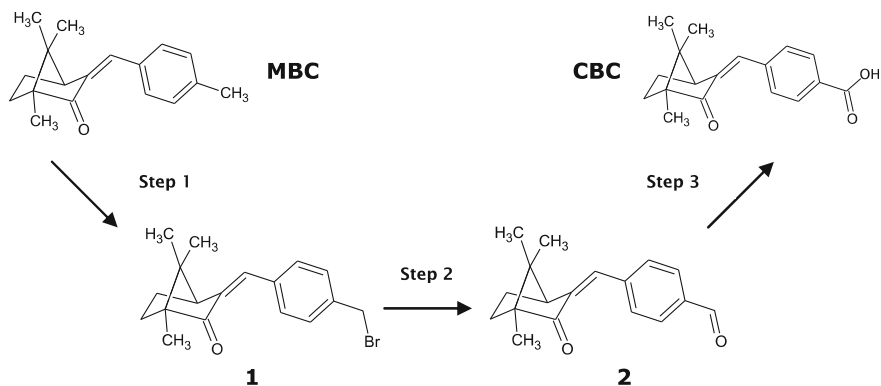
Fig. 9.1 Scheme of the CBC synthesis from MBC. Experimental conditions are described in Sect. 9.2.3

All reactions were performed using glassware dried in oven at 120 °C for at least 12 h and monitored by TLC analysis. Samples were dissolved in CDCl$_3$ and TMS was used as internal standard (δ 0 ppm) in both ^{1}H and ^{13}C NMR experiments.

9.2.3.1 Step 1: Bromination

1.45 g (5.7 mmol) of MBC and 1.06 g (5.95 mmol) of N-bromosuccinimide were added to a 10 mL of carbon tetrachloride in a round bottom flask equipped with a refrigerator and a septum under nitrogen atmosphere.

The mixture was stirred at 80 °C under reflux and exposed to the action of a lamp (150 W) that provided white light. The reaction was monitored by TLC until MBC reacted completely (48 h). Then, the suspension was filtered and the filtrate was concentrated in vacuo. The solid residue [see Fig. 9.1, compound (**1**)] was washed with deionized water and recrystallized from isopropanol. 0.823 g of a pale yellow solid in the form of needles was obtained.

9.2.3.2 Step 2: Kornblum Reaction

0.411 g (4.9 mmol) of sodium bicarbonate was added to the stirred solution consisting of 0.823 g (**1**) (2.5 mmol) in 7 mL (200 mmol) of dimethylsulfoxide and the reaction mixture was kept stirring at room temperature for 48 h.
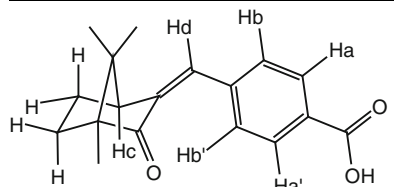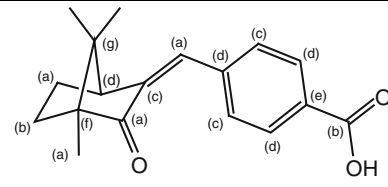
Next, the reaction mixture was diluted with deionized water (50 mL), and a liquid-liquid extraction (LLE) was proceeded in triplicate with 30 mL of ethyl acetate. The organic phases were washed with deionized water and dried with anhydrous magnesium sulfate. After concentrating in vacuo, 0.56 g of a brown oil [see Fig. 9.1, compound (**2**)] was obtained.

9.2.3.3 Step 3: Oxidation

The Jones reagent was prepared by adding 0.67 g (6.7 mmol) of chromium trioxide to 0.6 mL (11.3 mmol) of concentrated sulfuric acid, and the mixture was cautiously diluted with deionized water (5 mL).

1 mL of Jones reagent was added to a stirred solution consisting of 0.56 g of (**2**) (2 mmol) and 20 mL (272.9 mmol) of acetone. The reaction mixture was stirred at 15 °C and monitored by TLC until the reaction was completed (4 h). Next, 10 mL of an aqueous sodium hydroxide (1 M) was added and the reaction mixture was placed in an ice bath for 30 min. The organic phase obtained by LLE with diethyl ether (2 × 20 mL) was discarded and hydrochloric acid (2 M) was added to the basic aqueous phase until pH 1 conditions. Next, the reaction mixture was extracted with diethyl ether (3 × 20 mL) and the combined organic phases were dried with anhydrous magnesium sulfate. After evaporating in vacuo of the solvent, 0.258 g of CBC (see Fig. 9.1) was obtained. Spectral data for the characterization of CBC are shown in Table 9.2.

Table 9.2 Characterization of CBC by ^{1}H RMN and ^{13}C RMN analysis

^{1}H RMN			^{13}C RMN	
δ^a (ppm)	Multiplicity^b	Assignment	δ^a (ppm)	Assignment
0.84	s	–CH$_3$	9.3	–CH$_3$ (a)
1.02	s	–CH$_3$	18.3	–CH$_3$
1.05	s	–CH$_3$	20.7	–CH$_3$
1.58	m	–CH, –CH	25.9	–CH$_2$ (a)
1.82	m	–CH	30.5	–CH$_2$ (b)
2.23	m	–CH	46.6	–C (g)
3.11	d (J = 4.2)	H$_c$	49.3	–CH (d)
7.26	S	H$_d$	57.2	–C (f)
7.56	d (J = 8.4)	H$_b$, H$_{b'}$	126.1	–CH (a)
8.13	d (J = 8.4)	H$_a$, H$_{a'}$	129.1	–C (e)
			129.6	–CH (c)
			130.4	–CH (b)
			141.1	–C (d)
			144.5	–C (c)
			171.1	–C (b)
			207.9	–C (a)

^{a} Chemical shifts relative to residual undeuterated solvent (CDCl$_3$)
^{b} Expressed as singlet (*s*), doublet (*d*), multiplet (*m*). The coupling constants (J) are expressed in Hz
^{c} The assignment of the carbon multiplicity was determined by distortionless enhancement by polarization transfer (DEPT) experiments

9.2.4 Analytical Method for the Determination of Methyl Benzylidene Camphor and its Major Metabolite in Urine

9.2.4.1 Preparation of Solutions

Separate solutions at a concentration level of 400 μg mL^{-1} of both the analytical standards (MBC, CBC) and the internal standard (BZ-d) were prepared in EtOH and kept at 4 °C.

To correct the error caused by the matrix effects (see Sect. 9.3.2), the standard addition calibration was used. Thus, a BZ-d solution (4 μg mL^{-1}) in EtOH and a MBC and CBC solution (4 μg mL^{-1}) in the urine sample to be analyzed were prepared from the respective stock solutions.

The calibrate solutions were prepared fortifying five aliquots (4,750 μL) of urine containing 0, 30, 60, 90 and 120 μL of the MBC and CBC standard solution (4 μg mL^{-1}), to which 150, 120, 90, 60 and 30 μL of urine was added, respectively, to set the same final volume. Likewise, 50 μL of the BZ-d solution (4 μg mL^{-1}) was also added to each calibrate solution.

9.2.4.2 Enzymatic Hydrolysis

To determine the total analyte content, i.e., unconjugated and glucuronide conjugated forms, 50 μL of the commercial solution of ß-glucuronidase was added to each calibrate solution. Alternatively, urine samples were also treated with deionized water to determine only the unconjugated content of analyte. Urine solutions were stirred and subjected to enzymatic hydrolysis by incubation at 37 °C for 12 h with ß-glucuronidase.

Subsequently, 50 μL of FA was added to the urine samples to stop the reaction. Hence, The urine solutions were adjusted to pH 3 for the subsequent SPE procedure of the analytes.

9.2.4.3 Solid-Phase Extraction

The C18 solid-phase extraction cartridge was conditioned with 2 mL of EtOH followed by 2 mL of deionized water. Next, the cartridge was loaded with 4 mL of each incubated urine solutions at a flow rate of 0.5 mL min^{-1}, washed with 8 × 1 mL of deionized water and dried under vacuum for 10 min. The analytes were eluted with 3 × 0.35 mL of acetone. Then, the eluted fractions were evaporated to dryness in an oven at 50 °C (30 min) and reconstituted with 100 μL of deionized water:MeOH:ACN (2:1:1), (v/v/v) containing 0.1 % FA. Finally, the reconstituted samples were injected into the LC-MS/MS system.

Table 9.3 Elution gradient of the LC-MS/MS analysis to determine MBC and CBC in urine

Time (min)	0.1 % FA in deionized water (%)	0.1 % FA in MeOH:ACN (1:1, *v/v*) (%)
0	60	40
1	60	40
1.1	0	100
3.1	0	100

Table 9.4 SRM parameters of interest to determine MBC and CBC in urine

Compound	Parameter				
	ESI ionization mode	First quad voltage(V)	Collision energy (V)	SRM transition to quantify	SRM transition to confirm
BZ-d[a]	Positive	35	15	$193.2 \rightarrow 110.1$	$193.2 \rightarrow 82.1$
CBC	Negative	38	20	$283.2 \rightarrow 239.2$	$283.2 \rightarrow 257.2$
MBC	Positive	30	25	$255.2 \rightarrow 105.1$	$255.2 \rightarrow 119.2$

[a] Internal standard

9.2.4.4 LC-MS/MS Analysis

The mobile phase consisted of deionized water and MeOH:ACN (1:1, *v/v*), both containing 0.1 % FA. The elution gradient shown in Table 9.3 was used at a flow rate of 0.4 mL min^{-1} and at room temperature. The analytical column was equilibrated with a post-analysis time of 3 min. The injection volume was 5 μL.

The analytes were determined by selected reaction monitoring (SRM) in both positive and negative electrospray ionization (ESI) modes. The conditions of the triple quadrupole mass analyzer were as follows: temperature of gas ionization source, 120 °C; gas flow ionization source, 700 L h^{-1}; nebulizer gas temperature, 300 °C; nebulizer gas flow, 25 L h^{-1}; capillary voltage, 3,900 V. Dwell time was set to 37 ms. Two SRM transitions to quantify and to confirm were selected for each compound of interest. They are specified in Table 9.4, along with other relevant information.

Calibrates were obtained by representing the ratio of areas between each analyte and internal standard (BZ-d) versus the added concentration. Figure 9.2 shows the SRM chromatograms for the compounds of interest obtained by applying the described SPE-LC-MS/MS procedure to a urine sample containing no analytes, fortified (at 200 ng mL^{-1}) and unfortified.

9.3 Results and Discussion

9.3.1 SPE Procedure: Study of the Variables

Preliminary assays were carried out to verify the SPE conditions using the LC-UV/VIS system.
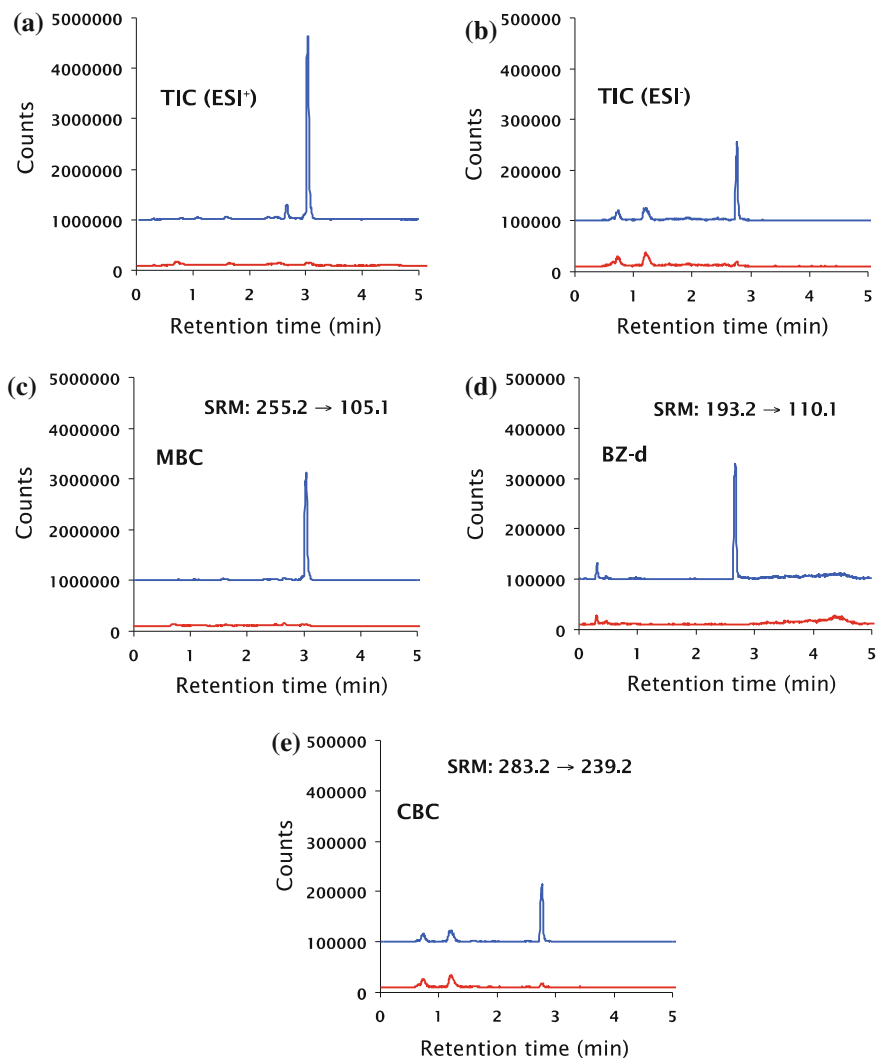
Fig. 9.2 Total ion chromatograms (TIC) in positive (**a**) and negative (**b**) ionization modes and selected reaction monitoring (SRM) chromatograms of MBC (**c**, ESI⁺), BZ- d (**d**, ESI⁺) and CBC (**e**, ESI⁻) obtained by analyzing a urine sample containing no compounds (red line) and the same urine sample fortified at a concentration of 200 ng mL⁻¹ (blue line). Experimental conditions are described in Sect. 9.2.4

C18 cartridges were used to show the suitable capacity to retain the compounds of interest. Thus, after duly conditioning of the cartridge with 2 mL of EtOH followed by 2 mL of deionized water, aqueous standard solution of MBC and CBC (1 μg mL⁻¹) under different conditions depending on the performed study was

loaded and quantitative elution of the analytes with 3×0.35 mL of acetone was proceeded. The effectiveness of the elution conditions was checked (R > 95 %).

The eluted fraction was evaporated under a stream of air, reconstituted with 100 μL of deionized water:MeOH:ACN, 2:1:1 (*v/v/v*) containing 0.1 % FA and injected into the LC-UV/VIS system. The mobile phase consisted of deionized water (0.1 % FA) and (MeOH:ACN, 1:1, 0.1 % FA). The employed gradient of elution at a flow rate of 1 mL min^{-1} and at room temperature was as follows: 0–5 min, 60 % (MeOH:ACN, 1:1, 0.1 % FA); 5–10 min, linear gradient from 60 to 70 % (MeOH:ACN, 1:1, 0.1 % FA); 10–10.5 min, linear gradient from 70 to 100 % (MeOH:ACN, 1:1, 0.1 % of FA), maintained for 5 min. The analytical column was equilibrated with a post-analysis time of 5 min. The injection volume was 20 μL. Under these conditions, the resulting retention times were 2.6 min for CBC and 8.2 min for MBC.

First, the retaining process of the SPE procedure was studied. Hence, 50 μL of FA, deionized water or sodium hydroxide was added to three aqueous standard solutions, resulting pH 3, 5 and 12, respectively. Then, 1 mL of each of these solutions was loaded into the C18 cartridges. The results obtained from triplicate LC-UV/VIS analysis showed that the signal of CBC at pH 3 was greater than at pH 5 (see Fig. 9.3).

At pH 12, no signal was observed for CBC, thus showing that no retention was produced for CBC in the C18 cartridges under these conditions. The findings are consistent with its pK$_a$ value (see Table 9.1), so that CBC remains quantitatively in its non-ionized form at pH below 3, enhancing hydrophobic interactions with C18 cartridges. The signal of MBC was kept constant at all three pH conditions, being slightly lower at pH 12. Therefore, urine solutions were adjusted at pH 3 prior to the SPE step.

To study the load capacity, different aliquots of aqueous standard solutions (1 μg mL^{-1}) were loaded into two C18 cartridges assembled in series. Thereafter, SPE cartridges were disassembled and the procedure described above was carried out in both cartridges. As the retention process in the first cartridge when loading 4 mL of solution was almost quantitative (R > 99 %), it was concluded that there
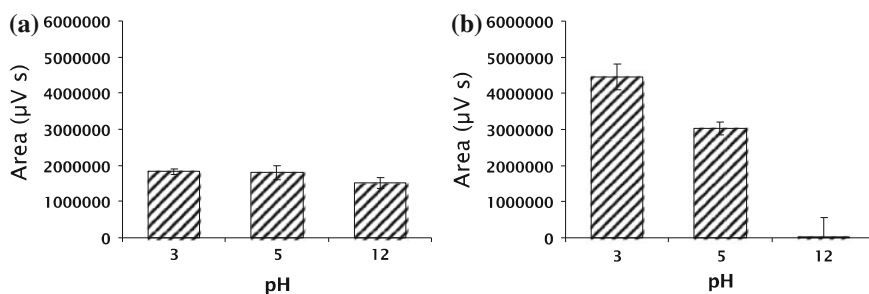
Fig. 9.3 Effect of pH during the retention process in the SPE procedure of MBC **(a)** and CBC **(b)** by analysing an aqueous solution (1 μg mL^{-1}). Error bars show the standard deviation of the results ($N = 3$)

was no analyte loss under these conditions. Recovery of the SPE procedure was estimated comparing the signal obtained from the eluted and reconstituted fraction and external standards. The obtained values were around 60 and 95 % for MBC and CBC, respectively. Due to the low obtained value of MBC, an alternative way to dry the eluted fraction from the SPE procedure was considered. Thus, the oven assisted evaporation at 50 °C for 30 min was studied and compared with the results obtained under the air stream evaporation. The recovery of MBC by using the oven assisted evaporation was improved up to 90 %, while CBC results were maintained constant (95 %). Therefore, SPE eluted fractions were dried before reconstitution with the mobile phase in the oven at 50 °C (30 min) to avoid the analyte losses showed when air stream was used to evaporate to dryness.

9.3.2 LC-MS/MS Analysis

The MS/MS spectra of the compounds of interest were acquired by CID experiments by direct infusion for standard solutions of each target compound (200 µg mL^{-1}) prepared in MeOH, operating with electrospray ionization in positive (ESI^{+}) or negative (ESI^{-}) modes.

   The most intense MS/MS transitions for MBC and BZ-d were obtained with positive electrospray ionization, through the corresponding protonated molecular ions ([M+H]$^{+}$). However, the corresponding deprotonated molecular ion ([M-H]$^{-}$) obtained by negative electrospray ionization was more intense for CBC. Under these conditions, tandem mass spectrometry parameters were optimized (see Table 9.4).

9.3.3 Study of Sample Stability

The stability of MBC and CBC in fortified urine samples stored at 4 °C was studied. As can be seen in Fig. 9.4, the reproducibility of the LC-MS/MS analysis from the first to the sixth day indicates the stability of the analytes during at least this period of time, under the study conditions. Therefore, the urine samples were kept at 4 °C until analysis.

9.3.4 Validation of the Analytical Method: Study of the Interferences

The matrix effects, which may occur in both extraction and detection processes, were evaluated. Hence, the differences between the responses obtained from a
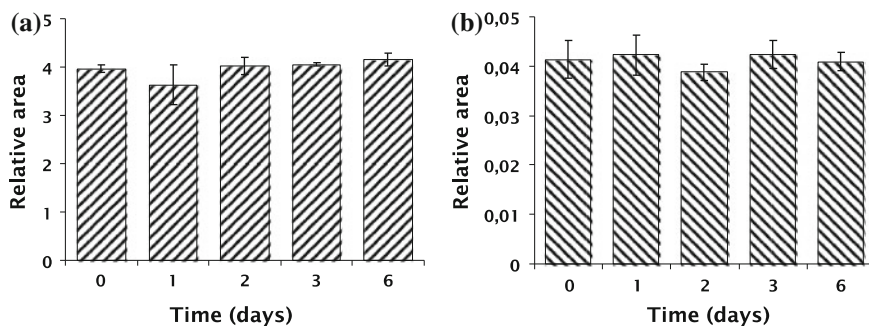
Fig. 9.4 Peak area ratios between MBC **(a)** and CBC **(b)** and the internal standard obtained by analyzing a urine sample fortified at 50 ng mL^{-1} and stored at 4 °C at different times. Experimental conditions are described in Sect. 9.2.4

series of standard solutions (30–150 ng mL^{-1}) prepared in a urine sample containing no analytes from a single volunteer (*single urine calibrate*) were statistically compared to those obtained from a series prepared in a pool of urine samples containing no analytes from five volunteers (*pool urine calibrate*). Moreover, all the solutions were fortified (50 μL) with BZ-d (4 μg mL^{-1}) and pH was adjusted by adding 50 μL of AF (pH 3). Then, the described SPE procedure followed by the LC-MS/MS analysis was carried out.

As can seen in Table 9.5, statistically different slopes were obtained when comparing both calibrates for MBC. In the case of CBC, they were statistically comparable.

Therefore, the developed analytical methodology considered standard addition calibration to correct the proportional errors caused by potential matrix interferences in order to correctly determine the content of the target compounds in the urine samples.

Table 9.5 Comparison of the MBC and CBC calibrates obtained from a single urine sample and a pool of urine samples

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}[f]
		Single[d]	Pool[d]		
MBC	a[a]	0.17 ± 0.19	0.11 ± 0.09		0.2
	b (mL μg^{-1})[b]	52 ± 2	36 ± 1	144	6.2
	R^{2c}	0.996	0.997		
CBC	a[a]	−0.001 ± 0.0002	0.000 ± 0.001		0.8
	b (mL μg^{-1})[b]	0.66 ± 0.02	0.70 ± 0.01	94	1.6
	R^{2c}	0.997	0.998		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the slopes obtained using both calibrates
[f] Statistical $t_{tab(0.05,(N1-2)+(N2-2=6)} = 2.45$ (see Annex III.4)

9.3.4.1 Accuracy

Accuracy was evaluated using the developed method by analyzing urine samples fortified with known amounts of MBC and CBC, from volunteers who had not applied any cosmetic product containing MBC. The obtained values are shown in Table 9.6.

Standard deviations were obtained as the standard deviation of the extrapolated value in the standard addition line (Miller and Miller 2005). The accuracy of the analytical method was statistically confirmed by Student's t-test, thus showing the absence of significant differences between concentration values found by the developed method and concentration values of the fortified urine samples (see Annex III.3).

9.3.4.2 Other Analytical Parameters

Calibration curves (N = 5) were found to be linear for CBC and MBC over a range of concentrations from 30 to 150 ng mL^{-1} (working range) with a regression coefficient greater than 0.995 in all cases.

The limits of detection (LOD) and quantification (LOQ) of the analytes were estimated based on the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) (see Table 9.7).

The intra-day variability (repeatability) was assessed by analyzing in the same batch five fortified (50 ng mL^{-1}) urine samples containing initially no analytes. The inter-day variability (reproducibility) was determined by analyzing in five different batches a single fortified (50 ng mL^{-1}) urine sample containing initially no analytes. The intra- and inter-day variabilities expressed as relative standard deviation (RSD) are shown in Table 9.8.

Table 9.6 Determination of MBC and CBC in fortified urine samples

Analyte	Parameter	Urine samples				
		1	2	3	4	5
MBC	μ^a	17	87	122	157	174
	$C \pm s^b$	19 ± 2	89 ± 4	119 ± 4	158 ± 4	173 ± 8
	t_{cal}^c	2.49	0.97	1.17	0.75	0.29
CBC	μ^a	17	83	116	149	166
	$C \pm s^b$	19 ± 1	81 ± 4	112 ± 4	148 ± 5	164 ± 10
	t_{cal}^c	3.09	0.99	2.11	0.37	0.47

[a] Fortified concentration (ng mL^{-1})

[b] Found concentration (ng mL^{-1}) by standard addition calibration

[c] Statistical $t_{tab(0.05, N-2=3)}$ = 3.18

Table 9.7 Limits of detection (*LOD*) and quantitation (*LOQ*) of the developed methodology to determine MBC and CBC in urine samples

Sample[a]	MBC		CBC	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	4	13	3	9
2	7	22	8	23
3	6	20	6	19
4	5	16	7	20
5	9	27	8	26

[a] Urine samples from different volunteers
[b] Estimated in the urine sample, taking into account the dilution factor during the treatment of the sample, as $3.3.s_{Y/X}/b$, where b is the slope of the standard addition calibration curve and $s_{Y/X}$ the residuals standard deviation of the calibrate. Values expressed as ng mL^{-1}
[c] Estimated in the urine sample as $10.s_{Y/X}/b$ (see annotation b)

Table 9.8 Precision parameters for the determination of MBC and CBC in urine samples

Analyte	Parameter	
	Repeatability (%)[a]	Reproducibility (%)[a]
MBC	2.0	7.7
CBC	1.3	4.5

[a] Expressed as RSD ($N = 5$)

9.3.5 Application of the Analytical Method

To apply the proposed analytical method to urine samples from users of cosmetic products containing MBC in their composition, a volunteer was instructed to apply 13 g of the cosmetic product in the morning and evening for three consecutive days, making a total of 6 applications. The cosmetic product was prepared in the laboratory according to an adapted protocol (Jordán and Jordán 1991) containing 4 % of MBC (see Annex II.5). The applied doses are in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007). A shower per day just before the second daily application was allowed to the volunteer. The reason to conduct this study was to simulate a real situation of sunscreen cosmetic product application, which usually involves the application of repeated doses over sun-bathing time.

During the first 4 days from the application of the cosmetic product, the total excreted urine was collected over periods of 24 h in sterile commercially available containers. The urine volume was measured and stored at 4 °C until analysis. Taking into account the total collected urine is possible to determine the content of MBC excreted by urine. Thus, urine sample 1 (U1) corresponded to the urinary excretions collected during the first 24 h, urine sample 2+3 (U2+3) was prepared
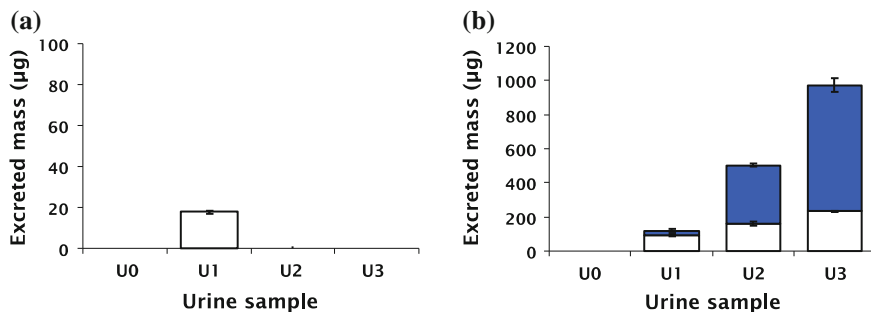
Fig. 9.5 Found amounts of MBC (**a**) and CBC (**b**) in urine samples collected at times specified in Sect. 9.3.3 from a volunteer after repeated topical application of a sunscreen cosmetic product containing MBC (4 %). Error bars show the standard deviation of the results. The results are expressed in terms of absolute mass considering the dilution factor corresponding to the total volume collected in each case. Unconjugated form content, (*square*); glucuronide conjugated form content, (*filled square*)

by mixing the excreted urine collected during the second and third day and finally, urine sample 4 (U4) corresponded to the urine excretion collected during the fourth day since the first application. An additional urine sample was collected just before the first application (U0) to verify the initial absence of MBC and/or CBC.

Urine samples (U1, U2+3 and U4) were divided into two fractions. To each urine fraction, deionized water or ß-glucuronidase solution were added, thus allowing the determination of the unconjugated analyte content or the total (i.e., unconjugated and glucuronide conjugate) analyte content, respectively. Thereby, each urine sample was analyzed in duplicate (with and without enzymatic treatment) by applying the SPE-LC-MS/MS analytical methodology described above.

As can be seen in Fig. 9.5, MBC was determined only in U1, thus concluding that MBC follows an extensive biotransformation process in human body. By contrast, increasing amounts of both unconjugated and glucuronide conjugated forms of CBC were detected along with the study. The highest concentration of CBC was found in U4, that corresponds to the urine sample collected the day after the last application of the cosmetic product, showing a clear process of bioaccumulation.

Figure 9.6 shows a SRM chromatogram obtained after the application of the SPE-LC-MS/MS methodology to the U2+3 urine sample, with and without enzymatic treatment.

The effectiveness of the deconjugation reaction is evident, thus showing that the glucuronide conjugated species of CBC contribute greatly to the total content of CBC excreted by urine when topical incorporation of MBC is increased.

New conditions were included into the LC-MS/MS analysis to consider the detection of another phase I metabolite of MBC described in literature, i.e., CBC-OH. SRM parameters were adapted from Schauer et al. (2006), applying the same conditions of voltage and collision energy as for CBC (see Table 9.9).
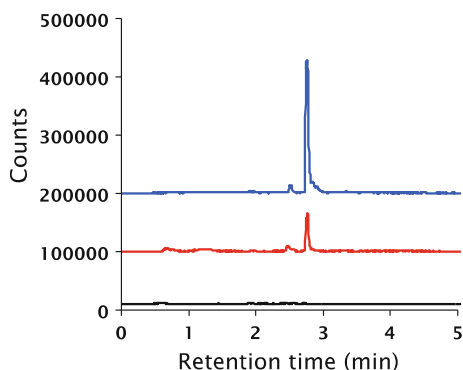
Fig. 9.6 SRM chromatograms for CBC obtained by analyzing U0 (black line) and U2+3 (red line) urine samples from a volunteer who had repeatedly applied a sunscreen containing MBC. U2+3 sample was also treated by enzymatic hydrolysis (blue line). Experimental conditions are described in Sect. 9.2.4 and the conditions of the study can be found in Sect. 9.3.3

Table 9.9 SRM parameters in electrospray ionisation mode to detect CBC-OH in urine

Compound	Parameter				
	ESI ionization mode	First quad voltage(V)	Collision energy (V)	SRM transition to quantify	SRM transition to confirm
CBC-OH	Negative	38	20	299.2 → 255.2	299.2 → 237.2
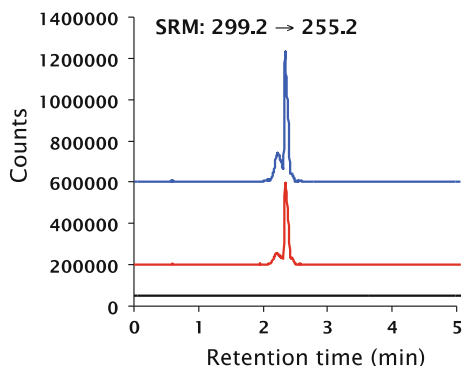
Fig. 9.7 SRM chromatograms for CBC-OH obtained by analyzing urine samples U0 (black line) and U2+3 (red line) from a volunteer who had repeatedly applied a sunscreen cosmetic product containing MBC. U2+3 sample was also treated by enzymatic hydrolysis process (blue line). Experimental conditions are described in Sect. 9.2.4 and the conditions of the study can be found in Sect. 9.3.3

The contribution of the different isomers of CBC-OH as a consequence of the *in vivo* biotransformation process of MBC can be seen in Fig. 9.7, with predominance of one isomer as described in the literature (Schauer et al. 2006).

Furthermore, it can be concluded that the contribution of the conjugated species by glucuronidation to the total content of this metabolite was also important, when comparing both enzymatic treated and untreated samples.

9.4 Conclusions

An analytical method based on the combination of SPE and a subsequent process by LC-MS/MS analysis to determine MBC and its metabolite, CBC, in human urine has been developed and validated. Since CBC was not commercially available, it was synthesized from MBC.

The standard addition calibration was employed to correct the proportional errors caused by the observed matrix effects. Under this approach, statistically accurate results were obtained by analyzing urine samples previously fortified with the analytes.

The methodology has been successfully applied to the analysis of urine samples collected from a volunteer that had applied repeatedly a sunscreen cosmetic product containing MBC. A low content of MBC in the urine sample collected 24 h after the first application was determined. However, the largest contribution to the MBC excretion corresponds to the CBC species conjugated by glucuronidation, which increased with the number of sunscreen applications. Furthermore, CBC-OH, another major metabolite of MBC, was identified and detected, and it was found that CBC-OH also prevailed mainly as glucuronide conjugated in urine samples.

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Part IV
Summary and General Conclusions

The use of high efficacy sunscreens is necessary to minimize the harmful effects that can be caused to the human body by an excessive sun radiation. Nowadays, dermatologists strongly recommend the use of sunscreen products not only when incidence of extreme sun exposure (i.e., beach, snow, etc.), but also in daily situations. However, these cosmetic formulations contain in their composition chemical compounds, commonly known as UV filters, which undergo some undesirable processes, such as percutaneous absorption and systemic effects.

The final aim of this PhD thesis has been the development and validation of analytical methods based on both *in vitro* (Chap. 2) and non invasive *in vivo* (Chaps. 3–9) procedures, which allow to gain more information about the adverse side effects that can be produced to the human body by the UV filters contained in sunscreen formulations. Hence, the described methodologies would help to contribute to the design of cosmetic formulations able to provide a high level of safety without any kind of risks to the users.

Chapter 10
Highlights of the Ph.D. Thesis

10.1 Part II: Methodology to Estimate the *In Vitro* Percutaneous Absorption Processes of the UV Filters Contained in Sunscreen Cosmetics

The most employed *in vitro* **methodology** to estimate percutaneous absorption processes of UV filters is based on the use of diffusion cells. This technique was used to study this type of processes associated to the most common water-soluble UV filters (i.e., phenyl benzimidazole sulfonic acid (PBS), disodium phenyl dibenzimidazole tetrasulfonate (PDT), terephtalydene dicamphor sulphonic acid (TDS) and benzophenone-4 (BZ4)) in sunscreen cosmetics (Chap. 2).

Hence, an analytical method based on ion-interaction liquid chromatography with UV/VIS detection (LC–UV/VIS) to determine PBS, PDT, TDS and BZ4 in the receptor fluid from the diffusion cells has been developed. The inclusion of an ion-pairing reagent in the mobile phase allowed the simultaneous determination of the analytes from the first hours after sunscreen application to human skin with a low limit of detection.

Although the estimation of the percutaneous absorption cannot be conclusive from this study due to the use of a low number of diffusion cells, it may give a general idea about how the analytes cross the epidermal surface. In this sense, no more than 1 % of the total cosmetic formulation applied dose was determined in the receptor solution after 48 h in all cases.

The studied chromatographic variables (i.e., mobile phase, ion-pairing reagent concentration, pH, temperature) provided well enough analytical features to consider this methodology very useful in future biokinetic studies. Hence, more exhaustive research can be performed into percutaneous absorption of these analytes by using the proposed analytical method with a large number of diffusion cells including different kind of skin and considering different times.

10.2 Part III: Methodology to Study Related Processes from the *In Vivo* Percutaneous Absorption of the UV Filters Contained in Sunscreen Cosmetics

In Vivo **procedures** include the determination of UV filters in biological fluids from users of sunscreens that applied cosmetic formulations at different extent containing the target compounds in their composition. Due to the fact that transdermal processes of UV filters in humans involve also biotransformation, accumulation and excretion mechanisms, it is better to refer to disposition processes, as a more general term including all these bioprocesses. The target UV filters that were studied in this Doctoral Thesis have been benzophenone-3, ethylhexyl dimethyl PABA and methyl benzylidene camphor.

10.2.1 Part III. 1 Benzophenone-3

To start with the *in vivo* study, an analytical method has been developed to determine simultaneously **benzophenone-3** (BZ3) and benzophenone-4 (BZ4) in human urine, which is considered the major route of excretion of these compounds, throughout an automated solid-phase extraction (SPE) process directly coupled to the LC-UV/VIS system (Chap. 3).

The combination of sequential injection (SI) analysis with liquid chromatography enhances robustness, ease in sample handling, precision, reproducibility and cost effectiveness. After full validation of the method, the obtained analytical parameters assure suitability for simultaneous quantification of BZ3 and BZ4 in both pharmacokinetic and pharmacodynamic *in vivo* studies.

The methodology was employed in the analysis of urine samples collected from volunteers that had applied a sunscreen cosmetic containing both UV filters.

During these analyses, some metabolites were observed and they instigated us to consider not only the target UV filters but also their biotransformation by-products produced after sunscreen application in order to estimate the whole body disposition process.

Hence, taking into consideration that the metabolism pathways of BZ3 were well-established, a sensitive analytical method based on SPE combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS) for the determination of BZ3 and its phase I metabolites, namely 2,4-dihydroxy-bezophenone (DHB), 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB) in human urine has been developed (Chap. 4).

In order to correct the observed matrix interferences, the standard addition calibration method has been used as a quantitative approach. Full validation of the method has been carried out, giving statistically accurate results for fortified analyte-free samples and the methodology has been satisfactorily applied to the analysis of urine samples collected from a volunteer that had applied a sunscreen

cosmetic containing BZ3. Urine samples have been also treated with ß-glucuronidase to carry out enzymatic hydrolysis, which has allowed the determination of the analytes content in both their no conjugate and glucuronide forms.

In comparison to existing methods, the developed method is the one to date that allows the simultaneous determination of the BZ3 and all its three metabolites in human urine in the low ng mL^{-1} range.

The described analytical method can be applied to carry out further required *in vivo* studies concerning the pharmacokinetics of BZ3, and especially of its metabolites, which might have more long-term adverse effects than the parent compound.

Indeed, considering the estrogenic activity showed not only by BZ3 but also by its metabolites, a deeper toxicological evaluation regarding the effects of these compounds on the male reproductive system could be of great interest. In this way, an analytical methodology based on SPE combined with LC–MS/MS has been developed and validated to determine BZ3, DHB, DHMB and THB in human semen (Chap. 5).

The application of the method to the analysis of semen samples collected from a volunteer that had applied a sunscreen cosmetic containing BZ3 supposes the first time that a UV filter and its metabolites have been determined in such biological fluid. Semen samples have been also treated with ß-glucuronidase to carry out enzymatic hydrolysis.

Furthermore, another point of interest of the developed method is the possibility to study how the estrogenic metabolites of this widely used UV filter do affect the variation of specific reproductive toxicity parameters by establishing relationships between the found amounts and semen quality.

10.2.2   Part III. 2 Ethylhexyl Dimethyl PABA

Among the UV filters allowed by the European legislation in force to be included in cosmetic products, metabolism and distribution processes in human body are known to only a few. **Ethylhexyl dimethyl PABA** (EDP), which is one of the most widely used UV filters in sunscreens, was not one of them. For this reason, there was a need in understanding these mechanisms for EDP.

Therefore, the *in vitro* biotransformation of EDP has been studied by means of metabolomic incubations with the cytochrome P450 system from rat liver microsomes. Two phase I metabolites of EDP, namely 4-(*N,N'*-dimethylamino) benzoic acid (DMP) and 4-(*N*-methylamino) benzoic acid (MMP), have been identified by both gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS) analysis (Chap. 6).

Although *in vitro* phase II metabolism has been also investigated by means of glucuronidation and acetylation, no acetylated or glucuronidated conjugates have been detected in the case of EDP. In both cases, PABA has been used as a well described reference compound, but only its acetylation product has been found.

Furthermore, a sensitive method based on SPE combined with LC–MS for the determination of EDP and its phase I metabolites at low ng mL^{-1} levels in human urine samples has been developed. The standard addition calibration has been used in order to correct the observed matrix effects. The analytical method has been fully validated, giving statistically accurate results.

The high sensitivity of the developed method may enable its application to *in vivo* studies concerning the urinary excretion of EDP and its phase I metabolites. In general, this should allow gaining further insights into the toxicology of this widely applied substance.

To confirm the *in vitro* metabolism pathway for EDP, a fully automated on-line SPE–LC–MS/MS method for the determination of EDP, DMP and MMP in human urine has been developed. The proposed hyphenated method requires minimal human intervention and is quite simple, sensitive and selective. Ultrasound-assisted enzymatic hydrolysis has been used to deconjugate possible phase II glucuronide metabolites of EDP (Chap. 7).

The analytical procedure has been validated statistically with accurate results, using standard addition calibration for quantitative correction of the matrix effects. The ensuing method has been used to determine the target compounds in urine from male and female volunteers after application of an EDP-containing sunscreen cosmetic.

Only DMP and MMP have been found in urine, mainly as glucuronide conjugates, which have been characterized by liquid chromatography-time-of-flight/mass spectrometry (LC–TOF/MS) analysis based on accurate mass measurements and confirmed by LC–MS/MS (QqQ) analysis.

This is the first reported instance of the presence of EDP metabolites in human urine samples. The reason why phase II metabolites of EDP have been only detected under *in vivo* conditions probably is based on the inherent differences between *in vitro* and *in vivo* experiments.

In this context, the potential of EDP and its metabolites to cause reproductive toxicity has not been evaluated yet. Apart from semen quality studies (i.e. spermatozoe motility, vitality, numbers and morphology tests) to ascertain the required histological parameters for assessment, the determination of EDP and its metabolites in human semen could be of great interest to establish relationships between semen concentrations of these substances and possible adverse effects on the male reproductive system.

Hence, a sensitive analytical method based on on-line SPE–LC–MS/MS method for the determination of EDP, DMP and MMP in human semen with previous ultrasound assisted enzymatic hydrolysis treatment has been developed (Chap. 8).

The standard addition calibration method has been used as a quantitative approach to correct matrix interferences. Full validation of the method has been carried out, giving statistically accurate results for fortified analyte-free samples.

Moreover, the methodology has been satisfactorily applied to the analysis of semen samples collected from volunteers who were subjected to single and repeated whole-body application of an EDP-containing sunscreen product.

DMP and MMP have been found at different extents in semen samples from a repeated whole-body application study, thus showing evidences for bioaccumulation in humans. Nevertheless, researchers in the field of male reproductive health should continue working to elucidate the magnitude of EDP and its metabolites to male reproductive health effects.

10.2.3 Part III. 3 Methyl Benzyliden Camphor

Finally, the other UV filter authorized by the European legislation whose biotransformation pattern is known to date is **4-methylbenzylidene camphor** (MBC).

Hence, 3-(4′-carboxybenzylidene) camphor (CBC) and four isomers of 3-(4′-carboxybenzylidene)-hydroxycamphor (CBC-OH), being the most predominant 3-(4′-carboxybenzyliden)-6-hydroxycamphor, were identified as *in vivo* phase I metabolites of MBC. However, clear evidences of estrogenic activity have been shown with this controversial compound that is not allowed to be used as cosmetic ingredient in the United States or Japan.

An analytical method has been developed and validated based on SPE procedure and LC–MS/MS analysis to determine MBC and its main metabolite, CBC, in urine from MBC-containing sunscreens users (Chap. 9). Due to the fact that CBC is not a commercially available substance, it has been synthesized from MBC and, after purification, used as analytical standard.

In order to correct the observed matrix effects, standard addition calibration methodology has been employed. By using this approach, statistically accurate results have been obtained when urine samples previously fortified were analyzed.

The methodology has been satisfactorily applied to the analysis of urine samples collected from a volunteer after repeated whole-body applications of a sunscreen cosmetic product containing MBC. Urine samples have been also treated with ß-glucuronidase to carry out enzymatic hydrolysis.

The parent compound has been determined in the urine sample that corresponded to the collected excretions 24 h after the first product application. The amount excreted of CBC increased as long as cosmetic product was applied, mainly as glucoronide conjugate. In fact, the highest concentration of CBC was determined in the sample collected the day after the last application, showing evidences of bioaccumulation processes. Furthermore, CBC-OH has been also identified and detected in urine samples. In this case, the contribution of the glucoronide conjugate was important too.

Annex I
Keys Used

The keys used throughout this PhD Thesis are shown below.

ACN	Acetonitrile
AR	Androgen receptor
ATP	Adenosine triphosphate
C8	Octyl SPE sorbent
C18	Octadecyl SPE sorbent
CBA	Carboxypropyl SPE sorbent
CBC	3-(4-carboxybenzilidene)camphor
DC	Diffusion cells
CN	Cyanopropyl SPE sorbent
DEA	Diethylamino SPE sorbent
DEET	*N,N*-diethyl-m-toluamide
DHB	2,4-Dihydroxybenzophenone
DHMB	2,2′-Dihydroxy-4-methoxybenzophenone
DMP	(*N,N*-dimethyl)-*p*-aminobenzoic acid
ER	Estrogen receptor
EtOH	Ethanol
FA	Formic acid
GC	Gas chromatography
GC−MS	Gas chromatography with mass spectrometry detection
GPC	Gel permeability chromatography
HCl	Chlorhydric acid
HF−LPME	Hollow fiber liquid-phase microextraction
HPLC	(see LC)
ICP	Induced coupled plasma
IR	Infrared espectrometry
IR−ATR	Infrared espectrometry-atenued total reflexion
LC	Liquid chromatography
LC−ECD	Liquid chromatography-electrochemical detection

LC−F	Liquid chromatography-molecular fluorescence detection
LC−UV/VIS	Liquid chromatography-ultraviolet/visible detection
LC−MS	Liquid chromatography with mass spectrometry detection
LC−MS/MS	Liquid chromatography with tandem mass spectrometry detection
LC−TOF/MS	Liquid chromatography with time-of-flight mass spectrometry detection
LOD	Limit of detection
LOQ	Limit of quantification
LLE	Liquid-liquid extraction
MALLE	Membranes assisted liquid-liquid extraction
MB-6HC	3-(4-Methylbenzilidene-6-hydroxy)camphor
MeOH	Methanol
MS	Mass spectrometry
MMP	(*N*-methyl)-*p*-aminobenzoic acid
MSTFA	*N*-methyl-*N*-(trimethylsylil)-trifluoroacetamide
NH2	Aminopropyl SPE sorbent
PEEK	Polyether ether ketone
PH	Phenyl SPE sorbent
PRS	Propilsulphonic acic SPE sorbent
RSD	Relative standar deviation
RX	X rays spectrometry
SAX	Strong anionic exchanger SPE sorbent
SBSE	Stir-bar sorption extraction
SDME	Single-drop microextraction
SI	Sequential injection system
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TD	Thermal desorption
TEM	Transmission electronic microscopy
THB	2,3,4-Trihydroxybenzophenone
TLC	Thin-layer chromatography
tRNA	Ribonucleic acid for transcription
TS	Tape-stripping technique
UV/VIS	Ultraviolet/visible spectrometry
2OH	Diol SPE sorbent

Annex II
Protocols to Develop the Sunscreen Cosmetic Products Used in this Ph.D. Thesis

The cosmetic ingredients used in the elaboration of sunscreen products, which were provided by Guinama SL (València) or RNB SL (València), are shown below:

- **Avocado oil** is a natural mixture of saturated fatty acids, oleic and linoleic, rich in vitamins A, B1, B2, D and E. Among its functions is to protect the skin.
- **Tanning accelerator** is a preparation of tyrosine derivatives and riboflavin which promotes melanin formation process.
- **Base PFC o/w cream** is a base which allows the production of oil/water emulsions and is composed of a mixture of myristyl myristate, cetyl alcohol, monoglyceryl laurate, cetearyl octanoate, isopropyl myristate and other components of lipophilic nature.
- **Dimethicone 350** consists of a mixture of polymers including dimethylsilicone and dimethylsiloxane. Among its functions, enhancement of the emulsions formation, providing a greater smoothness and softness on the product application and acting as a carrier for the active components.
- **Disodium salt EDTA** is a complexing agent that capture metals contained in the cosmetic product preventing from the formation of coloured complexes.
- **Glycerin** has main functions to protect the skin and provide softness.
- **Hidroviton**® is a preparation consisting of a mixture of amino acids, sodium lactate, urea, allantoin, alcohols, humectants and sodium chloride, having the main function of skin moisturizer.
- **Kathon**® is an aqueous solution of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one with preservative properties.
- **Perfume** brings a pleasant fragrance to the product.
- **Phenonip**® comprises a mixture of parabens and phenoxyethanol whose main function is preservative.
- **Polysorbate-80** is a synthetic nonionic emulsifying agent.
- **Propylene glycol** is an emulsifying agent which prevents dryness of the horny layer of the skin.
- **Vitamin E** has antioxidant properties, and protects against the formation of free radicals, softens the skin by moisturizing and provides greater elasticity.

Annex II.1: Development of a Sunscreen Containing the Most Widely Used Hydrosoluble UV Filters Approved by the European Union Law

A sunscreen cosmetic was developed by adapting a manufacturing protocol (Jordán and Jordán 1991). The composition and method of preparation is indicated below.

Phase A:	Kathon®	0.1 %
	Dimethicone 350	0.75 %
	EDTA disodium salt	0.1 %
	Glycerin	0.75 %
	Water	up to 100 %
Phase B:	PBS	3 %
	PDT	3 %
	BZ4	3 %
	TDS	3 %
	Water	25 %
	Sodium hydroxide solution	up to 10 %
Phase C:	Tanning accelerator	0.25 %
Phase D:	Polysorbate-80	0.5 %

For the preparation of the sunscreen, Phase B was prepared by adding the sodium hydroxide solution 10 % until completely solution of the hydrosoluble UV filters. Then, Phases A, C and D were mixed and added to Phase B. Finally, pH of the lotion was adjusted to a value close to 7 with a 10 % sodium hydroxide solution.

Annex II.2: Development of a Sunscreen Cosmetic Containing BZ3 and BZ4

A sunscreen cosmetic was developed by adapting a manufacturing protocol (Jordán and Jordán 1991). The composition and method of preparation is indicated below.

Phase A:	PFC Base o/w	18 %
	Avocado oil	5 %
	Dimethicone 350	5 %
	Vitamin E	0.5 %
	BZ3	8 %
	BZ4	4 %
Phase B:	Propylene glycol	5 %
	Hidroviton®	4 %
	Water	up to 100 %
Phase C:	Phenonip®	0.5 %

For the preparation of the sunscreen cosmetic, components of Phases A and B were weighed in separate beakers, which were covered and immersed into a water bath ($\sim$70 °C) until Phase A was melted. Then, Phase B was poured over Phase A very slowly under continuous stirring. Next, the beaker was removed from the water bath and allowed to reach 30–35 °C under continuous stirring. At that moment, Phase C was added and stirring was maintained until complete homogenization. Finally, the cosmetic was allowed to cool at room temperature.

Annex II.3: Development of a Sunscreen Cosmetic Containing BZ3

A sunscreen cosmetic was developed by adapting a manufacturing protocol (Jordán and Jordán 1991). The composition and method of preparation is indicated below.

Phase A:	PFC Base o/w	18 %
	Avocado oil	5 %
	Dimethicone 350	5 %
	Vitamine E	0.5 %
	BZ3	10 %
Phase B:	Propylene glycol	5 %
	Hidroviton®	4 %
	Water	up to 100 %
Phase C:	Phenonip®	0.5 %
	Perfume	0.5 %

To prepare the sunscreen cosmetic, see Annex II.2

Annex II.4: Development of a Sunscreen Cosmetic Containing EDP

A sunscreen cosmetic was developed by adapting a manufacturing protocol (Jordán and Jordán 1991). The composition and method of preparation is indicated below.

To prepare the sunscreen cosmetic, see Annex II.2.

Phase A:	PFC o/w base	18 %
	Avocado oil	5 %
	Dimethicone 350	5 %
	Vitamin E	0.5 %
	EDP	8 %
Phase B:	Propylene glycol	5 %
	Hidroviton	4 %
	Water	up to 100 %
Phase C:	Phenonip®	0.5 %
	Perfume	0.5 %

Annex II.5: Developing a Sunscreen Cosmetic Containing MBC

A sunscreen cosmetic was developed by adapting a manufacturing protocol (Jordán and Jordán 1991). The composition and method of preparation is indicated below.

Phase A:	PFC o/w base	18 %
	Avocado oil	5 %
	Dimethicone 350	5 %
	Vitamin E	0.5 %
	MBC	4 %
Phase B:	Propylene glycol	5 %
	Hidroviton®	4 %
	Water	up to 100 %
Phase C:	Phenonip®	0.5 %
	Perfume	0.5 %

To prepare the sunscreen cosmetic, see Annex II.2.

Annex III
Statistical Tests

The statistical tests (Miller and Miller 2005) used in this PhD Thesis are shown below.

Annex III.1: Grubbs Test to Outliers

The Grubbs test compares statistically the difference between the suspect value (x_i) and the mean of the data set ($\overline{x}$), with the standard deviation of the data set (s).

For a given outlier, the contrast is used to test the null hypothesis (H_0), that considers that all measures come from the same normal population. The statistic G is calculated as:

$$G_{calculated} = \frac{x_i - \overline{x}}{S}$$

where *s* is calculated including the suspect value. If the calculated **G** value exceeds the critical value for a given level of significance (P_0) and N number of data, the null hypothesis is rejected and therefore the suspicious data is abnormal.

Annex III.2: Fisher Test to Compare Variances

The F contrast is used to compare the variances of two sets of data, considering the relationship of the two variances. So, to test whether the difference between the two variances is significant, that is, to test the null hypothesis (H_0), which considers that both variances are equal (H_0: $s_1^2 = s_2^2$), the statistical F is calculated as:

$$F_{calculated} = \frac{s_1^2}{s_2^2}$$

where data sets 1 and 2 are displayed so that F is always ≥ 1. If the $F_{calculated}$ value exceeds the critical value ($F_{tabulated}$) for a given significance level (P_o) and $N_1 - 1$ y $N_2 - 1$ degrees of freedom (DoF) for each data series, the null hypothesis is rejected and it is accepted that there is a difference between the standard deviations of both sets of data.

Annex III.3: Comparison of a Experimental Mean with a Known Value: Student's t-Test

To decide if the difference between the experimental mean ($\bar{x}$) of a set of values (N) and a known value (μ) is meaningful, that is to test the null hypothesis (Ho), that considers no differences between the experimental mean and the known value ($Ho : \bar{x} = \mu$), the statistic is calculated as:

$$t_{calculated} = \frac{(\bar{x} - \mu)\sqrt{N}}{S}$$

where **s** is the standard deviation of the experimental mean.

If the absolute value of $t_{calculated}$ exceeds the critical value ($t_{tabulated}$) for a given significance level (P_o) and $N - 1$ degrees of freedom, that is, if $|t_{calculado}| > t_{tabulado}$, the null hypothesis is rejected and it is accepted that there is a significant difference at the level of significance between the mean and the known value obtained experimentally.

Annex III.4: Comparison of Two Experimental Means: Student's t-Test

To test whether two experimental means differ significantly, the Student's *t*-test is used. The null hypothesis to be tested is that there is no significant difference between the two experimental means ($\bar{x_1}$ and $\bar{x_2}$) of two sets of data. This *t*-statistic is calculated as:

$$t_{calculated} = \frac{(\bar{x_1} - \bar{x_2})}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}}$$

where s_1 and s_2 are the standard deviations and N_1 and N_2 are the number of data series **1** and **2**, respectively, and the degrees of freedom (DoF) are given by:

$$DoF = \frac{\left(\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}\right)}{\left(\frac{s_1^4}{N_1^2(N_1-1)} + \frac{s_2^4}{N_2^2(N_2-1)}\right)}$$

The obtained DoF value is rounded to the nearest whole number.
If the two samples do not have standard deviations significantly different (see Annex II.2), a common standard deviation can be calculated (**s**), so that:

$$s = \sqrt{\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{(N_1 - 1) + (N_2 - 1)}}$$

and the **t**-statistic is calculated as:

$$t_{calculated} = \frac{(\overline{x_1} - \overline{x_2})}{s\sqrt{\frac{1}{N_2} + \frac{1}{N_2}}}$$

with $(N_1 - 1) + (N_2 - 1)$ degrees of freedom, or $(N_1-2) + (N_2-2)$ degrees of freedom, in the case of comparing intercepts or slopes.
If the statistic $t_{calculated}$ exceeds the critical value $t_{tabulated}$ to given significance level and degrees of freedom, there is a significant difference between the means of both data sets and the null hypothesis is rejected.

Annex III.5: Comparison of Two Sets of Results Using a Linear Regression Model

The use of a linear regression model to test whether the values of two data sets are statistically comparable is very useful when comparing the added concentration values versus the obtained concentration values by the proposed method.
If representing the values obtained by the proposed method (*vertical axis*) versus the added concentration values (*x-axis*) yields a straight line whose intercept (a) is statistically equivalent to zero and whose slope (b) is statistically equivalent to one, it can be conclude that both sets of data do not differ significantly.
To test this hypothesis, the Student's *t*-test is used, so that:

$$\left[(t_{calculated})_a = \frac{|a - 0|}{s_a} \qquad (t_{calculated})_b = \frac{|b - 1|}{s_b} \right]$$

where s_a is the standard deviation of the intercept and s_b is the standard deviation of the slope of the regression line. Thus, if **t** calculated values for both intercept and slope are lower than the critical value of **t** for a particular significance level and **N** − 2 degrees of freedom (where **N** is the number of points on the line

regression), then the null hypothesis must be accepted, as there are no significant differences between the results obtained by the proposed method and the real values.