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STUDY OF UV FILTERS IN COSMETIC PRODUCTS AND ENVIRONMENTAL SAMPLES

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Dna. Marlene Vila González

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STUDY OF UV FILTERS IN COSMETIC PRODUCTS AND ENVIRONMENTAL SAMPLES

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ABBREVIATIONS

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2EHMC: 2-ethylhexyl methoxycinnamate

4MBC: 4-methylbenzylidene camphor

ACN: acetonitrile

ANOVA: analysis of variance

BMDM: butyl methoxy dibenzoyl methane

BP1: benzophenone-1

BP2: benzophenone-2

BP3: benzophenone-3

BP4: benzophenone-4

BP6: benzophenone-6

BP8: benzophenone-8

BS: benzyl salicylate

CCl_4: carbon tetrachloride

CLF: chloroform

DAD: diode array detector

DHHB: diethylamino hydroxybenzoyl hexyl benzoate

DRT: drometrizole trisiloxane

DVB/CAR/PDMS: divinylbenzene/carboxen/polydimethylsiloxane

EHPABA: ethylhexyl dimethyl p-aminobenzoic acid

EHS: ethylhexyl salicylate

EtAc: ethyl acetate

Eto: etocrylene

GC: gas chromatography

HMS: homosalate

HPLC: high performance liquid chromatography

HSSPME: headspace solid-phase microextraction

IAMC: isoamyl p-methoxycinnamate

IDL: instrumental detection limits

INCI: International Nomenclature of Cosmetic Ingredients

LC: liquid chromatography

LOD: limit of detection

LOQ: limit of quantification

MA: menthyl anthranilate

MeOH: methanol

MS: mass spectrometry

MS/MS: tandem mass spectrometry

OCR: octocrylene

PA: polyacrylate

PBSA: ensulizole

PDMS/DVB: polydimethylsiloxane/divinylbenzene

PLE: pressurized liquid extraction

RSD: relative standard deviation

SIM: selected ion monitoring

SPME: solid-phase microextraction

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SRM: selected reaction monitoring

TCE: trichloroethane

US: ultrasounds

USAEME: ultrasound-assisted emulsification microextraction

UV: ultraviolet

w/w: weight to weight

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ABSTRACT

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The main objective of this thesis is to develop analytical methods to determine a kind of cosmetic ingredients, the UV filters, in the cosmetics themselves, and in environmental matrices such as waters and beach sand. For that purpose, microextraction techniques and chromatographic analysis were employed.

This work is divided in four chapters. The first one, justification and objectives, expose the problems that exist in relation to the use of UV filters and their presence in the environment, and the necessity of developing methods to analyse them in these matrices, which is the aim of this thesis.

Chapter II includes an introduction of different aspects addressed in the thesis. On one hand, UV filters are defined and some general aspects such as types of UV filters, their toxicity and some physicochemical properties are also commented. On the other hand, it is dealt with the matrices studied in this work, both cosmetics and environmental samples (waters and beach sand). In these points, a brief introduction of cosmetics, regulatory aspects, the risks of these emerging contaminants in the environment and some analytical background are presented. Finally, the sample preparation techniques and analysis instrumentation employed in this thesis are discussed.

In Chapter III, the experimental work carried out during this period is exposed. The first two methods presented were developed to analyse UV filters in cosmetics, and they are based on pressurized liquid extraction (PLE) followed by gas or liquid chromatography-tandem mass spectrometry. The following three methods are related to the analysis of the same compounds in water samples by ultrasound-assisted emulsification microextraction (USAEME) and solid phase microextraction (SPME). Finally, different methods dealing with the analysis of UV filters in beach sand are presented.

In the last chapter, the conclusions resulting from the studies included in this thesis are presented.

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I. JUSTIFICATION AND OBJECTIVES

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I. Justification and objectives

In recent years, the concern of the population about the adverse health effects of ultraviolet radiation (mainly sunburns and the appearance of cancer), have caused the consumption of sunscreens to be significantly increased. In addition to other families of compounds usually added to cosmetic products, such as preservatives, fragrances, etc., UV filters are included in sunscreen formulations, since they are the substances responsible for protecting the skin against solar radiation. Because of the great awareness of how important is to protect the skin against solar radiation, nowadays UV filters are not only included in solar range formulations, also in daily use cosmetics such as moisturizing creams, make up, lipsticks, hand creams, etc.

However, these compounds, despite being necessary to fulfil the function of protecting the skin against the solar radiation, are suspected to cause adverse health effects such as endocrine disruption. Therefore, it is important to control these compounds in cosmetics to maintain consumer safety. In fact, there are different regulations according to each country. In the case of Spain, cosmetics must comply with the European Regulation 1223/2009 [1]. In Annex VI are gathered the UV filters allowed in cosmetic formulations with their maximum permitted concentration. Below that concentration it is assumed that its use is safe for consumers. With the new discoveries regarding its toxicity, the legislation is permanently revised. Consequently, it is necessary the development of analytical methods useful to verify that the cosmetics comply with current legislation and, in addition, they must include the largest number of these compounds as possible and be prepared for possible new restrictions.

Furthermore, UV filters enter the environment directly through aquatic activities or indirectly with domestic discharges. In fact, they are considered emerging pollutants [2]. With the increasing use of solar products, as discussed before, it is logical that their presence in the environment is increased. Moreover, sewage treatment plants are not always effective in removing these chemicals [2, 3]. UV filters suppose

a hazard to the aquatic organisms where it has been demonstrated that they are bioaccumulated, and in consequence to humans, since is biomagnified up the food chain. Although nowadays there is not a specific regulation for these compounds in the environment, one UV filter, the 2-ethylhexyl methoxycinnamate, has been included during the development of this thesis (in 2015) in a watch list for its monitoring in water samples (although also recommends its monitoring in sediments) and its future consideration as priority contaminant. Therefore, not only is necessary the development of analytical methods for UV filters in cosmetics, but also in environmental samples such as all types of waters, sediments, soils, sand, etc. In the case of environmental matrices, above all water samples, the compounds are found at levels of ng L^{-1}, so sensitive methods are necessary.

For the reasons set out above, this thesis was focused on the development of sensitive, selective and environmental friendly methods that include the largest possible number of analytes to analyse UV filters in cosmetic and environmental samples.

Microextraction techniques were used with the aim of extracting (and in some cases also enriching) the compounds from the corresponding matrices avoiding organic solvents or using the smallest amounts as possible and using a small amount of sample. For cosmetic samples, pressurized liquid extraction (PLE) was employed, since it is an adequate extraction method for solid samples. In the case of environmental samples, for liquid matrices such as waters, ultrasound-assisted emulsification microextraction (USAEME) and solid-phase microextraction (SPME) were utilized, while for solid samples such beach sand ultrasounds and vortex assisted extractions, on-column lixiviation and SPME were employed. All the extraction techniques are commented in Section 4, Chapter II.

Regarding the analysis of UV filters, both gas and liquid chromatography were used. The aim is to develop sensitive and selective methods, so tandem mass spectrometry was employed as detector of both chromatographic systems.

In all cases, once the analytical method was optimized, it was validated and applied to the corresponding type of samples.

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II. INTRODUCTION

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II. Introduction

1. UV filters

1.1. DEFINITION

According to the definition given by the European Regulation [1], UV filters are "substances which are exclusively or mainly intended to protect the skin against certain UV radiation by absorbing, reflecting or scattering UV radiation".

It is well known that solar radiation may cause cancer, sunburn, premature aging of the skin (wrinkles or spots) or other diseases. The solar radiation that arrives Earth is composed by UVA and UVB radiation, both of which pass through the ozone layer, unlike UVC radiation. UVA radiation is responsible for skin pigmentation and premature skin aging, and it is subdivided into UVA I (wavelength range 320-340 nm) and UVA II (wavelength range 340-400 nm). UVB radiation can also induce the pigmentation but it has higher energy due to its shorter wavelength, between 290-320 nm and is the mainly responsible for skin sunburns and the subsequent reddening of the skin (erythema) [4]. As to the cancer risk, although UVB radiation is the main contributor, the risk generated through UVA radiation cannot be neglected. Research also suggests that excessive exposure to UVB radiation as well as UVA radiation impacts on the body's immune system [5].

Therefore, the presence of UV filters in cosmetics is necessary and, in addition, sunscreen products should be sufficiently effective against UVB and UVA radiation to ensure a high protection of public health, so a mixture of different UV filters must be added to the cosmetics, although no sunscreen can guarantee total protection against risks that ultraviolet (UV) radiation entails for health [5]. These compounds are also used to enhance product stability (UV blockers), for example in textiles, plastics, fabrics, coatings, adhesives, and optical products [2].

As a consequence of the great awareness of the population about the prevention of the appearance of cancer, the use of sunscreens is more and more increased, not only in specific solar products, but in daily use moisturizing creams, make-up, etc. However, despite being required with this objective, there are some studies that report that these compounds may produce adverse effects such as endocrine disruption [6]. Consequently, an equilibrium between providing protection and avoiding their negative health consequences must be achieved, and for this reason they are included in cosmetic regulations, where the maximum allowed concentrations of UV filters are indicated. This aspect will be later commented in section 2.2.

1.2. TYPES

UV filters can be classified into organic (chemical) and inorganic (physical) based on their mechanism of action [4, 7].

Inorganic UV filters: they are also called physical, because their mode of skin protection against solar radiation is scattering and reflecting UV radiation, a physical phenomenon. The most frequently used inorganic UV filters are titanium dioxide (TiO_2) and zinc oxide (ZnO).

Inorganic UV filters present some advantages over organic filters, such as photostability, non-irritability and broad-spectrum protection. Nevertheless, inorganic filters have a whitening effect in the skin, so they are not so accepted by the population for aesthetic reasons. In addition, the nanomeric forms of inorganic UV filters are now in the spotlight because they might be able to penetrate the skin.

Organic UV filters: they are also called chemical filters, as their mode of action is related to chemical changes in their molecules that prevent UV radiation reaching the skin by absorbing the UV light. As a consequence of its mechanism of action, organic UV filters can release free radicals and consequently cause damage to collagen, elastin or skin cell DNA. In addition, they can be absorbed through the skin, being able

of causing endocrine disruption or other adverse effects. As commented before, they absorb the UV radiation, so they can be degraded and, therefore, lose their photoprotective function and, the most important, lead to other molecules that can be even worse than initials. Thus, the use of organic UV filters could be questioned from the point of view of human health.

Chemical UV filters are organic compounds that usually possess single or multiple aromatic structures, sometimes conjugated with carbon–carbon double bonds and/or carbonyl moieties. Among they are benzophenone derivatives, p-aminobenzoic acid derivatives, salicylates, cinnamates, benzotriazole derivatives, benzimidazole derivatives, camphor derivatives, triazine derivatives, and others [8].

Sunscreen products should be sufficiently effective against UVB and UVA radiation to ensure a high protection of public health. Not all the organic UV filters absorb both UV bands and many absorb only UVB radiation, so a mixture of UV filters must be added to the sunscreen products to ensure a good protection. Among the UVB agents highlight the p-amino benzoic acid (PABA) derivatives, cinnamates, ensulizole, salicylates and octocrylene. Benzophenones and avobenzone are examples of UVA absorbers [9].

As discussed in this section, organic UV filters are those that present major problems from the point of view of health, so this thesis will focus on these compounds.

1.3. TOXICITY

As said before, despite of being necessary for protecting the skin against solar radiation, UV filters are under scope due to their toxicity and adverse effects, mainly their endocrine disrupting effects.

Some UV filters react with some protein residues in the skin, causing allergic reactions [10-12].

It is proved that these compounds are able to penetrate the skin, since they have been found in human blood or plasma [13-16], urine [13-19], placental tissue [20-22], semen [17, 23] and breast milk [24]. Most of the publications found in literature dealing with the analysis of UV filters in biological fluids are focused on the study of benzophenone-3, although also can be found results for 4-methyl benzylidene camphor, 2-ethylhexyl methoxycinnamate, ethylhexyl PABA, and to a lesser extent for p-aminobenzoic acid, butyl methoxy dibenzoyl methane, ethylhexyl salicylate, homosalate, 3-benzylidene camphor, ensulizole and disodium phenyl dibenzimidazole tetrasulfonate.

As consequence of the penetration through the skin they can induce endocrine disrupting effects and there are studies that demonstrate so. A recent review published by Wang et al. [6] summarizes the studies found in the literature concerning endocrine disrupting effects of UV filters. It is mainly focused in benzophenones, camphor and cinnamate derivatives since, as commented before, they are for which more studies have been published. These effects are related with oestrogen, androgen, progesterone, thyroid hormone and other nuclear receptors, amongst are included activation of ERα and ERβ (ER: oestrogen receptor), inhibition of the activity of 17β-Oestradiol, induction of proliferation of MCF-7 cell, reduce of the uterine weight in rats, induction of vitellogenin in fathead minnows and fish, antagonists of human androgen and progesterone receptor, inhibition of testosterone in HEK-293 cells and in rats, inhibition of human recombinant thyroid peroxidase and decrease of thyroxine level, amongst others. Also, a review published in 2012 by Krause et al. [25] show a complete list of endocrine disrupting properties of UV-filters, and, alarmingly, there is a large number of contributions on this topic.

In addition, it is suspected that UV filters may induce reactive oxygen species (ROS) generation in epidermis [26] and in aqueous solution [27, 28]. In general, harmful effects of ROS on the cells are most often damage of DNA or RNA, oxidations of polyunsaturated

fatty acids in lipids, oxidations of amino acids in proteins and oxidative deactivation of specific enzymes by oxidation of co-factors [29].

Another aspect to take into account is the possibility of organic UV filters undergo degradation, mainly by photolysis, but also as consequence of reaction with chlorine in chlorinated media (e.g., the sea or the swimming pool) [30, 31]. It is important the identification of the degradation products to determine their environmental and human health effects since they may be even more toxic than the parent UV filters [32]. Some studies showed some possible degradation products or by-products [31, 33-36], although the research in this field is still scarce.

These studies are key in the modification of regulations, since the use of cosmetics containing these compounds must be safe for humans. Thus, as new adverse effects are discovered, certain compounds can be prohibited, or their maximum allowed concentration is reduced to a level where their use does not compromise health. In section 2.2., regulatory aspects will be further discussed.

1.4. STRUCTURE AND PHYSICOCHEMICAL PROPERTIES

Table 1 shows the list of UV filters under study in this doctoral thesis (INCI: International Nomenclature of Cosmetic Ingredients), along with their CAS number, molecular weight (MW), and some physicochemical properties of analytical interest such as the negative decimal logarithm of the acid dissociation constant (pK_a) boiling point, vapor pressure (VP), the decimal logarithm of the octanol-water partition coefficient ($\log K_{ow}$) and the molar solubility in water. All data comes from Scifinder ((Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2018 ACD/Labs)) and from ChemSpider (Predicted data is generated using the US Environmental Protection Agency's EPISuite™).

These compounds usually have single or multiple aromatic structures, sometimes conjugated with carbon–carbon double bonds

and/or carbonyl moieties. They are usually soluble in fatty matrices, although some of them contain ionizable moieties, such as sulphonic (-SO_3H) or carboxylate (-COOH), which enables their solubility in water [8]. The structures of the UV filters studied in this thesis are shown in Figure 1.

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Table 1. INCI name, CAS number, molecular weight and some physico-chemical properties of the studied UV filters

INCI name	CAS No.	MW (g/mol)	pK_a	Boiling Point (°C)	VP (mTorr)	log K_{ow}	Solubility in water (mol L^{-1})
Benzophenones							
Benzophenone-1 (BP1)	131-56-6	214.22	7.72±0.4	409±14	$2.84x10^{-4}$	3.2±0.4	pH 1-5: 1.8×10^{-3} pH 7: 2.4×10^{-3} PH 10: 3.62
Benzophenone-2 (BP2)	131-55-5	246.22	6.98±0.4	531±25	$6.69x10^{-9}$	3.1±0.4	pH 1-4: 4.0×10^{-3} pH 7: 0.013 pH 10: 4.06
Benzophenone-3 (BP3)	131-57-7	228.24	7.56±0.4	370±27	0.00526	4.0±0.4	pH 1-4: 4.4×10^{-4} pH 7: 5.7×10^{-4} pH 10: 0.10
Benzophenone-4 (BP4)	4065-45-6	308.31	-0.7±0.5	498	$1.34x10^{-8}$	0.9±0.4	pH 1: 2.72 pH 2-10: 3.24
Benzophenone-6 (BP6)	131-54-4	274.27	6.8±0.4	439±45	$2.49x10^{-5}$	4.8±0.4	pH 1-5: 2.4×10^{-4} pH 7: 7.0×10^{-4} pH 10: 3.15
Benzophenone-8 (BP8)	131-53-3	244.24	7.1±0.4	375±0	0.00373	4.3±0.4	pH 1-4: 4.9×10^{-4} pH 7: 1.0×10^{-3} pH 10: 4.09

Table 1 (cont.). INCI name, CAS number, molecular weight and some physico-chemical properties of the studied UV filters

INCI name	CAS No.	MW (g/mol)	pK_a	Boiling Point (°C)	VP (mTorr)	log K_{ow}	Solubility in water (mol L^{-1})
Cinnamates							
2-Ethylhexyl methoxycinnamate (2EHMC)	5466-77-3	290.40	-	405±20	$8.89x10^{-4}$	5.9±0.5	2.2×10^{-5}
Isoamyl p-methoxycinnamate (IAMC)	71617-10-2	248.32	-	363±17	0.0189	4.5±0.2	$2.4x10^{-4}$
Salicylates							
Ethylhexyl salicylate (EHS)	118-60-5	250.33	8.1±0.3	332±15	0.0807	5.9±0.2	pH 1-5: $6.1 x10^{-5}$ pH7: $.6.6 x10^{-5}$ pH 10: $4.4 x10^{-3}$
Benzyl salicylate (BS)	118-58-1	228.24	8.1±0.3	320±0	0.175	4.2±0.3	pH 1-6: $3.8x10^{-4}$ pH 10: 0.028
Homosalate (HMS)	118-56-9	262.34	8.1±0.3	341±15	0.0417	5.9±0.3	pH 1-6: $7.9 x10^{-5}$ pH 10: $6.1 x10^{-3}$

Table 1 (cont.). INCI name, CAS number, molecular weight and some physico-chemical properties of the studied UV filters

INCI name	CAS No.	MW (g/mol)	pK_a	Boiling Point (°C)	VP (mTorr)	log K_{ow}	Solubility in water (mol L^{-1})
Others							
4- Methylbenzylidene camphor (4MBC)	36861-47-9	254.37	-	3712±22	9.99 x10^{-3}	3.4±0.3	6.5 x 10^{-5}
Butyl methoxy dibenzoyl methane (BMDM)	70356-09-1	310.39	9.7±0.1	464±35	8.9 x10^{-6}	4.2±0.4	pH1-8: 1.4 x 10^{-5} pH10: 4.0 x10^{-5}
Diethylamino hydroxybenzoyl hexyl benzoate (DHHB)	302776-68-7	397.51	7.6±0.5 2.7±0.4	525±40	1.2 x10^{-8}	6.9±0.4	pH1: 2.6 x10^{-5} pH7: 8.1 x10^{-7} pH10: 1.6 x10^{-4}
Drometrizole trisiloxane (DRT)	155633-54-8	501.84	8.4±0.5 0.7±0.3	531±60	7.1 x10^{-9}	8.3±1.2	pH1: 2.5 x10^{-7} pH7:1.9 x10^{-7} pH10:1.3 x10^{-5}
Ensulizole (PBSA)	27503-81-7	274.30	-0.9±0.4 4.2±0.1	566	7.3 x10^{-12}	-0.2±0.8	pH 1-3: 5.5 x10^{-2} pH 5: 0.25 pH 7-10: 3.6
Ethylhexyl dimethyl PABA (EHPABA)	21245-02-3	277.40	2.4±0.1	383±25	0.00457	5.4±0.2	pH1: 4.1 x10^{-4} pH 5-10: 1.7x10^{-5}
Etocrylene (Eto)	5232-99-5	277.32	-	408±33	7.46 x10^{-4}	4.0±0.3	9.5 x10^{-5}
Menthyl Anthranilate (MA)	134-09-8	275.39	2.2±0.1	384±15	4.38 x10^{-3}	6.1±0.3	pH1: 2.0 x10^{-4} pH 4-10: 1.3 x10^{-5}
Octocrylene (OCR)	6197-30-4	361.48	-	479±33	2.56 x10^{-6}	6.9±0.3	1.0 x 10^{-6}

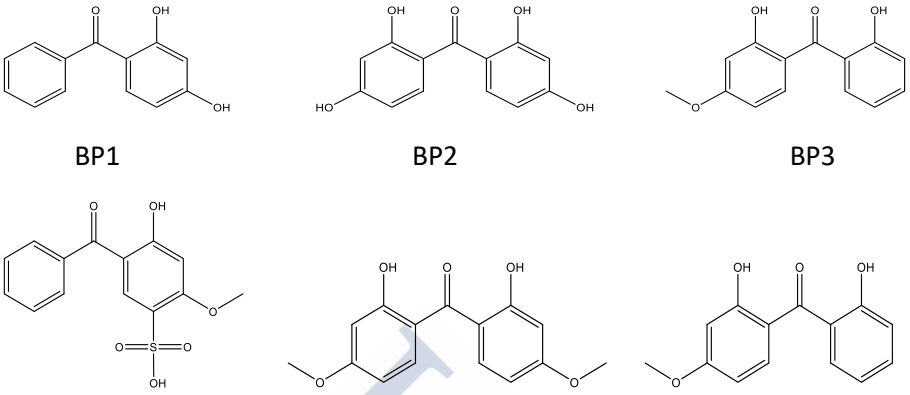

Figure 1.1. Benzophenones

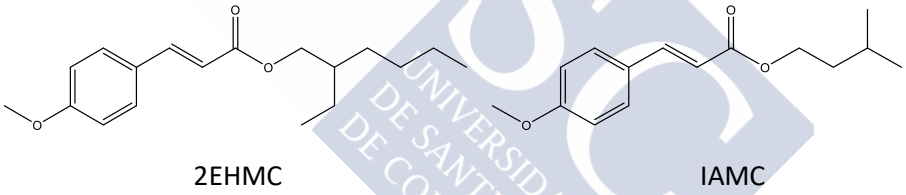

Figure 1.2. Cinnamates

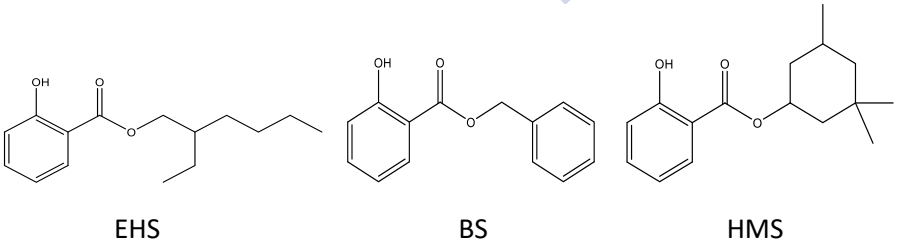

Figure 1.3. Salicylates

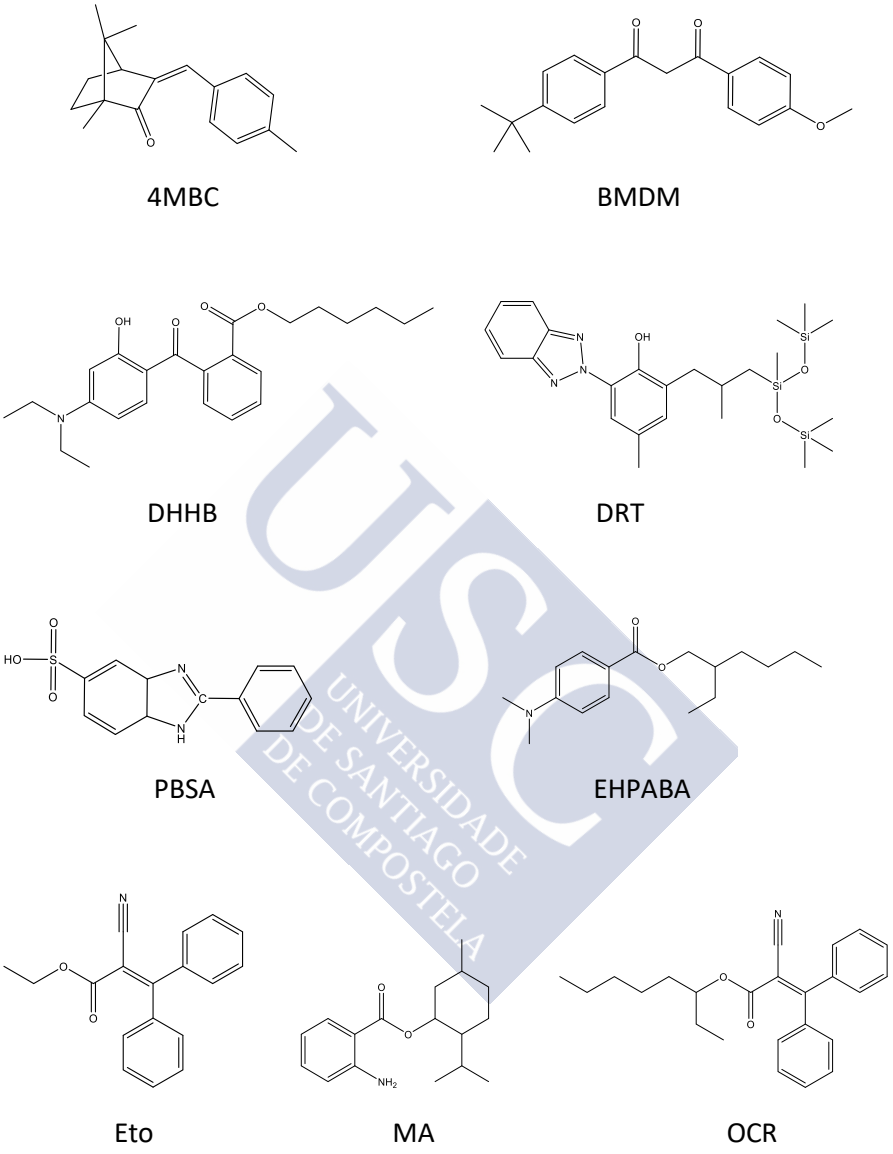

Figure 1.4. Others

2. Cosmetics

2.1. DEFINITION AND HISTORY

One of the matrices under study in this thesis have been cosmetics, the initial consumer product where the UV filters are included. The European Regulation [1] defines as cosmetic "any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours".

Cosmetics are present in daily life, and it is estimated that each person uses between 10 and 20 cosmetics in a day, since products such as soap, shampoo, deodorant, moisturizing creams, aftershave, etc. are considered cosmetics.

Their use is increasingly growing, involving the consumption of perfumes and cosmetics an increase of 3.25% during 2016 up to 6,660 million euros in Spain. The consumption of skincare products increased by 3.9% in 2016, being this category of cosmetics the most used (28%). In particular, the products that most substantially increased its consumption were solar range products, reaching 9%, due to the awareness of the population of the need to protect itself against solar radiation. Spain is at the forefront of solar protection worldwide.

The use of solar protection is relatively new. It was in the 1920s when photoprotection was firmly established with the arrival of the fashion phenomenon. The French designer Coco Chanel during a trip to Cannes and the famous Parisian singer Josephine Baker, both tanned, were role models. Since then, being tan is fashionable and with it the need to protect the skin emerged. The first solar protectors were based on olive and almonds oil. In 1933 the first photoprotective agent based on benzimidazole appeared. In 1935 the French chemist Eugène

Schueller, founder of L'Oreal, realized the need to protect the skin from sun exposure not only in the beach, but also in some sports such as sailing. In this way, Schueller developed a UVB sunscreen filter, responsible for sunburn. During the Second World War, the soldiers displaced in the Pacific suffered burns due to sun exposure and it was then discovered that the paraffin applied to the skin created a layer that prevented the ultraviolet rays from penetrating the skin. The paraffin would be patented in 1950 and its protector marketed under the name of 'Coppertone'. In the 80s, sunscreen protected only against UVB rays, with very low protection filters because, at that time, it was believed that if you protected yourself from the sun it would be impossible to tan. In 1983, the filter capable of absorbing UVA radiation was approved by the European Union. The industry of the European Union becomes a world leader in UV sun protection filters when in the 90s the European cosmetic legislation regulated its use. At the beginning of the 21st century, new formats of sunscreen appeared such as sprays, products with a gel texture, etc. In 2006, with an intense collaboration with the Industry and based on the recommendation that Cosmetics Europe had made, the European Commission published in September its Recommendation (2006/647/EC) "Concerning the efficacy of sunscreen products and declarations about them" [5]. This initiative aimed to standardize and simplify to the maximum the way in which solar protection products are tested and labelled throughout Europe. In 2009, Cosmetics Europe recommends that the indication of compliance with the Commission Recommendation be made through the letters "UVA" printed in a simple circle.

The sector continues to evolve, and sunscreens can now be found on the market for other parts of the body such as hair or lips, polyvalent products such as BBcreams, make-up, daily moisturizing creams, etc. [37].

2.2. REGULATORY ASPECTS

As mentioned before, UV filters used in cosmetics are regulated to ensure consumers health by different legislations according to the country, among which highlight the ones recommended by the European Regulation [1], the Food and Drug Administration (FDA) in United States of America [38] and the proposed by Japan [39].

In the case of the European Regulation (1223/2009), the UV filters allowed in cosmetics are found in Annex VI. Any other filter not included in this list is forbidden in cosmetics. Benzyl salicylate can act as a filter as well as a fragrance. This compound does not appear in Annex VI, but it does in Annex III since it has some restrictions related to the labelled.

This regulation is constantly changing, being usually increasingly restrictive as suspected or proved that a compound has a detrimental effect on health. For example, PABA and 3-benzylidene camphor, initially allowed in 2009, have been forbidden in 2013 and 2015, respectively. Sometimes, new substances are also included in this list, as is the case of zinc oxide. It can also happen that a substance remains allowed, but its maximum permitted concentration is diminished, as occurred for benzophenone-3 in 2017, whose maximum permitted level was reduced since 10% to 6%. Nowadays, 25 organic and 2 inorganic UV filters are allowed by the European Regulation. Table 2 shows all of them and their maximum permitted concentration.

Table 2. List of UV filters allowed in cosmetics by the European Regulation

INCI name	Maximum allowed concentration
Camphor Benzalkonium Methosulfate	6 %
Homosalate	10 %
Benzophenone-3 [(1)]	6 %
Phenylbenzimidazole Sulfonic Acid	8 % (as acid)
Terephthalidene Dicamphor Sulfonic Acid	10 % (as acid)
Butyl Methoxydibenzoylmethane	5 %
Benzylidene Camphor Sulfonic Acid	6 % (as acid)
Octocrylene	10 % (as acid)
Polyacrylamidomethyl Benzylidene Camphor	6 %
Ethylhexyl Methoxycinnamate	10 %
PEG-25 PABA	10 %
Isoamyl p-Methoxycinnamate	10 %
Ethylhexyl Triazone	5 %
Drometrizole Trisiloxane	15 %
Diethylhexyl Butamido Triazone	10 %
4-Methylbenzylidene Camphor	4 %
Ethylhexyl Salicylate	5 %
Ethylhexyl Dimethyl PABA	8 %
Benzophenone-4, Benzophenone-5	5 % (as acid)
Methylene Bis-Benzotriazolyl Tetramethylbutylphenol	10 %
Disodium Phenyl Dibenzimidazole Tetrasulfonate	10 % (as acid)
Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine	10 %
Polysilicone-15	10 %
Titanium Dioxide [(2)]	25 %
Diethylamino Hydroxybenzoyl Hexyl Benzoate	10 %
Tris-biphenyl triazine Tris-biphenyl triazine (nano) [(3)]	10 %
Zinc Oxide Zinc Oxide (nano)	25 % [(4)]

[(1)] It must be indicated in the label 'Contains benzophenone-3' if the concentration exceeds 0.5 % (not for product protection purposes).
[(2)] For use as a UV filter
[(3)] Not to be used in sprays. Only nanomaterials having the following characteristics are allowed: median primary particle size > 80 nm; Purity ≥ 98 %; Uncoated
[(4)] In case of combined use of zinc oxide and zinc oxide (nano), the sum shall not exceed 25 %. They have some extra specifications, not commented here because inorganic UV filters are not in the scope of this thesis.

2.3. ANALYTICAL BACKGROUND

Most of the published methods about the determination of UV filters in cosmetic samples performed sample dilution in different solvents, sometimes with the aid of ultrasounds (US) or agitation [40, 41]. However, the use of microextraction techniques has been proposed in recent years with the aim of using methods environmental friendly and to avoid all the sample components are introduced in the analysis instruments. In this sense, the hollow fibre liquid-phase microextraction (HFLPME) [42], bar adsorptive microextraction (BAμE) [43], dispersive solid-phase microextraction (DSPE) [44] and ionic liquid dispersive liquid-liquid microextraction (IL-DLLME) [45] have been proposed to extract UV filters from cosmetic samples. In this thesis, two methods based on pressurized liquid extraction (PLE) will be presented (Section 1, Chapter III).

Regarding the methods of analysis for UV filters in cosmetics, liquid chromatography with UV detection was mainly employed. Recent works show the use of LC coupled to MS/MS, which is a detector much more sensitive and selective than UV. In this sense, a method based on LC-MS/MS was developed in this thesis to determine fifteen UV filters in cosmetics (Section 1.2., Chapter III). Also, it can be found in the literature some references that perform the analysis of these compounds by means of micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary electrochromatography (CEC), the thin layer chromatography (TLC) and other less common approaches such as the electroanalytical methods. GC is not a common choice to analyse UV filters in cosmetics, and only one work based on GC coupled to MS was found in the literature to analyse UV filters and preservatives in cosmetics [46]. In this thesis, a method to analyse sixteen UV filters in cosmetics by GC-MS/MS is proposed (Section 1.1., Chapter III).

More information can be found in the review published by our research group [47] dealing with the analysis of different families of

cosmetic ingredients, including the UV filters. It gathers the studies published between 2005 and 2015 in the field of cosmetics.

There is an European Standard (EN-16344) [48], in force since 2013, where a method for the screening of UV filters listed in the Annex VI of the European Regulation 1223/2009 and quantitative determination of 10 UV filters in cosmetic products by high performance liquid chromatography-diode array detector (HPLC-DAD) is described. The proposed method is based on the extraction of the cosmetics with a mixture of acetone/methanol (or other mixtures for specific compounds) with the aid of US and centrifugation.

3. Environment

UV filters are considered as emerging contaminants [2]. They enter the environment directly via wash-off from the skin surface during recreational aquatic activities of cosmetic users (e.g. swimming, bathing, etc), as well as indirectly through domestic and industrial discharges, and by the effluents from wastewater treatment plants (since they are not always effective in removing UV filters) and sewage sludge, that may be used as a fertilizer in agriculture [3].

3.1. WATER

The most efforts with regard to the analysis of UV filters in environmental matrices were focused in water samples [49, 50]. In this field, different types of waters such as lake [51], river [52, 53], seawater [54-57], swimming pool [52, 54], wastewater [3, 52], well [58], bottled [58] and tap [58] waters were studied. Nevertheless, solid samples such as sediments and soils were also analysed, although to a lesser extent [49, 59].

This contamination in the environment involves a risk for the marine biota. Moreover, due to their high lipophilicity, UV filters are

readily concentrated and accumulated in living aquatic organisms. There are studies that demonstrate the presence of UV filters in aquatic organisms such as different species of fish (salmon, carp, codfish, etc), mussels, clams, prawns, crabs, and dolphins, and also in birds [49, 60].

This is not a problem that only concerns to aquatic biota, but also to humans. Species higher in the food web can be exposed to all the chemicals that lower-order species accumulate (biomagnification) [60].

3.1.1. Regulatory aspects

Regarding the legislation of UV filters in the field of environmental samples, no regulations exist to limit maximum concentrations of these compounds. In 2015, the UV filter 2-ethylhexyl methoxycinnamate was included in a "watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council" [61]. The purpose of this directive is supporting future prioritization of all substances included on it. The regulation does not provide a maximum concentration above which a substance is forbidden, only recommends methods to analyse them in these matrices and a maximum acceptable method limit of detection. In the case of 2-ethylhexyl methoxycinnamate, it should be monitored in whole water samples and also in suspended particulate matter or sediment, because of its tendency to partition into this matrix. For this compound, the maximum limit of detection is established in 6000 ng L^{-1} for waters and 200 ng kg^{-1} for suspended particulate matter and in sediments.

3.1.2. Analytical background

As earlier mentioned, it can be found in the literature several articles dealing with the analysis of UV filters in water samples. Most of these works performs the extraction by solid phase extraction (SPE) [49]. However, microextraction techniques are preferred, since they require less volumes of sample and organic solvents. Therefore, some studies reference the use of dispersive liquid-liquid microextraction

(DLLME) [52, 56], ultrasound-assisted emulsification microextraction (USAEME) [62], single drop microextraction (SDME) [63], hollow fibre liquid phase microextraction (HF-LPME) [64], solid-phase microextraction (SPME) [65, 66], stir bar sorptive extraction (SBSE) [67-69], microextraction by packet sorbent (MEPS) [70], membrane-assisted liquid–liquid extraction (MALLE) [71] and bar adsorptive micro-extraction (BAμE) [43].

3.2. SOLID SAMPLES

In the same way that for waters, UV filters are deposited in environmental solid samples such as soils, sediments, sludge, etc. In addition, most of these compounds have high octanol-water partition coefficients, which means that they tend to accumulate in solids. In fact, some publications reported the presence of these compounds in river, lake and coastal sediments and soils [49].

3.2.1. Analytical background

Regarding the analysis of UV filters in environmental solid samples, the sample preparation technique mainly selected was the pressurized liquid extraction (PLE) [72-74]. Microwave assisted extraction (MAE) [75], matrix solid phase dispersion (MSPD) [76], pressurized hot water extraction–stir bar sorptive extraction (PHWE-SBSE) [77], Soxhlet [78] or a solid-liquid extraction by shaking [79] or assisted by ultrasounds [80] were also reported.

One of the matrices under study in this thesis was marine beach sand. It is an environmental matrix scarcely studied. In fact, only one study deals with the analysis of UV filters in beach sand [81]. In this case, a dispersive liquid-liquid extraction (DLLME) was applied to an acetone extract of the beach sand obtained by vortex assisted extraction. Concerning other kind of compounds, musks were extracted from beach sand using QuEChERS (Quick, Easy, Cheap, Effective, Rugged, y Safe) [82].

4. Sample preparation

The sample preparation step typically consists on the extraction of components of interest from the sample matrix. It is a fundamental part in the analytical process, since most analytical instruments cannot handle the matrix directly. In addition, this essential step often produces enrichment of the compounds, that means, it brings them to higher concentration levels, allowing reducing the limits of detection [83].

New sample preparation methods are in continuous development with the aim of reducing or eliminating organic solvents and to try to miniaturize this process.

In the case of cosmetic products, as commented before, the sample cannot be introduced directly in the analysis equipment. For this reason, an extraction step must be performed. Regarding organic UV filters, they are allowed in cosmetics by the European Regulation up to 15 % (15 mg g^{-1}) (w/w), so there is not the necessity of applying techniques that provide high concentration factors.

Concerning environmental samples, and more specifically water samples, UV filters can be found at very low concentration levels (ng mL^{-1}), consequently extraction techniques with high enrichment factors are mandatory.

Therefore, in this doctoral thesis pressurized liquid extraction (PLE), ultrasound-assisted emulsification microextraction (USAEME), solid-phase microextraction (SPME), and ultrasounds (US) and vortex assisted extractions were used with the aim of extracting UV filters from personal care products and environmental matrices.

4.1. PRESSURIZED LIQUID EXTRACTION (PLE)

4.1.1. Introduction

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), pressurized fluid extraction (FPE) or pressurized solvent extraction (PSE), is an extraction technique that combines high temperatures (50-200 ºC) and pressures (up to 2000 psi) to extract compounds from solid matrices [83-85]. In this way, the extraction solvent can be in liquid phase at temperatures higher than its boiling point, which makes it a very powerful extraction technique.

The first commercial instrument to perform PLE was developed by Dionex Corporation in 1995 [86]. Regardless of the model and the commercial house, the PLE equipment consist of a pump to boost the solvent, an oven where the steel cells are introduced to maintain the samples at the selected temperature, a collector vial where the liquid extract is collected and nitrogen to purge the cell once the extraction process is finished. A schematic diagram of a PLE can be found in Figure 2.

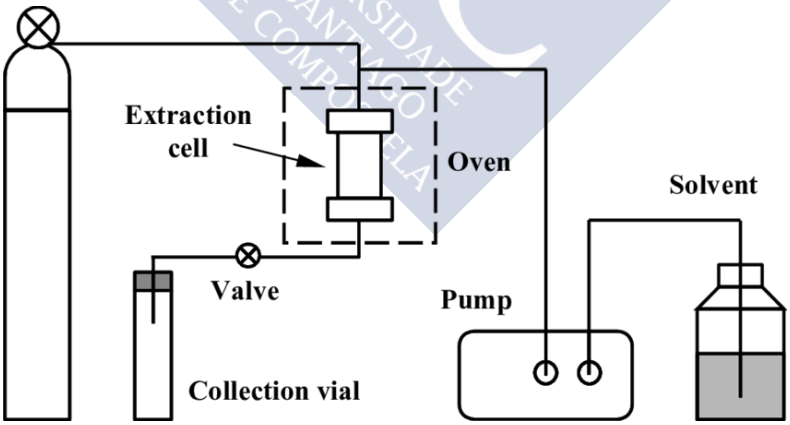

Figure 2. Schematic diagram of a PLE system

Among its main advantages with respect to classical procedures such as Soxhlet, it stands out its speed and high extraction efficiency,

the use of smaller volumes of solvents, being a "greener" technique, and that it is a highly automated technique; therefore, it is especially useful when carrying out routine analysis.

PLE has been shown to be comparable to other classical extraction techniques such as Soxhlet [87] in terms of recovery and precision, so its use has been proposed in the Method 3545 recommended by the United States Environmental Protection Agency (EPA) "for extracting water insoluble or slightly water soluble organic compounds from soils, clays, sediments, sludges, and waste solids" [88].

4.1.2. PLE procedure

First of all, it is convenient to disperse the sample in a desiccant and/or inert sorbent, such as sodium sulphate, sand, Florisil® or diatomaceous earth, since the diffusion of the analytes from the sample to the solvent can be increased considerably by decreasing the particle size due to the increasing in the contact surface between the sample and solvent, and avoiding aggregation of the sample particles. The sample must be dried to avoid the presence of water that difficults the penetration of the extracting solvent in their pores. Then, this mixture is introduced in the extraction cell. Usually, to prevent the metal frits located at both ends of the extraction cell from becoming clogged, cellulose filters are placed at both ends. Also, to make extraction more efficient, it is advisable to fill the dead volume of the cell with an inert material, usually sand or diatomaceous earth.

Once the cell is introduced in the system, the extraction takes place. This process can be performed in two different ways:

i. Static mode: the cell is heated, with the sample inside, to an appropriate temperature during a time of equilibrium (approximately 5 min), which is followed by a static extraction process where the solvent is introduced into the cell and this is maintained at constant pressure for a certain time. This process can be repeated several times if low recoveries are obtained in a single stage.

ii. Dynamic mode: the solvent is continuously passing through the pressurized cell. Although in this way the transfer of matter is improved, this type of extraction is scarcely used, mainly due to the high solvent consumption compared to the static mode.

After extraction, the extract is transferred to a collection vial while the cell is rinsed with several portions of new solvent (flush). The entire system is then purged with pressurized nitrogen for 1-2 minutes.

4.1.3. Factors affecting the extraction efficiency

Different parameters can be modified to optimize the efficiency of the extraction. The most important are extraction solvent, temperature, pressure and static extraction time [86, 89].

i. Temperature

It must be high enough to promote kinetics of the extraction, but without degrading the analytes. When the temperature increases, the solvent decreases its viscosity, so it penetrates more easily in the pores of the matrix, favouring the diffusion of analytes.

ii. Pressure

As commented before, the main reason for using high pressures in PLE is to maintain the solvent in liquid state at high temperatures well above its boiling point. The high pressures also favour the penetration of the solvent into the pores of the sample, which increases the recovery of the analytes. In addition, its solvation power is higher due to the increase in density. However, the increase in density causes, in turn, a decrease in diffusion coefficients, which could lead to a decrease in recovery values, due to a slow kinetics of the extraction process.

iii. Solvent

In general, the polarity of the solvent should be similar to that of the analytes to be extracted, although normally, polar and non-polar solvent mixtures offer higher recoveries.

iv. Extraction time

Extraction times in PLE are very short compared to those of conventional solid-liquid extraction techniques (e.g. Soxhlet). In the case of analytes strongly retained in the matrix, the extraction time can be increased in order to achieve a better recovery of the analytes. The extraction time chosen should be the minimum possible to achieve a complete extraction. Usually, the extraction times are of 15 minutes or lower.

4.1.4. Applications

Until the moment this thesis was started, there were not references regarding the extraction of UV filters from cosmetics with PLE. However, it was successfully used to extract these compounds from soils [72], sludge [73], sediments [74], dolphin liver tissue [90] and fish [91]. PLE had been applied to cosmetics samples with the aim of extracting other families of compounds such as fragrances [92] and preservatives [93], and later on, to fragrance allergens, musks, phthalates and preservatives in baby wipes [94].

Therefore, the use of PLE applied to the analysis of cosmetics to extract UV filters seemed a good approach, and for this reason in this thesis two methods were developed to analyse UV filters in cosmetics based on this technique followed by GC-MS/MS and LC-MS/MS.

4.2. ULTRASOUND-ASSISTED EMULSIFICATION MICROEXTRACTION (USAEME)

4.2.1. Introduction

This extraction technique was proposed for the first time in 2008 by Regueiro et al. [95] in the investigation group where this thesis has been carried out. USAEME is an extraction and preconcentration technique based on the emulsion of a small volume of organic solvent in an aqueous matrix by ultrasounds. The tiny droplets of solvent are dispersed into water, increasing the surface contact, favouring the pass of the analytes from the sample to the solvent and improving the kinetics of the process. Then, by centrifugation, both phases are separated, and the organic extract is collected for subsequent analysis. In contrast to dispersive liquid-liquid microextraction (DLLME), this procedure avoids the use of a disperser solvent and is consequently more environmentally friendly [96].

USAEME is a fast, easy and low-cost extraction technique whose main advantage is the low organic solvent volumes required (less than 200 µL), what makes it a green extraction technique, and also low sample volumes are needed (≈ 10 mL). In addition, it is a quantitative extraction technique.

4.2.2. USAEME procedure

The extraction is performed in a conical bottom glass tube, where the aqueous sample is introduced and a microvolume of an immiscible organic solvent (generally halogenated) with density higher than that of the water is added. Then, the tube is immersed into an ultrasonic water bath, where the emulsification of the system is produced. Disruption of the emulsion is achieved by centrifugation, and the organic extract, sedimented at the bottom of the tube, is collected with a syringe and is ready for analysis. Figure 3 shows a scheme of the USAEME procedure.

Figure 3. Scheme of the USAEME procedure

4.2.3 Factors affecting the extraction efficiency

With the aim of obtaining good extraction efficiencies, some parameters involved in USAEME must be studied. Amongst these are the type and volume of organic solvent, the ratio between sample and solvent volumes, the ionic strength of the medium (which can be modified by adding a salt) or the extraction time and temperature.

4.2.4. Applications

Regarding the extraction of UV filters by means of USAEME, only one work had been published before the beginning of this thesis [62], with the particularity that an ionic liquid was used as extracting solvent. In that work four UV filters (three benzophenones (BP, BP1, BP3) and 4MBC) were analysed in environmental water samples.

Concerning the analysis of other compounds in water samples by USAEME, the first application of this extraction technique was in 2008, involving the extraction of emerging contaminants (musks and phthalates) and pesticides [95]. Since that moment, it was applied to preservatives [97], polybrominated flame retardants [98],

polychlorinated biphenyls (PCB) [99], fragrance allergens [100], polycyclic aromatic hydrocarbons (PAH) [101], etc and to metals such as cadmium [102], nickel [103] or lead [104].

The extracting solvent is usually a high density organic solvent such as chloroform [95, 97-100]. Sometimes, it can be a low density organic solvent such as toluene [101], staying the organic solvent at the top of the tube. Other kind of solvents such as ionic liquids have also been proposed [62].

4.3. SOLID-PHASE MICROEXTRACTION (SPME)

4.3.1. Introduction

Solid phase microextraction (SPME) is an extraction technique which integrates sampling, extraction, concentration and sample introduction into a single step. SPME was invented in early nineties by Prof. Janusz Pawliszyn. It was developed to address the need for rapid sample preparation procedures both in the laboratory and field research.

The SPME is based on the use of a fused silica fibre, chemically inert, covered with a stationary ab/adsorbent phase of polymeric nature. This fibre is placed inside a stainless-steel needle. The needle is part of a syringe specially designed to house the fibre. Figure 4 shows the scheme of the most used device in SPME, introduced by Supelco in 1993.

To carry out the extraction process, the fibre is exposed to the sample to be analysed for a selected time. At that point, the transport of analytes from the matrix to the coating begins. It is a non-exhaustive extraction technique. SPME is usually completed when the analyte concentration has reached distribution equilibrium between the sample matrix and the fibre coating. At that moment, although the extraction time is increased, the amount of analyte extracted remains constant [105]. Then, the analytical determination must be performed. This technique is often followed by gas chromatography since the

ab/adsorbed analytes may be thermally desorbed in the injector of a gas chromatograph (GC) for separation and further quantification.

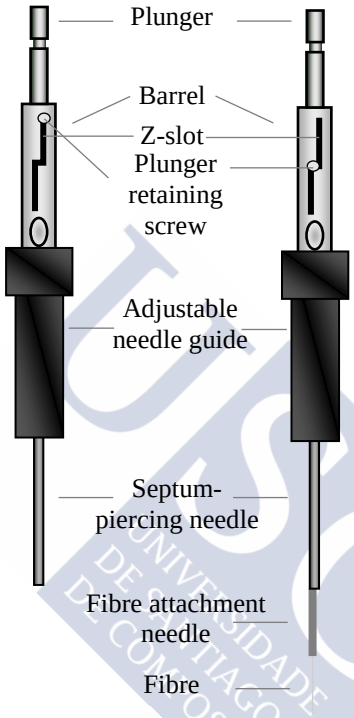

Figure 4. Scheme of a SPME device

Among the main advantages of the SPME highlights that:

- The sampling, extraction and concentration of the analytes takes place in a single stage.

- It allows a quick extraction and a direct transfer to the separation and analysis equipment, which allows obtaining high sensitivity since the extracted analytes are concentrated in the fibre and all the extracted compounds are introduced in the equipment.

- Does not require the use of solvents, so it is a technique that respects the environment. In addition, it generally uses low sample amounts.

-Easily automated

4.3.2. SPME procedure

The SPME process is carried out in two basic stages [105]:

- Extraction: The fibre is exposed to the sample contained in a sealed vial allowing the migration of the analytes to the fibre occurs during a given time.

- SPME fibre desorption: The fibre is introduced into the injector of an analytical system (gas chromatograph) where the analytes are thermally desorbed, or they can be desorbed with a solvent (liquid chromatograph).

Regarding the extraction, it can be performed in two different ways (see Figure 5):

1. Direct or immersion extraction (SPME): the fibre is directly introduced into the sample with the direct migration of the analytes from the matrix to the fibre. It is usually selected when the samples are relatively simple, and/or the analytes are slightly volatile.

2. Headspace extraction (HSSPME): The fibre is exposed to the head space over the sample, so the analytes pass from the sample to the headspace, and from there to the polymeric coating. It is suitable for volatile and semivolatile compounds in complex matrices.

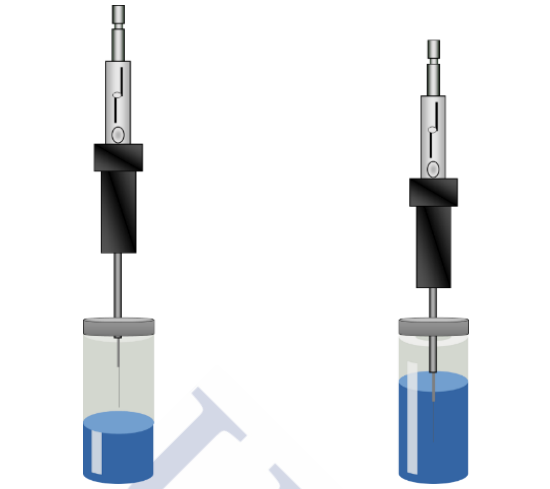

Figure 5. SPME sample modes

4.3.3. Factors affecting the extraction efficiency

There are some factors that can affect the passage of the analytes to the fibre such as [106]:

i. Fibre coating

There are different types of commercial polymer phases with different polarities, and also with different thickness of the phase. Usually, fibres with a polarity similar to that of the analytes will be chosen.

ii. Extraction time

The maximum amount of analyte will be extracted once the equilibrium is reached, at a determined time, and higher extraction times do not represent a benefit. In the case of compounds with low distribution constants more time is needed to achieve equilibrium. In

these cases, times shorter than equilibrium can be selected to save time, but when this occurs, the exposure time of the fibre requires strict control in order to obtain satisfactory reproducibility.

iii. Extraction temperature

The increase of the extraction temperature favours the diffusion of the analytes towards the fibre. In addition, in HSSPME, the temperature facilitates the transfer of analytes to the headspace. However, since the absorption stage is an exothermic process, an increase in temperature reduces the fibre / sample distribution constant (K_{fs}) of the analytes.

iv. Salting-out effect

The addition of salts (NaCl, KCl, etc) causes an increase in the ionic strength, varying the distribution constants of the analytes between the sample and the fibre. There is therefore a decrease in the solubility of the analytes in water, favouring its passage to other phases of the system, such as headspace and fibre.

v. pH of the sample

The pH of the sample affects the dissociation equilibrium of the analytes with acidic or basic groups. In these cases, in order to obtain maximum extraction efficiency, the pH must be two units below the pK_a in case of acidic compounds, and two units above in the case of the basic ones.

vi. Volume of sample

The partition of the analytes in the system is governed by the respective distribution coefficients between the different phases. In the case of a system consisting of three phases (the sample, the fibre and the headspace), the amount of analyte extracted follows the next equation (eq.1):

$$n=\frac{K_{fs}V_fV_s}{K_{fs}V_f+K_{hs}V_h+V_s}C_0$$

where n are the moles of analyte extracted; K_{fs} and K_{hs} are the distribution constants between the fibre and sample, and between the headspace and sample, respectively; V_f, V_s, and V_h are the volumes of fibre, sample and headspace, respectively; C_0 is the initial concentration of the sample.

Therefore, when increasing the volume of sample, the amount of analyte extracted is increased until a certain volume, where the amount of analyte extracted does not increase even though the volume of sample does. When the V_s is high, the equation would be (eq. 2):

$$n=K_{fs}V_fC_0$$

And, therefore, the volume of sample does not influence the amount of analyte extracted.

vii. Headspace volume

As can be observed in the equation eq. 1, when the volume of the headspace is increased, the amount of analyte extracted decreases.

viii. Agitation of the sample

The agitation favours the diffusion of the analytes from the matrix to the fibre, accelerating the kinetics of extraction.

ix. Addition of solvent

The addition of organic solvents to aqueous samples usually reduces the amount of analyte extracted. However, for solid samples the extraction efficiency usually increases with the addition of a solvent, since it favours the diffusion of analytes from the matrix to the fibre.

4.3.4. Applications

All the literature found dealing with the extraction of UV filters using SPME is practically focused on the analysis of these compounds in water samples. There are commercially available fibre coatings, although in the last few years, new fibre coatings based on nanoparticles of Au [107, 108], Ti–TiO_2 [66, 109], Zn–ZnO [110], Ti–TiO_2–ZrO_2 [111], nitrogen-containing carbon nanoparticles [112] C_{12}–Ag [113] or polymeric ionic liquids [114] were synthetized to analyse some UV filters in environmental water samples.

The papers published until the beginning of this thesis in the field of analysis of UV filters in waters by SPME involved the determination of five or less UV filters simultaneously [65]. In section 2.2 and 2.3. (Chapter III) are presented two methods developed during this thesis concerning the analysis of almost fourteen UV filters in water samples.

Regarding solid environmental samples (sediments, soils, etc.), there are not references of the use of SPME to extract UV filters from them. However, it was applied to extract other compounds such as polyaromatic hydrocarbons [115], polychlorinated biphenyls [116] or pesticides [117] from this kind of matrices. For these reasons, it might be a good option to extract also UV filters from beach sand, and it was applied in Section 3, Chapter III.

5. ANALYSIS

5.1. INTRODUCTION

Regarding the analysis of UV filters, it was mainly carried out by gas and liquid chromatography. Regarding gas chromatography (GC), all the published articles used mass (MS) or tandem mass spectrometer (MS/MS) as detector, while with liquid chromatography (LC) both UV detection and mass spectrometry were employed. However, UV detection was mostly utilized when determining these compounds in cosmetic samples, and mass spectrometry for environmental matrices. It is true that in cosmetic samples, UV filters are allowed at very high concentration levels (up to 15% for organic UV filters), and it is not need as much sensitivity as for environmental samples, where these compounds can be found at ng L^{-1} concentration levels. Nevertheless, cosmetic formulations are complex matrices, containing mixtures of substances of very diverse nature, not only UV filters, but fragrances, preservatives, musk, phthalates, etc. Therefore, in order to avoid interferences, the use of mass spectrometry is recommended since it provides greater selectivity. In addition, in the case of aiming to determine forbidden substances, a great sensitivity is required, since with the finding of a forbidden compound at trace levels, the cosmetic product could no longer be commercialized.

In the present doctoral thesis both chromatographic modalities have been used coupled to MS and tandem mass spectrometry (MS / MS) as detection systems. Some characteristics of both techniques are described below, and some applications of both determination techniques to the analysis of UV filters will be mentioned.

5.2. GAS CHROMATOGRAPHY

5.2.1. Introduction

Gas Chromatography is based on the distribution of the analytes between a gaseous mobile phase (the carrier gas) and a stationary phase,

generally liquid, immobilized on the surface of a solid (the chromatographic column). This technique is selected for the separation of thermally stable volatile or semi-volatile compounds, since the sample is usually introduced into the column in gas phase, so the liquid samples must undergo a previous stage of volatilization at elevated temperatures.

5.2.2. Derivatization

In the case of polar and / or thermosensitive compounds, the analysis by GC is conditioned to a previous process of derivatization by means of which a certain functional group of the analyte is modified, generating less polar and sufficiently volatile species to elute at a reasonable temperature without thermal decomposition or molecular reorganization. This procedure improves the chromatographic resolution and increases the response of the analytes in the detection system [118].

Acetylation is one of the most common procedures for the derivatization of phenolic compounds. It consists on the substitution of a hydrogen belonging to a hydroxyl group by an acetyl group, through the formation of a carbon-oxygen bond. The advantages lie in the high efficiency obtained during the process using low price reagents, especially compared to the cost of the reagents used in other derivatizing processes such as silylation. Acetylation with acetic anhydride as an acetylating reagent, and pyridine as a catalyst, has been used in this doctoral thesis for the derivatization of some UV filters in organic medium, while the acetylation reactions in aqueous medium were carried out using potassium carbonate instead of pyridine (see sections 1.1. and 2.3., Chapter III).

5.2.3. Mass spectrometry

The coupling of gas chromatography to a mass spectrometer is very easy since both techniques work in the gas phase and they need a very small amount of sample for analysis, so they are very compatible.

When using GC, the only data available is the retention time of the corresponding chromatographic peaks and this data is not sufficient for unambiguous identification. Mass spectrometry has advantages such as the ability to identify in a virtually unambiguous way, by providing a characteristic spectrum of each molecule, high sensitivity and structural information. The GC-MS coupling is probably the most widely used hybrid technique, since it combines the high resolving power of gas chromatography with the high sensitivity and structural information provided by mass spectrometry.

A mass spectrometer is composed of three fundamental elements: the source of ionization, the mass analyser and the detector. In the case of the GC-MS coupling, the entrance of the sample in the MS is directly through the gas chromatograph. Within the mass spectrometer, the sample is ionized. The most frequent ionization system is the electronic impact (used in this thesis) in which the molecules are bombarded with electrons of a certain energy, capable of provoking the stimulated emission of an electron from the molecules and thus ionizing them. In addition to ionized molecules or molecular ions (M +), fragment ions are also formed due to the decomposition of molecular ions with excess of energy. The type and relative proportion of each of these fragments are characteristic of the molecules analysed and the conditions of the ionization process. Once the molecules are ionized, they are accelerated and conducted to the collector system by electric or magnetic fields. The speed reached by each ion will be dependent on its mass. The consecutive detection of the ions formed from the molecules of the sample, assuming that it is a pure substance, produces the mass spectrum of the substance, which is different for each chemical compound and which constitutes a practically unambiguous identification of the compound analysed.

There are different types of mass analysers, such as time of flight, ion trap, magnetic sector, but those used in this thesis were quadrupoles. The quadrupole analyser consists of four parallel cylindrical bars that act as electrodes, electrically connected to each other in opposite pairs.

A variable radiofrequency voltage is applied to these pairs (poles), which tunes to a certain ion. When there is harmony between the ion that is passing through them and the applied frequency, the ion continues its path, deviating all the others not tuned out of the quadrupole without reaching the detector. Figure 6 shows a scheme of the functioning of a quadrupole.

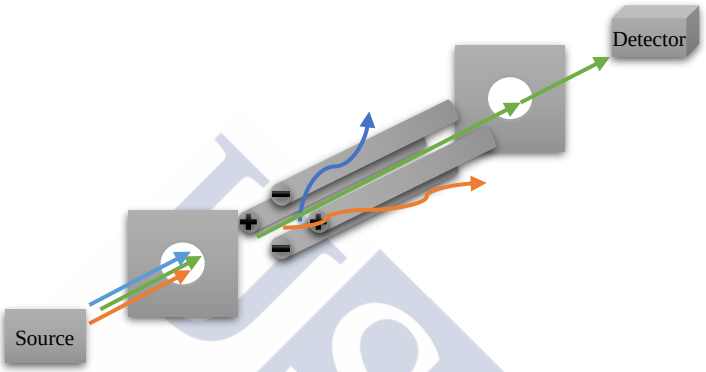

Figure 6. Scheme of a quadrupole

There are different ion monitoring modes:

-Full scan: there is a continuous filtration of ions, registering all the masses.

-Selected Ion Monitoring (SIM): only ions with a specific value of m/z get cross completely the analyser.

-Selective Reaction Monitoring (SRM): this mode is possible when triple quadrupole is available, that is, three quadrupoles in series. The first and third quadrupoles work in SIM mode, selecting some precursor ions in the first quadrupole and, after their fragmentation in the second quadrupole, some specific product ions are selected in the third quadrupole. In this way, interferences can be minimized or even eliminated, reducing the chemical noise of the chromatograms, reaching excellent selectivity and sensitivity. This was the principal scan mode used in this thesis.

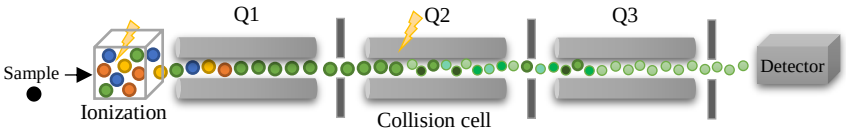

Figure 7. Scheme of the SRM acquisition mode in a triple quadrupole

5.2.4. Applications

Gas chromatography coupled to mass or tandem mass spectrometry was used to analyse UV filters mainly applied to environmental matrices, and, in particular, to water samples [52, 53, 65]. Also, studies dealing with the analysis of UV filters by GC-MS or GC-MS/MS in sediments [75, 80], soils [72, 79, 80], sludge [73], indoor dust [119], urine [120, 121] and molluscs [122] are found in the literature. Until 2014, only one paper reported the analysis of UV filters (eight) in cosmetics by GC-MS [46]. Few contributions can be found regarding gas chromatography with flame ionization detector (FID), concretely one for the analysis of the UV filter 2-ethylhexyl methoxycinnamate [123], and another one for benzophenone-3 and ethylhexyl p-aminobenzoic acid [124] in waters.

5.3. Liquid chromatography

5.3.1. Introduction

Liquid chromatography (LC) is an analytical technique used to separate a mixture in solution into its individual components [125]. It is based on the distribution of analytes between a solid stationary phase and a liquid mobile phase, and is the technique par excellence for separating non-volatile, polar and / or thermally unstable compounds.

A LC instrument consists of an injector, a pump, a column and a detector. There are different types of detectors, such as UV, fluorescence, etc, but the one used in this thesis was the mass spectrometer. As commented before, there are different types of mass

analysers, and, once again, the employed in this thesis coupled to LC was the quadrupole. In this case, the coupling of LC and MS is not so easy since the injected sample leaves the column in the liquid state, so it cannot be directly introduced in the quadrupole. Therefore, an ionization source that transforms the molecules in solution into ions in the gas phase without producing its thermal degradation and eliminating the large amount of gas and vapor from the liquid phase before entering into the high vacuum region of the mass spectrometer is necessary. The sources of atmospheric pressure ionization are a good solution for this problem. Among them, the electrospray ionization (ESI), which has been used in this thesis (see Section 1.2, Chapter III) is the one most frequently employed. This ionization source nebulizes the sample in solution through a capillary needle which is maintained at a potential of several kV with respect to a cylindrical electrode surrounding. The resulting charged micro droplets are desolvated by coulombic repulsions and with the additional help of gas and heat flows, originating ions in gas phase with one or multiple charges.

5.3.2. Applications

Concerning the determination of UV filters by LC, most of the contributions are in the field of water analysis [49, 126]. In this case, although the main detector selected was mass spectrometry [68, 69, 71, 126-129], also UV detection was widely used [51, 62, 63, 66]. Regarding the analysis of UV filters in cosmetics, at the beginning of this thesis it only was carried out by LC-UV [40, 41]. Other matrices where the LC-UV was implemented were biological matrices (urine [19, 130, 131], blood/plasma [13, 132] and skin [13, 132]) and packaging [133]. LC-MS and LC-MS/MS were used to analyse UV filters in sediments and sludge [74, 134, 135], human fluids or tissues (placenta [20-22], urine [15, 17], blood [15], semen [17] and breast milk [24]) and different organisms such as dolphins [90], fish [91], different aquatic organisms [136] and others [60].

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III. RESULTS AND DISCUSSION

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III. 1. Determination of UV filters in cosmetics

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UV filters are substances included in personal care formulations with the aim of protecting the skin against certain UV radiations by absorbing, reflecting or scattering them.

Nowadays, there is a great awareness of how important it is to protect the skin from the UV radiation to prevent the occurrence of cancer. For this reason, the use of sunscreens is increasing not only in specific solar product range but also in daily creams, lip balms, etc. However, in spite of being required for this reason, they can cause some adverse effects. Therefore, the EU established a specific Regulation (EC) Nº 1223/2009 laying down the rules that must follow all marketed cosmetic products in order to ensure a high level of protection of human health. Sunscreens allowed for use in cosmetic products are gathered in Annex VI of this regulation. The analytical control of these compounds is necessary to guarantee compliance with these rules.

Up to the moment this work was developed, the analysis of UV filters in cosmetics was based on the dissolution of the sample in a solvent and subsequent filtration. However, in this way the matrix is introduced in the chromatographic system, which could damage injector, column and detector. For this reason, it was looked for a sample preparation technique that allows obtaining cleaner extracts. Pressurized Liquid Extraction (PLE) was selected, since it can be considered a green extraction technique, and an automated procedure.

Regarding the analysis of these compounds, until the beginning of this thesis it was mainly addressed by liquid chromatography with UV/Vis detector. LC-UV has the inconvenience of possible coelutions, which may difficult correct identification of the compounds, so in this thesis it is proposed, on one hand, their analysis by GC-MS/MS and, on the other hand, by LC-MS/MS. In addition to the selectivity provided by mass spectrometry, it offers more sensitivity, which can be interesting to detect forbidden substances such as certain benzophenones. With the aim of including more polar compounds such as benzophenones, some of them hardly determined by GC-MS/MS, a derivatization step was implemented. In the case of LC-MS/MS this

step is not necessary. However, other compounds could not be detected with the ionization source available in the laboratory (Heated Electrospray Ionization, HESI). Therefore, both methods are complementary.

Next, the two methods developed to analyse UV filters in cosmetic samples are presented.

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1.1.

Optimization of an analytical methodology for the simultaneous determination of different classes of ultraviolet filters in cosmetics by pressurized liquid extraction–gas chromatography tandem mass spectrometry

Marlene Vila, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart

Journal of Chromatography A, 1405 (2015) 12-22

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1.2.

Determination of fifteen water and fat-soluble UV filters in cosmetics by pressurized liquid extraction followed by liquid chromatography tandem mass spectrometry

Marlene Vila, Rocio Facorro, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac and Maria Llompart

Analytical Methods, 8 (2016) 6787-6794

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III. 2. Determination of UV filters in water samples

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UV filters are considered emerging contaminants. They enter the environment directly through the bath, swimming, etc or indirectly by domestic wastewater discharges. Although nowadays they are not regulated in the water policy field, they are starting to be considered in the EU watch list for its monitoring, as it is the case of the UV filter 2-ethylhexyl methoxycinnamate. Some of these compounds also show adverse health effects like estrogenic activity and the lipophilic UV filters can bioaccumulate and biomagnify through the food chain. For these reasons, it is important to control these compounds not only in personal care but also in different water systems.

As commented before, these compounds are found in environmental waters at very low levels (ng mL^{-1}), consequently extraction techniques that provide high concentration factors and analytical methods with high sensitivity.

Concerning sample preparation, solid phase extraction (SPE) has been the technique more frequently used. SPE requires high volumes of sample and organic solvent. In this thesis, the use of ultrasound-assisted emulsification microextraction (USAEME) and solid-phase microextraction (SPME) was proposed. USAEME was developed in 2008 by the group where this thesis has been carried out. It is a fast, easy and low-cost technique that provides high concentration factors and only 100-200 µL of organic solvent are needed, so it is a technique respectful with the environment. SPME does not involve the use of organic solvents, so it is even a more environmental friendly technique. Also provides a concentration of the sample, since all the compounds extracted are directly introduced in the chromatography system without dilution. It has also as advantage the possibility of performing an in-situ derivatization reaction, which allows analysing more polar compounds by GC without involve more steps. This advantage was utilised in the third paper which will be presented later, with the aim of analysing some benzophenones. Some of them are forbidden by the cosmetic EU regulation when they are used as UV filters, but they can appear in other products such as nail polishes, plastics, furniture, etc. for

photoprotective purposes. Therefore, they can be found finally in the environment.

Then are presented the methods developed concerning the analysis of UV filters in environmental water samples.

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2.1.

Ultrasound-assisted emulsification microextraction followed by gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry for the analysis of UV filters in water

Marlene Vila, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart

Microchemical Journal, 124 (2016) 530-539

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2.2.

Determination of fourteen UV filters in bathing water by headspace solid-phase microextraction and gas chromatography-tandem mass spectrometry

Marlene Vila, Maria Celeiro, J. Pablo Lamas, Thierry Dagnac, Maria Llompart, Carmen Garcia-Jares

Analytical Methods, 8 (2016) 7069-7079

DOI: 10.1039/C6AY01787H

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2.3.

Simultaneous in-vial acetylation solid-phase microextraction followed by gas chromatography tandem mass spectrometry for the analysis of multiclass organic UV filters in water

Marlene Vila, Maria Celeiro, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart

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III. 3. Determination of UV filters in beach sand

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As commented before, UV filters are considered emerging contaminants since they are detected in environmental samples, and they may have a negative impact in the aquatic life. They arrive the environment directly through recreational activities such as bathing or swimming or indirectly through domestic and industrial discharges. In addition, in the case of beach sand, these compounds can finish on it by applying the sunscreen in the beach or by contact of people impregned in sunscreen with it.

UV filters can induce some adverse health effects such as endocrine disruption (toxicological effects are commented in Section 1.3., Chapter II), so they are regulated in cosmetics, although not in beach sand. Nevertheless, their presence in this matrix can be harmful for human beings, and of course for marine organisms.

Despite of the importance of controlling these compounds in beach sand for the above-mentioned reasons, there is hardly any literature in this field, and only one study was found in the bibliography.

Therefore, this thesis was also focused on the development of methods for the analysis of UV filters in beach sand. In this sense, different methodologies were optimized, validated and applied to real beach sand samples.

The method of analysis was in all cases GC-MS/MS, with the aim of having high selectivity and sensitivity. Regarding sample preparation, different techniques were employed including ultrasounds (US) and vortex assisted extractions, on-column lixiviation, and the SPME. Concerning this last technique, two alternatives where tested. On one hand, an US extract of the sand was subjected to the SPME procedure, and on the other hand, direct SPME of the sand sample dispersed in water was performed. Once the different methods were validated, it was verified that all the methods were suitable, excluding lixiviation, since it does not allow quantitative extraction of the compounds. Concerning the rest of methods developed, the most convenient according to the needs of the analysis can be selected. US

and vortex extractions are rapid and low-cost, while with SPME the limits of the detection can be improved. Between the two methods based on SPME, the direct SPME of the sand does not require organic solvents and allows reducing LODs for some of the compounds.

Next, the publications derived from these studies are presented.

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3.1.

Different miniaturized extraction methodologies followed by GC-MS/MS analysis for the determination of UV filters in beach sand

Marlene Vila, Maria Llompart, Carmen Garcia-Jares, Thierry Dagnac

Journal of Separation Science (2018)

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3.2.

Development and optimization of a solid-phase microextraction gas chromatography tandem mass spectrometry methodology to analyse UV filters in beach sand

Marlene Vila, Maria Llompart, Carmen Garcia-Jares, Vera Homem, Thierry Dagnac

Journal of Chromatography A (2018)

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IV. CONCLUSIONS

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During the development of this thesis, new sample preparation procedures were optimized and applied to the extraction of UV filters from cosmetics and environmental samples, such as waters and beach sand.

The sample preparation techniques employed were fast and simple, and easy to implement in laboratories. In addition, the developed methods can be considered environmental friendly, since they require minimum consumption of organic solvents and sample amount, allowing reducing costs, residues, and risks. The techniques employed were: pressurized liquid extraction (PLE) for the analysis of cosmetic samples, solid-phase microextraction (SPME) and ultrasound-assisted emulsification microextraction (USAEME) for water samples, and ultrasounds (US) and vortex assisted extractions, lixiviation, and SPME, for beach sand samples.

The extraction procedures have been optimized applying chemometrics tools. Thus, experimental designs were used in order to evaluate the influence of the main factors on the extraction process, as well as the interactions between factors, employing the minimum required number of experiments.

The methods of analysis were based on gas and liquid chromatography, both coupled to tandem mass spectrometry, which provides high sensitivity and selectivity. This is especially useful to detect UV filters in the environment, where they are found at trace levels, but also to detect forbidden substances in cosmetics, which can also be found at very low concentrations.

Methods quality parameters were evaluated in terms of linearity, repeatability, reproducibility, accuracy, precision and the LODs. In general, satisfactory results have been obtained in all cases.

In summary, the research developed during this PhD Thesis, contributed by far to the development and validation of new analytical methods based on micro-extraction techniques and chromatographic

analysis to analyze UV filters in different matrices. The general conclusions that can be extracted from each field of study are drawn below:

1. Determination of UV filters in cosmetics

- Until the start of this thesis, the sample preparation consisted of a dilution of the samples. However, cosmetic samples are very complex matrices made up of mixtures of tens of ingredients. The direct dilution of the samples is not recommendable since all the cosmetic ingredients would enter the chromatographic system producing damage in the injector, column and detector. In this thesis, the use of PLE to extract UV filters from cosmetic samples was proposed for the first time.

- In one of the works, a PLE cell of 1 mL was employed. It allowed reducing the volume of extracting solvent used (10 mL) and the amount of dispersing agent used to fill the cell. The amount of sample was only 100 mg. It was proved that more polar compounds such as some benzophenones remained retained in the Florisil, leading to low recoveries for these compounds. It was solved using sand instead of Florisil, although it forced the change to a 10 mL cell, since the mixture of the sample with sand was not so easy to handle to introduce it in a cell of as small diameter as for 1 mL, and, in addition, recoveries lower than 80% were obtained for all compounds.

- The main method of analysis of UV filters in cosmetics found in the literature was LC–UV, which has the inconvenience of possible coelutions (as explained before, the cosmetics are made of multiple components, not only the target compounds), that may interfere in the results obtained. In this thesis, the use of GC and HPLC coupled to MS/MS was proposed. It provides more selectivity and allows reducing LODs, which is interesting to detect forbidden substances.

- The analysed samples were composed by several target UV filters, and the concentrations found were up to 10 % (w/w), in some cases close to the maximum concentration allowed by the European Cosmetic Products Regulation.

- Extracts were analysed by GC-MS/MS and HPLC-MS/MS. GC required a derivatization step to determine some polar compounds (mainly benzophenones) and HPLC allowed analysing these compounds and other additional compounds such as ensulizole and avobenzone, that can be hardly or not detected by GC. However, with the HESI source, used in this work in the case of LC, salicylates could not be detected. Therefore, two methods based on GC and HPLC were developed, and both can be selected depending on the requirements.

2. Determination of UV filters in water samples

Some general and coinciding conclusions can be drawn of the three works developed based on USAEME and SPME in the field of analysis of UV filters in water samples presented in this doctoral thesis and are:

- USAEME and SPME are microextraction techniques, and only 10 mL of water sample were necessary.

- The use of MS/MS provides the sensibility required for this kind of matrices, where the compounds can be found at levels above the ng mL^{-1}, and selectivity, since the samples contain diverse compounds, not only the UV filters, so they can interfere with the target compounds.

- The concentrations found in the analysed real samples were between the low part per trillion up to 4 µg mL^{-1}. The most frequent compounds found were OCR, EHS, HMS and 2EHMC. OCR and the salicylates EHS and HMS came out with

the highest concentrations, particularly in open-air swimming pools and aquaparks.

- USAEME and SPME applied to the analysis of real water samples provided similar concentrations of the target UV filters, so they are equivalent.

Some conclusions that can be derived from each single work are:

-USAEME:

- Only 100 µL of organic solvent are used, being an environmental friendly extraction technique.

- No matrix effects were observed, so external calibration can be performed with standards prepared in chloroform, simplifying the method.

-SPME:

- No matrix effects were observed, so external calibration can be performed, but carrying out the entire SPME process.

- With the aim of including more polar compounds such as benzophenones, and in-vial acetylation can be performed.

3. Determination of UV filters in beach sand

- The study of UV filters in beach sand is very scarce in the literature, and only one work was found in this field in the bibliography. In this thesis, different methods were developed to fulfil this lack of methodology in this issue, all of them new in this topic. The methods proposed are based on US and vortex assisted extractions, on-column lixiviations and two varieties of SPME (performing the US extraction and then the SPME (USSPME), or directly carrying out the SPME of sand wet with water).

- Lixiviation offered low recoveries, so it was discarded to analyse UV filters in sand samples. The other four techniques provided good recoveries and good precisions, so all of them are perfectly valid. From the point of view of time, difficulty and price, US and vortex assisted extractions would be the selected ones. However, if it is necessary to detect low concentration levels, the use of SPME, in its two modalities, would be the best option, because it achieves lower LOQs. In addition, when using SPME, external calibration can be performed with standards prepared in water, while for the rest of methods matrix match calibration is needed. The advantages of the direct SPME of the sand is that not organic solvents are needed, being an even more environmental friendly extraction technique than the USSPME, and in some cases LOQs can be reduced with respect to USSPME. Therefore, four valid methods were developed, and the most convenient can be selected according to the needs.

- The differences between the concentrations found for four samples using the methods based on US, vortex and USSPME were statistically evaluated, and it can be concluded that all of them give rise to results statistically comparable.

- Beach sand samples from the Atlantic Ocean (Galicia (Spain), North of Portugal and Canary Islands) and Mediterranean Sea (Mallorca, Spain) were analysed with the validated developed methods. The concentrations of the UV filters studied found were up to 2 mg g^{-1}, being very high levels.

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ANNEX I

RESUMEN EN ESPAÑOL

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En los últimos años, el consumo de protectores solares se ha incrementado significativamente debido a la preocupación de la población sobre los efectos perjudiciales que la radiación ultravioleta puede provocar en la salud humana (principalmente las quemaduras solares y la aparición de cáncer). Entre los ingredientes de las formulaciones de los protectores solares se incluyen los filtros UV, ya que son las sustancias responsables de proteger la piel contra los rayos UV. Debido a la gran conciencia de cuán importante es proteger la piel contra la radiación solar, previamente comentada, hoy en día los filtros UV no solo se incluyen en formulaciones de rango solar, sino también en cosméticos de uso diario como cremas hidratantes, maquillaje, barras de labios, cremas para manos, etc.

Sin embargo, se sospecha que estos compuestos, a pesar de ser necesarios para cumplir la función de proteger la piel contra la radiación solar, pueden tener efectos adversos para la salud, como la disrupción endocrina, entre otros. Por lo tanto, es importante controlar estos compuestos en cosméticos para garantizar la seguridad del consumidor. De hecho, hay diferentes regulaciones propuestas por diferentes organismos y aplicables a diferentes regiones donde se recogen los ingredientes permitidos, prohibidos o con restricciones en cosméticos. En el caso de España, los cosméticos deben cumplir el Reglamento Europeo 1223/2009. En el Anexo VI se recogen los filtros UV permitidos en formulaciones cosméticas con su concentración máxima permitida. Por debajo de esa concentración, se supone que su uso es seguro para los consumidores. Con los nuevos descubrimientos sobre su toxicidad, la legislación se actualiza constantemente. En consecuencia, es necesario el desarrollo de métodos analíticos útiles para verificar que los cosméticos cumplan con la legislación vigente y, además, deben incluir el mayor número de estos compuestos como sea posible y estar preparados para posibles nuevas restricciones.

Además, los filtros UV están considerados contaminantes emergentes. Estos compuestos entran en el medio ambiente directamente a través de actividades acuáticas o indirectamente con las descargas domésticas. Con el uso creciente de productos solares,

comentado anteriormente, es lógico que la presencia de estos compuestos en el medio ambiente también se incremente. Además, las plantas de tratamiento de aguas residuales no siempre son efectivas para eliminar estos productos químicos. Los filtros UV suponen un peligro para los organismos acuáticos, ya que se ha demostrado que se bioacumulan en ellos y, en consecuencia, para los seres humanos, ya que se biomagnifican a través de la cadena alimentaria. Aunque hoy en día no existe una regulación específica para estos compuestos en el medio ambiente, un filtro UV, el metoxicinamato de etilhexilo, ha sido incluido durante el desarrollo de esta tesis (en 2015) en una lista de vigilancia para su monitorización en muestras de agua (aunque también recomienda su seguimiento en sedimentos) y su futura consideración como contaminante prioritario. Por lo tanto, no solo es necesario el desarrollo de métodos analíticos para filtros UV en cosméticos, sino también en muestras ambientales como todo tipo de aguas, sedimentos, suelos, arena, etc. En el caso de matrices ambientales, sobre todo muestras de agua, los compuestos se encuentran en niveles de ng L^{-1}, por lo que se necesitan métodos muy sensibles.

Por tanto, derivada de esta problemática surgió la necesidad de desarrollar métodos analíticos para determinar un tipo de ingredientes cosméticos, los filtros UV, en los propios cosméticos y en matrices ambientales como aguas y arena de playa, y este ha sido el objetivo principal de esta tesis.

En el capítulo II de esta tesis se incluye una introducción donde se explica de forma más extensa algunos aspectos relacionados con los compuestos objeto de estudio en este trabajo, los filtros UV, como los tipos existentes, sus propiedades fisicoquímicas y su toxicidad. También se hace una breve introducción de los cosméticos, regulación existente en este campo, y los antecedentes analíticos de la determinación de los filtros UV en estas matrices. Por otro lado, también se presenta la problemática de estos compuestos en el medioambiente, comentando los riesgos que entrañan estos contaminantes emergentes a organismos marinos, y por tanto a humanos a través de la cadena alimenticia, y algunos antecedentes

analíticos con respecto al análisis de estos compuestos en matrices ambientales, tanto aguas como muestras sólidas.

En el capítulo III se expone el trabajo experimental llevado a cabo durante este período. Los dos primeros métodos presentados, desarrollados para analizar filtros UV en cosméticos, están basados en la extracción con disolventes presurizados (PLE) seguido de cromatografía de gases o líquidos y espectrometría de masas en tándem. Los tres siguientes trabajos están relacionados con el análisis de los mismos compuestos, pero en aguas, mediante microextracción-emulsificación asistida por ultrasonidos (USAEME) y microextracción en fase sólida (SPME). Finalmente, se presenta el desarrollo de diferentes métodos para el análisis de filtros UV en arena de playa.

En todos los casos, se intentó desarrollar métodos sensibles, selectivos y respetuosos con el medio ambiente, y que abarquen el mayor número posible de analitos en un solo análisis. Por ello, se aplicaron técnicas de preparación de muestra como las anteriormente expuestas, que utilizan poca cantidad de muestra y ningún o poco volumen de disolvente orgánico. En cuanto al análisis de filtros, a lo largo de toda la tesis se ha abordado empleando técnicas cromatográficas, tanto de gases como de líquidos. El detector en todos los casos ha sido la espectrometría de masas en tándem con triple cuadrupolo (en un trabajo, además, se compara con la espectrometría de masas simple con un único cuadrupolo). El método de adquisición fue la monitorización de reacciones seleccionadas (SRM, del inglés Selected Reaction Monitoring). Este tipo de detector y modo de adquisición ofrecen una alta selectividad, por lo que es muy buena opción en este tipo de matrices donde puede haber una cantidad muy grande de compuestos que pueden provocar interferencias con los analitos que se pretenden determinar y, además, sensibilidad, necesaria en casos donde los compuestos se encuentran a niveles traza (como puede ser el caso de muestras ambientales).

En el primer trabajo presentado en esta tesis (Sección 1.1., Capítulo III), se desarrolló un método basado en extracción con disolventes

presurizados (PLE) y análisis mediante cromatografía de gases acoplada a espectrometría de masas en tándem (GC-MS/MS) para el análisis de 16 filtros UV pertenecientes a diferentes familias en productos de cuidado personal, no solo en productos de gama solar sino también cremas hidratantes, maquillaje, pintalabios, pintauñas, etc. Entre estos grupos de compuestos se encuentran los metoxicinamatos, salicilatos, derivados del ácido p-aminobenzoico, benzofenonas y otros. El análisis de las benzofenonas mediante cromatografía de gases ha sido posible gracias a una etapa de derivatización sobre el extracto derivado de la PLE. La reacción elegida fue la acetilación y fue optimizada mediante un diseño factorial completo. En este diseño se estudió el volumen de anhídrido acético (agente derivatizante) y piridina (una base que favorece la reacción) y el tiempo de derivatización. Las condiciones finalmente seleccionadas fueron 200 µL de anhídrido acético, 10 µL de piridina y 1 hora de reacción a 100 ˚C. Otra ventaja de la derivatización es que la forma de pico obtenida es mucho mejor para los derivados acetilados. La etapa de extracción también fue optimizada mediante un diseño multifactorial categórico en el que se estudió el disolvente y la temperatura de extracción. Ya que los cinco disolventes estudiados daban respuestas similares (a excepción del metanol, que daba peor resultados para los compuestos acetilados), se decidió elegir el acetonitrilo por su compatibilidad con cromatografía de gases y líquidos. La temperatura de extracción elegida fue 90 ˚C. Cabe destacar que esta es una técnica miniaturizada, puesto que solo utiliza 100 mg de muestra y se lleva a cabo en una celda de PLE de 1 mL, obteniendo extractos de menos de 10 mL. El agente dispersante utilizado fue el florisil, y el desecante sulfato de sodio anhidro. Finalmente, esta metodología fue validada en términos de linealidad, con un rango lineal para estándares entre 0,1 y 5000 ng mL^{-1} para la mayoría de los compuestos y R^2>0,9971. Es importante mencionar que el calibrado es externo, preparado sobre estándares en disolvente. Los límites de cuantificación estuvieron entre 0,090 y 19 ng g^{-1}, muy por debajo de los límites establecidos para los compuestos permitidos por el reglamento europeo para cosméticos, pero suficientemente bajos para determinar trazas de sustancias prohibidas como algunas benzofenonas. Se evaluaron también las recuperaciones y se observó un efecto matriz

positivo que se solucionó añadiendo una pequeña cantidad de un aceite de almendras comercial sobre los estándares. Así, las recuperaciones obtenidas fueron cuantitativas excepto para 3 benzofenonas (entre 37 y 52 %). Como estas están prohibidas por el reglamento europeo para cosméticos, serían suficientes esas recuperaciones para demostrar la presencia o ausencia del compuesto. La precisión fue evaluada como la desviación estándar relativa de varias réplicas de la misma muestra y fue menor del 10 %. Finalmente, este método se aplicó a diferentes muestras reales.

El siguiente trabajo desarrollado (Sección 1.2., Capítulo III) también está basado en PLE, pero esta vez seguido de un análisis mediante cromatografía líquida acoplada a espectrometría de masas en tándem (HESI-LC-MS/MS). Esta técnica permite detectar todas las benzofenonas sin un paso previo de derivatización y otros compuestos nuevos como ensulizole y avobenzona. Sin embargo, no se detectaron 3 salicilatos incluidos en el trabajo previo. La fase móvil utilizada fue metanol-agua (ambas con 1 % ácido fórmico y 3 mM de formiato amónico) en gradiente, comenzando en 50:50 y aumentando el % de metanol. En cuanto a la etapa de extracción, se observó que el acetonitrilo, previamente seleccionado en el trabajo anterior, no daba buenos resultados para uno de los nuevos compuestos, el ensulizole, por lo que se probaron nuevos disolventes. Finalmente, una mezcla 1:1 de metanol:acetona dio buen resultado para todos los compuestos, excepto, de nuevo, para algunas benzofenonas. Con el fin de solventar este problema, se cambió el florisil por arena y se observó que las recuperaciones mejoraban, posiblemente debido a que estos compuestos son muy polares y quedaban adsorbidos al florisil. Sin embargo, fue necesario cambiar la celda de 1 mL por la de 10 mL, ya que el manejo de la muestra dispersada con arena para introducirla en una celda tan pequeña era complicado y, además, las recuperaciones resultaron menores del 80 %. De esta forma, el volumen de extracto final fue menor de 20 mL. El método fue validado en términos de linealidad, con rango lineal entre 0,1 y 1000 ng mL^{-1} para la mayoría de los compuestos y R^2>0,9910. Los límites de cuantificación fueron menores que 0,1 µg g^{-1}. Las recuperaciones medias estuvieron entre

81,7 y 102 % para todos los compuestos bajo las condiciones óptimas con %RSD menores del 12 %. Finalmente, se aplicó el método a leches corporales, cremas de manos, maquillaje, pintauñas, crema solar, entre otras.

En cuanto al análisis de filtros UV en muestras medioambientales acuosas (Sección 2., Capítulo III), se han desarrollado tres métodos basados en técnicas de microextracción como son la microextracción-emulsificación asistida por ultrasonidos (USAEME) y la microextracción en fase sólida (SPME). Con estas técnicas, el volumen de muestra utilizado ha sido de 10 mL, muy por debajo de la requerida para técnicas habitualmente empleadas en aguas como es la extracción en fase sólida (SPE), que puede utilizar hasta 1L de muestra. Además de reducir el volumen de muestra también se reduce el uso de disolventes orgánicos, ya que con la USAEME solo son necesarios 100 µL de disolvente extractante y la SPME ni siquiera requiere de disolvente, mientras que con la SPE se utilizarían en torno a unos pocos mL y con otro tipo de técnicas, como el Soxhlet, podríamos hablar de cientos de mL. La técnica de análisis utilizada en los tres métodos desarrollados para el análisis de filtros UV en aguas (uno para USAEME y dos con SPME) fue GC-MS/MS. El método de adquisición fue la monitorización de la reacción seleccionada (SRM), lo que nos permite reducir todavía más los límites de detección debido a su gran selectividad y sensibilidad.

En cuanto a la USAEME, esta se llevó a cabo añadiendo 10 mL de la muestra en un tubo de centrífuga de fondo cónico y 100 µL de disolvente extractante. Luego, se realizó una etapa de ultrasonidos y, por último, una etapa de centrifugación. Algunos factores que influyen en la eficacia de extracción como el tipo de disolvente, la temperatura y el tiempo de la etapa de extracción con ultrasonidos, y la adición de sal se optimizaron mediante un diseño multifactorial. Las condiciones seleccionadas fueron adición de un 20 % de cloruro sódico a la muestra, cloroformo como disolvente y 5 min de US a 25 ˚C. Las condiciones de centrifugación fueron 10 minutos a 3500 rpm. Por último, se recogió el cloroformo con una jeringa, se pasó a un vial con inserto y se analizó

mediante GC-MS/MS. Con este método es válida una calibración externa con patrones preparados en cloroformo. Se demostró linealidad en un rango entre 0,1 y 1000 ng mL^{-1} con R^2>0,9910. Los límites de cuantificación estuvieron entre 0,27 y 9,7 ng L^{-1}. Las recuperaciones medias estuvieron entre 64,8 y 105 % con RSD menores de 9,1 %. El método se aplicó a aguas de piscina, aquapark, mar, spa, agua de grifo y río.

Se desarrolló también un método para el análisis de 14 filtros UV en aguas mediante SPME-GC-MS/MS. Para obtener una buena eficacia de extracción, se estudiaron mediante un diseño experimental de cribado el tipo de fibra empleada, el modo y temperatura de extracción, y la adición de sal (NaCl). Las condiciones finalmente seleccionadas para la extracción fueron un 35 % de sal en 10 mL de muestra, y SPME en espacio de cabeza a 100 °C durante 20 minutos con la fibra de poliacrilato. Con este método se consiguieron unos límites de detección entre 0,068 y 12 ng L^{-1}. El rango fue lineal para la mayoría de los compuestos entre 1 y 2000 ng L^{-1} con R^2>0,9937 y la precisión del método, calculada como la desviación estándar relativa, menor de un 20 %. Se evaluaron las recuperaciones en 4 tipos de aguas a 3 niveles, y resultaron ser entre 64 y 128 %. Se analizaron diferentes aguas de piscinas, ríos, mar, spa, etc. y se detectaron concentraciones entre 0,061 y 497 ng L^{-1} de los filtros UV estudiados.

Con el objetivo de incluir otros compuestos más polares, como son las benzofenonas, se desarrolló un nuevo método basado en SPME-GC-MS/MS, pero incluyendo una etapa de derivatización in-situ. Se llevó a cabo una reacción de acetilación con anhídrido acético y se probaron diferentes bases que permitieran acelerar la reacción. Las condiciones óptimas de acetilación fueron 100 mg de K_2CO_3 y 200 µL de anhídrido acético en 10 mL de muestra de agua y a 100 °C durante 30 minutos. De nuevo se estudiaron las condiciones óptimas de extracción en estas condiciones mediante un diseño multifactorial categórico. Las condiciones seleccionadas fueron la fibra triple (divinilbenceno/ carboxen/polidimetilsiloxano) y el modo de inmersión. El rango lineal fue similar al obtenido sin derivatización, pero se redujeron los límites

de detección para la mayoría de los compuestos. Esto es debido a la mejor forma de pico obtenida para los derivados acetilados. Se obtuvieron recuperaciones cuantitativas para todos los compuestos, entre 79,9 y 106 %. El análisis de aguas mediante este método demostró la presencia de 11 de los filtros estudiados en concentraciones entre 0,010 y 540 ng mL^{-1}.

Por último, los trabajos presentados en la sección 3 del capítulo III están relacionados con el análisis de los filtros UV en muestras de arena de playa.

En el primer artículo mostrado relacionado con este tema, se presentaron y optimizaron cuatro diferentes metodologías miniaturizadas aplicadas al análisis de once filtros UV en muestras de arena. Estos métodos se basaron en extracciones con ultrasonidos (US) y vórtex, lixiviación en columna y extracción de ultrasonidos seguida de microextracción en fase sólida (USSPME). La cantidad utilizada de muestra fue de 1 g y el volumen de disolvente orgánico empleado para la extracción fue de 1 mL. Para los tres primeros métodos, el disolvente utilizado fue el acetato de etilo, mientras que para la USSPME se eligió el metanol ya que es miscible con agua, necesaria para diluir el extracto y llevar a cabo la SPME. Se utilizó la cromatografía de gases acoplada a espectrometría de masas en tándem en todos los casos para el análisis cuantitativo. Una vez validados los métodos en términos de linealidad, recuperaciones, precisión y límites de cuantificación, la lixiviación se descartó debido a que proporcionó las recuperaciones más bajas y los límites más altos de cuantificación. Sin embargo, las extracciones con ultrasonidos y vórtex, y la extracción de ultrasonidos seguida de microextracción en fase sólida fueron adecuadas, con recuperaciones en general superiores al 85% y límites de cuantificación en la baja parte por billón (ng g^{-1}). Además, la extracción con ultrasonidos seguida de microextracción en fase sólida permitió el uso de calibración externa con patrones preparados en agua, y proporcionó mayor sensibilidad, con límites de cuantificación en general un orden de magnitud más bajos que los logrados con las otras técnicas.

En el último trabajo incluido en esta tesis, se desarrolló una metodología basada en microextracción en fase sólida (SPME) seguida de cromatografía de gases-espectrometría de masas en tándem (GC-MS / MS) para el análisis simultáneo de once filtros UV pertenecientes a diferentes familias en arena de playa. Esta es la primera vez que esta técnica de extracción se aplica al análisis de filtros UV en muestras de arena y en otro tipo de muestras sólidas ambientales. Se optimizaron los principales parámetros de extracción, como el modo y la temperatura de extracción, la cantidad de muestra, la adición de sal, el recubrimiento de fibra, el volumen de agua añadida a la arena y el tiempo de extracción. Los tres primeros parámetros se eligieron tras llevar a cabo unas pruebas previas y el resto mediante un diseño experimental. Las condiciones finalmente seleccionadas consistieron en agregar 1 ml de agua a 1 g de muestra seguido de la SPME en espacio de cabeza durante 20 minutos a 100 °C, usando la fibra de polidimetilsiloxano/ divinilbenceno (PDMS/DVB). El método de SPME-GC-MS/MS se validó en términos de linealidad, precisión, límites de detección y cuantificación, y precisión. Los estudios de recuperación también se realizaron a tres niveles de concentración en muestras reales de arena del Atlántico y el Mediterráneo. Las recuperaciones fueron generalmente superiores al 85 % y las desviaciones estándar relativas inferiores al 11 %. Los límites de detección estuvieron en el nivel de pg g^{-1}. La metodología validada se aplicó con éxito al análisis de muestras reales de arena recolectadas en playas del Océano Atlántico situadas en la costa noroeste de España y Portugal y en las Islas Canarias (España), y playas del Mar Mediterráneo (Mallorca, España). Los filtros UV encontrados con mayor frecuencia fueron el etilhexil salicilato (EHS), homosalato (HMS), 4-metilbencilideno alcanfor (4MBC), metoxicinamato de etilhexilo (2EHMC) y octocrileno (OCR), con concentraciones de hasta 670 ng g^{-1}.

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ANNEX II

LIST OF PUBLICATIONS

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List of publications

During the development of this doctoral thesis have been published in scientific journals the following works:

- Marlene Vila, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart, Optimization of an analytical methodology for the simultaneous determination of different classes of ultraviolet filters in cosmetics by pressurized liquid extraction–gas chromatography tandem mass spectrometry, Journal of Chromatography A 1405 (2015) 12–22
  DOI: 10.1016/j.chroma.2015.05.061

- Marlene Vila, Rocio Facorro, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart, Determination of fifteen water and fat-soluble UV filters in cosmetics by pressurized liquid extraction followed by liquid chromatography tandem mass spectrometry, Analytical Methods 8 (2016) 6787-6794
  DOI: 10.1039/C6AY01195K

- Marlene Vila, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart, Ultrasound-assisted emulsification microextraction followed by gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry for the analysis of UV filters in water, Microchemical Journal 124 (2016) 530–539
  DOI: 10.1016/j.microc.2015.09.023

- Marlene Vila, Maria Celeiro, J. Pablo Lamas, Thierry Dagnac, Maria Llompart, Carmen Garcia-Jares, Determination of fourteen UV filters in bathing water by headspace solid-phase microextraction and gas chromatography-tandem mass spectrometry, Analytical Methods 8 (2016) 7069–7079
  DOI: 10.1039/C6AY01787H

- Marlene Vila, Maria Celeiro, J. Pablo Lamas, Carmen Garcia-Jares, Thierry Dagnac, Maria Llompart, Simultaneous in-vial acetylation solid-phase microextraction followed by gas chromatography tandem mass spectrometry for the analysis of multiclass organic UV filters in water, Journal of Hazardous Materials 323 (2017) 45–55
  DOI: 10.1016/j.jhazmat.2016.06.056

- Marlene Vila, Maria Llompart, Carmen Garcia-Jares, Thierry Dagnac, Different miniaturized extraction methodologies followed by GC-MS/MS analysis for the determination of UV filters in beach sand, Journal of Separation Science (2018) (in revision)

- Marlene Vila, Maria Llompart, Carmen Garcia-Jares, Vera Homem, Thierry Dagnac, Development and optimization of a solid-phase microextraction gas chromatography-tandem mass spectrometry methodology to analyse UV filters in beach sand, Journal of Chromatography A (2018)
  DOI: 10.1016/j.chroma.2018.06.016