APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.

Laura Vallecillos Marsal

Dipòsit Legal: T 72-2016

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Laura Vallecillos Marsal

DOCTORAL THESIS

Supervised by

Prof. Francesc Borrull and Dra. Eva Pocurull

Departament de Química Analítica i Química Orgànica

UNIVERSITAT ROVIRA I VIRGILI

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2015

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i Química Orgànica

El Prof. FRANCESC BORRULL I BALLARÍN, Catedràtic del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. EVA POCURULL I AIXALÀ, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

FEM CONSTAR:
Que aquest treball, titulat "APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE DETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES", que presenta LAURA VALLECILLOS MARSAL per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Química Analítica i Química Orgànica d'aquesta universitat. Tots els resultats presentats són fruit d'experiències realitzades per l'esmentada doctoranda, i compleix els requeriments per a poder optar a la menció europea.

I, per a que consti, expedim aquest certificat a Tarragona, 1 de setembre de 2015.

Prof. Francesc Borrull i Ballarín

Dra. Eva Pocurull i Aixalà

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Ha arribat el moment d'escriure aquestes línies i de tancar una etapa que va començar ja fa més de quatre anys en el laboratori de preparativa d'aquest grup de recerca. Un lloc estrany podríeu pensar, però tothom que ha passat pel grup sap que n'és el punt neuràlgic. Allí és on conviuen a diari estudiants de grau, de màster i els doctorands, punt d'origen de grans amistats, de converses surrealistes i també de les discussions més acalorades que ens puguem imaginar. Doncs bé, va ser allí, on el Prof. Francesc Borrull em va oferir la possibilitat d'optar a una beca predoctoral al seu grup de recerca.

En primer lloc vull donar el meu més sincer agraïment als meus directors de Tesi, el Prof. Francesc Borrull i la Dra. Eva Pocurull. Gràcies per deixar-me aprendre al vostre costat, per la vostra dedicació, els valuosos consells i per la confiança i suport dipositats en mi durant aquests anys. També, vull agrair l'atenció, l'ajuda i els consells de la resta de persones que formen part del grup, les Dres. Rosa Maria Marcé, Carme Aguilar, Marta Calull, Núria Fontanals i Sandra Peñalver, amb les quals he compartit reunions de grup i converses disteses al office i a congressos. Un agraïment especial a la Rosa Maria i a l'Eva per l'experiència viscuda al congrés d'Itàlia, per tots els moments compartits i per la magnífica ruta amb cotxe! No obstant, quan penso en aquell congrés la imatge que se'm apareix el cap és la cara que se'ns va quedar quan el cotxe no arrancava de camí a casa. Tota una aventura!

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Arribats a aquest punt, ara és el moment de donar les gràcies a totes aquelles persones que han compartit el dia a dia amb mi al laboratori tots aquests anys. Que sapigueu que per mi més que companys de laboratori sou com part de la família, moltes gràcies a tots pels moments viscuts tant a dins com a fora del laboratori i per ajudar-me sempre que ho he necessitat. Gràcies de tot cor Alejandro G., Alejandro M., Antonio, Carol, Cristian, Daniela, Dominika, Estefania, Henry, Igor, Irene, Jordi, Julio, Maarten, Marta Palomo, Marta Pedrouzo, Martí, Mireia N, Montse, Núria G, Núria M, Paula, Pol, Sameer, Silvia E, Sílvia M i Tatiana. Especialment vull donar les gràcies a aquelles

persones amb les que he passat més temps, les meves companyes de gasos. A les meves mestres l'Anna i la Noelia per introduir-me en el món de la cromatografia de gasos i la seva paciència i dedicació durant els primers mesos, a la Mireia A. gràcies per l'ajuda, els consells i els ànims al llarg d'aquesta tesi i a la Laura T i l'Alba les meves companyes de viatge en aquests últims mesos gràcies de tot cor pel vostre recolzament i per omplir el laboratori de rialles i alegria.

Finalment vull donar les gràcies a la meva família, per creure en mi i donar-me ànims i per estar sempre el meu costat de manera incondicional. Sense el seu esforç i constancia al llarg dels anys jo no hagués arribat a ser la persona que sóc avui en dia. Moltes gràcies especialmente pel recolzament dels últims mesos per tal que aquesta tesi arribés a la seva fi. Per tot això i molt més aquesta tesi va dedicada a vosaltres.

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INDEX

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1. INTRODUCTION

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The production and use of chemicals continues to grow worldwide, particularly in developing countries, either to improve the quality of life or achieve more efficient and economically suitable industrial processes. Over recent decades, the world has experienced the adverse consequences of uncontrolled development of multiple human activities in, for example, industry, transport, agriculture, and urbanization. The increase in living standards and higher consumer demand have amplified pollution of the air with, for example, CO_2 and other greenhouse gases, NO_x, SO_2 and particulate matter; of water with a variety of chemicals, nutrients, leachates and oil spills, among others; and of the soil due to the disposal of hazardous wastes, spreading of pesticides and sludge, as well as the use of disposable goods or non-biodegradable materials and the lack of proper facilities for waste [1]. As an example of the number of chemicals currently used in European Union, more than 100,000 chemicals are registered and their consumption is estimated to be around 300 million tonnes per year [2]. Thus, it is obvious that these large amounts of chemical substances may result in an unhealthy impact on the environment and living species. This is shown in a report by the World Health Organization (WHO), which estimates that a significant number of human diseases are caused as a result of prolonged exposure to environmental pollutants. In particular, environmental hazards affect over 80% of the communicable and non-communicable diseases and injuries monitored by the WHO. Overall, environmental hazards are responsible for about quarter of the total burden of disease worldwide [3]. The WHO works to establish the scientific basis for the sound management of chemicals and to strengthen national capabilities and capacities for chemical safety through the International Programme on Chemical Safety (IPCS) [4]. Chemical safety is achieved by undertaking all activities involving chemicals in such a way as to ensure the safety of human health and the environment. It covers natural and manufactured chemicals, and the full range of exposure situations from the natural presence of chemicals in the environment to their extraction or synthesis, industrial production, transport, use and disposal.

Emerging organic compounds (EOCs), which is a term encompassing chemical substances whose continuous emission into the environment may be hazardous for the recipient ecosystems and mankind, comprise a wide range of man-made chemicals (such as pesticides, personal care products (PCPs), pharmaceuticals, illicit drugs or drugs of abuse, brominated flame retardants, disinfection by-products, among others), which are in use worldwide and which are indispensable for modern society [5]. It has been shown that, between 1930 and 2000, global production of anthropogenic chemicals increased from 1 million to 400 million tonnes per year [6]. Statistics published by EUROSTAT in 2015 reveal that, between 2004 and 2013,

environmentally harmful compounds accounted for over 50% of the total production of chemicals. As shown in Figure 1, these chemicals can also be divided into five main representative groups: chronic environmental impacts, moderate, severe or significant chronic environmental impacts and acute environmental impacts [7]. Over 35% of the environmentally harmful compounds produced are chemicals that can cause severe chronic environmental impacts. Meanwhile, the compounds that generate a moderate chronic environmental impact account for only 6% of the total amount of environmentally harmful compounds produced.

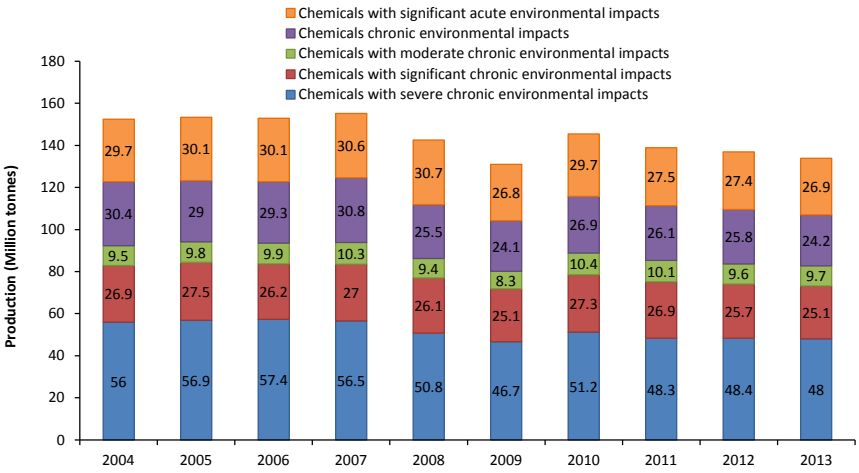

Figure 1. Production of environmentally harmful chemicals in EU-27, by environmental impact class (million tonnes) [7].

EOCs can be found in various environmental compartments and/or in areas where they have never been used, mainly due to their persistence over long transportation distances. The sources and pathways of these EOCs can be increasingly associated with the waste and wastewater resulting from industrial, agricultural or municipal activities [8]. Because of their particular characteristics, these pollutants require changes in the conventional approach to pollution prevention and control, although they result from similar domestic, commercial and industrial activities to conventional contaminants [9]. Chemical micropollutants are often generated through the degradation of organic compounds, resulting in the accumulation of persistent metabolites [10], or from the disposal of products such as pharmaceuticals in the natural environment. The increased appearance of biological pollutants throughout the production and distribution of drinking water may be related to several factors including changes in human

demographic behaviour. In addition, changes in agricultural practices towards intensive farming and spreading of manure or sludge on agricultural fields may cause leaching to surface and groundwater, and the consequent health problems [11,12].

Within the group of EOCs, there is a lot of interest in certain categories of environmental contaminants, with particular chemical structures and properties, which interfere with endogenous hormone systems. These contaminants, known as endocrine disrupting compounds (EDCs), are poorly inventoried and regulated and insufficient information exists regarding their occurrence, fate and impact in the environment. Moreover, as many have pharmaceutical, personal care and household uses (hormones, glucocorticoids, analgesics such as ibuprofen, additives in drugs and cosmetics such as parabens and synthetic musk fragrances, and household cleaners), more information about their ecotoxicological effects is essential for their monitoring and removal. Apart from the aforementioned EDCs, other products such as fire retardants, heavy metals (cadmium, mercury and lead), widely used industrial chemicals and certain pesticides have been shown to affect natural endocrine systems [13-16].

In consequence, EOCs in general and EDCs and their degradation products in particular constitute a topic of extensive research for the scientific community [13,14]. Studies indicate that toxicity data are not yet available for all these compounds, which originate from various sources (see Figure 2), the most relevant of which include direct releases into waters, wastewater treatment plants (WWTPs) (effluent and sludge, or domestic septic systems), landfill sites, and surface water run-off [17]. Pharmaceuticals, for instance, are more concentrated in the wastewater discharged from hospitals, long-term care facilities and other medical facilities [18-20], while veterinary pharmaceuticals used in animal feeding operations are present at higher concentrations in sludge or soils.

In the last few decades, technical advances in analytical chemistry in terms of the development of more sensitive and selective methodologies have helped the scientific community to confirm that lots of chemical compounds, including EOCs, are present in environmental matrices at trace levels, as certain EOCs are only partially removed during sewage treatment. WWTPs were basically designed to remove pathogens and organic and inorganic matter. Therefore, the conventional sewage treatments are not efficient enough to remove EOCs and other micropollutants from sewage, as demonstrated by several studies and reviews published about the source, occurrence, environmental behaviour and fate of EOCs that can be found in the literature [22-25].

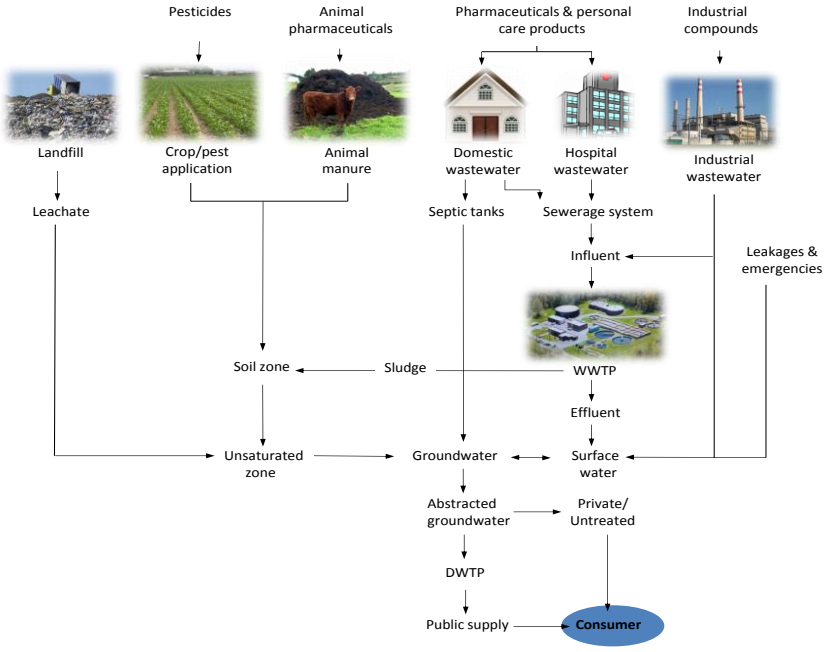

Figure 2. Schematic pathways of some EOCs from sources to receptors [21]. WWTP: wastewater treatment plant and DWTP: drinking water treatment plant.

However, the pathway of these pollutants from sources to receptors continues to be an essential subject for advanced research. This is because information is still poor, mainly due to the problems generated by the physico-chemical properties of the target compounds and the complexity of environmental characteristics, among others, which may determine the unexpected behaviour of EOCs in air, water or soil [13,15,22,26].

In light of the above, this Thesis focuses on the determination of a family of cyclic PCPs known as synthetic musk fragrances in environmental samples using different microextraction techniques and gas chromatography (GC) and mass spectrometry (MS) as separation and detection techniques. Firstly, the main characteristics of these contaminants are reviewed, as well as their occurrence and main environmental and health effects. This is followed by an overview of the most widely used analytical techniques for aqueous and solid samples. After the introduction, the main objectives of this Doctoral Thesis are set out. The third chapter presents the results and discussion of the studies derived from the experimental research included in paper format. Finally, the main conclusions that can be drawn from the studies are presented.

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1.1. Synthetic musk fragrances

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Musk is a class of aromatic substances commonly used as base notes in perfumery. They include glandular secretions from animals such as the musk deer, numerous plants emitting similar fragrances and artificial substances with the same odours. Originally, the name was given to substances with a penetrating odour obtained from a gland of the male musk deer. This musk has been used as a popular perfume fixative since ancient times and is one of the most expensive animal products in the world. Although harvesting natural musk was not banned outright until 1979, its cost soon gave rise to early aroma chemical experiments to look for more cost-effective replacements [27,28]. Nowadays, synthetic musks, known as white musks in the perfume industry, are the aroma chemical used to emulate the scent of deer musk or natural musk. Synthetic musks have a clean, smooth and sweet scent lacking the faecal/'animalic' notes of natural musks and are sometimes attributed as having notes of blackberry, ambrette or ambergris. These compounds are essential in modern perfumery and form the base note foundations of most perfume formulas. Moreover, they are also added in high quantities to scent a wide range of consumer products, such as detergents and cosmetics, as well as in the food industry. Synthetic musks can be divided in four main groups according to their chemical structure: nitro, polycyclic, macrocyclic and alicyclic musk fragrances [27,29,30]. The general characteristics of the four groups of musks are their substantive properties related to their lipophilic character and their relatively low volatility. The lipohilic character is reflected in the relatively high log Kow values, between 4.3 and 6, and low solubility in water. This property implies a high solubility in organic solvents and tissues, and adsorption into organic matter [30]. In the following sections, the compounds included in each group are described in more detail, as well as their chemical characteristics and their ecotoxicological risk. Furthermore, their occurrence in environmental samples such as wastewater, sewage sludge and biological samples is discussed.

1.1.1. Nitro musk fragrances

Nitro musk (NM) fragrances, which were the first synthetic musks to be produced, were simply discovered by accident [31]. Albert Bauer had actually been searching for new explosives and had alkylated trinitrotoluene (TNT) by Friedel-Crafts reactions with *tert*-butyl halides [32]. Together with the *Société des Produits Chimiques de Thann et de Mulhouse,* in 1888, Albert Bauer patented and produced the first NM fragrance, known as musk Bauer. In the years that followed, encouraged by its immediate success, he systematically studied further derivatives. In short succession, he discovered musk xylene (MX), musk ketone (MK) [33] and musk ambrette (MA) [34], three compounds that soon replaced musk Bauer and became the archetype of musk fragrances for the

following decades. Many perfumes of that period, such as *Chanel No. 5* by Ernest Beaux (Chanel, 1921) for instance, used a cocktail of the abovementioned NM fragrances with MK primarily. MA was the key note of *L'Air du Temps* by Francis Fabron (Nina Ricci, 1948), while MX was somewhat harsher and, due to its cheaper price, found more applications in the soap and detergent segment.

Extensive research on nitroarenes led to the introduction of three further NMs: musk tibetene (MT) [35], musk alpha and musk moskene (MM) [36]. Due to its unique blend of marine and musky tonalities, MT became the most extensively used NM fragrance, while MM was the first NM with an indane skeleton. As can be seen in Figure 3, all NM fragrances are characterized to be two- or three- fold nitrate benzene derivatives with additional alkyl, keto or methoxy groups.

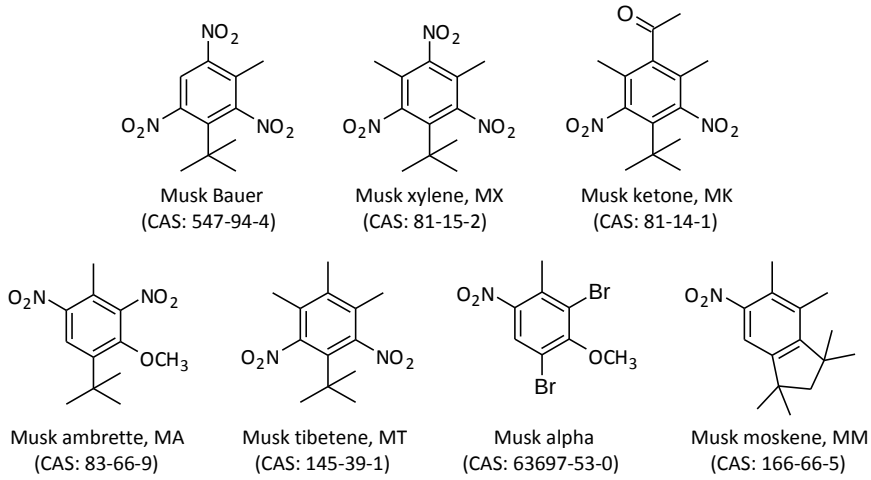

Figure 3. Chemical structures and CAS numbers of the NM fragrances.

Discussions on the toxicology of NM fragrances soon began due to the presence of a nitroaromatic compound in their structure and their bioaccumulation in environmental matrices [37]. The toxicity of NM fragrances is surprisingly different. Whereas MA was shown to be clearly neurotoxic and mutagenic, MX, MM, MK and MT showed neither effect [38,39]. However, MX was found to be carcinogenic under long-term exposure [40,41] and adverse reproductive effects were caused by MK [42]. All of the NM fragrances are photochemically degradable [38,43]. In response to this situation, the 1995 (95/34/EC) amendment of European Directive 76/768/EC relating to cosmetic products prohibited the use of MA [44]. MM and MT were also banned in Europe by

European Directive 98/62/EC [45], while the use of MX and MK in the cosmetic industry was restricted by European Directive 2002/34/EC. MX content is limited to 1% in fine fragrances, 0.4% in eau de toilette and 0.03% in other products. Moreover, MK is limited to 1.4%, 0.56% and 0.042%, respectively [46]. Recently, the European Commission, under the new REACH chemical regulations (Registration, Evaluation, Authorization and Restriction of Chemicals), has classified MX as a very persistent and highly bioaccumulative substance and therefore decided to ban it as well [47]. Furthermore, the reduction of the nitro groups of NM fragrances to the corresponding amines is a well known pathway of biological and environmental transformation processes of nitroaromatic substances [48]. This interconversion involves the intermediate production of the corresponding nitroso and hydroxylamine derivatives, which are very reactive and in many instances display higher toxicity [49] and higher hormone disrupting potential [50] than the respective parent molecules. This led to a significant decrease in the use of these compounds and an increase in the production of polycyclic musk fragrances at first, followed by macrocyclic and alicyclic musk fragrances in the recent years. Data on the consumption of the NM fragrances MX and MK in Europe have been estimated by the Oslo and Paris Commission (OSPAR) 2004 [30]. These data are related to the volumes used in fragrance compounding, for instance, and the preparation of mixtures that are used in the consumer product formulations across Europe. The results are presented in Figure 4.

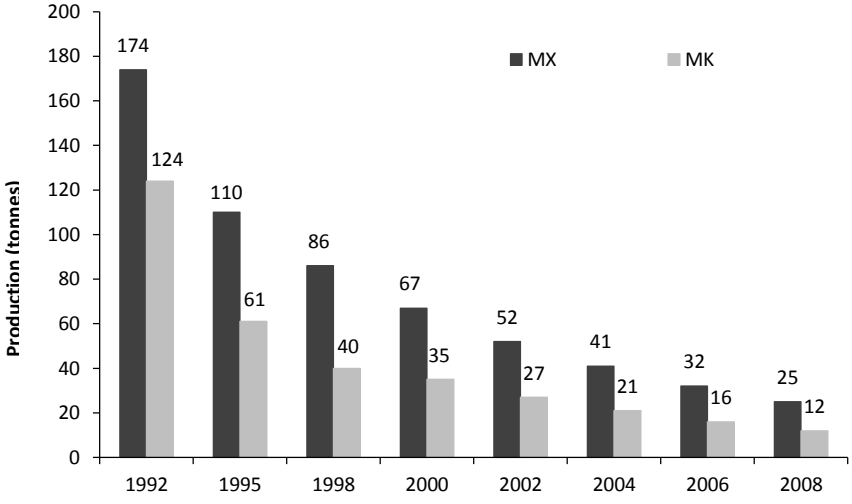

Figure 4. Estimated volumes (tonnes/year) of MX and MK used in Europe [30].

Therefore, there is currently no production of NM fragrances in the European Union (EU). Most European companies have stopped manufacturing them over course of the last decade. Nowadays, China is the major producer of NM fragrances in the world and is the most important source off European imports [30,51,52].

Although, all NM fragrances are banned by the European Commission with the exception of MK, they have been found in surface waters [53-55], influent and effluent wastewater [53-56], in sewage sludge [57-59] and fish [60-63], due to their bioaccumulative properties.

Specifically, MX and MK were determined in influent and effluent wastewater samples from Shanghai at concentrations of 80.4±7.1 ng L^{-1} for MX and 72.1±3.2 ng L^{-1} for MK in influent samples, while a decrease in NM fragrance concentrations was observed in effluent samples, dropping to 8.4±3.2 ng L^{-1} in the case of MK, while MX was not detected [56]. More recently, Ramírez *et al.* [64,65] found NM fragrances at concentrations in the range of 22-232 ng L^{-1} for MX and 20-84 ng L^{-1} for MK in influent wastewater samples from Tarragona (NE Spain). After the WWTP treatment, the concentrations decreased to values in the range of 20-84 ng L^{-1} and 20-53 ng L^{-1} for MX and MK, respectively. Concentrations of MX between 0.3 and 1.1 ng L^{-1} were also found in effluent samples from a tertiary treatment based on reverse osmosis (RO) [64,65]. The analysis of river samples from the River Ebro showed the presence of the NM fragrances MX (0.6-0.9 ng L^{-1}) and MK (0.8-7.2 ng L^{-1}) at significantly lower concentrations than those found in influent and effluent WWTP samples [64,65]. Furthermore, Llompart *et al.* [58] reported an MX concentration of 16±2 ng g^{-1} dry weight (d.w.) in secondary sludge from a WWTP located in Galicia (NW Spain) and Guo *et al.* [57] found MK concentrations in the range of 10-190 ng g^{-1} (d.w.) in sludge samples from different WWTPs in Korea. Higher concentrations of MX and MK were found by Liu *et al.* [66] in sludge from the secondary treatment of the WWTP in Hangzhou (China), with maximum levels of 360 ng g^{-1} (d.w.) for MX and 2,500 ng g^{-1} (d.w.) for MK. In addition, due to their bioaccumulative properties, NM fragrances can reach the food chain through fish specimens. In this respect, MX and MK concentrations in the range of 1.8-1.2 ng g^{-1} lipid weight (l.w.) for MX and 2-2.9 ng g^{-1} (l.w.) for MK were found in fish samples from Switzerland [61] and higher concentrations of the same compounds (<3-273 ng g^{-1} (l.w.) for MX and <13-295 ng g^{-1} (l.w.) for MK) were reported in fish specimens from German rivers by Rüdel *et al.* [62]. MX and MK were also detected in mussel samples from the North Sea and Baltic Sea at concentrations in the range of <0.1-0.34 ng g^{-1} wet weight (w.w.) and <0.1-0.60 ng g^{-1} (w.w.) for MX and MK, respectively.

As well as being persistent or pseudo-persistent, NM fragrances are also semi-volatile. As a result, another route of exposure is their vaporization in the air [67-71]. In a recent study, Ramírez *et al.* [67] evaluated and compared the presence of MX, MM and MK in indoor and outdoor air samples from the area of Tarragona (NE Spain). In the indoor samples (chemical laboratory, secretary's office, medical centre, pharmacy, hairdresser and flower shop), MX and MK were detected at concentrations in the range of 2.9-766.5 ng m^{-3} and 1.9-68.4 ng m^{-3}, respectively. Meanwhile, in outdoor samples, regardless of whether they were taken in urban and suburban locations, the concentrations decreased significantly to values in the range of 1.6-4 ng m^{-3} for MX and 0.8-1.5 ng m^{-3} for MK. MM was not detected in any of the samples analysed. These results are in line with those reported by Sofuoglu *et al.* [70] for air samples from a primary school classroom (9.89 ng m^{-3} for MX and 0.12 ng m^{-3} for MK) and a women's sport centre (3.24 ng m^{-3} of MX) in Turkey. In both studies and also a third one conducted in kindergartens in Germany [68], MK was not detected in indoor air samples.

1.1.2. Polycyclic musk fragrances

The first polycyclic musk (PCM) fragrance was discovered by Carpenter *et al.* [72] at the beginning of 1948. Although it was never introduced to the market, it became known as ambral and inspired chemists to synthesize many related nitro-free aromatic musk fragrances. The creation of this class of musks was largely prompted by the need to eliminate the nitro functional group from NM fragrances due to their photochemical reactivity and their instability in an alkaline medium, causing discolouration problems in functional products. In addition, the possibility of synthesizing aromatic musks without nitro groups led to the creation of stable musks for functional perfumery, cosmetics, laundry and detergents, a huge market with enormous potential [27].

It was not until 1951, that the first nitro-free aromatic musk to be introduced to perfumery was discovered by Kurt Fuchs. Even without knowing of its correct structure, it was introduced to the market one year later under the trade name of phantolide (AHMI). Four years later, when the structure of tonalide (AHTN) was clarified as acetyl indane [73], polycyclic arenes became the new lead structure for musk odorants. Although AHTN did not have a very strong odour (6.7 ng L^{-1} in air of odour threshold), due to its stability and hydrophobicity, its performance in washing powders and detergents was outstanding. In the following years, besides hedonics and odour strength, the evolution in musk odorants was mainly focused towards stability in harsh media and hydrophobicity, a key factor in the deposition of odorants on the fabric

during the washing process. This evolution resulted in numerous PCM fragrances (see Figure 5): traseolide (ATII), celestolide (ADBI), vulcanolide (FHTN), versalide (AETT), musk 89, novalide, okoumal, moxalone, nebulone, cashmeran (DPMI), with galaxolide (HHCB) as the culmination of this evolution in terms of stability and hydrophobicity.

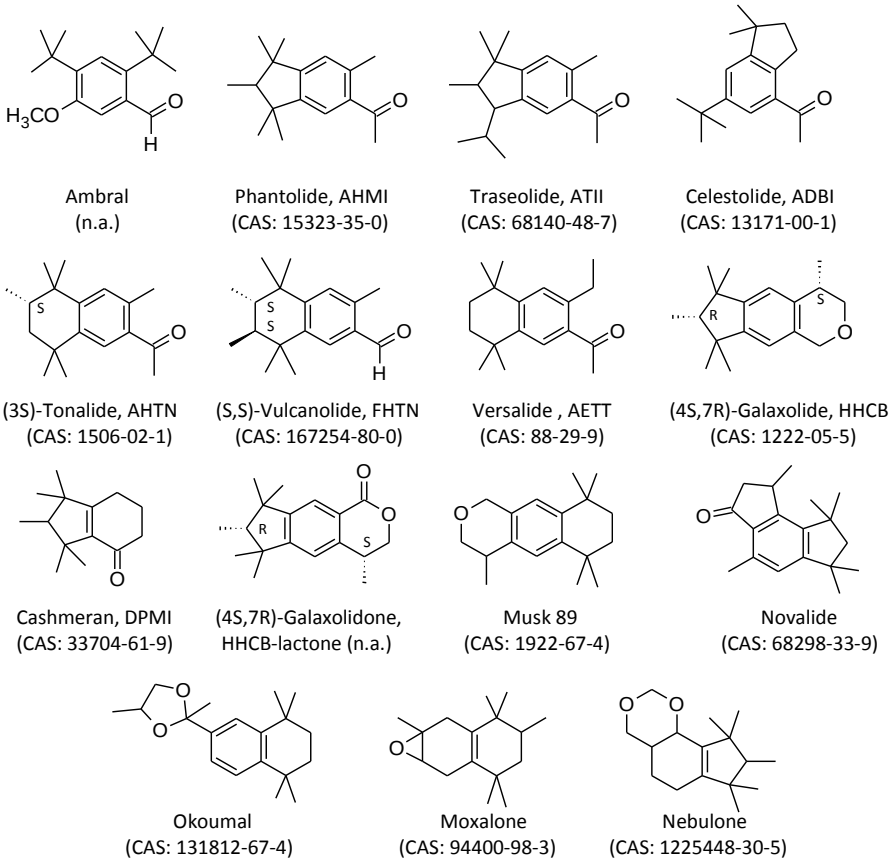

Figure 5. Chemical structures and CAS numbers of the most common PCM fragrances. (n.a.: not available).

HHCB was the result of systematic studies by Heeringa and Beets of the company International Flavors & Fragrances (IFF) on the osmophoric group of PCMs fragrances, the most polar functional group of odorants. In short, Heeringa and Beets wanted to replace the carbonyl group presented in all of the known PCM fragrances by other functional group to make them even more stable and hydrophobic. Specifically, the replacement of the carbonyl group with ether oxygen placed in a rigid tetrahydropyran ring resulted in the synthesis of HHCB in 1965 [74]. From this moment onwards, already

in the late 1960s, HHCB was used in dosages up to 40% in fabric softeners such as *Comfort* and *Softlan*, and in detergents like *Coral* at 27%. Moreover, high concentrations of HHCB were also incorporated in perfumes, such as *Trésor* by Sophia Grojsman (Lancôme, 1990) with 21.4% of HHCB.

Defined by the International Fragrance Association (IFRA) as fragrance ingredients with musk as the predominant odour note, PCM fragrances are characterized to be a bi-cyclic aromatic structure of the indane or tetraline type, which is substituted with a combination of an acetyl group or a pyran ring in combination with methyl, isopropyl and/or t-butyl groups. Due to their structure, some of the PCM fragrances, such as HHCB and AHTN, are commercially available as an isomeric mixture. Nevertheless, the various stereoisomers differ significantly in terms of their threshold [75,76], and this provides some interesting insight into the molecular features of musk fragrances. For instance, although the four stereoisomers of HHCB are synthesized by the Friedel-Crafts reaction [27], the configuration at C-4 is much more important than that at C-7 and that is reflected in the odour threshold values of the four isomers [75]: 0.63 ng L^{-1} in air for (4S,7R)-HHCB, 130 ng L^{-1} in air for (4R,7S)-HHCB, 1.0 ng L^{-1} in air for (4S,7S)-HHCB and 440 ng L^{-1} in air for (4R,7R)-HHCB. In short, the (4R)-HHCB isomers are much weaker and do not contribute to the odour profile of the commercial product. In the same way, the most powerful stereoisomers of the PCM fragrances, AHTN and Vulcanolide, which are shown in Figure 5, were studied in order to establish the exact odour threshold of each isomer. However, no exact threshold data have been reported in the literature for these compounds [77,78].

As a consequence of the massive production volumes of PCM fragrances, their excellent chemical stability, non-biodegradability and high octanol/water partition coefficients, PCMs have been found in air [67], wastewater [55], sewage sludge [58] and soil [79], and they have also been bioaccumulated in fish and other marine organisms [80]. Although no apparent toxicity has been reported for most of the PCM fragrances [31], with only AETT being banned due to its neurotoxicity [81], possible long term effects [82] are difficult to foresee and more and more PCM-free formulations increasingly appear on the market. Seinen *et al.* [83] and Schreurs *et al.* [84] found evidence that exposure to PCM fragrances can cause hormone-disrupting effects. HHCB and AHTN can bind to and stimulate human oestrogen receptors [83], and both musk fragrances have been shown to affect androgen and progesterone receptors [84]. AHTN has also been reported to increase the proliferation of oestrogen-responsive human breast cancer cells [85]. Moreover, AHTN has been identified as a photosensitizer, a chemical that becomes more toxic when exposed to sunlight on the skin [86], and has

also been linked to liver toxicity [87]. As a result, HHCB and AHTN are included on the fourth Priority List of substances for review by the EU. This Priority List was the fourth compiled by the EU of high volume substances that were to be evaluated by the Competent Authorities of the Member States, as part of the requirements of EU Council Regulation 793/93 "on the evaluation and control of the risk of existing substances" [88]. The EU published the final reports on HHCB [89] and AHTN [90] in 2008 and concluded that, for all human and health environmental endpoints, "there is at present no need for further information and/or testing and no need for risk reduction measures". In the particular case of AHTN, the report recommended the limitation of use of this fragrance due to its photosensitizing effects. The use of AHTN in the cosmetic industry has, in fact, been regulated by European Directive 2008/42/EC [91].

Currently, the PCM fragrances HHCB, AHTN and ADBI are economically the most significant musk fragrances. Specifically, HHCB and AHTN represent about 95% of the market in Europe for musk fragrances. For this reason, both compounds are included on the EU's REACH list [92] and also on the EPA's high production volume chemical list [93]. The PCM fragrances AHMI, ATII, musk 89 and novalide are much rarer. DPMI is also considered part of the PCM fragrance group even though its scent has a distinctive woody character. Okoumal, a propylene glycol acetal of an otherwise non-commercialized polycyclic compound, has an intense ambergris note. Moxalone, vulcanolide and nebulone represent the most important discoveries of the past 30 years in this musk category, though their volumes are in no way comparable to those of the above mentioned stars of the group.

At the present time, the entire production of HHCB and AHTN thakes place at one plant in Europe, with a production volume of between 1,000 and 5,000 tonnes/year. An overview of the production of HHCB and AHTN between 1992 and 2004 is shown in Table 1 [89,90].

Table 1. Production of HHCB and AHTN in Europe.

Year	Production (tonnes/year)	
	HHCB[1]	AHTN[2]
1992	2,400	885
1995	1,482	585
1998	1,473	385
2000	1,427	358
2003	1,441*	265*
2004	1,307	247

* Indicative.
[1] Data from European Union Risk Assessment Report of HHCB [89].
[2] Data from European Union Risk Assessment Report of AHTN [90].

About 63% of the production volume (HHCB undiluted) is exported outside the EU, of which 25% (HHCB undiluted) is in undiluted form and 37.5% (HHCB undiluted) after dilution. As shown in Figure 6a, 37.5% (HHCB undiluted) of the HHCB produced is consumed by the EU after dilution, of which 86% is for fragrance oil compounding and 14% is for formulating consumer products [89]. Meanwhile, 62% of the total AHTN production is exported outside Europe. The rest of the AHTN produced is consumed in Europe (see Figure 6b), with 80% being used for fragrance oil compounding and 20% for formulating consumer products [90].

HHCB, commercialized as a pourable liquid, and AHTN, as crystallized product, are both used as ingredients in fragrance oils. HHCB is the largest volume product of the fragrance materials known collectively as PCM fragrances, followed in the second place by AHTN. The IFRA Code of Practice for the Fragrance Industry [94] defines fragrance oils as complex mixtures prepared by blending many fragrance ingredients in varying concentrations. A vast majority of these ingredients are liquids, in which HHCB is mixed and AHTN has to be dissolved.

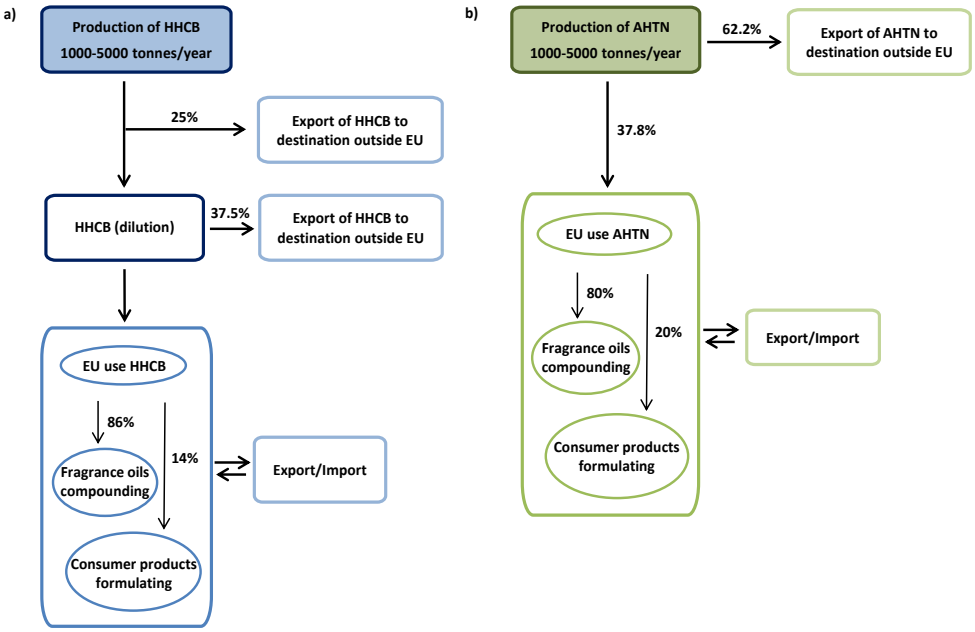

Figure 6. Material flow of a) HHCB and b) AHTN based on IFRA Production/Sales history data 2000 [30].

Applications of fragrance oils include consumer products such as perfumes, cosmetics, soaps, shampoos, detergents, fabric conditioners, household cleaning products and air fresheners, etc. The distribution between the various categories is shown in Figure 7. For instance, Llompart *et al.* [95] reported HHCB and AHTN concentrations in the range of 0.0374-0.134 μg g^{-1} and 0.0115-0.0483 μg g^{-1} in rinse-off products, such as shower gels and shampoos. Meanwhile, HHCB and AHTN were found in 11 of the 19 leave-on samples (body milk, hand creams and sun milk) analysed, and the concentration of these PCMs fragrances was very high in several samples, with values about 1,000 μg g^{-1}. However, the use of PCM fragrances in cosmetics and household cleaning products for the European market significantly decreased during the second half of the nineties. As a consequence of the bad publicity about these PCM ingredients, most of the producers of PCPs and detergents with European wide brands asked their fragrance suppliers to avoid using PCM fragrances. This led to PCM fragrances being replaced by macrocyclic musk and alicyclic musk fragrances, and these European wide brands no longer contain PCM fragrances. Specifically, this trend was followed by many producers of locally marketed cosmetics and household cleaning products in Northern European countries such as the Netherlands, Germany, Austria, Switzerland and Scandinavia. In contrast, in southern European countries (i.e. Portugal, Spain, Italy, France and Greece), local producers followed this trend to a lesser extent.

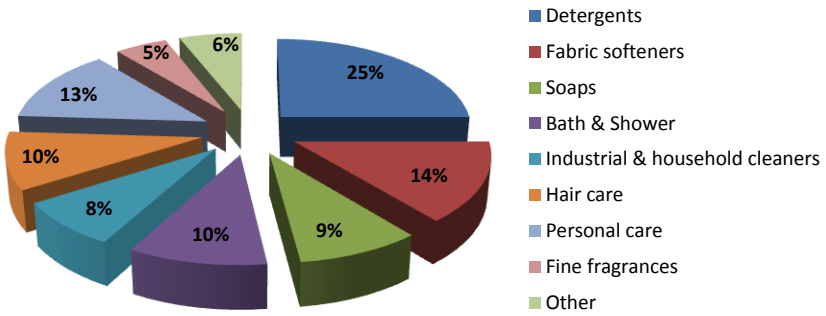

Figure 7. The use of PCM fragrances HHCB and AHTN in the EU [30].

Mainly, there are two factors that may cause an unequal distribution of the use volume of PCM fragrances *per capita* in Europe. A 'cultural' factor of different use volumes of detergents may cause higher use of detergents per capita by a factor of 1.25 in southern EU (Italy, Spain, Portugal and France) with 166 million inhabitants, whereas an average volume is found in Belgium/Luxembourg, Greece, UK and Ireland, with 84.6 million inhabitants. In the northern countries of Germany, Austria, the Netherlands,

Denmark, Sweden and Finland (125.5 million inhabitants), detergent use is below average by a factor of 0.7 [89,90]. The second factor is the development of the market in which, since 1995, PCM fragrances have gradually been replaced by other fragrance ingredients, such as macrocyclic and alicyclic musk fragrances, for instance.

In line with this trend, recent studies focusing on the determination of PCM fragrances in environmental samples were conducted in countries of the south of the EU, mainly in Spain. For instance, Ramírez *et al.* [64] found HHCB and AHTN concentrations in the range of 476-2,069 ng L^{-1} and 17.7-78.7 ng L^{-1}, respectively, for influent wastewater samples from the Tarragona region (NE Spain). Meanwhile the remaining PCMs studied (DPMI, ADBI, AHMI and ATII) were present at lower concentrations in the range of 3.6-87.7 ng L^{-1}. A decrease in the concentration of all of the PCM fragrances studied was reported, with effluent samples concentrations in the range of 233-1432 ng L^{-1} for HHCB, 25.4-93.6 ng L^{-1} for AHTN and 4.15-43.3 ng L^{-1} for the rest of PCM fragrances. The authors also reported concentrations of HHCB (1.40-26.2 ng L^{-1}), AHTN (0.34-0.37 ng L^{-1}), DPMI (0.49-1.72 ng L^{-1}) and AHMI (0.27 ng L^{-1}) in river water. In addition, Arbulu *et al.* [55] found HHCB, DPMI and ADBI at concentrations in the range of 220-2184 ng L^{-1}, 277-1,377 ng L^{-1} and 41-96 ng L^{-1}, respectively in water samples from the River Alegría (Alava, Spain). The same PCM fragrances, as well as AHTN, were also found in influent and effluent samples from the WWTP in Vitoria-Gasteiz with HHCB (influent: 900-3,568 ng L^{-1} and effluent: 800-3,021 ng L^{-1}) and DPMI (influent: 70-530 ng L^{-1} and effluent: 100-400 ng L^{-1}) being the most abundant PCM fragrances [55]. Moreover, HHCB and AHTN have also been detected in influent and effluent samples from WWTPs in China (Beijing and Shanghai) [56,59] at concentrations in the range of 1,251-3,003 ng L^{-1} of HHCB and 111-378 ng L^{-1} of AHTN in influent samples. Meanwhile in effluent samples, the concentrations of HHCB and AHTN went down to values in the range of 212-1,258 ng L^{-1} and 3.8-14.1 ng L^{-1}, respectively. In addition, some of the most widely used PCM fragrances, such as HHCB (4.7 ng L^{-1}), AHTN (1 ng L^{-1}), ATII (0.11 ng L^{-1}), ADBI (0.029 ng L^{-1}) and AHMI (0.52 ng L^{-1}), were also found in Lake Michigan (USA) by Peck and Hornbuckle [96].

Due to their stability and non-biodegradability, PCM fragrances have also been found in sewage sludge and sediments all over the world [58,79,97]. For example, Clara *et al.* [98] reported HHCB and AHTN concentrations in the range of 5-21,000 ng g^{-1} (d.w.) in sewage sludge from WWTPs in Austria, while other PCM fragrances, such as DPMI (5-260 ng g^{-1} (d.w.)) and ADBI (7.5-61 ng g^{-1} (d.w.)), were also present at minor concentrations. Meanwhile, DPMI (1.2-2.5 ng g^{-1} (d.w.)), HHCB (1.4-2.8 ng g^{-1} (d.w.)) and AHTN (0.5-0.7 ng g^{-1} (d.w.)) concentrations were detected in sewage sludge from a

WWTP in Taiwan [99] and in sediments surrounding the effluent of the WWTP (HHCB: 0.2-4 ng g^{-1} (d.w.) and AHTN: 0.1-3 ng g^{-1} (d.w.)). In addition, due to their bioaccumulative properties, PCMs were quantified at trace levels in fish samples. In this respect, HHCB concentrations in the range of 42-230 ng g^{-1} (l.w.) and AHTN concentrations in the range of 20-54 ng g^{-1} (l.w.) were reported in fish from lakes in Switzerland [61]. ADBI (8.7-35 ng g^{-1} (l.w.)), AHMI (0.79-3.3 ng g^{-1} (l.w.)) and ATII (1.3-2.1 ng g^{-1} (l.w.)) were also detected at minor concentrations [61]. Higher concentrations of HHCB in the range of 14-9,750 ng g^{-1} (l.w.) and AHTN in the range of 14-1,520 ng g^{-1} (l.w.), as well as ADBI (13-600 ng g^{-1} (l.w.)), AHMI (3-642 ng g^{-1} (l.w.)) and ATII (13-1,230 ng g^{-1} (l.w.)), were found in fish samples from Lake Belou and Rivers Danube and Rhine [62].

As with all of the semi-volatile compounds, PCMs fragrances can also be found in indoor [67-70,100] and outdoor [67,71,101] air samples at ng m^{-3} levels. However, the concentrations found in indoor air samples are significantly higher than those recorded in outdoor samples. The concentrations of HHCB and AHTN found in air samples from the Great Lakes (USA) [71] and the Artic and the North Sea [101] are in the range of 0.012-2.03 ng m^{-3} and 0.003-0.965 ng m^{-3}, respectively. However, in indoor samples from a sports centre in Turkey [70], the concentrations of HHCB and AHTN increased to maximum values of 144 ng m^{-3} and 39.5 ng m^{-3}, respectively. Other PCM fragrances such as DPMI (16.90 ng m^{-3}), ADBI (1.01 ng m^{-3}), AHMI (0.08 ng m^{-3}) and ATII (31 ng m^{-3}) were also detected in indoor samples from the sports centre [70].

1.1.3. Macrocyclic musk fragrances

In the quest to identify and artificially recreate substances responsible for the musky smell, systematic studies of natural Tonkin musk began in the early 20th Century [102]. Heinrich Walbaum of Schimmel & Co. was the first to find the principal musk odorant of Tonkin musk in 1906, and he established that it was a ketone of the empirical formula $C_6H_{30}O$ with two double bond equivalents. He gave it the name of 'muscone' [103]. However, he had no idea about the chemical structure of muscone, the content of which ranged from 0.5% to 2% in Tonkin musk (musk grains). The rest of the brown grains were various aromatic or non-smelling organic compounds, such as cholesterol esters, steroid compounds, fatty acids, proteins and waxes, etc.

The chemical structure of muscone was discovered by Professor Leopold Ruzicka in his work (1922-1926) that earned him the Nobel Prize in Chemistry in 1939. He found the structure of muscone to be a 15-membered ring ketone with one methyl substituent in the 3-position (3-methylcyclopentadecan-1-one) [104]. Ruzicka also

investigated and clarified the structure of civetone (9-cyclopentadecen-1-one), the musk odorous principle of the glandular secretion of the civet cat, which had been isolated in 1915 [105]. These works started a chapter of macrocyclic chemistry and also the hunt for macrocyclic ketones in order to find other synthetic musks similar to those found in nature. Eventually, scientists learned to make muscone and other natural macrocyclic musks (MCMs) such as civetone and exaltone (see Figure 8) in the laboratories, so the musks appeared on the market. Therefore, in 1926, cyclopentadecanone (exaltone) was introduced on to the market instead of muscone, at a prohibitive price of 50,000 CHF Kg^{-1} [106] due to the low yields of its synthesis and difficult purification.

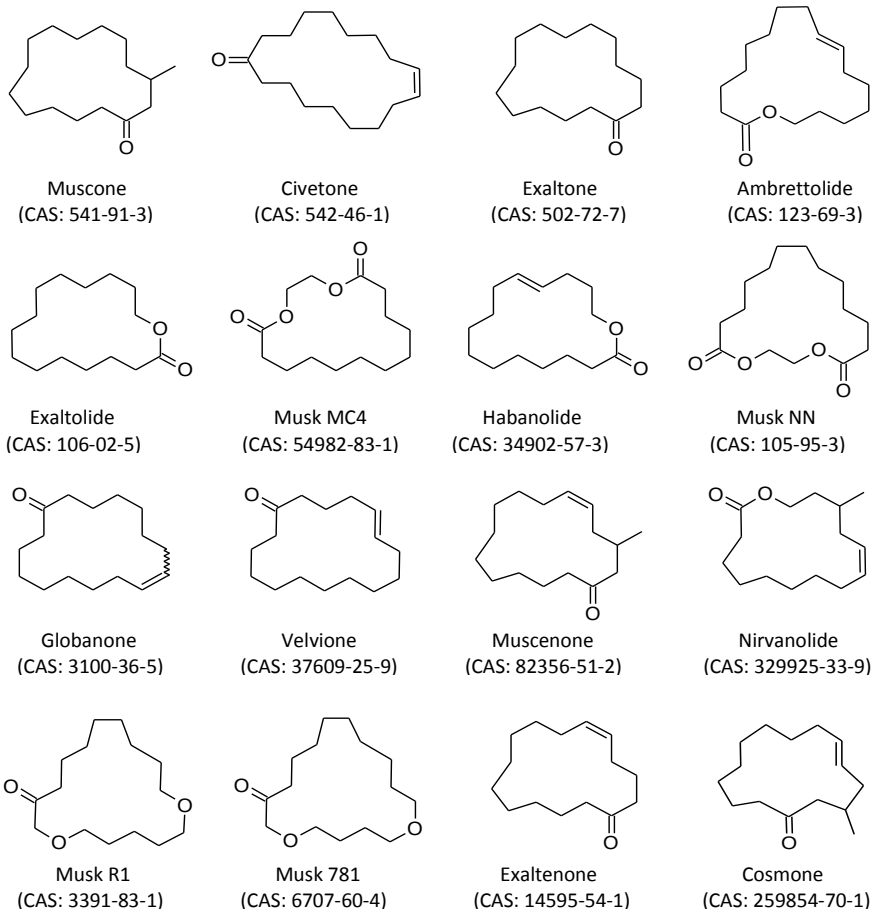

Figure 8. Chemical structures and CAS numbers of the most common MCM fragrances.

In the plant kingdom, the substances responsible for the odour strength are exclusively macrolides, macrocyclic lactones. The most common of these, 15-pentadecanolide (exaltolide), was discovered in 1927 in angelica root oil by Kerschbaum [107]. Exaltolide has a more distinct and delicate musk note and is less animalic than exaltone and, like most macrolides, it smells slightly floral. It was also introduced into perfumery by Firmenich at the exorbitant price of 100,000 CHK Kg^{-1} [106]. Kerschbaum [107] also found other important musk-smelling lactones in ambrette seed oil (ambrettolide and musk NN) that smell similar to exaltolide: powerful musky, warm-aromatic with fruity-floral nuances and some reminiscence of genuine ambrette seed oil.

Therefore, MCMs have been known to scientists and perfumers since the 1920s, but were too expensive for mass production. After World War II, chemists were able to find cheaper ways to get musks found in nature. For example, perfectly neutral musk exaltolide, mentioned earlier, is produced industrially at a price of around 60 CHK kg^{-1} [108], and it is sold under various trade names by many suppliers. Givaudan calls it thibetolide, while at Firmenich it is called exaltolide and it bears the name macrolide in Symrise and pentalide at Soda Aromatics. In the same way, the price of exaltone dropped from 50, 000 CHF Kg^{-1} in 1926 to about 500 CHF Kg^{-1} in 1947 [106]. In addition, chemists were able to find more similar macrocyclic molecules with a musky smell as well as how to manufacture them. One example is habanolide, that has a neutral musk background with shades of linen and cotton, hot iron, wood, metal and hot candle wax and it plays an important role in the 'white musk accord'.

Nowadays, to replace or even outperform PCM and NM fragrances in diverse applications, one can either lower the production price of macrocyclic or increase their odour intensity, which means lowering their odour threshold. The latter option allows for more complex synthetic approaches and, accordingly, higher production costs. The second generation of MCM fragrances, including globanone, velvione, muscenone, nirvanolide, exaltenone and cosmone, are the results of the advances made in MCM fragrance synthesis over the last 20 years. Muscenone, which was first reported in 1971 [109] and had its debut in perfumery in 1993 with the floriental feminine fine fragrance *Jean-Paul Gaultier* [27], is one of the most widespread of the second generation MCM fragrances. It also possesses a very elegant and diffusive musk odour reminiscent of the NM fragrance MK, and, with an olfactory threshold of 0.9 ng L^{-1} in air, it is as powerful as the PCM fragrance HHCB. Globanone [110] and velvione [111] have also been introduced to perfumery. Velvione was extensively used in the fine fragrance *Velviona* (Helmut Lang, 2001), but it is also economic and substantive enough to provide powdery

volume and musky softness in laundry-care products. Most recently, nirvanolide [77], with its clean and sweet, powdery and persistent, and slightly animalic musk odour, is also quite close to MK in terms of odour. With an odour threshold of 0.1 ng L^{-1} in air, it has proven to be more powerful than muscenone. 6.7% of nirvanolide is present in the composition of the perfume *Forever Elizabeth* created by David Apel [27]. Although second generation MCM fragrances such as muscenone and nirvanolide are considered to be the perfumery materials of choice when it comes to replacing PCM and NM fragrances in older formulations, currently MCM exaltolide and musk NN are the most extensively used MCM fragrances [112,113].

It is expected that, in the coming years, the advances made in the synthesis of MCM fragrances will allow their synthesis prices to be reduced, making them more and more widely available [77,114,115]. As they seem to be more easily degradable in the environment and smell more intense than PCM fragrances less mass is needed to achieve the same performance in perfumery [116]. In addition, the chemical structure of MCM fragrances suggests easy microbial decomposition, which has yet to be confirmed. Despite the lack of information on the use of MCM fragrances in Europe, it is indicated that less than 25% of the musk produced worldwide in 1998 were MCM fragrances. This percentage grew to 60-65% by 2008 due to the risk associated with NM and PCM fragrances [113]. In this respect, Sommer and Juhl [117] analysed eight MCM fragrances in 146 cosmetics collected during 1999 and 2001 in Germany. Musk NN and exaltolide were predominant compounds, at concentrations in the range of 100-33,600 μg g^{-1} and 100-12,800 μg g^{-1}, respectively. They indicated that the number of cosmetics containing MCM fragrances increased particularly in the low-price products in 2001. A TNO (Toegepast-natuurwetenschappelijk onderzoek) report in 2005 referring to the presence of phthalates and artificial musks in perfumes [112] indicated that MCM fragrances are gradually replacing PCM fragrances, just as the latter replaced NM fragrances. The fact that there are also products that contain both MCM and PCM fragrances may indicate that this replacement is not always straightforward. This study also confirms that the MCMs exaltolide and musk NN, which were found in 11 and 15 of the 29 samples analysed, respectively, are the most extensively used MCM fragrances. The presence of ambrettolide, civetone, muscone and musk MC4 was also confirmed in some of the samples analysed. In 2008, the usage volumes of musk NN, habanolide, exaltolide and musk MC4 in EU ranged from 100 to 1,000 tonnes/year for each compound [118]. These values were mostly comparable with those of HHCB (1427 tonnes/year) and AHTN (358 tonnes/year) [89,90]. Meanwhile, the global usage volumes of these MCM fragrances in 2008 were higher than 1,000 tonnes/year for musk NN and between 100-1,000 tonnes/year for habanolide, exaltolide and musk MC4 [118].

Recently, Nakata *et al.* [119] evaluated the concentrations of the MCM fragrances musk NN, habanolide, exaltolide, ambrettolide, musk MC4, exaltone and muscone in household commodities (perfume, fabric softener, shampoo, body lotion, body soap, laundry detergent, etc.) collected in Japan. The results showed maximum concentrations in the range of 190-11,000 μg g^{-1} for musk NN, 160-2,200 μg g^{-1} for habanolide, 26-6,700 μg g^{-1} for exaltolide, 9.5-880 μg g^{-1} for ambrettolide, 13-34 μg g^{-1} for musk MC4, 22-67 μg g^{-1} for exaltone and 21-77 μg g^{-1} for muscone, depending on the product analysed. This highlighted the high levels of production and usage of MCM fragrances in cosmetics and household commodities in Japan nowadays.

Furthermore, different studies have focused on the determination of MCM fragrances in environmental samples such as wastewater samples [55,120], sewage sludge [121] and house dust [119]. For example, García-Jares *et al.* [120] evaluated the presence of synthetic musk fragrances, including the MCM fragrance ambrettolide, in effluent wastewater samples, while Arbulu *et al.* [55] studied the concentrations of the MCM fragrances ambrettolide, muscone and musk NN in wastewater influent/effluent samples, river samples and groundwater samples. Although concentrations of PCM fragrances and alicyclic musk fragrances were reported in both studies, none of the samples analysed contained MCM fragrances at concentrations higher than the method detection limits (MDLs). In contrast, Matamoros *et al.* [121] reported ambrettolide concentrations in the range of 10-150 ng g^{-1} (d.w.) in sludge from a 20 years old sludge treatment reed bed system placed in Nakskov (S Denmark). Maximum concentrations of musk NN of 220 ng g^{-1} and 1,300 ng g^{-1} in house dust and beauty shop dust, respectively, were reported by Nakata *et al.* [119].

1.1.4. Alicyclic musk fragrances

Alicyclic musks (ACM), otherwise known as cycloalkyl ester or linear musks, are a class of musk fragrances, neither macrocyclic, polycyclic nor benzenoid, that certainly possess a notable musk odour. The first compound of this class, introduced to perfumery as cyclomusk, was discovered by Hoffmann and Fraunberg of BASF in 1975 [122]. Though similar structures were noted earlier in citronellyl oxalate and rosamusk [123], cyclomusk emanates a warm, powdery musk odour with fruity, strawberry-like nuances [122]. Although its industrial synthesis, which started with the thermal cyclization of dehydrolinalool, was not too expensive, cyclomusk had no chance against HHCB in the late seventies and, as a result, was withdrawn from the market.

Development of the group of ACM fragrances was continued by the discovery and introduction of helvetolide (a fruity blackberry musky smell) at Firmenich in 1990 [123]. Helvetolide, the structure of which is shown at Figure 9, was the first ACM fragrance produced at a commercial scale and it was, for instance, added in percentages between 3.8% and 8.8% in widely known perfumes such as *Emporio Armani White for Her* (Armani, 2001), *Miracle* (Lancôme, 2000) and in *Flower* (Kenzo, 2000). Ten years later, one dimethyl group of helvetolide could be replaced by a carbonyl group without losing the musk odour and that resulted in the ACM fragrance commercially known as romanolide being developed by Firmenich [124]. With a delicate, fresh, berry-like camphoraceous musk smell, romanolide was integrated into the formula of a number of perfumes. By way of illustration, it is used in the perfumes *Absolu* (Rochas, 2002) and *Murmure* (Van Cleef & Arples, 2002), in proportions of 5.0% and 1.0%, respectively.

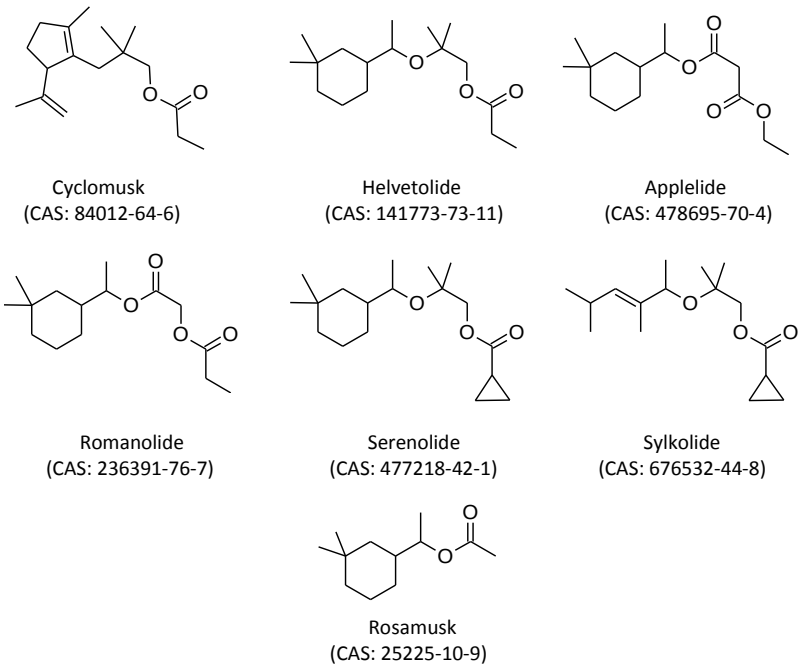

Figure 9. Chemical structures and CAS numbers of the most common ACM fragrances.

Serenolide, sylkolide and applelide are other linear musks, constituting the fourth generation of musks, and they can be tuned to be either substantive or very volatile. Serenolide (Givaudan, 2006) is an elegant ACM fragrance with sweet fruity connotations providing warm and soft velvety notes that blend well with all kinds of trendy fruit

accords [125]. It can be found in perfumes such as *Polo Double Black* (Ralph Lauren, 2006) and *John Galiano* (John Galiano, 2008). Thus, sylkolide, which was introduced by Givaudan in 2010, is a top-note musk. It brings a modern musky backbone that is noticeable throughout a fragrance and combines wonderfully with the red fruit facets that characterize this ingredient [126]. It was used by Tommy Hilfiger in the formulation of *Hilfiger Woman Cheerfully Pink* (2013). In contrast, applelide, which was first synthesized by IFF in 2008, is a sensuous ACM fragrance with a touch of fresh, fruity green apple and creamy, powdery textures.

It is also possible to combine ACM fragrances with MCM fragrances in order to avoid some anosmias towards ACM fragrances. The combination of ACM and MCM fragrances is very common, especially in the case of what is known as the white musk accord. Defined as the harmonious combination of several MCM and ACM fragrances that give a fresh and pure feeling, some shine, some air and cleanliness. The first combination of white musks consisted of the mixture of ACM fragrance helvetolide and MCM fragrance habanolide (*Emporio Armani White*, Alberto Morillas, 2001) [125].

At present, ACM are still only used in PCPs to a very limited degree [55]. However, in the coming years, due to the biodegradable properties of ACM fragrances and their low cost of manufacture compared to MCM fragrances, ACM fragrances are considered to be the future of synthetic musk fragrances. Although no information is yet available about the production and uses of ACM in Europe, the increasing number of research articles concerning the development of reliable analytical methods to determine the presence of ACM fragrances in environmental samples [55,127,128] is clear evidence of the importance that ACMs are gaining in perfumery. In this regard, Arbulu *et al.* [55] monitored the presence of the ACM fragrances helvetolide and romanolide in groundwater, river water and influent and effluent wastewater in Vitoria (N Spain) for a year (2009-2010). The results showed that romanolide was the only ACM found in river water at a concentration ranging between 73 ng L^{-1} and 306 ng L^{-1}. Moreover in groundwater, neither helvetolide nor romanolide were detected. In contrast, when influent/effluent wastewater samples were analysed, both helvetolide (45-58 ng L^{-1}) and romanolide (45 ng L^{-1}) were reported in influent samples. They were also found in effluent samples (21-70 ng L^{-1} for helvetolide and 56 ng L^{-1} for romanolide) at concentrations similar than those reported in influent samples. However, to date, most ACM fragrance studies still focus on the synthesis and identification of these compounds, rather than their determination in environmental samples in general and, specifically, in solid samples.

Currently, the research for new musk fragrances continues in every perfume company. As shown in the timeline of musk fragrances (see Figure 10), there has been a continuous evolution of musk fragrances over the years. Therefore, the ideal synthetic musk does not yet exist. Some musk fragrances have a pleasant scent but are dangerous and have been banned as NM fragrances. Others, as in the case of PCM fragrances, are too stable and non-biodegradable (though are not known to be toxic to date), and some are still very expensive to manufacture (MCM fragrances).

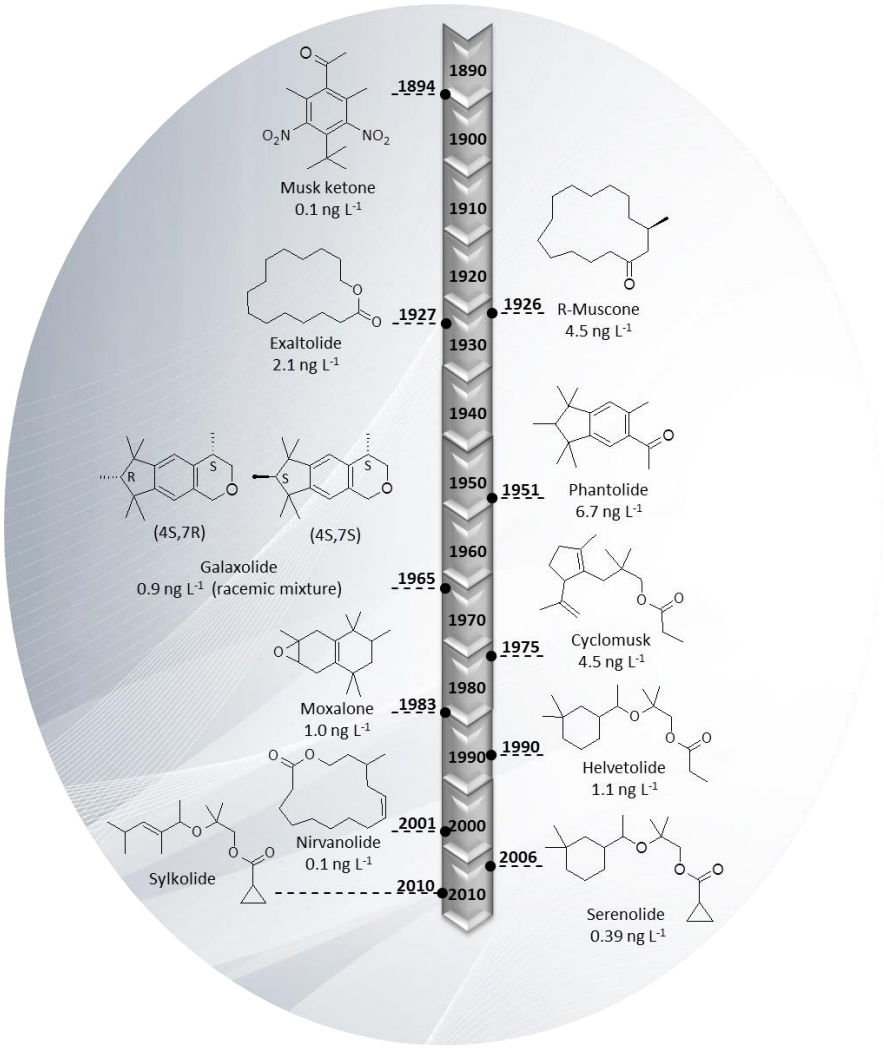

Figure 10. Evolution of synthetic musk fragrances over the years.

New musk fragrances should be non-toxic and biodegradable, have high persistence and chemical stability in different environments, lower production costs and new smell profiles [129]. In terms of productivity, if an inexpensive musk fragrance such as musk NN is used, the musk sensation might be somewhat diffuse. However, very potent molecules, such as nirvanolide, muscenone and MK, make a clear statement and they are also affordable. The intensity of the second generation MCM fragrances nirvanolide and muscenone does not yet compensate for their high price, but they give a crisper, more modern, radiant sensation than using a large amount of musk with a high threshold (first generation MCM fragrances), which would act as a filler.

1.2. Determination of musk fragrances in environmental samples

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

PCPs, including antimicrobial agents, insect repellents, preservatives, UV filters and fragrances, are widely used as additives in a broad range of everyday products such as cosmetics, foods and drinks [24,130]. They are included within the group known as EOCs, which are present in the environment as a result of their widespread use throughout the world for several applications [24,131,132]. Synthetic musk fragrances are a family of cyclic PCPs with widespread use, incorporated in several personal care and household products (e.g. lotions, perfumes, softeners) as fragrance additives and fixative elements [64,133]. Following their application, most of these compounds are released via household effluents, reaching WWTPs, where higher concentrations of musk fragrances (parts per billion) have already been found in water [120]. The conventional processes applied in WWTPs are not always capable of removing synthetic musk fragrances. In fact, this is a group of compounds with a chemical structure that it is not readily biodegradable [134,135]. For that reason, their removal in WWTPs occurs mainly due to the transfer from the liquid to the solid-phase and sorption onto sludge particles [136]. As a result, they are not completely removed during wastewater treatment [133,137,138] and are therefore frequently found in surface waters [56,138,139] at concentration levels ranging between parts per billion and parts per trillion. In fact, the application of sludge and/or biosolids to agricultural fields is a direct input of musks into the soil [140], and thus into the food chain [141], whereas the discharge of effluents is the major route for water and aquatic biota contamination [142-144]. Apart from being persistent or pseudo-persistent, synthetic musk fragrances are also semi-volatile, which may explain their detection in remote areas after long-range atmospheric transport [55,64]. Therefore, specific sample pretreatment techniques involving preconcentration and clean-up processes are necessary, before selective and sensitive analysis techniques for their determination in environmental matrices [24,114].

Conventional extraction techniques such as liquid-liquid extraction (LLE) [96] and solid-phase extraction (SPE) [135,145,146], which also allow the preconcentration of the analytes of interest, have successfully been used for the determination of synthetic musk fragrances in aqueous matrices. However, these extraction techniques involve the use of organic solvents, which constitutes a pollution problem in itself. For this reason, in order to reduce or eliminate the use of organic solvents during the preconcentration step and to obtain more environmentally friendly analytical methods [147,148] new microextraction techniques have been developed over the last few decades. Dispersive liquid-liquid microextraction (DLLME) [53,149], ultrasound-assisted emulsification-microextraction (USAEME) [139], solid-phase microextraction (SPME) [120,150], single-drop microextraction (SDME) [151,152] and microextraction by packed sorbents (MEPS)

[54,153] are just a few examples. However, although fully automated SDME has successfully been used for the determination of some EOCs such as UV filters [151] or persistent organic compounds such as chlorobenzenes [152] with GC-MS, no reports have been found for the application of SDME for the determination of synthetic musk fragrances. In the case of solid matrices, such as sewage sludge or sediment samples as well as biological samples ultrasound assisted extraction (USAE) [59,154], microwave assisted extraction (MAE) [155-157] and pressurized liquid extraction (PLE) [57,79] are the preferred extraction techniques, but high analyte preconcentration is not usually obtained. However, due to the lack of selectivity of the extraction techniques for the abovementioned solid samples, extracts from complex samples (e.g. sludge) have to be subjected to time-consuming clean-up steps, usually SPE [57,59] or gel permeation chromatography (GPC) [57,98], to the detriment of analysis time and increasing solvent consumption.

After the extraction step, a chromatographic technique is required due to the similar physicochemical properties of the synthetic musk fragrances. Furthermore, an effective chromatographic separation is able to reduce the matrix effect as much as possible through the separation of the compounds of interest and undesired co-eluting matrix components. Currently, the most widely employed techniques for the determination of EOCs are GC and liquid chromatography (LC). The choice between these techniques depends on the physicochemical properties of the analytes, such as their volatility, thermal stability and polarity, among other factors. In the particular case of synthetic musk fragrances, as they are considered semi-volatile compounds, lipophilic and thermostable, they are mainly separated by GC [114,158]. In addition to the separation technique used, powerful detection techniques in terms of sensitivity and selectivity are also needed. In this respect, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are the detection techniques most widely used for the determination of synthetic musk fragrances in environmental samples.

In the following sections, there is an overview of the different extraction and analysis techniques currently employed for the determination of the synthetic musk fragrances included in this Thesis, with a particular emphasis on the replacement of conventional extraction techniques with microextraction techniques in order to obtain more environmentally friendly analytical methods.

1.2.1. Extraction techniques

As mentioned earlier, the extraction technique selected for the development of an analytical methodology is strongly related to the matrix analysed. In fact, extraction techniques have been designed based on the physical state of the matrix, as it can be gaseous, aqueous, solid or semi-solid. The following sections focus on the most commonly used extraction techniques when working with aqueous and solid/semi-solid matrices, because these are the matrices studied in this Thesis. However, synthetic musk fragrances are also present in gaseous samples, as discussed previously.

1.2.1.1. Extraction techniques for aqueous matrices

In general, the extraction techniques used for aqueous matrices are based on the partitioning equilibrium between the sample matrix (aqueous phase) and another phase, which can be a non-miscible solvent for LLE or a solid sorbent for SPE. Until the 1980s, LLE was the most extensively used technique for extracting different EOCs, including synthetic musk fragrances, from environmental waters. Specifically, Bester *et al*. [159,160] developed a method based on LLE followed by GC-MS for the determination of the most widespread PCMs fragrances, HHCB, AHTN and the degradation product HHCB-lactone, in influent/effluent wastewater samples from an urban WWTP and river water from the Ruhr catchment area, both in Germany. With 1 L of sample volume, 10 mL of toluene as the extraction solvent and extracts concentrated by a rotary evaporator to 1 mL, the developed method provided recovery values of 75% for HHCB, 78% for AHTN and 100% for HHCB-lactone and method quantification limits (MQLs) of 5, 10 and 100 ng L^{-1} for HHCB-lactone, AHTN and HHCB, respectively, in wastewater samples and down to values of 1, 3 and 9 ng L^{-1} in river water. However, LLE has several well-known pitfalls such as the use of large volumes of toxic organic solvents, limited enrichments factors and a laborious and time consuming procedure.

Miniaturization and improvement of LLE was therefore a challenge tackled by several researchers in recent years in order to overcome some of the drawbacks mentioned above. Recently, liquid-phase microextraction (LPME) has been introduced. This is a solvent-minimized sample pretreatment procedure of LLE in which only several µL of solvent are required to concentrate analytes from liquid samples rather than hundreds of mL such as in traditional LLE. LPME can be performed with three main approaches: single-drop microextraction (SDME), hollow-fibre liquid-phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME). As shown in Figure 11, in order to increase the potential applicability of SDME, two main approaches can be used:

direct immersion (DI)-SDME and headspace (HS)-SDME. In DI-SDME, the drop is directly immersed in the aqueous phase, while in HS-SDME, the drop is exposed to the headspace above the sample. Applications of DI-SDME are normally restricted to medium polar, non-polar analytes and those with polarities that can be reduced before the extraction. The organic solvents used for DI-SDME are immiscible with water. Moreover, HS-SDME is usually used to extract volatile analytes and, unlike in DI-SDME, water can be used as the extraction solvent to extract volatile and water-soluble analytes [161]. This significantly enhances the range of extractable analytes, as well as the range of analytical methods that can be coupled to SDME. Generally, high enrichment factors can be obtained in SDME as a result of the great reduction in the extractant phase-to-sample volume ratio [148]. Initial projects involving SDME were carried out using a microdrop of an organic solvent. However, the instability of the drop limits the usable volume of the extracting medium and directly affects the precision and sensitivity of the determinations. This limitation is more marked when HS-SDME is performed at high temperatures because of the evaporation of the organic solvent during the extraction [162,163]. However, greener solvents such as ionic liquids (ILs), which are ionic media resulting from the combination of organic cations and various anions, have been proposed as alternative to organic solvents because of their low vapour pressures and high viscosity, which allows the use of larger and more reproducible extracting volumes [164,165]. Both DI-SDME [163] and HS-SDME [152,162,164] have successfully been used to determine EOCs in environmental samples. For instance, Vidal *et al.* [162] developed a method to determine chlorobenzenes in water (10 mL) with HS-SDME followed by GC-MS, with 2.5 µL of toluene as the extraction solvent and 5 min of extraction time. The method provided MDLs in the range of 3-31 ng L^{-1} and repeatabilities in the range of 2.1-12.9% for tap and well water. Meanwhile, He *et al.* [163] determined the presence of chlorobenzenes in water (4 mL) by means of DI-SDME (1 µL toluene as the extractant and extraction time of 15 min) followed by GC-MS. The validation parameters showed MDLs in the range of 100-300 ng L^{-1} with a repeatability of 9.7% with ultrapure water. The suitability of ILs for the extraction of benzene, toluene, ethylbenzene and xylene isomers by HS-SDME followed by GC-MS was also investigated by Aguilera-Herrador *et al.* [164]. Using 2 µL of the IL 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]) as the extraction solvent exposed in the HS of 8 mL water sample for 30 min at 30 °C, the developed method provided MDLs between 20 ng L^{-1} and 91 ng L^{-1} and repeatability values varied between 3.0% and 5.2% for river, drinking, tap and well water.

In the particular case of synthetic musk fragrances, HS-SDME and DI-SDME based methods have not yet been reported. However, a variation of DI-SDME known as in-syringe dynamic SDME, followed by GC-MS, has recently been evaluated by Wang *et al.* [166] for the determination of PCMs in surface waters of the Pearl River estuary and South China Sea. The extraction procedure consisted of withdrawing 1 µL of *n*-hexane into a 10 µL microsyringe and then immersing the needle tip in the aqueous sample (10 mL) and withdrawing a 6 µL aqueous sample with a dwell time of 30 s, before pushing out 6 µL of sample with a dwell time of 30 s. The same process was repeated 50 times within 25 min. Finally, the aqueous sample was pushed out with 1 µL of toluene left in the syringe. The method provided MDLs ranging between 3.4 and 11 ng L^{-1} and repeatabilities with relative standard deviations (RSDs) lower than 11.1%.

With respect to HF-LPME, it was introduced by Pedersen-Bjergaard and Rasmussen [167] in 1999 and consists of hanging a polypropylene HF membrane in the HS of a vial filled with the aqueous sample. The HF may be either a rod with a closed bottom or a u-shape where both ends are connected to guiding tubes. Figure 11 illustrates HF-LPME with a u-shape HF membrane. Prior to extraction, the HF membrane is dipped in the organic solvent, which is immiscible with water to ensure that it remains within the pores during the extraction, to immobilize the solvent in the pores, and excess solvent is removed. After that, the extraction solvent fills the lumen of the HF membrane. This extraction solvent can be an organic solvent (the same used for the HF membrane pores), resulting in a two-phase extraction system, or it may be an acidic or alkaline aqueous solution, resulting in a three-phase extraction system. Typically, two-phase HF-LPME involves the use of toluene or *n*-octanol as the extraction solvent, whereas three-phase HF-LPME is conducted with HCl and NaOH to adjust the pH of the extraction solution and the sample and *n*-octanol and dihexyl ester are the organic solvents used to impregnate the pores of the HF membrane. In HF-LPME, the extraction efficiency is governed by partitioning of the analytes between the sample matrices and the immobilized solvent and by partitioning between the extraction solvent and the immobilized solvent. So, hydrophobic analytes or, in other words, volatile and semi-volatile analytes are easily extracted into organic solvents from aqueous sample solutions. Moreover, hydrophobic ionic analytes have large solubility differences in acidic and basic aqueous solutions and, consequently, they are extracted well into the aqueous extraction solvent. Polar analytes are, therefore, difficult to extract by HF-LPME due to their low solubility in water-immiscible organic solvents and small differences in their solubility in acidic and basic aqueous solutions. The ability of HF-LPME to exclude the extraction of polar analytes contributes to the selectivity of the method. In addition,

the small size of pores in the walls of the HF membrane (200 nm) give rise to a better clean-up of the sample, especially for those of a biological nature, since large molecules are unlikely to be extracted.

The first report on HF-LPME utilized three-phase extraction with methamphetamine as a model drug [167]. Meanwhile, two-phase HF-LPME was firstly used by King *et al.* [168] for extracting polycyclic aromatic hydrocarbons (PAHs) from soil. Nowadays, HF-LPME is carried out in two modes: dynamic and static. In dynamic HF-LPME, the extraction solvent is repeatedly withdrawn into and discharged from the HF by a syringe pump and, for this reason, it is claimed to provide better extraction efficiency and improve the reproducibility compared to the static mode [169]. In this respect, Zhao and Lee compared dynamic and static HF-LPME for the extraction of PAHs in aqueous samples [170]. The results showed 35-fold enrichment in 10 min and good repeatability (4%) for static HF-LPME, while higher enrichment (75-fold) in 10 min and even better repeatability (3%) were obtained working with dynamic HF-LPME. Dynamic HF-LPME followed by high performance liquid chromatography-UV detection (HPLC-UV) has also successfully been applied for the determination of paraben preservative in water samples by Esrafili *et al.* [171]. With *n*-octanol as the extraction solvent and 20 mL of sample volume, the developed method provided MDLs in the range of 2,000-5,000 ngL^{-1} and repeatability values of 3.0-16.2%. As in the case of DLLME, in recent years, the use of ILs as extraction solvents in HF-LPME has also been evaluated [172]. For instance, Ma *et al.* [172] used the IL 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) as an extraction solvent for the HF-LPME extraction of benzene, toluene, ethylbenzene and xylene present in water samples (20 mL). With GC and a flame ionization detector (FID) as the separation and detection techniques, MDLs in the range of 2,200-4,000 ng L^{-1} and repeatabilities of 1.3-5.4% were obtained.

To the best of our knowledge, only a few research papers focus on the determination of synthetic musk fragrances using HF-LPME [173,174]. Einsle *et al.* [173] applied HF-LPME followed by GC-MS for the determination of HHCB and AHTN in aqueous samples with a polyethylene bag filled with 500 µL of chloroform exposed in the HS of 50 mL of aqueous sample for 60 min, obtaining MDLs of 20 ng L^{-1} and RSDs of 6% for HHCB and 5% for AHTN. Meanwhile, Posada-Ureta *et al.* [174] applied HF-LPME and large volume injection (LVI)-GC-MS for the determination of a wide range of synthetic musk fragrances including NMs and PCMs in aqueous samples. The developed method consisted of exposing a polyethylene bag filled with 200 µL of *n*-hexane in the HS of 150 mL of sample for 240 min. This method provided MDLs in the range of 4-25 ng L^{-1} and good precisions (<20%).

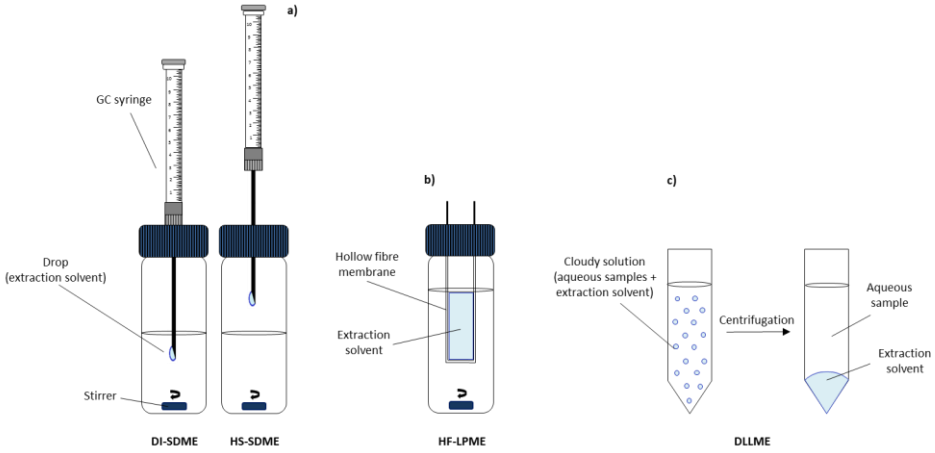

Figure 11. Schematic of a) DI-SDME and HS-SDME, b) HF-LPME and c) DLLME.

Another type of LPME is DLLME, which was firstly described by Rezaee and co-workers in 2006 [175] for the determination of PAHs in aqueous samples. It consists of two main steps: the injection of appropriate mixture of extraction and disperser solvents into aqueous samples, and the subsequent centrifugation of the cloudy solution [176,177]. The extraction steps of DLLME are also illustrated in Figure 11. In the first step, the extracting solvent is dispersed into the aqueous sample as very fine droplets and the analytes are enriched into it. Owing to the large surface area of contact between the extracting solvent and the aqueous sample, equilibrium state is achieved quickly and the extraction is independent in terms of time. Then, after centrifugation, the cloudy solution is separated into the aqueous phase and extraction solvent.

The main advantages of DLLME are the negligible consumption of extraction solvent (microlitre volumes), the short extraction time due to the rapid achievement of the equilibrium state and the high enrichment factor due to the high phase ratio of the donor phase (aqueous sample) and the acceptor phase (extraction solvent). Consequently, DLLME can be considered as a simple, quick and efficient technique that meets the requirements of green chemistry. However, this technique also has certain limitations, which primarily result from requirements related to the extraction and disperser solvents. The extraction solvent must have good extraction capability with respect to the target analytes, low solubility in the aqueous phase, and a greater density than water. Moreover, the disperser solvent must be miscible with both the extraction solvent and the aqueous phase. For this reason, the selection of the extraction solvent becomes the most restrictive aspect because few organic solvents meet such

requirements. Normally, organic solvents are selected as extraction solvents for DLLME on the basis of their higher density compared to water, extraction capability of interested compounds and good chromatographic behaviour. Halogenated hydrocarbons, such as chlorobenzenes, chloroform, carbon tetrachloride and tetrachloroethylene, are usually selected as extraction solvents. Acetone, methanol and acetonitrile are usually selected as disperser solvents. In addition, volumes of extraction solvent, as well as the disperser solvent, pH value and salt addition, may crucially influence the extraction results and must also be optimized. The extraction solvent volume has a significant effect on the preconcentration factor (PF). By increasing the extraction solvent volume, the volume of sedimented phase obtained by centrifugation increases, resulting in a decrease in the PF. Therefore, the optimal extraction solvent volume should ensure both the PFs and enough volume of the sedimented phase for the subsequent analysis after centrifugation. The disperser solvent volume directly affects the formation of the cloudy solution (water/disperser solvent/extraction solvent), the dispersion degree of the extraction solvent in aqueous phase and, subsequently, the extraction efficiency. The suitable volume of disperser solvent for a suitably cloudy solution depends on the volume of both aqueous phase and extraction solvent.

As water-immiscible solvents are generally used in DLLME, the preferred technique for the analysis of extracts is GC. The versatility of DLLME-GC is seen in relation to the variety of applications in a broad range of areas. For instance, Rezaee *et al.* [175] applied DLLME for the determination of PAHs in water samples, with 1 mL of acetone (as the disperser solvent) containing 8.0 µL of chloroform (as the extraction solvent) injected into a 5 mL aqueous solution. After centrifugation, 2 µL of sedimented phase was injected into the GC-FID. The developed method provided MDLs in the range of 7-30 ng L^{-1}, recovery values of 82-111% and RSD values of 1.4-10.2%. DLLME has also successfully been applied for the determination of UV filters in environmental samples by Negreira *et al.* [178]. The method consisted of mixing 1 mL of acetone containing 60 µL of chlorobenzene with 10 mL of aqueous solutions and the subsequent centrifugation and injection of 2 µL of sediment phase in the GC-MS. The method validation parameters results showed LOQ of 2-10 ng L^{-1}, repeatability values in the range of 5-11% and recoveries of 95-74%. NM fragrances can also be extracted by DLLME, as demonstrated by López-Nogueroles *et al.* [53] in 2011. With 1 mL of acetone as the disperser solvent containing 50 µL of chloroform as the extraction solvent injected into 5 mL of aqueous solution, after centrifugation, 1µL of sedimented phase was injected into the GC-MS. The developed method provided recovery values in the range of 87-116%, depending on the water matrix analysed, MDLs of 7-33 ng L^{-1} and RSD values below 5% for all of the target analytes. DLLME has also been applied for the

determination of PCPs, including 5 PCM fragrances and 2 phthalates, in environmental waters [149]. With 0.62 mL of methanol (as the disperser solvent) containing 250 µL of chloroform (as the extraction solvent) injected into 5 mL aqueous sample, after centrifugation, 1 µL of sedimented phase was injected into the GC-MS. The PCM validation results showed recovery values between 77.4% and 92.9%, depending on the kind of water analysed, MDLs of 28-63 ng L^{-1} and repeatabilities that did not exceed 9.7%.

Since the development of DLLME in 2006, significant efforts have been made with respect to DLLME in order to obtain more efficient and simpler approaches and to further expand the range of applications of this technique. In this respect, the need to eliminate toxic solvents, such as chlorinated hydrocarbons, has led to the search for alternative solvents to be applied in DLLME technique. The application of solvents less dense than water as extraction solvents has been reported in a number of recently published studies. Such solvents enable the extraction of target analytes without a centrifugation step. For example, Guo and Lee [179] developed a method based on low-density solvent-based (LDS)-DLLME followed by GC-MS for the determination of 16 priority PAHs in environmental samples. Moreover, Chen *et al.* [180] applied LDS-DLLME GC-MS/MS for the determination of carbamate pesticides present in aqueous samples. Both authors used *n*-hexane (50 µL) as the extraction solvent and acetone (500 µL) as the disperser solvent to extract the target compound present in 5 mL of aqueous samples. The emulsification was broken by the addition of 500 µL of acetone. The methods provided MDLs in the range of 3.7-39.1 ng L^{-1} for PAHs and 1-50 ng L^{-1} for carbamate pesticides, with repeatability values lower than 10% in both cases. There is also a significant number of applications that use ILs as the extraction solvent in DLLME [181-183]. For instance, Pena *et al.* [182] developed a DLLME based method followed by HPLC-fluorescence detection (FD) to determine PAHs in water samples (10 mL) using 50 µL of [OMIM][PF_6] as the extraction solvent. The developed method obtained MDLs in the range of 0.03-2 ng L^{-1}, repeatabilities of 1.2-4.5% and recoveries values of 90.3-103.8%. However, to date, neither LDS-DLLME nor IL-DLLME have been applied for the determination of synthetic musk fragrances in water samples. At present, the increasing trend is to avoid the process of dispersion/emulsification using ultrasound treatment (ultrasound-assisted emulsification-microextraction (USAEME) and ultrasound dispersion liquid-liquid microextraction (US-DLLME)) [139,184,185]. Fontana *et al.* [185] developed a method to determine polybrominated flame retardants in water samples by USAEME followed by GC-MS. The aqueous sample (10 mL) was mixed with 500 µL of sodium chloride solution and 100 µL of chloroform as the extraction solvent.

The resulting mixture was immersed in an ultrasonic bath for 5 min before the centrifugation step. The developed method provided MDLs in the range of 1-2 ng L^{-1}, recovery values of 96-106% and repeatabilities of 4-20%. USAEME followed by GC-MS has also successfully been applied for the determination of a group of EOCs including phthalates, PCM and NM fragrances and pesticides in environmental waters [184]. The developed method consists of putting a mixture of 100 µL of chloroform (extraction solvent) with 10 mL of aqueous solution into an ultrasonic bath and the subsequent centrifugation of the emulsification to separate the aqueous phase from the extraction solvent. The validation parameters obtained showed recovery values of 87-114%, a repeatability range of 3-11% and MDLs in the range of 6-133 ng L^{-1}. Meanwhile, Yang *et al.* [139] developed a USAEME GC-MS method focused on the determination of PCMs fragrances present in aqueous samples. In this case, 10 mL water sample containing sodium chloride was mixed with 1 mL of isopropyl alcohol (disperser solvent) and 10 µL of carbon tetrachloride (extraction solvent) to form a cloudy solution that was sonicated for 1 min prior to the centrifugation step. The method performance results showed recovery values of 70-95%, MDLs near 0.2 ng L^{-1} regardless of the target compounds and repeatability values lower than 4% in all cases.

Before the explosion in the number of LLME techniques, SPE had already replaced LLE and, nowadays, it is still the most commonly used extraction technique for aqueous samples [186]. SPE emerged in the mid-1970s and has been widely used for extracting a broad range of compounds with different properties from aqueous samples. SPE is a sorption-based extraction in which the analytes present in aqueous sample interact with a solid sorbent on which they are retained. Afterwards, they are generally eluted using an organic solvent. In the case of SPE, the most important parameter to be optimized is the extraction sorbent. Initially, surface-modified silica particle based sorbents, such as C18 or C8, were the SPE sorbents selected to retain volatile and semi-volatile compounds present in aqueous samples efficiently. However, silica-based sorbents display a limited working pH range and the sorbent needs to remain wet during sample loading in order not to lose retaining capabilities. The inclusion of polymeric materials for SPE on the market has solved these problems, as they can work in all pH ranges and the polymeric material remains wet for more time, thereby increasing extraction reproducibility. Polymeric materials also have a higher surface area than silica-based sorbents and, therefore, they have greater capacity. At present, the most widely used sorbents for extracting EOCs from aqueous matrices are balanced hydrophilic/hydrophobic polymeric compounds with different polarities. One such example is Oasis HLB (Waters®), which is a macroporous copolymer made from

divinylbenzene (hydrophobic) and N-vinylpyrrolidone (hydrophilic) monomers [187]. The combination of two classes of monomers with different properties makes this sorbent capable of retaining both polar and non-polar compounds.

SPE sorbents come in the form of extraction cartridges and disks. The disk format provides a large surface area for sorbent/sample contact and faster flow rates and higher throughput compared to cartridges. For instance, Simonich *et al.* [146,188] developed a method to extract a group of sixteen fragrances using SPE with a C18 Empore disk and GC-MS. The method provided recoveries (relative to the deuterated internal standard) on the range of 97-115% with MQLs ranging from 0.5 to 35 ng L^{-1}. Meanwhile, Buerge *et al.* [189] focused on monitoring HHCB and AHTN present in water samples from lakes in Switzerland. In this particular case, the SPE extraction sorbent was a macroporous polystyrene-divinylbenzene copolymer (BioBeads SM-2, Bio-Rad Laboratories) and the recovery values obtained were 81% and 86% for HHCB and AHTN, respectively. The MDLs found in surface waters were 2 ng L^{-1} for HHCB and 1 ng L^{-1} for AHTN and up to 10 ng L^{-1} for both compounds when working with WWTP samples. In addition, Osemwengie and Gerstenberge [145] and Lv *et al.* [56] developed two methods using SPE as the extraction technique and a mixture of polymethyl methacrylate: polystyrene cross-linked with 50% divinylbenzene (1:1, Nexus, Varian) and a polymerically bonded octadecyl (17% C), encapped (ENV-18, Supelco) as extraction sorbents, respectively, to determine PCM and NM fragrances in environmental water samples. Slightly better recovery values, ranging between 93% and 116%, were obtained by Osemwengie and Gerstenberg [145], while Lv *et al.* [56] obtained recoveries in the range of 88.3% to 104.1%. With GC-MS as separation and detection techniques, both methods provided MDLs at low ng L^{-1} levels (0.09-0.18 ng L^{-1}). SPE with Oasis HLB as the extraction sorbent followed by GC-MS was also used by Zhou *et al.* [59] to determine HHCB and AHTN in WWTP water samples. The authors reported recoveries of 83.5% for HHCB and 92.4% for AHTN and MDLs of 0.4 ng L^{-1} for both target compounds.

The on-line coupling of the sample preconcentration process (SPE) and the separation and detection process (LC or GC) allows automation, and prevents contamination from external sources. Moreover, the automation of SPE minimizes sample losses and contamination during handling, and improves the reproducibility and MDLs, since the total amount of components introduced into the analytical system is transferred to the chromatograph. However, the on-line coupling of SPE to GC requires the injection of relatively large volumes of organic solvents, while conventional GC injectors only allow a few microlitres [190]. Therefore, the use of an injection technique is required, such as partially concurrent solvent evaporation (PCSE) using an on-column

interface [191-193] or programmed temperature vaporizer (PTV) [194,195]. As shown in Figure 12, for the injection of large volumes (up to at least 100 µL) to the GC using an on-column interface, the use of a deactivated uncoated fused-silica retention gap is a must. Moreover, in order to speed up the evaporation process and to protect the detector from the large solvent cloud, the solvent vapour is generally released from the GC system through a solvent vapour exit placed just before the GC analytical column. In the particular case of the PTV injector, it allows the injection of large volumes without the need for retention gaps, and the solvent vapour is released from the GC system via a split vent. The main advantage of the on-column interface is that it can be used for non-volatile and volatile analytes, while alternative techniques for large volume sampling, such as PTV, are restricted to compounds eluting at relatively high temperatures [190,196].

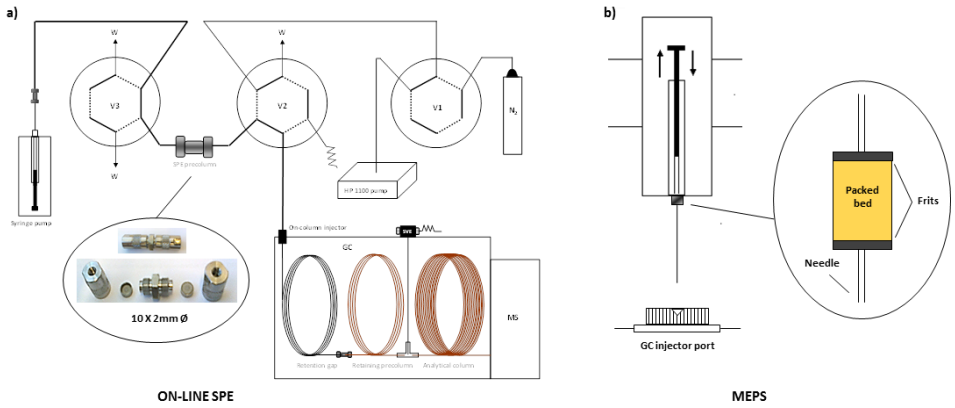

Figure 12. Illustration of a) on-line SPE GC set-up and b) MEPS device.

Pocurull *et al*. [192] and Brossa *et al.* [193] developed two methodologies based on on-line SPE followed by GC-MS with an on-column interface. Specifically, Pocurull *et al.* [192] developed a method to determine a group of pesticides with different chemical structures. With polystyrene-polyvinylbenzene copolymer (PLRP-S) as the SPE sorbent, a sample volume of 10 mL (30% methanol) and 100 µL of ethyl acetate as the elution solvent, the developed method provided MDLs between 2 and 20 ng L^{-1}, recovery values higher than 90% for the vast majority of the target pesticides and RSD values in the range of 8-13%. Meanwhile, Brossa *et al.* [193], applied a number of modifications to the abovementioned methodology, such as using a sample volume of 15 mL (50% methanol), to optimize the on-line SPE GC-MS method for the determination of a group of endocrine-disrupting agents, including pesticides, phthalates and PAHs, in water

samples. The validation parameters results showed MDLs of 0.1-20 ng L^{-1}, repeatabilities of 8-28% and recovery values in the range of 10-78% for all of the target analytes. The PTV interface, has also successfully been applied for the determination of pesticides present in water samples by Sasano *et al.* [197] and Koning *et al.* [198]. In particular, the on-line SPE method developed by Sasano and co-workers [197] consisted of the preconcentration of 2 mL of water in a PLRP-S sorbent and the subsequent elution of the target compounds (30 pesticides) with 20 µL of acetone as the elution solvent. Employing this system, the recoveries and RSD of most compounds were greater than 75% and below 10%, respectively. Meanwhile, Koning *et al.* [198] focused on the determination of a small group of Nitrogen/Phosphorus (NP)-pesticides (11 compounds) in surface water samples. The developed method involves the preconcentration of 7.5 mL of sample in a PLRP-S sorbent and the subsequent desorption of the target analytes with 50 µL of ethyl acetate as the elution solvent. The developed method provided recovery values in the range of 71-96%, MDLs at low ng L^{-1} (0.7-9.5 ng L^{-1}) and repeatabilities of 1.7-6.0%. Mostly, on-line SPE coupled to GC-MS applications focus on the determination of pesticides [192,193,197,198], phthalates [193] and PAHs [191,193]. The application of on-line SPE followed by GC-MS for the determination of synthetic musk fragrances in water samples has not yet been reported.

In recent years, a new miniaturization of conventional SPE known as microextraction by packed sorbent (MEPS), which was introduced by Abdel-Rehim in 2004 [199], has attracted the interest of scientists [200,201] because it can be connected on-line to GC or LC without any modification and significantly reduces the volume of solvents and sample needed. In MEPS, the sorbent, 1-2 mg, is either inserted into the syringe barrel (100-250 µL) as a plug or between the needle and the barrel as a cartridge, as shown in Figure 12. Sample extraction and enrichment can be accomplished on the packed sorbent. MEPS can be fully automated. The sample processing, extraction and injection steps are performed on-line in a single device composed of the following two parts: the MEPS syringe and the MEPS cartridge, also known as the BIN. The BIN contains the packed MEPS bed, a solid support that retains the target analytes when the sample passes through it and is built into the syringe needle. The BIN is used with a 100 µL or 250 µL gas tight MEPS syringe that allows fluid handling at normal SPE pressures. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Any sorbent material can be used, such as silica-based (C2, C8, C18), strong cation exchanger (SCX) using sulphonic acid bonded silica, restricted access material (RAM), hilic, carbon, polystyrene-divinylbenze copolymer (PS-DVB) or molecular

imprinted polymers (MIPs) [200]. However, only the silica based sorbents and the polymeric phases are commercially available, while more specific MEPS BINs filled with MIPs are made in-house.

The most important parameters in MEPS performance can be divided into the following categories: selection of a solvent for the conditioning, loading, washing and elution steps, sample flow rate, washing solution and volume and type of elution solvent [199-201]. Beginning with the solvent and sample flow, it can accurately be controlled to modulate the interaction of the target analyte with the sorbent. Presumably, a lower flow allows a better interaction between the analyte and the sorbent. This is, however, a drawback in the case of manual MEPS extraction, as the flow is not measured and the repetitive handling procedure is user-dependent and prone to experimental errors. Regardless of the conditioning step, the solvent volumes and the number of loading cycles should be optimized to avoid carry-over from previous extractions. Samples can be loaded directly (environmental waters) or properly diluted if they are too viscous or concentrated (biological fluids such as blood or urine) [202]. Moreover, the sample volume should be optimized to obtain the best equilibrium between good analytical performance and a good extraction methodology. Therefore, ideally, the proper concentration factor for the target analytes should be obtained in the minimum sample volume loading cycles, but it is common for MEPS extraction protocols to use up to ten or more extraction cycles. This creates another optimization opportunity when the sample is loaded several times. The aliquot can be discarded or reloaded several times. The selection of the best conditions will depend on the nature of the matrix being used and the retention capacity and specificity of the sorbent. The washing step is usually performed with the same solvent used to equilibrate the sorbent during the conditioning step, but once again, the number and volume of washing cycles should be optimized. Lastly, the elution solvent and its volume should be carefully optimized in order to allow the release of the analytes from the sorbent and to be directly injected to the LC or GC systems. For instance, in a standard MEPS procedure, two volumes of the total syringe capacity are recommended for the conditioning step, starting with a strong organic solvent followed by equilibration with water (or acidified water). Then, the sample is drawn once or more through the sorbent manually or by an autosampler (draw-eject in the same vial or draw and eject into waste). The solid phase is then washed once by 50-100 µL water to remove the interferences. The analytes are then eluted with a solvent (20-50µL) directly into the injector of the instrument. The process can be manual, semi-automated or fully automated [201]. To reuse an MEPS cartridge,

the sorbent is washed 3-4 times with water and 4-5 times with elution-solution solvent to eliminate carry-over. Depending on the complexity of the matrix being processed, MEPS sorbents can be reused several times, up to 100 or more.

MEPS has been employed for the analysis of a wide variety of organic pollutants present in environmental water samples such as chlorobenzenes [203], pesticides [204], fragrances [54,153] and PAHs [205,206], among others [207,208]. For instance, Fu *et al.* [205] and El-Beqqali *et al.* [206] developed two methodologies with MEPS followed by GC-MS for the determination of PAHs in aqueous samples. Fu *et al.* [205] passed 2 mL of water sample (40 times x 50 µL) through a C18 MEPS BIN at a flow rate of 5 µL s^{-1} and the analytes were directly eluted with 50 µL of methanol directly into the GC. Meanwhile, El-Beqqali *et al.* [206] passed 3 mL of water sample (60 times x 50 µL) through a C8 MEPS BIN at a flow rate of 20 µL s^{-1} and the analytes were directly eluted with 30 µL of methanol directly into the GC. The developed methods provided comparable MDLs ranging between 1 and 8 ng L^{-1}, good intra-day precisions of 1.6-14% and recovery values in the range of 72-117%. Fluoroquinolones have also successfully been preconcentrated with MEPS by Prieto *et al.* [208]. In this case, the authors synthesized an MIP in order to improve the selectivity of the method, as well as the recoveries of the target analytes. The developed method consisted of the extraction of 1,600 µL of sample (16 times x 100 µL) at a speed of 5 µL s^{-1} and the subsequent elution with two portions of 25 µL of methanol:acetic acid (50:50, v/v). The elution portions were evaporated to dryness, reconstituted with 100 µL of an aqueous solution (water:methanol, 70:30, v/v) and finally injected for LC-MS/MS analysis. The validation data obtained showed MDLs in the range of 0.5-3.8 ng L^{-1}, recovery values of 93-115% and precisions of 7.7-12.6%. Some of the compounds that have been studied in this Thesis have also successfully determined using MEPS as the extraction and preconcentration technique [54,153]. Specifically, Moeder *et al*. [153] optimized a MEPS-based method to determine a group of compounds including UV filters, caffeine and the PCM fragrances HHCB and AHTN using both C8 and C18 as extraction sorbents and ethyl acetate as the elution solvent. With the preconcentration of 800 µL of aqueous sample (8 times x 100µL) at a flow rate of 10 µL s^{-1} and the subsequent elution with two portions of 25 µL of ethyl acetate directly into the LVI-GC-MS instrument, the method provided recoveries in the range of 46-114% and 65-109% for C8 and C18, respectively. It also showed MDLs of 34-96 ng L^{-1} (C8) and 35-87 ng L^{-1} (C18), which enable the determination of the analytes at common environmental concentrations levels. Both sorbents showed linear calibration curves for most of the analytes up to a

concentration level of 20 ng mL^{-1}. Meanwhile, Cavalheiro *et al.* [54] developed a new procedure for the simultaneous determination of nine NM and PCM compounds in environmental water samples using MEPS followed by LVI-GC-MS. The method consists of passing 5.5 mL of aqueous sample (55 times x 100 µL) through a C18 sorbent BIN at a flow rate of 10 µL s^{-1} and the elution with two portions of 25 µL of an ethyl acetate: *n*-hexane mixture (50:50, v/v) directly into the LVI-GC-MS. MDLs ranged from 5 to 25 ng L^{-1}, 7 to 39 ng L^{-1} and 8 to 84 ng L^{-1} for influent, effluent and estuarine samples, respectively. Apparent recoveries were higher than 75% for all of the target compounds in all of the matrices studied.

Other approaches that have been evaluated for extracting EOCs from water samples include the use of solvent free microextraction techniques based on the partitioning of analytes between the gaseous or liquid phase and stationary phase, such as SPME. Since the development of SPME in 1990 by Arthur and Pawliszyn [209], it has become widespread not only because of its solventless nature but also due to its ability to facilitate rapid, convenient sample preparation in the laboratory and on site. The geometry of the SPME technique was optimized to improve speed, sensitivity and convenience of use. As a result, during its development, different SPME configurations were introduced. SPME configurations can be classified into static and dynamic techniques. The most common static procedures, which are typically carried out in stirred samples, include SPME fibre, rotating disk sorptive extraction (RDSE), stir bar sorptive extraction (SBSE) and thin-film microextraction (TFME). These are shown in Figure 13. Dynamic techniques include needle trap microextraction (NTME) and solid-phase dynamic extraction (SPDE) [210].

Conventional SPME fibres are made with fibre cores of fused silica or quartz, but the inherent fragility of fibre cores led to the introduction of metal or other alloy wires as the support, in order to improve durability and thermal stability of SPME fibres. Meanwhile, coated stir bars and rotating disks were used for stirred configurations with larger amounts of extraction phase (or sorbent) than the SPME fibre. In TFME, a flat sheet of film with a large surface area-to-volume ratio is used in the extraction phase. The SPDE device is also more robust than the fragile SPME fibre. However, the main obstacle of this extraction technique is the carry-over effect, because the analytes tend to remain on the inner wall of the needle after TD [211]. Another configuration of SPME is NTME. In this case, the sorbents or fibres are packed into a removal needle mounted on a gas-tight syringe and the compounds are extracted from the aqueous samples in HS mode.

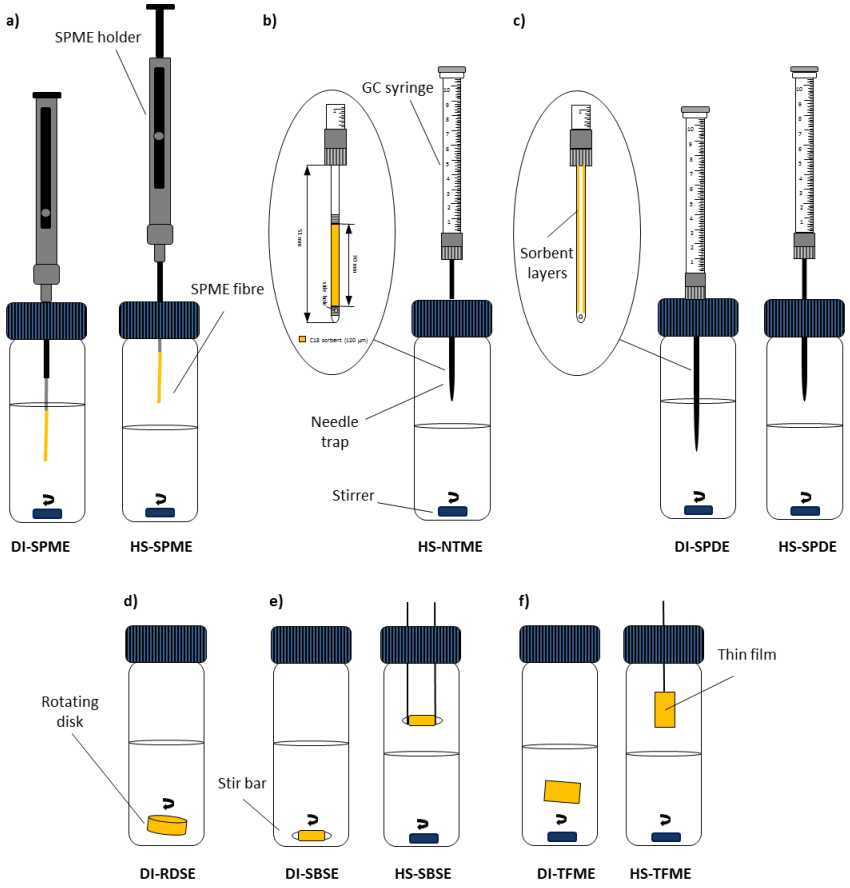

Figure 13. Different configurations of SPME: a) SPME fibre, b) NTME, c) SPDE, d) RDSE, e) SBSE and f) TFME.

Regardless of the SPME configuration used, the principle behind SPME is the diffusion of analytes from the aqueous matrix or HS above it to the extraction phase, for the purpose of reaching equilibrium between the phases. The extraction process generally follows the profile shown in Figure 14, which was firstly introduced by Ouyang and Pawliszyn [212] for understanding the kinetics of the SPME procedure. The extraction selectivity and efficiency of SPME mainly depend on the coating's properties and size as well as its interactions with the analytes. Recently, efforts have been made to obtain high extraction efficiency in SPME, including the use of porous coatings for SPME characterized by high distribution constants [213,214] or simply using a larger volume of extraction phase [215,216]. New extraction materials, such as sol-gel [217] and poly(pyrrole) [218-220] have also been utilized, due to their porous extraction phases and multifunctional properties. Higher extraction selectivity than commercial

SPME fibres has been achieved for some compounds. More selective microextraction coatings have also been developed based on artificial polymers, such as restricted access materials (RAMs) [210,221,222] and MIPs [210,223,224] for the applicability of SPME for complex samples (urine, blood, etc.).

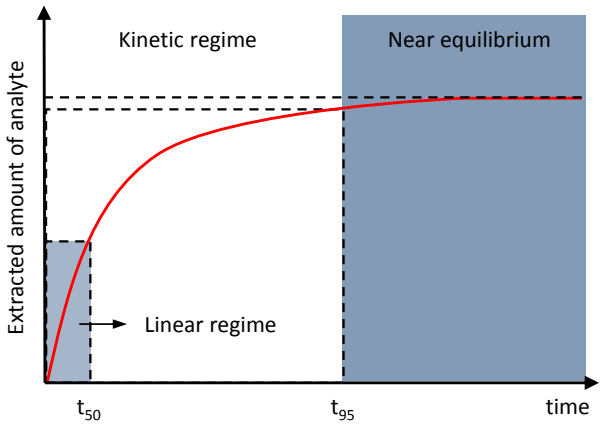

Figure 14. Extraction-time profile of SPME [212].

The extraction efficiency in SPME techniques can therefore be enhanced by increasing the distribution coefficient of the target analytes between the coating and sample matrix or increasing the volume of active surface area of the extraction phase. An increase in the coating thickness can increase the volume of the extraction phase and therefore improve the sensitivity of the method, as demonstrated by coated stir bar [215] and coated multifibre methods [216] or by other SPME methods using thick coatings [225]. However, longer equilibration time (t_e) is required because the extraction rate is controlled by the diffusion from the sample matrix through the boundary layer to the extraction phase, as illustrated by Equation 1 [226].

$$t_e=t_{95\%}=3\delta k_{es}(b-a)/D_s \quad \text{(Equation 1)}$$

where $(b-a)$ is the coating's thickness, D_s is the diffusion coefficient of the analytes in the sample matrix, and δ is the thickness of the boundary layer surrounding the extraction phase. In addition, the initial rate of SPME extraction is proportional to the surface area of the extraction phase [227] (Equation 2):

$$dn/dt=(D_sA/\delta)C_s \quad \text{(Equation 2)}$$

where *n* is the mass of analyte extracted over the sampling time *t* and *A* is the surface area of the extraction phase. So, in view of the fact that, in a typical widely used SPME fibre such as 100 µm polydimethylsiloxane (PDMS), the volume of extraction phase is approximately 0.5 µL, different configurations have been evaluated to increase the amount of extraction phase. SBSE with an extraction phase volume 50-250 times larger than that of SPME fibre is just one example. RDSE also immobilizes a larger amount of PDMS than in SBSE [228]. Compared with SBSE, RDSE displays better or similar recoveries [228,229], less equilibrium time [228] and similar reproducibility [229] under the same conditions for the extraction of different analytes. The main drawbacks of SBSE and RDSE are long extraction time and low portability. TFME increases the volume of the extraction phase, while the thickness of the coating remains constant or is even thinner than a commercial SPME fibre. In this regard, the thin-sheet PDMS membrane displays higher extraction efficiency for different analytes than a PDMS-coated fibre [230] and a PDMS stir bar [231]. Therefore, the ideal way to increase the volume of the extraction phase and, thus, the sensitivity of the method is to use a thin extraction phase with a large surface area. In other words, the extraction phase should have a large surface area-to-volume ratio. This results in enhanced sensitivity without sacrificing analysis time.

The main experimental parameters affecting the SPME process, namely extraction mode, sample volume, ionic strength, stirring rate, extraction temperature and extraction time, have to be optimized in order to achieve good sensitivity values. There are two different ways of optimization: the evaluation of the effect of each variable individually [150,232] or the use of a multifactorial experimental design [233-236]. For instance, Machado *et al.* [233] optimized a SPME fibre-based method to determine synthetic musk fragrances in aqueous matrices with a careful study of six different kinds of commercially available SPME fibres, including two metal alloy core fibres. The sample volume and stirring rate were fixed at 10 mL and 250 rpm, respectively. The extraction time (10-70 min), temperature (30-90 °C) and ionic strength (0-20%) were optimized through a multifactorial design with 5 levels for each factor. The final optimal extraction conditions were: polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 µm fibre exposed in the HS of 10 mL aqueous sample (20% NaCl) for 40 min at 70°C. Meanwhile, Ramírez *et al.* [64] used a multifactorial design of 2^3 with 2 levels for each factor for the optimization of a SBSE methodology to determine synthetic musk fragrances in water samples. Once the amount of organic phase (no addition), the volume of sample (100 mL) and the stirring rate (900 rpm) were fixed, the influence of other factors that

play an important role in the efficiency of the SBSE extraction were studied by multifactorial screening. These factors included ionic strength (0% and 20% of NaCl), extraction time (3 h to 12 h) and extraction temperature (25 °C and 60 °C). The best recovery values were obtained working with a PDMS stir bar (20 mm length x 0.5 µm film thickness, with 48 µL of PDMS coating), 100 mL of sample adjusted to pH 7, stirred at 900 rpm for 4 h at 25 °C.

Although the use of a multifactorial design for the study of the variables is the preferred option for SPME fibre and SBSE, in the case of other SPME configurations, such as TFME [231], RDSE [229], SPDE [237] and NTME [238], the study of each variable individually is preferred. For example, Giordano *et al.* [229] developed a method for the determination of pesticides in river water using RDSE as the extraction technique. The variables studied were the rotational velocity of the disk (0-1,250 rpm), extraction time (30 min-24 h) and the ionic strength (0-20% NaCl), as well as the desorption solvent (2-10 mL of methanol). The optimal variables for extraction of all analytes were: a PDMS disk of 1.5 cm Ø, extraction time of 3 h, sample volume of 25 mL, rotational velocity of the disk at 1,250 rpm and 2 mL of methanol as the desorption solvent (30 min). Apart from the variables optimized when working with static SPME configurations, the extraction flow rate and the desorption flow rate have to be taken into account when working with dynamic SPME configurations, such as SPDE and NTME. For this reason Jochmann *et al.* [237], when describing the optimal SPDE experimental conditions for the determination of volatile organic compounds (VOCs) in aqueous samples, specified the extraction flow rate (125 µL s^{-1}) and desorption flow rate (50 µL s^{-1}). The rest of the SPDE parameters were: 10 mL sample volume (30% NaCl) stirred at 500 rpm, extraction temperature of 50 °C and 125 mL of headspace vapours percolated via 50 strokes of 2.5 mL.

In terms of the applicability of SPME techniques for the determination of EOCs present in aqueous samples, SPME fibre and SBSE are the most widely used techniques to date. Applications for the determination of synthetic musk fragrances [150,232,233,236], phthalate esters [234], flame retardants [235,239], UV filters [150] and parabens [240,241] with SPME fibre have been published in recent years. In addition, SBSE applications include the determination of parabens [65], synthetic musk fragrances [55,64], UV filters [242], EDCs [243], insect repellent [244] and flame retardants [245]. Other organic compounds, such as PAHs [246,247], VOCs [248,249] and pesticides [250,251], have also been determined with SPME fibre and SBSE.

In the particular case of NM fragrances, Polo *et al.* [236] developed a SPME fibre-based method followed by GC-micro electron capture detection (μECD) for the determination of MX, MM, MK and MT in wastewater samples. With a polydimethylsiloxane-divinylbenzene (PDMS/DVB) 65 μm fibre and under the optimal extraction conditions (HS mode, 10 mL sample (stirred), extraction temperature of 100 °C, extraction time of 25 min and 270 °C for 2 min as desorption parameters), the developed method provided MDLs in the range of 0.25-1.05 ng L^{-1} and repeatability values of 2.4-11%. Furthermore, a 30 μm PDMS SPME fibre was selected by Liu *et al.* [150] for the determination of PCM fragrances in river water. Under the optimal conditions (3 mL sample volume, 10% NaCl, 90 min extraction time and 24 °C as extraction temperature) and with GC-MS as separation and detection techniques, the method achieved MDLs in the range of 0.2-9.6 ng L^{-1} and repeatability values lower than 10% in all cases. Heating the water sample increased the extraction efficiency of HS-SPME. However, conventional heating using an external source, e.g. water bath, is slow and poorly efficient. The use of MAE-HS-SPME, which has recently been developed as a simple, efficient and rapid extraction process for the determination of various semi-volatile compounds from water [252,253], was tested by Wang *et al.* [232] for the extraction of PCM fragrances present in wastewater samples. The authors poured 20 mL of water sample in a 40 mL sample vial containing 4 g of NaCl and a PDMS/DVB 65 μm SPME fibre was placed in the HS when the system was microwave irradiated at 180 W for less than 4 min. Afterwards, the SPME fibre was retracted and desorbed into the GC-MS instrument. The MDLs ranged from 0.05 to 0.1 ng L^{-1}, and the LOQ were less than 0.2 ng L^{-1}. Good repeatability values were also found with RSD percentages in the range of 1.2-6.9% (n=3, 10 ng L^{-1}). More recently, a 65 μm PDMS/DVB metal alloy fibre was successfully applied by Machado *et al.* [233] for the determination of HHCB, AHTN, MX and MK present in different kinds of water samples (groundwater, surface water, wastewater and drinking water). The final optimal extraction conditions were: HS extraction of 10 mL of sample volume (20% NaCl) at 70 °C for 40 min. The MDLs obtained after GC-MS/MS ranged from 2 ng L^{-1} to 4 ng L^{-1}, while the repeatability values were between 9.0% and 19.6 % (n=6, 100 ng L^{-1}).

PDMS stir bars have also successfully been applied by Arbulu *et al.* [55] and Ramírez *et al.* [64] for the determination of the most widespread synthetic musk fragrances present in wastewater and river water samples. In the case of Arbulu *et al.* [55], the authors include PCMs, NMs, MCMs and ACMs on the target analyte list. The optimal conditions were achieved when the PDMS stir bar (10 mm length and 0.5 mm film thickness) was introduced into a 30 mL water sample (stirred at 7,500 rpm) that contained 10% of NaCl for 4 h at 30 °C. Afterwards, the stir bar was thermally desorbed

into the GC-MS. Acquiring in full scan mode, the MQLs range between 5 ng L^{-1} and 80 ng L^{-1} and the precision was evaluated in terms of repeatability and reproducibility (n=5, 80 ng L^{-1}), with the values obtained ranging from 3.7% to 23.5% and 16.2 to 27.5% for intra- and inter-day studies, respectively. Meanwhile, Ramírez *et al.* [64] focused their study on PCM and NM fragrances and used a PDMS stir bar of 20 mm length and 0.5 mm film thickness. With 100 mL of sample volume (pH 7) stirred at 900 rpm, the best recovery values (81.6-94.6%) were obtained after 4 h of extraction at 25 °C. After TD, the target analytes were separated using GC and detected with MS in SIM mode. The developed method provided low MDLs of 0.02-0.30 ng L^{-1}, as well as good repeatability and reproducibility values (n=5, 100 ng L^{-1}) in the range of 2.2-8.2% and 2.3-6.9%, respectively.

The rest of the SPME configurations, including TFME, RDSE SPDE and NTME have successfully been applied for the determination of EDCs [254], pesticides [224,228,229], PAHs [230,231,238], anti-inflammatory drugs [255], parabens [256] and polar VOCs [237]. However, no information is available on the applicability of these microextraction techniques for the determination of synthetic musk fragrances present in water samples.

A comparison of the most commonly used extraction techniques for the determination of semi-volatile organic compounds shows that the perfect extraction technique does not exist. In Table 2, there is a summary of the advantages and disadvantages of the extraction techniques discussed above, as well as their main characteristics. To summarize, conventional extraction techniques, namely LLE and SPE, are versatile and provide high extraction recoveries. SPE also displays high sorptive capacity and high selectivity due to the great variety of SPE sorbents commercially available and the inclusion of MIPs on the market. However, the large volume of organic solvent used during the extraction and the laborious, time consuming methodologies are the principle disadvantages of these conventional extraction techniques. The microextraction techniques based on the miniaturization of LLE tend to reduce the extraction solvent from hundreds mL to hundreds µL for DLLME and HF-LPME and even to 1-3 µL for SDME. The sample volume and extraction time are also minimized. However, the main handicap of these techniques is the risk of water mixture or the instability of the organic solvent drop, causing repeatability problems. In the case of MEPS or on-line SPE, the decrease in terms of the sorbent amount and the elution volume used facilitate the automation of the overall process with good recovery values. Future efforts have to focus on resolving carry-over and blockage problems. Lastly, the disadvantages of SPME fibre have gradually been resolved or reduced by the

development of new configurations, such as SBSE, TFME, RDSE, etc., but the equilibrium time of these techniques is still long and, in most cases, automation is not possible.

Although there is no agreement among the scientific community about which is the best extraction technique, LLE has now practically been replaced by DLLME because of the comparable recoveries and MLDs obtained and the significant reduction in the sample and solvent volume, as well as extraction time. However, scientists still prefer SPE to on-line SPE or MEPS because of the great variety of commercially available sorbents, which allow better recovery values and validation parameters to be obtained. In terms of solventless techniques, SPME fibre is the most extensively used configuration due to the possibility of automating the whole process and the variety of commercially available fibres.

Table 2. Comparison of extraction techniques for the determination of semi-volatile organic compounds in aqueous samples.

Technique	Sample volume (mL)	Extraction solvent (mL)	Extraction time (min)	Multiple step	Advantages	Disadvantages	References
LLE	100-10,00	10-250	5-120	Yes	Versatility, high extraction recovery	Large volume of solvent, limited enrichments factors, time consuming	[133,134]
DLLME	5-10	0.05-0.25	≤1	Yes	Small volume of solvent, fast and easy, good extraction recovery	Risk of water mixture, limited precision and sensitivity	[27], [126], [150], [153]
SDME	8-20	0.001-0.003	5-25	One step adsorption/injection	Small volume of solvent, on-line, high enrichment factor	Instability of the solvent drop, limited precision and sensitivity	[136-138], [140]
HF-LPME	50-150	0.5-0.2	60-240	One step adsorption/injection	Small volume of solvent, high enrichment factor, good extraction recovery	Only for volatile compounds, limited precision	[148,149]
SPE	500-85,000	10-50	≥200	Yes	High sorptive capacity, chemical or physical mechanical stability, high selectivity	Large volume of solvent, time consuming	[30], [73], [121], [123]
ON-LINE SPE	2-15	0.02-0.1	2-7.5	Yes	Small volume of solvent, on-line, good extraction recovery, high selectivity, sensitivity	Instrumental requirements, injection, time consuming	[166], [167], [171], [172]
MEPS	0.8-5.5	0.025-0.05	1.5-9	Yes	High throughout, sensitivity, fast and easy, good extraction recovery	Blockage and carry-over desorption	[28], [130], [179], [180], [182]
SPME	3-20	TD*	4-90	One step adsorption/desorption	Solvent-free, on-line, suitable for volatile compounds	Losing and breakable fibre, desorption, low sensitivity	[127], [206], [207], [210]
SBSE	30-100	TD*	240	One step adsorption/desorption	High sensitivity, high extraction recovery, packed and coated sorbent	Coating loss, drying and desorption, on-line disability, long equilibrium time	[29], [42]
TFME	20-1,000	0.1 or TD*	30-120	Yes or One step adsorption/desorption	Large surface area, high sensitivity, high extraction recovery	on-line disability, low portability, long equilibrium time	[204], [205], [228]
RDSE	50-250	5-10	20-90	Yes	Coating protection, high sensitivity, high extraction recovery	on-line disability, low portability, long equilibrium time	[202], [203], [229], [230]
SPDE	10	TD*	15-30	One step adsorption/desorption	Solvent-free, on-line, suitable for volatile compounds	desorption, limited precision, pneumatic restrictions	[198], [211]
NTME	5	TD*	15	One step adsorption/desorption	Solvent-free, on-line, suitable for volatile compounds	desorption, limited precision, pneumatic restrictions	[212]

* TD= thermal desorption.

1.2.1.2. Extraction techniques for solid matrices

Extraction techniques for solid matrices are based on partitioning analytes between a solid sample matrix and a liquid phase, which is usually an organic solvent. Generally, some sample pretreatment is needed before the extraction process. This includes freezing, lyophilization and homogenization and, as such, the amount of analyte refers to sample dry weight (d.w.) [57,59,79,154]. However, other pretreatment techniques, such as centrifugation or filtration and the subsequent drying step with sodium sulphate to remove the water content of the sample, have also successfully been used by scientists [97,99]. Classical extraction techniques for the determination of EOCs including synthetic musk fragrances present in solid matrices include shaking [98,257,258] and Soxhlet [97,143,159,259] extraction. For example, Clara *et al.* [98] optimized a method for the determination of the most widespread PCMs, including HHCB, AHTN, ATII, DPMI, ADBI and AHMI, in sewage sludge samples from 14 WWTPs in Germany. The method consisted of a solid-liquid extraction by shaking 1 g (d.w.) of sludge with 20 mL of *n*-hexane for 150 min followed by 3 min in an ultrasonic bath. After repeating the process twice, *n*-hexane extracts were centrifuged and concentrated to 5 mL under a gentle stream of nitrogen, prior to cleaning up the extract with an aluminium oxide column. The eluate, 35 mL of *n*-hexane:ethyl acetate (90:10, v/v), was concentrated to a final volume of 1 mL and 1 µL was injected into the GC-MS. This method provided good recovery values ranging between 92% and 108% and MDLs of 5 to 25 ng g^{-1} (d.w.), while the highest LOQ was 50 ng g^{-1} (d.w.) for HHCB. Meanwhile, Reiner and co-workers [143] developed a method for the determination of HHCB, AHTN and HHCB-lactone in sediments from the Hudson River (New York, USA), with Soxhlet used as the extraction technique. After the dehydration of 40 g of sediment, the sample was subjected to Soxhlet extraction with 400 mL of a mixture of *n*-hexane and dichloromethane (1:3, v/v) as the extraction solvent for 16 h. Afterwards, the extract was concentrated to 1 mL with a rotary evaporator and 1 µL was then injected into the GC-MS. Recovery values between 85% and 98% were obtained, with MQLs of 5 ng g^{-1} (d.w.) for HHCB and AHTN and 15 ng g^{-1} (d.w.) for HHCB-lactone.

Although classical extraction techniques are still used in some analytical methods and provide good results, they are gradually being replaced by new extraction techniques which provide more efficient extraction and consume less solvent and time. USAE [59,154], supercritical fluid extraction (SFE) [260], MAE [155-157] and PLE [57,79] followed by a clean-up step, usually by SPE [59,79,154] or GPC [57,97,98], are the most extensively used extraction techniques when working with solid samples. However, in recent years, environmentally friendly extraction techniques such as HS-SPME and

HS-SBSE have successfully been applied for the determination of EOCs present in solid samples. QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction, a consolidated extraction methodology used in the field of food analysis [261] and gaining growing interest in the field of environmental analysis, was also successfully applied for the determination of EOCs in solid samples [262,263].

As an alternative to Soxhlet extraction, ultrasound energy has been widely used for the leaching of EOCs in solid matrices. The cavitation process produced by the ultrasound bath considerably reduces the extraction time required in Soxhlet, although it is less reproducible [264]. A wide range of analytes have been studied using USAE. For instance, the literature reports applications to pharmaceutical and EDCs [265,266], PAHs [267,268], PCBs [269,270], musk fragrances [59,154,271] and pharmaceutical and personal care products (PPCPs) [271].

Although similar solvents to those used in Soxhlet are employed in USAE, both the volume of the extraction and the extraction time are reduced to 4-60 mL and 10-30 min, respectively [272]. In most of the studies, the temperature of the ultrasound bath is not controlled. However, in the case of the determination of a group of EDCs including bisphenol A (BPA) [266], the extraction temperature was studied in the range of 40-70 °C range, with 50 °C being the optimal temperature. Based on the results, Samaras and co-workers [273] set the temperature at 50°C for the extraction of pharmaceuticals and phenolic EDCs. USAE methods applied for the determination of musk fragrances in solid samples mainly focus on the determination of AHTN and HHCB in sludge [59,154,271]. For instance, Ternes *et al.* [154] extracted the HHCB and AHTN present in 0.5 g (d.w.) of sludge by sonication for 5 min with 2 mL and 4 mL of methanol successively and then two times with acetone. The sample extracts were centrifuged and the supernatants combined and concentrated to 200 µL. Afterwards, the extracts were diluted to 150 mL with groundwater and subjected to a clean-up step with SPE, followed by a silica gel column clean-up before injection into the GC-MS. The developed method provided recovery values of 78±15% and 74±20% for AHTN and 87±10% and 64±12% for HHCB in activated sludge and digested sludge samples, respectively. The MQLs obtained with GC-MS were 250 ng g^{-1} (d.w.). Meanwhile, Zhou *et al.* [59] extracted the HHCB and AHTN present in sewage sludge using USAE according to the method developed by Ternes *et al.* [154] with certain modifications. Specifically, 0.02 g (d.w.) of sludge was extracted with 5 mL of methanol:water (5:3, v/v), and then successively extracted three times with 5 mL hexane:acetone (85:15, v/v). In each extraction step, the sample slurry was ultrasonicated for 10 min. The sample extract was filtered with a glass fibre filter prior to concentration to a final volume of 200 µL. After dilution with 500 mL of water, an SPE-based clean-up step was performed.

Recovery values of 74.8±10.6% for HHCB and 60.7±10.9% for AHTN were obtained. Meanwhile, the MQLs obtained, 670 ng g^{-1} for HHCB and 590 ng g^{-1} for AHTN, were significantly higher than those obtained by Ternes *et al.* [154].

In the recent years, focused ultrasound assisted liquid extraction (FUSALE) has been developed, which is based on the application of high-power focused ultrasonic waves using a microtip immersed directly into the extraction solvent [264]. The cavitation process of ultrasound probes is the same as in ultrasonic baths but the energy emitted by the probes is more reproducible and remains constant for a longer time, improving one of the main disadvantages of the classical ultrasound bath. Probe devices undoubtedly provide the most efficient method for transmitting ultrasonic energy in an analytical process or step [264]. To summarize, when ultrasound waves cross the liquid solvent, a large amount of tiny gas bubbles (cavitation bubbles) implode producing very high local temperatures, pressures and velocities of solvent microjets. Although only a few reports of applying FUSALE for the extraction of organic compounds in solid samples can be found in the recent literature [274-276], it can be described as a potential technique for the extraction of organic compounds including synthetic musk fragrances from sludge.

SFE has become an important technique in analytical sample preparation, as is apparent from the number of publications dedicated to the subject in recent years [260,277,278]. In particular, the field of environmental analysis has been the focus of the majority of SFE research. SFE has many advantages over conventional liquid extraction: (i) it requires far less time to achieve extraction and uses a fraction of the organic solvent, (ii) the solvent used, typically carbon dioxide (CO_2), is non-toxic and can have its solvation characteristics altered by changing either the pressure or temperature of the fluid. This leads to the ability of supercritical fluids to extract target analytes selectively from a mixture. Unlike conventional extraction methods, SFE requires no preconcentration step and offers the possibility of eliminating any clean-up stage often required using conventional extraction protocols prior to the detection [279,280].

The SFE extraction can be divided into three sequential steps. The first step consists of the initial partitioning of the analytes from the matrix active sites into the supercritical fluid. This is frequently the step that limits the rate (and ultimate recovery) of SFE for heterogeneous environmental matrices. The second step, chromatographic elution, depends on the amount of fluid flow versus sample size and conventional chromatographic partitioning between the extraction fluid 'mobile' phase and the

matrix [281]. Finally, the third step, collection, is the most instrument-specific step, and depends heavily on the restrictor system and trapping system used.

In practice, more than 90% of all analytical SFE is performed with CO_2 for several practical reasons. Apart from having relatively low critical pressure (74 bar) and temperature (32°C), CO_2 is non-poisonous, not flammable or explosive, chemically relatively inactive, available in high purity at relatively low cost, is easily removed from the extract, and creates non environmental problems when used for analytical purposes. In the supercritical state, CO_2 has a polarity comparable to liquid pentane and is, therefore, best suited for lipophilic compounds. For instance, Bielská *et al.* [278] determined the presence of PAHs in soil samples by SFE with CO_2 as the supercritical fluid followed by GC-MS. The SFE extraction was run as follows: extraction pressure of 30 MPa, extraction temperature of 50 °C, restrictor temperature of 120 °C and extraction time of 120 min. Supercritical CO_2 extraction, with methanol as the modifier, was also applied for the determination of ubiquinones and menaquinones in activated sludge. Four ubiquinones and 12 menaquinones species were identified based on the retention time and UV spectrum in 0.1 g (d.w.) sludge. The optimal extraction conditions were a pressure of 25 MPa, a temperature of 55 °C and 10% (v/v) methanol for 15 min. In addition, supercritical CO_2 extraction was used by Lee *et al.* [260] for the determination of synthetic musk fragrances in sludge samples (0.1 g (d.w.)) followed by GC-MS. Optimal SFE conditions were found to be a pressure of 35.8 MPa, a temperature of 80 °C and an extraction time of 20 min. The mean recoveries of the target synthetic musk fragrances were between 87% and 97% with RSD% better than 5%. The estimated MDLs were 30 ng g^{-1} (d.w.) for the target PCMs and 1 ng g^{-1} (d.w.) for the target NMs. The main drawback of CO_2 is its lack of polarity for the extraction of polar analytes.

The second most common choice of extraction fluid for analytical SFE is nitrous oxide (N_2O). This fluid is considered better suited for polar compounds because of its permanent dipole moment [282,283]. One of the applications where N_2O has shown significant improvements when compared to CO_2 is for extraction of polychlorinated dibenzodioxins from fly ash [284,285]. Unfortunately, this fluid has been shown to cause violent explosions when used for samples with high organic content and should therefore only be used when absolutely necessary [277]. Other less used supercritical fluids which have been used for environmental SFE are SF_6 and freons. SF6 is a very polar molecule and as a supercritical fluid, it has been shown to selectively extract aliphatic hydrocarbons up to around C-24 from a mixture containing both aliphatic and aromatic hydrocarbons [286]. Freons, especially $CHClF_2$ (Freon-22), have on several

occasions been shown to increase extraction efficiency compared to CO_2. For example, Hawthorne *et al.* [283] showed that $CHClF_2$ extracted PCBs from river sediments with higher efficiency than pure CO_2. Although supercritical H_2O has often been used for the destruction of hazardous organics [287], the high temperature and pressure needed (T>374 °C and P>221 bar), together with the corrosive nature of H_2O under these conditions, has limited the possible practical applications in environmental analysis. H_2O at subcritical conditions has, however, been shown to be an effective fluid for the extraction of several classes of environmental pollutants [288]. Hawthorne *et al.* [288] showed subcritical H_2O (250°C and 350 bar) to be capable of effectively extracting PAHs from soil and urban air particulates in 15 min.

The use of microwave energy in sample preparation first emerged in the early 1970s [289]. Specifically, the extraction of organic compounds by microwave irradiation was first reported in the work of Ganzler *et al.* [290] in 1986. Since then, the technique has attracted growing interest and has been widely used in analytical chemistry. In MAE, microwave energy is used to heat solvents in contact with solid samples or liquid samples and to promote partition of the analytes from the sample matrix into the solvent (the extractant). Thus, the principle of MAE is based on the direct effect of microwaves on molecules of the extraction system caused by two mechanisms, ion conduction and dipole rotation [291,292]. The ion conduction generates heat due to the resistance of medium to ion flow. Meanwhile, dipole rotation generates multiple collisions from the agitation of molecules and energy release, thereby increasing the temperature [293].

The technical application of microwave energy to the samples may be performed using either closed vessels (under controlled pressure and temperature), commonly known as pressurized MAE (PMAE), or open vessels (at atmospheric pressure), referred to as focused MAE (FMAE). Both systems are shown in Figure 15. Whereas, in open vessels, the temperature is limited by the boiling point of the solvent at atmospheric pressure, in closed vessels the temperature may be elevated by simply applying the adequate pressure. It should be noted that, unlike usual conventional forms of heating, microwaves heat the extracted system directly, leading to a very short extraction times.

Consequently, the effect of microwave energy is strongly dependent on the nature of both the solvent and the matrix. Other important parameters influencing MAE performance include solvent volume, microwave power, exposure time and temperature [291,292,295]. In MAE processes, analytes can be extracted into a single solvent or a mixture of solvents that absorb microwave energy strongly; into a

combined solvent containing both high and low dielectric losses mixed in various proportions; and into a microwave transparent solvent from a sample of high dielectric losses [291]. However, most MAE applications involve a mixture of non-polar solvent and water, including the humidity of biological matrices themselves. In contrast to conventional extraction techniques, in MAE, the use of high volumes of extraction solvent may lead to lower recoveries, probably due to inadequate mixing of the solvent with the matrix by the microwaves.

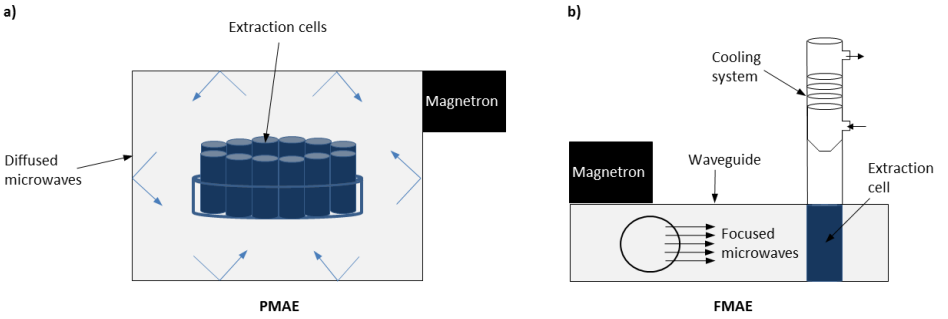

Figure 15. Illustration of devices for a) PMAE and b) FMAE. Redrawn from Sanchez-Prado *et al.* [294].

The selection of solvent volume depends on the type and the size of sample, but, on average, the amounts of solvent may be about 10-fold lower than those used in classical extractions, always taking into consideration that the solvent volume must be sufficient to ensure that the whole sample is immersed [292]. With respect to the nature of the matrix, water content is a key factor because of the high dipole moment of the water molecules, which leads to high efficiency in heating the sample. The inconvenience is the need to control the matrix water content to obtain reproducible results. The selection of microwave power and the corresponding irradiation time depends on the type of sample and solvent used. In theory, the use of high-power microwaves should allow the exposure time to be reduced. However, in some cases, very high-power microwaves decrease extraction efficiency by degrading the sample or rapidly boiling the solvent in open-vessel systems, which hinders contact with the sample. Generally, extraction times in MAE are much shorter than those in classical extraction techniques. Usually, increasing extraction times above the optimal range does not improve extraction efficiency, and, in some cases, may even decrease analyte recoveries (e.g. thermolabile compounds). In most cases, elevated temperatures result in improved extraction efficiency as a result of increased diffusivity of the solvent into the internal parts of the matrix, and the enhanced desorption of the component from the active sites of the matrix. In closed systems, pressure is also an important variable.

Because of its advantages, the use of microwave energy for the extraction of analytes in various matrices has become popular over the last 20 years or so. The main advantages of MAE from the point of view of green chemistry are a significant reduction of the solvent required, which reduces waste generation, shorter extraction times, and a smaller amount of sample required, with the corresponding reduction in energy input and cost [147,296]. One of the main advantages of using MAE is the reduction in extraction time when applying microwaves. This can mainly be attributed to the difference in heating performance involved in the microwave technique and conventional heating. In the case of conventional heating, a finite period of time is needed to heat the vessel before the heat is transferred to the solution, while microwaves heat the solution directly. This keeps the temperature gradient to a minimum and accelerates the speed of heating. Additionally, MAE allows for a significant reduction in organic solvent consumption as well as the possibility of running multiple samples simultaneously. These are, of course, minimal criteria for modern sample preparation techniques and are all fulfilled to a great extent by MAE. Consequently, MAE is an attractive alternative to conventional techniques [297].

Applications of MAE, at first, focused on the determination of PAHs [298,299] and polychlorinated biphenyls (PCBs) [300,301] from soils and sediments. Since then, numerous other compounds have been extracted efficiently, such as pesticides [302], phenols [303] and organometallic components [304]. In the last few years, flame retardants [305,306], surfactants [307,308], pharmaceuticals [309,310] and PCPs including triclosan [309,311,312] and musk fragrances [155-157,309], among others, have been the object of numerous environmental studies. In the particular case of musk fragrances, the presence of PCMs and NMs in sewage sludge samples of between 1 and 2.5 g (d.w.) was investigated by Svoboda *et al.* [313] and Smyth *et al.* [155] using MAE and a silica gel column for the clean-up of the extracts followed by GC-MS. The recovery values obtained ranged from 80% to 105% and the MDLs obtained were between 27 and 41 ng g^{-1} for the PCMs and 4 ng g^{-1} for NMs. Later on, Smyth *et al.* [156] reported the equivalence of two extraction methods: SFE and MAE in the analysis of PCMs and NMs in sludge samples. They found no significant differences between the SFE and MAE extraction methods. Nevertheless, the air-drying sample preparation step in SFE has the potential to cause degradation and/or volatilization of the PCMs and NMs. Moreover, MAE of centrifuged or filtered sludge resulted in recoveries that compare well with those reported in the literature. With respect to the application of MAE for the detection and quantification of musk compounds in soil and sediments, Rice and Siddhartha [309] developed a time and cost-effective MAE-based method for the simultaneous analysis of eight structurally diverse PPCPs, including MK, in these

matrices. The method consisted of optimizing the following variables: derivatization of the polar target analytes, silica gel open column clean-up, and GC-MS analysis of sample extracts for analysis and detection of the target compounds. The final multi-residue PPCP method was applied to both standard-amended soil samples and to natural sediment samples collected directly outside a WWTP effluent pipe. Good recovery values were achieved for MK (89.6±2.89%) in the case of both soil and sediment samples (3 g (d.w.)). In addition, Regueiro *et al.* [157] developed a high-throughput method for the determination of NMs (together with organochlorinated compounds and pyrethroid insecticides) in indoor dust. MAE was proposed as the extraction technique in this work in which several clean-up procedures were tested. An on-batch clean-up step, avoiding other more complex multi-step clean-up procedures reduced sample manipulation while increasing the throughput of the analysis. MDLs were at the low nanogram per gram level for most of the compounds. The proposed method was then applied to the analysis of real house dust samples, with NMs being found in most cases at concentrations between 14.94 and 2,303 ng g^{-1}(d.w.).

Likewise, the rapid acceptance of PLE as a US Environmental Protection Agency (EPA) method [314] in 1995 probably contributed to its swift acceptance as a relatively simple, fast, efficient, and reasonably green exhaustive extraction technique that essentially works equally well regardless of the analyte and matrix. In PLE, or what is commonly known as accelerated solvent extraction (ASE), the solid sample is typically dispersed in a drying or inert sorbent, such as sodium sulphate, hydromatrix or diatomaceous earth, and packed in a stainless steel cell (Figure 16). Once inserted into a closed flow-through system, pressure is applied to allow the use of extraction solvent or mixtures at temperatures higher than their normal boiling point. The increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. Moreover, the high temperature decreases the viscosity and the surface tension of the solvents, which helps to reach areas of the matrices more easily, thereby improving the extraction rate [315].

Many applications of PLE for the extraction of organic pollutants from sewage sludge can be found in the literature [272]. PLE has been applied to a wide variety of target analytes, since both polar and non-polar extraction solvents or mixtures can be used. For instance, methanol has been applied for the extraction of perfluorinated acids (PFAs), perfluorosulphonates (PFSs) and perfluoroctanesulphonamide (PFOs) [316], synthetic musk fragrances [154] and a group of PCPs including four UV filters, four preservatives and two antimicrobials [317], dichloromethane (DCM) in the case of BPA [274] and synthetic musk fragrances [318], toluene for PBDEs [319], PAHs [320] and

PBBs [321], and *n*-hexane for PAHs [322]. A wide variety of mixtures can also be found in the literature. Many of these combine water with other solvents, such as in the case of acetonitrile:water (7:3, v/v) for the analysis of polar organic contaminants [323], water:isopropyl alcohol (1:1, v/v) for non-polar organic compounds [323] methanol:water (1:1, v/v) for UV filters and derivatives [324], and methanol:water (1:2, v/v) for pharmaceuticals [325]. When water is used as the extraction solvent, the pH is also controlled in the case of analytes with acid-base properties, as in the case of the analysis of pharmaceuticals (methanol:water H_3PO_4 50 mM, 1:1, v/v) [326]. Strong acids (e.g. hydrochloric or nitric acid) must be avoided for the pH adjustment since they oxidize the steel components of the extraction cell [327]. Other solvent mixtures non containing water are found in the literature, such as DCM:*n*-hexane (1:1, v/v) for extracting PAHs [328] or musk fragrances [79], acetone:*n*-hexane (1:1, v/v) for musk fragrances [57], acetone:methanol (1:1, v/v) for alkylphenols [329], nonylphenols and nonylphenol carboxylates [330] and musk fragrances [331], as well as DCM:acetone (1:1, v/v) for chlorpyrifos and diazinon and their major metabolites [332].

Apart from the extraction solvent, the particular variables of PLE that are mostly studied include temperature, pressure, number of cycles and extraction time. Other parameters are the flush volume (volume of solvent flushed into the cell after the extraction) and the purge time (time that a stream of N_2 is passed through the cell to dry the sample). In the case of temperature, the range found in the literature is between 60 °C and 200 °C. The lowest temperature used was 70 °C for perfluorinated compounds [316] and 60 °C for musk fragrances [318]. Kinney *et al.* [323] used two extraction temperatures during the extraction of non-polar compounds. Firstly, in order to avoid thermal degradation as well as hydrolysis of certain analytes, extraction was carried out at 120 °C and, after that, the same extraction cell was extracted again with water: isopropyl alcohol (1:4, v/v) at 200 °C. Thermally stable compounds were collected in a vial containing 3 mL of pentane to minimize problems associated with collecting hot extracts.

In the case of extraction pressure, this is set in the 1,000-2,000 psi range, while static extraction time is usually within 1-15 min [79,316,318,319,328], although 5 min of extraction time is mostly used [79,154,320,322,324,325,329,332,333]. In the case of extraction cycles, or the number of times that fresh solvent gets into the cell and is in contact with the sample, the studied range is within 1-4 cycles [79,320,322,327], although 2 cycles are mostly used [57,154,316,318,320,322,324-327,329-333]. Currently, the combination of two extraction cycles of 5 min is very common [154,320,324,325,329,330,332,333].

PLE extraction is effective but sometimes displays low selectivity. Therefore, the extracts, rich in co-extracted materials, often have to be cleaned-up before instrumental analysis. For instance, Guo *et al.* [57] used a glass chromatography column, packed with 2 g of anhydrous sodium sulphate, 8 g of 5% deactivated silica gel and 2 g of anhydrous sodium sulphate for the purification of sewage sludge extracts obtained by PLE, prior to the determination of PCMs and NMs by GC-MS. Meanwhile, in a previous study, Ternes *et al.* [154] purified the sewage sludge extracts with a reverse phase (RP)-C18 SPE cartridge prior to the determination of the same compounds by GC-MS. To avoid these subsequent clean-up steps, some strategies for the PLE technique have been developed that have increased the selectivity and efficiency of PLE. These strategies are called in-cell clean-up and are summarized in Figure 16. In-cell clean-up also reduces process time by performing clean-up and extraction simultaneously and increases total sample preparation throughout, not just in terms of the number of samples extracted.

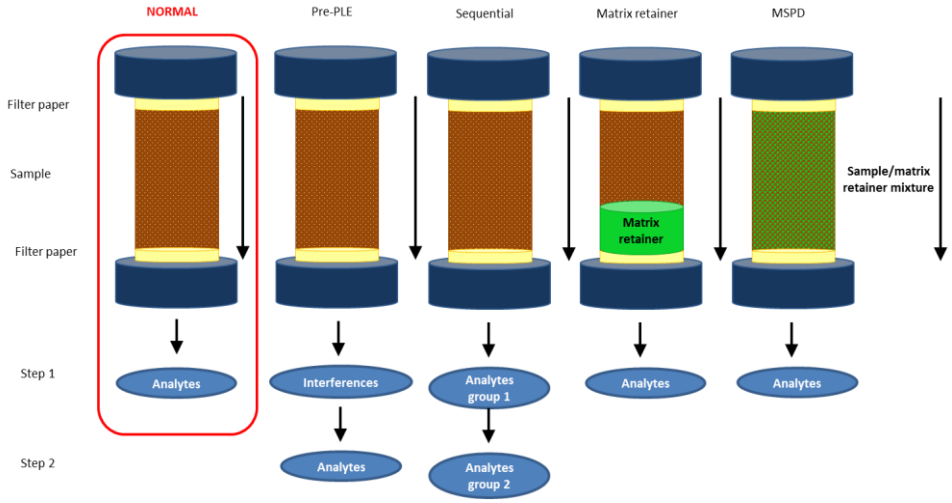

Figure 16. Extraction schemes for: normal PLE, pre-PLE, PLE with matrix retainer and matrix solid-phase dispersion (MSPD) PLE. Redrawn from Ramírez *et. al*. [334].

The first in-cell clean-up strategy is the pre-extraction of the interferences (pre-PLE) with a weak solvent to remove less strongly sorbed compounds before the extraction of the compounds of interest. The second strategy uses a sequential extraction procedure with solvents of increasing strength to separate the analytes of different polarity groups. Other strategies consist of the addition of an in-cell clean-up sorbent, which can retain the interferences and allows cleaner extracts, or the use of a matrix solid-phase dispersion (MSPD) technique in which the retainer sorbent is mixed with the sample

before the PLE extraction. The sorbents used in removing interferences from specific analyte groups include: carbon, copper, ion-exchange resins, C18 resins, silica gel, alumina and florisil. In Table 3, there is a list with the most suitable in-cell clean-up sorbents depending on the target analytes and the interferences that have to be removed from the extract. Some of these in-cell clean-up strategies have been used for determining EOCs in sewage sludge or solid samples. For instance, DiFrancesco *et al.* [318] dispersed between 7 and 10 g (w.w.) of sewage sludge with 2-4 g of diatomaceous earth loaded in a PLE cell (33 mL) containing 3 g of silica gel as a clean-up sorbent. During the extraction, which was performed with DCM at 60 °C and 2,000 psi (2 cycles of 15 min), lipid interferences were retained in the silica gel and the target fragrances were extracted efficiently. Meanwhile, Canosa *et al.* [335] dispersed 0.5 g of house dust with 3 g of florisil loaded in PLE cells (11 mL) containing 1 g of the same material as a clean-up sorbent. The non-polar interferences were firstly removed with *n*-hexane (40 °C and 500 psi) and then parabens were extracted with ethyl acetate at 100 °C and 2,000 psi in 3 static cycles of 1 min. Under the optimized conditions, recoveries of the target compounds ranged from 76% to 98%.

Table 3. Relation of the sorbents used for in-cell clean-up.

Sorbents	Interferences	Analyte group
Carbon	Organics	Non-polar compounds, dioxins
Copper	Elemental sulphur	Multi-residue pesticides
Ion-exchange resins	Organics, metals, and ionic interferences	Anions, cations, metal speciation (arsenic)
C18 resins	Organics, lipids, chlorophyll	Non-polar compounds
Silica gel	Lipids and oils	PCB and brominated flame retardants
Alumina	Lipids, chlorophyll, petroleum, waste	Amines, perchlorates, and PCB's
Florisil	Oils, lipids, and waxes	Pesticides and aromatics

The choice of the right extraction solvent and clean-up sorbent are the main parameters to optimize in these applications. For instance, Herrero *et al.* [336] tested two different ways of PLE in-cell clean-up for the analysis of polyether ionophores in sewage sludge: pre-PLE and MSPD. Several solvents were tested for pre-PLE, including DCM, ethyl acetate, *n*-hexane, methyl *tert*-butyl ether and isooctane. However, as a proportion of the polyether ionophores were extracted by these solvents, the recovery values decreased and, consequently, pre-PLE was ruled out. With respect to the MSPD assays with different sorbents (florisil, alumina and silica), florisil was the only one that provided enhancement of the recovery values and was the sorbent selected for the in-

cell clean-up. Under the optimal conditions, the extraction cell was filled with 1 g of diatomaceous earth, 1 g of florisil, 1 g (d.w.) sludge mixed with 1 g florisil and 1 g of diatomaceous earth. The extraction was carried out with 1 cycle of acetone at 80 °C and 1,500 psi for 10 min. Recoveries of the target polyether ionophores ranged between 82% and 95% with RSD% values always below 10%.

Water can also be used as an extraction solvent for PLE. This is called pressurized hot water extraction (PHWE) or subcritical water extraction (SWE), and it reduces or eliminates the use of the organic solvents. Temperature is the main factor that affects extraction efficiency during PHWE, since at elevated temperatures, high diffusions, low viscosities and low surface tensions are achieved. At high temperature and pressure, the dielectric constant of water decreases, reaching a polarity close to those of alcohols. This allows water to dissolve a wide range of medium and low polarity analytes [337]. In addition, the vapour pressure of the compounds increases at high temperatures and thermal desorption from the solid matrix occurs. However, degradation, hydrolysis and oxidation of the target compounds can also occur at high temperatures. Another variable studied during PHWE is the pH of the water phase when analytes with acid-base properties are studied, since the charged species are more soluble in the water phase and increased extraction efficiency can be obtained under those circumstances. PHWE is used in combination with other extraction techniques to concentrate and extract analytes from the aqueous solution prior to analysis, such as SPE, SPME and SBSE. For example, Llop *et al.* [338] developed a PHWE followed by the HS-SPME and GC-MS/MS method to determine N-nitrosamines in sewage sludge samples. The best peak areas results were obtained under the following PHWE conditions: ultrapure water at pH 7.5, extraction temperature of 125 °C, extraction pressure of 1,500 psi and 2 cycles of 5 min. Parabens present in house dust were also successfully extracted by PHWE followed by SBSE and GC-MS, as demonstrated Ramírez *et al.* [339]. The highest extraction efficiencies were obtained with ultrapure water at pH 7, extraction temperature of 80 °C, extraction pressure of 1,500 psi and 4 cycles of 5 min. Besides the reduction in the consumption of organic solvents, water is easily available, non-toxic and can be recycled or disposed with minimal environmental problems. Hence, PHWE is becoming an efficient and low cost technique of extraction for less polar organic analytes from solid matrices, such as sewage sludge [340], sediments [341,342] and house dust [339].

Furthermore, the direct application of solventless techniques, such as SPME and SBSE in the HS mode, have been investigated for the determination of EOCs in solid samples in order to avoid the pretreatment step and achieve simpler and faster extraction methodologies that are easier to automate. SPME and SBSE have successfully

been applied for the direct determination of organic contaminants, including synthetic musk fragrances [58,99], PCBs [343], chlorophenols [344] and EDCs [243], in sludge samples. For instance, Llompart *et al.* [58] developed an HS-SPME based method for the determination of PCMs and NM fragrances in sewage sludge samples. The optimal experimental conditions were achieved with a PDMS/DVB fibre exposed in the HS of 3 g of fresh sludge stirred at 600 rpm and 100 °C for 15 min. In addition, MA-HS-SPME followed by GC-MS was used by Wu and Ding [99] to determine PCMs in sewage sludge. The effects of the extraction parameters were systematically investigated (microwave irradiation power, extraction time, water amount added, pH value and addition of NaCl). The dewatered solid sample mixed with 20 mL of ultrapure water was efficiently extracted by a PDMS/DVB fibre placed in the HS when the extraction slurry was microwave irradiated at 80 W for 5 min. Meanwhile, DI-SBSE was applied for determining EDCs in biosolids and sludge samples. The DI-SBSE consisted of placing 1 g (w.w.) of sludge in a 20 mL headspace vial and adding 10 mL of ultrapure water and 0.5 g of sodium carbonate for pH adjustment. The DI-SBSE was performed at room temperature for 5 h, while stirring at 500 rpm.

Another extraction technique attracting increasing interest in recent years due to its simplicity is Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction, which has been used for extracting solid matrices. This extraction technique involves two basic steps: firstly, a single step buffered acetonitrile extraction and simultaneously salting out water from the aqueous sample using anhydrous magnesium sulphate to induce liquid-liquid partitioning and, secondly, a clean-up step using a dispersive solid-phase extraction (dSPE) in which a little amount of sorbent material (primary-secondary amine (PSA), C18 or graphitized carbon black) is dispersed in the organic extract obtained during extraction to clean up the mixture and remove undesired sample components. Both the European Standard Method EN 15662 [345] and the AOAC Official Method 2007.01 [346] are the most widely used QuEChERS methodologies (Figure 17). Thus, the main advantages of this technique are that sophisticated equipment is not required, there is a minimal solvent requirement and low cost when compared with instrumental extraction techniques.

This extraction technique has been widely used for the determination of pesticides in food matrices, mainly in fruits and vegetables [261,347-349], but other compounds can be extracted from other matrices using this technique. To induce solvent partitioning in QuEChERS extraction when environmental solid samples are analysed, small amounts of water are added, as these kind of samples are usually dry. However, its application to environmental matrices is still limited to very few papers regarding the determination of

pharmaceutical compounds [350,351] and the determination of benzothiazoles [352] in sewage sludge, and PAHs [353] and chlorinated compounds [354] from soil samples. Recently, a number of authors have used QuEChERS for the determination of UV filters and musk fragrances [263] and organochlorine pesticides [355] in mussels and shrimps, respectively. Núñez *et al.* [356] applied QuEChERS for the determination of pharmaceuticals in bivalves, such as lagoon cockles and coquina clams from different locations in Spain (Galicia and the River Ebro Delta) and also the Atlantic Coast of France, followed by LC-MS/MS. The developed method provided MDLs at ng g^{-1} (d.w.) levels and MQLs between 5 and 100 ng g^{-1}, while apparent recoveries ranged from 35% to 77%. Meanwhile, Trabalón *et al.* [80] used QuEChERS followed by GC-IT-MS/MS for the determination of synthetic musk fragrances, including PCMs and NMs, in 10 widely consumed fish and shellfish species from Tarragona (Catalonia, Spain). The method, which was validated for three different kind of matrices (hake, salmon and mussel), provided MDLs ranging between 1 and 10 ng g^{-1} (d.w.), MQLs from 5 to 30 ng g^{-1} (d.w.) and apparent recoveries between 47% and 109%. In the particular case of synthetic musk fragrances, QuEChERS has also successfully been applied for the determination of PCMs and NMS in PCPs such as deodorants, body washes, toilet soaps and toothpastes by Homem *et al.* [262]. The method was robust for the studied matrices and showed high precision (RSD% <15%) and accuracy (average recovery 85%), allowing the detection of synthetic musk fragrances at minimal concentrations between 0.01 ng g^{-1} for HHCB and 15.80 ng g^{-1} for MX.

1.2.2. Separation and determination techniques

As mentioned earlier, organic contaminants can be found at trace levels in environmental samples. Therefore, efficient and highly sensitive detection techniques that allow low MDLs are required for their reliable determination. Nowadays, LC and GC are the predominant techniques for the identification and quantification of organic contaminants, their metabolites and transformation products in the environment. GC is the preferred analytical technique for determining volatile and semi-volatile contaminants. However, for the determination of more polar and less volatile organic contaminants, a derivatization step is often needed prior to GC analysis. Hence, LC is the preferred technique for analysing these contaminants because of the good chromatographic signals and the high levels of sensitivity achieved. Both GC and LC can be used in combination with several detection techniques. MS is currently the most widely used because of its high sensitivity and selectivity. Other analytical techniques, such as capillary electrophoresis (CE), can also be found in the literature. Recent advances in CE coupled with MS detection make this hyphenated technique more

competitive in the analysis of environmental samples [357]. CE can also be applied for the enantiomeric separation of chiral organic contaminants. For instance, Martínez-Girón *et al.* [358] developed a CE method for the enantiomeric separation of PCMs HHCB, ATII, AHMI and AHTN, obtaining the complete separation of all the enantiomers, the 4 enantiomers of HHCB and the 2 enantiomers of AHTN, ATII and AHMI, respectively. In this section, there is a presentation of the most relevant features of the separation techniques used and MS detection for the determination of the compounds covered in this Thesis in environmental samples.

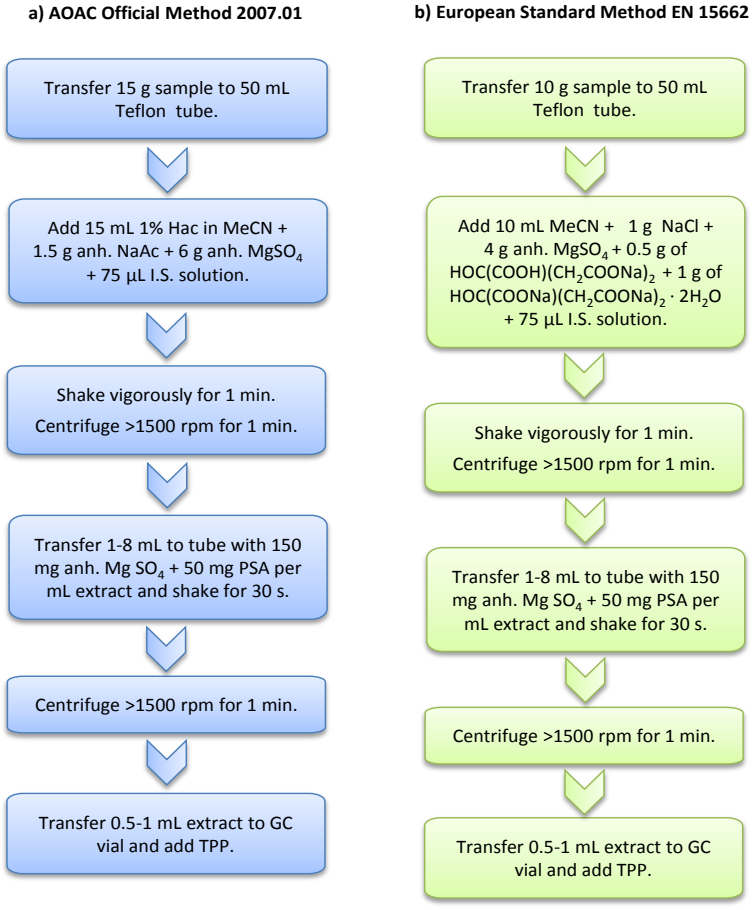

Figure 17. Schematic of a) AOAC Official Method 2007.07 and b) European standard Method EN 15662. MeCN= acetonitrile, HAc=acetic acid, anh. NaAC= anhydrous sodium acetate, anh. $MgSO_4$= anhydrous magnesium sulfate, NaCl=sodium chloride, $HOC(COOH)(CH_2COONa)_2$=sodium citrate dibasic sesquihydrate, and $HOC(COONa)(CH_2COONa)_2$= sodium citrate dehydrate.

HPLC with fluorescence and UV detection was first applied by Schüssler and Nitschke [359] for the determination of traces of HHCB in aqueous samples, with MDLs of 5 µg L^{-1} and 1.5 mg L^{-1} for fluorescence and UV detection, respectively. It should be noted that, in comparison to GC/MS, the HPLC method has only low separation efficiency and the typical HPLC detectors provide only moderate sensitivity and specificity for the synthetic musk fragrances. Nevertheless, some authors have used HPLC methods for bioaccumulation studies [360,361]. Recently, Herrera-López *et al.* [362] used LC coupled to hybrid quadrupole time-of-flight (QTOF)-MS to evaluate the degradation products of HHCB generated under oxidative and irradiation processes. The structure of 18 degradation compounds of HHCB have been clarified based on LC-QTOF-MS analysis, most of which has not previously been reported. In the same way, Lung and Liu [363] developed an ultra-high performance liquid chromatography-atmospheric pressure photoionization (UHPLC-APPI)-MS/MS based method that allowed the determination of six synthetic musk fragrances at concentrations lower than 6 pg with a linearity of 5-500 pg μl^{-1}.

GC-MS has been proven to be a versatile and widely used analysis technique for the determination of synthetic musk fragrances in environmental matrices because these compounds have high thermal stability and lipophilicity [82,114]. The electron impact (EI) mass spectra show several characteristic mass fragments [64,69,82] that can be used for identification and routine analysis. In addition, they are well suited as indicative mass ions in the selected ion monitoring (SIM) mode. Thus, GC/EI/MS in the SIM mode permits the screening of synthetic musk fragrances at a high level of specificity and sensitivity. This sensitivity can be increased by chemical ionization (CI) techniques only in the case of NM fragrances [158,364,365]. For the trace analysis of synthetic musk fragrances, various MS systems are used, such as quadrupole mass spectrometers (Q) [55,61,71], ion trap systems (IT) [68,69], high resolution mass spectrometers (HRMS) with time of flight (TOF) as an analyzer [362,366,367], and a tribrid system [368]. The GC-MS/MS technique with both IT and triple quadrupole (QqQ) as analyzers, which were successfully applied for the determination of NMs and PCMs, considerably reduced the background noise and, as a consequence, the analytical sensitivity and selectivity were increased remarkably [80,263,365]. Due to their chemical structure, PCM compounds, as well as MCMs and ACMs, cannot be detected with sufficient sensitivity by specific GC detectors, namely electron capture detectors (ECD) and nitrogen-phosphorous detectors (NPD), in contrast to the NM fragrances [158].

The simultaneous determination of PCMs and NMs in water matrices [54,369], sludge [59,370] and biota [61,80] is mainly performed using GC as the separation

technique and MS detection. A disadvantage of these methods may be the insensitive detection of NMs by EI-MS. In an investigation of Norwegian air samples focused on the determination of NMs and PCMs, GC/NCI/MS and GC/EI/MS methods were compared. NMs easily form negative ions resulting in high sensitivity of the GC/NCI/MS/SIM technique [371]. The analysis of PCMs by NCI/MS is not as sensitive as by EI/MS [364], but nevertheless, in most of the environmental samples, the concentration levels of PCMs are distinctly higher than those of NMs, compensating for this loss of sensitivity. PCMs and NMs were also determined simultaneously in mussel samples [263] using QuEChERS as the extraction technique and GC/HRMS as separation and detection techniques. Working with a QqQ analyzer, the developed method provided MDLs in the range of 0.5-50 ng g^{-1} (d.w.).

Due to their chemical structure, some PCM fragrances possess enantiomers and diastereoisomers. For instance, HHCB occurs in two diastereomeric forms [75], which could not be separated in the low polar 5% phenyl/95% dimethylpolysiloxane capillary columns commonly used in musk fragrances analysis (DB-5MS, ZB-5, etc.). However, a mid-polarity 50% phenyl/50% dimethylpolysiloxane was successfully applied by Ramírez *et al.* [64,65,67] for the enantioselective separation of the two enantiomeric forms of HHCB in both air and water samples. HHCB was also separated in these diastereoisomers in extracts of human adipose tissue [372] and in fish extracts [373] using a methylpolysiloxane phase with 12-15% phenyl groups and a polyethylene glycol phase, respectively. The ratio of the isomers seems to change in the extract of human samples in comparison to the standard substance [372]. Most recently, it was possible to separate the diastereoisomers of HHCB into the two pairs of enantiomers and also the two enantiomers of AHTN and ATII on chiral GC columns as heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin followed by GC/MS detection [374]. The same column was also used by Berset *et al.* [375] and Bester [160] for the study of the enantioselective transformation of the PCM fragrances HHCB, AHTN and the degradation product HHCB-lactone in wastewater, treated wastewater and sewage sludge. Wang *et al.* [376] evaluated four chiral columns, namely 20% hexakis-(2,3,6-per-*O*-methyl)-α-cyclodextrin, 20% hexakis-(2,3,6-per-*O*-methyl)-β-cyclodextrin, 20% hexakis-(2,3,6-per-*O*-methyl)-ϒ-cyclodextrin and 30% heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin, for the determination of PCM fragrances in different kinds of water samples (drinking water, wastewater, etc.). Of the columns tested, a 30% heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin column was shown to produce the optimal resolution of enantiomers for each PCM. This enantioselective chromatographic process, combined with MS/MS detection, provides a robust means of quantitative analysis for all of the target PCMs in environmental and wastewater

samples. MDLs between 1.01 ng L^{-1} and 2.39 ng L^{-1} were achieved for single enantiomers in the different water matrices. These chiral analyses seem to be helpful as a support tool for environmental research on bioaccumulation and the metabolism of PCM fragrances.

Meanwhile, in order to overcome the problems of separation and quantification of target musk fragrances that arise when working with complex environmental samples, as well as obtaining a good separation of the stereoisomers, one dimensional GC separation has been replaced by two dimensional GC (GCxGC) [362,366,367,377]. In this respect, Vetter and Bester [377] used GCxGC to obtain a good separation of AHTN and HHCB enantiomers. Specifically, in this study, the primary column was an enantioselective heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin) of 25 m length and 0.25 mm i.d., while a 1 m length DB-wax column with an inner diameter of 0.1 mm was used as a secondary dimension column with a TOF-MS as a detector. TOF-MS can give the resolution within the time needed for these high speed secondary column chromatograms. GCXGC-TOF-MS has also successfully been applied for the study of the degradation products of HHCB and AHTN by Santiago-Morales *et al.* [366] and Herrera-López *et al.* [362] and for the determination of a group of synthetic musk fragrances present in surface waters by Gómez *et al.* [367]. With SBSE as the extraction technique and a Rtx-5 (10 m x 0.18 mm i.d., 0.2 µm) as the primary column and a Rxi-17 (1 m x 0.1 mm i.d., 0.10 µm) as the secondary column, the GCxGC-TOF-MS methodology provided MDLs between 0.02 ng L^{-1} and 1.08 ng L^{-1} for the PCM fragrances studied and up to 2.54 ng L^{-1} for MX.

The introduction part of this Thesis ends with a review of the main analytical methods currently employed for the determination of synthetic musk fragrances in environmental samples. The review comprises instrumental aspects, and procedures for the extraction and clean-up. Special attention is paid to the replacement of conventional extraction techniques by microextraction techniques to obtain environmentally friendly methodologies. Degradation assays for the determination of the main transformation products of PCMs are also described. This review has already been published in TrAC Trends in Analytical Chemistry 72 (2015) 80-92.

1.2.3. Recent approaches for the determination of synthetic musk fragrances in environmental samples

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
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RECENT APPROACHES FOR THE DETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES

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Abstract

We review the main analytical methods currently employed for the determination of synthetic musk fragrances in air, aqueous and solid samples, such as sewage sludge or sediments, and biological samples. The review covers instrumental aspects, and procedures for extraction and clean-up. We pay special attention to current trends, such as the replacement of conventional extraction techniques (e.g., liquid-liquid extraction and solid-phase extraction) by microextraction techniques (e.g., solid-phase microextraction and microextraction by packed sorbents) in order to obtain environmentally friendly methodologies. We also discuss the applicability of comprehensive two-dimensional gas chromatography and mass spectrometry or high resolution mass spectrometry to enhance the separation of co-eluting compounds and to decrease the matrix effect. Further, we describe a number of degradation assays for the determination of the main transformation products of polycyclic musks fragrances.

Keywords: *air sample, biological sample, degradation, gas chromatography, mass spectrometry, microextraction, solid sample, synthetic musk fragrance, two-dimensional gas chromatography, water sample.*

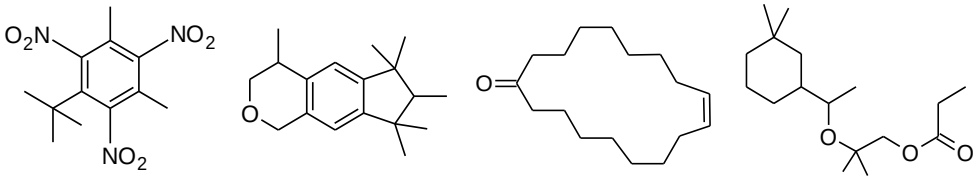
1. Introduction

Synthetic musk fragrances are a family of cyclic personal care products (PCPs) widely used as additives in a broad range of daily products such as cosmetics, flavourings, body oils, soaps, foods and drinks. These fragrances, which were synthesized to replace expensive natural musk fragrances, include a broad range of compounds that can be divided in four main groups according to their chemical structure: nitro, polycyclic, macrocyclic and alicyclic musk fragrances [1,2]. Fig. 1 shows the structures of a representative synthetic musk fragrance from each group.

Nitro musk (NM) fragrances are two-fold or three-fold nitrate benzene derivatives with additional alkyl, keto or methoxy groups. These musk fragrances were the first to be produced, but concerns about their toxicology soon arose because of the presence of a nitro aromatic compound in their structure. In this respect, European Directives 98/62/EEC [3] and 1223/2009/EEC [4], relating to cosmetic products, prohibit the use of musk ambrette (MA), musk moskene (MM) and tibetene (MT) in cosmetics and limits musk xylene (MX) and musk ketone (MK) content. Recently, the European Commission under the new chemical regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), considered MX a very persistent, very bioaccumulative substance, so decided to ban it as well [5]. This has led to a significant decrease in their use in recent decades due to their accumulation in environmental matrices and their potential carcinogenic effects [1]. Furthermore, NMs can be transformed in wastewater treatment plants (WWTPs), or in biota, into amino metabolites [6], which display higher toxicity and higher hormone disrupting potential [7,8].

Nowadays, polycyclic musk (PCM) fragrances are the most widely used. Compared with NMs, PCMs have better properties, such as a higher resistance to light and alkali [9]. The most representative PCMs are the commercially named galaxolide (HHCB) and tonalide (AHTN), which account for 95% of the commercially used PCMs [10]. For this reason, both compounds have been included on the EPA's high production list [11]. The use of AHTN in the cosmetic industry has in fact been regulated through European Directive 2008/42/EC [12].

In contrast, macrocyclic musk (MCMs) fragrances, which are 15- or 17-membered ring systems that can be found in nature or synthesized, are not as widely used as PCMs because of the cost of their synthesis. However, they are becoming more generally available because of advances made in synthesis methods in recent years [13,14]. We expect that, over the next few, years,



Musk xylene Galaxolide Civetone Helvetolide

Fig. 1. Representative structures of four synthetic musk fragrances: musk xylene (nitro musk), galaxolide (polycyclic musk), civetone (macrocyclic musk) and helvetolide (alicyclic musk).

the decreasing cost of synthesizing MCMs and their properties, such as stability with respect to light and alkalis, high fixation and quality odours and being easily degradable in the environment [15], will mean that they will replace PCMs on the market.

Alicyclic musk (ACMs) fragrances, which are considered the fourth generation of synthetic musk fragrances and are known as the linear musks (e.g., helvetolide, Fig. 1), are still used in PCPs to a very limited degree [16]. However, due to their biodegradable properties and low cost of manufacture compared to MCMs, ACMs are considered to be the future of synthetic musk fragrances.

On account of their widespread use, PCMs and NMs can be found everywhere in the world and, due to their lipophilic characteristics and slow biodegradation, they can accumulate in sediments [17], sludge [17,18], surface water [19,20] and fish species living in contaminated rivers and estuaries [21,22].

2. Instrumental analysis

2.1 . Chromatographic approaches

Gas chromatography (GC) is a versatile technique suitable for the determination of thermally-stable volatile and semi-volatile organic compounds, and is the preferred technique for determining synthetic musk fragrances, because these compounds have high thermal stability and lipophilicity [1]. One of the main advantages of GC is that it is compatible with sample preparation techniques that require subsequent liquid desorption or thermal desorption of the analytes, so it can be used in combination with a wide range of sample preparation techniques. In addition, all common injectors, including split/splitless (SSL), on-column (OC) and programmed temperature vaporizer (PTV), have successfully been applied for determining synthetic musk fragrances in environmental samples [17,23,24].

The most commonly used injection system is SSL, which allows the injection of 1-4 µL. However, the use of large volume injection techniques (LVI) through OC and PTV injectors is one of the strategies currently used to improve sensitivity. Nonetheless, due to the large volumes that can be injected in OC and PTV injectors (50 µL or more), they are only recommended when clean samples are used. Otherwise, the chromatographic system can easily become polluted and give unreliable results [1].

The separation of the synthetic musk fragrances is normally performed in low polar 5% phenyl/95% dimethylpoly-siloxane GC columns (e.g., DB-5MS, HP-5MS) [17,21,25,26]. However, as the separation of HHCB enantiomers/ diastereoisomers is not possible with these columns (see Fig. 2a), a mid-polarity 50% phenyl/50% dimethyl-polysiloxane column, such as ZB-50, has successfully been used for this purpose (see Fig. 2b) [27-29]. The columns used are usually 30 m in length, with a 0.25 mm internal diameter and 0.25 µm film thickness and the separation is performed in 20-45 min. Furthermore, as shown in Fig. 2c, the enantiomers/ diastereoisomers of the PCMs and their metabolites can be separated by enantioselective GC using a heptakis-(2,3-di-*O*-methyl-6-*O*-t- butyl-dimethyl-silyl)-β-cyclodextrin (25 m x 0.25 mm ID, 0.2 to 0.3µm film thickness) [10,30-33] column. In this case, the separation of the PCMs was extended up to 130 min.

However, the complexity of environmental matrices can cause several problems with the identification and quantification of target musk fragrances. The chromatographic peaks of the synthetic musk fragrances can sometimes co-elute with matrix components, so their separation by one dimensional GC is very difficult. To overcome this difficulty, GCxGC has been applied [34-36]. For example, Gómez *et al*. [36] developed a method based on GCxGC to determine a group of priority emerging organic contaminants in wastewater and river water, including 15 polycyclic aromatic hydrocarbons (PAHs), 27 pesticides and 13 PCPs; six of them were synthetic musk fragrances. Excellent results have been obtained in terms of separation efficiency as well as compound identification. Also, reliable confirmation of analyte identity was possible at low concentration levels (ng L^{-1}), even for typically troublesome compounds, such as PAHs [36]. As demonstrated by Herrera-López *et al*. [34] and Bester [31], both GCxGC and GC can be used for the study of HHCB and AHTN degradation products generated in degradation processes.

2.2. Detection

Although a flame ionization detector (FID) or electron capture detector (ECD) have been used for the determination of NMs [37], mass spectrometry (MS) is the most commonly used detection technique for determining synthetic musk fragrances. In GC analysis, the full scan mode of MS is employed in screening or as an untargeted approach to overcome the restrictions encountered with target analysis, though the reduction in acquisition

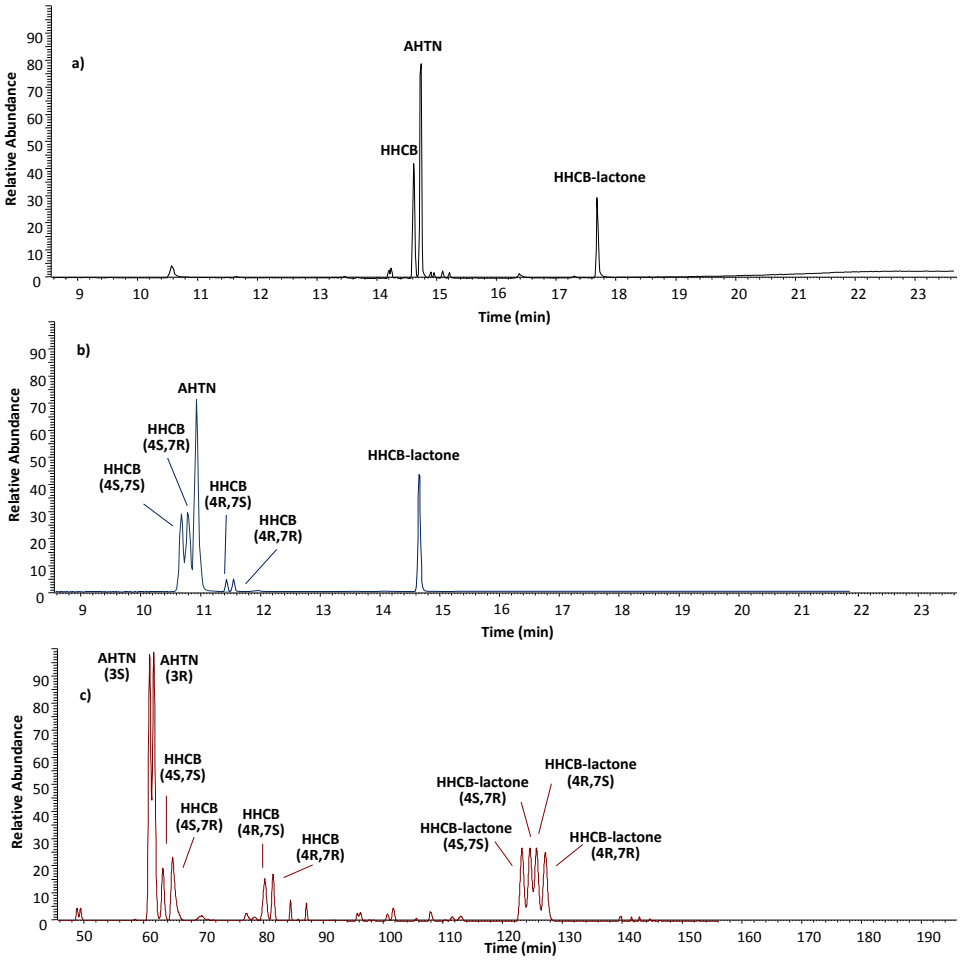


Fig. 2. Chromatographic separation of a mixture of musk fragrances containing galaxolide (HHCB), tonalide (AHTN) and galaxolidone (HHCB-lactone) by using: a) HP-5MS column (30 m x 0.25 mm ID, 0.25 µm film thickness), b) ZB-50 column (30 m x 0.25 mm ID, 0.25 µm film thickness) and c) heptakis -(2.3-di-O-methyl-6-O-t-butyldimethyl-silyl-β-cyclodextrin column (25 m x 0.25 mm id, 0.2-0.3 µm film thickness).

speed, poor response and interferences limit the suitability of such approach.

Whereas selective ion monitoring (SIM) and tandem MS (MS/MS) limit the number of compounds that can be analysed due to the need to select ions (SIM) or transitions (MS/MS) and, consequently they are usually applied to achieve high sensitivity for quantification in targeted analysis. PCMs, MCMs nor ACMs have no functional groups, so MS with electron ionization (EI) is routinely used for the detection of these fragrances [16,38-40]. However, GC coupled to chemical ionization (CI) MS is more sensitive for the NM fragrances and their amino metabolites [37]. For example, Herren *et al.* [41] found that negative (N) CI and quadrupole MS in SIM mode showed similar sensitivities compared to ion trap (IT) MS/MS in the case of NM fragrances and amino metabolites present in sewage sludge.

Quadrupole (Q) MS is so far the most widely used analyzer for the determination of synthetic musk fragrances. It has successfully been applied for determining synthetic musk fragrances in different kinds of samples, including air [38], wastewater [16], sewage sludge [41] or fish [40]. IT in MS [42,43] or tandem MS/MS [44,45] modes is the second most selected analyzer to determine PCMs, NMs and MCMs in different kinds of samples. For example, IT and MS/MS was the preferred option to determine PCMs and NMs fragrances present in complex and dirty samples due to its ability to achieve higher selectivity and sensitivity. The method developed by Llompart *et al.* [47] for the determination of NMs and PCMs fragrances in sewage sludge by SPME followed by GC-IT-MS as well as the method developed by Rüdel *et al.* [48] to determine PCMs and NMs fragrances in fish samples by PLE followed by GC-IT-MS/MS are two examples of the suitability of the IT. However, it is inadvisable to apply MS/MS when working with MCMs due to excessive fragmentation of the parent ions [28].

HRMS with a time-of-flight (TOF) analyzer was utilized by Gómez *et al.* [36] for the determination of PCMs and NMs in wastewater and river water. In addition, since TOF-MS includes acquiring full range, non-skewed, mass spectral information for all peaks, it is a powerful tool for the screening of non-targeted compounds. In this sense, Herrera *et al.* [34] and Santiago-Morales *et al.* [35] used TOF-MS for screening the degradation products of HHCB and AHTN generated under oxidative and irradiation processes. Fig. 3 show the enantiomeric separation of HHCB-lactone the degradation product of HHCB generated under oxidative and irradiation processes by Herrera *et al.* [34.]

3. Sample preparation

3.1. Air samples

Because of the high vapour pressure of most synthetic musk fragrances, an important route of exposure is their vaporization in air. For this reason, several analytical methods for determining synthetic musk fragrances in air were developed in recent years. Samples were typically collected by active sampling in high volumes (2-100 m^3) using polystyrene copolymer resins (XAD-2 resins) [38], poly-urethane foams (PUFs) [25,42,48] or combinations of both [50] (see Table 1). The samples were then extracted with organic solvents, such as ethyl acetate or dichloromethane by Soxhlet extraction, pressurized liquid extraction (PLE) or sonication. A clean-up step with chromatographic columns of de-activated silica, silica/alumina (2:1) or Florisil is commonly performed before the chromatographic analysis [48,49]. These methods are time-consuming and sample manipulation is required, meaning that there is a high risk of samples being contaminated by the presence of the synthetic musk fragrances in the environment. Moreover, these methodologies also involve the use of large amounts of organic solvents, which constitutes a pollution problem in. To overcome this, Regueiro *et al.* [43] and Ramírez *et al*. [27] recently developed two methods based on headspace (HS)-SPME and thermal desorption (TD), respectively. Specifically, in the HS-SPME method developed by

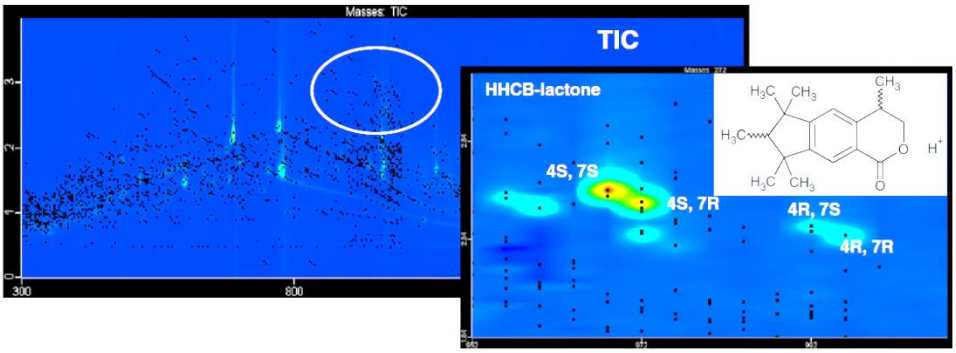


Fig. 3. GCxGC-TOFMS contour plot of a sample with ozone treatment that show the enantiomeric separation of the degradation product of HHCB, *trans*-HHCB-lactone (4S,7S and 4R, 7R) and *cis*-HHCB-lactone (4S,7R and 4R,7S), obtained with a Rtx-5 (10m x 0.18 mm i.d., 0.2 µm) as first column and Rxi-17 (1 m x 0.1 mm i.d., 0.10 µm) as second column.

Regueiro *et al.* [43], PCMs and NMs present in the air were adsorbed onto 25 mg of a porous polymer based on 2,6-diphenylene oxide (Tenax) by active sampling of 5 m^3 and then transferred to a SPME fibre in the HS mode and thermally desorbed in splitless mode in the GC. For sampling, Ramírez *et al*. [27] tested three kinds of desorption tube packaging: graphitized carbon (carbograph), Tenax, and a mixture of Tenax and carbograph. Tenax was shown to be the best desorption tube packaging for the analysis of PCMs and NMs present in the air. TD of the synthetic musk fragrances retained on the tubes was performed in a Unity Thermal Desorption system combined with an Ultra A autosampler. Both methods were rapid and simple, and provided enhanced sensitivity because the whole sample was analysed, and minimal manipulation of the sample was required. Nevertheless, as can be seen in Table 1, most of the methods developed for monitoring PCMs and NMs in air are still based on organic solvent extraction rather than solventless techniques such as HS-SPME or TD due to the high price of the SPME fibres or the need of specific equipment to work with TD, respectively.

Due to the widespread use of synthetic musk fragrances, their presence has been detected in air samples throughout the world. For example, PCMS and NMs were detected in urban and rural air [27,38] in Iowa and the Great Lakes (USA) at concentrations of up to 0.8 ng m^3 for HHCB, 0.33 ng m^3 for AHTN, and, in urban and suburban air samples from the area of Tarragona (NE Spain), at concentrations of 5.9 to 10.5 ng m^3 and 1.1 to 2.4 ng m^3 for HHCB and AHTN, respectively. They have also been found at minor concentrations in air samples from the North Sea (28 pg m^3 HHCB and 18 pg m^3 AHTN) and the Arctic (4 pg m^3 HHCB and 8 pg m^3 AHTN) [49].

However, most of the determinations of synthetic musk fragrances in air have focused on indoor air, where musk concentrations are much higher [25,27,42,43,48]. For example, Ramírez *et al.* [27] analysed the presence of PCMs and NMs in indoor air from different origins including a laboratory, a pharmacy, a flower shop, a classroom, a secretary's office, a medical centre and a hairdresser's. The results showed HHCB and AHTN as the most abundant PCMs with concentrations ranging between 47.1 ng m^{-3} for HHCB and 5.3 ng m^{-3} for AHTN found in pharmacy samples and 1256 ng m^{-3} for HHCB and 138.1 ng m^{-3} for AHTN found in samples from the hairdresser. The remaining PCMs were present at minor concentrations (between lower than limit of quantification (<LOQ) and 35.3 ng m^{-3}). In terms of NM

Table 1. Overview of methods for air samples.

Compounds	Samples	Sampling	Extraction	Clean-up	GC injector	GC separation column	Detector	References
PCMs	Outdoor samples	XAD-2 resin	Soxhlet	Pasteur Pipette with 0.75g	Splitless	HP-5MS	MS (quadrupole)	[38]*
NMs	urban and rural		Hexane:acetone	Florisil (100-200 mesh)		(30 m x 0.25 mm id,	EI	
			(50:50, v/v)	Ethyl acetate		0.25 µm film thickness)		
HHCB	Outdoor samples		Soxhlet	Silica gel column	Pulsed	HP-5MS	MS (quadrupole)	[49]*
AHTN	Northeast Atlantic and the Arctic	PUF/ XAD-2 resin	Hexane:diethyl eter	F2: Hexane:diethyl eter (3:1, v/v)	Splitless	(30 m x 0.25 mm id,	EI	
		column	(4:1, v/v)	F3: Hexane:diethyl eter (1:1, v/v)		0.25 µm film thickness)		
PCMs	Indoor samples	PUF	Sonication	Florisil column	PTV	HP-5MS	MS (quadrupole)	[25]*
NMs	sport centre and classroom		Acetone:hexane	Ethyl acetate		(30 m x 0.25 mm id,	EI	
			(50:50, v/v)			0.25 µm film thickness)		
PCMs	Indoor samples	PUF	Soxhlet	Silica:alumina column (2:1)	Splitless	HP-5MS	MS (quadrupole)	[48]*
	Cosmetic plant		DCM	DCM		(30 m x 0.25 mm id,	EI	
						0.25 µm film thickness)		

*MDLs not found in the literature.
Polyurethane foams= PUF.
Polyester copolymer= XAD-2.
Dichloromethane= DCM.

Table 1. (Cont.).

Compounds	Samples	Sampling	Extraction	GC injector	GC separation column	Detector	References
PCMs	Indoor samples	PUF	PLE	Splitless	HP-5MS	MS (ion trap)	[42]*
NMs	Apartments and kindergartens		Hexane:diethyl eter (95:5, v/v)		(30 m x 0.25 mm id, 0.25 µm film thickness)	EI	
PCMs	Indoor samples	TA sorbent	HS-SPME	Splitless	VF-5MS	MS (ion trap)	[43][a]
NMs	Homes		DVB/CAR/PDMS fibre		(30 m x 0.25 mm id, 0.25 µm film thickness)	EI	
PCMs	Outdoor samples	TA tubes	Thermal desorption		ZB-50	MS (quadrupo	[27][b]
NMs	urban and suburban		Tenax TA cryogenic trap		(30 m x 0.25 mm id, 0.25 µm film thickness)	EI	
	Indoor samples chemical laboraoty, secretary's office, medical centre, pharmacy, hairdresser's and flower shop						

*MDLs not found in the literature.

[a] MDLs: 0.029-0.380 ng m^{-3}.

fragrances, MM was not detected in any of the samples analysed, while MX was found in all of the samples, with concentrations between 2.9 ng m^{-3} at the flower shop and 766.5 ng m^{-3} at the hairdresser.

In summary, as shown in Table 1, all the literature has focused on the development of reliable analytical methods to determine PCMs and NMs in outdoor/indoor air samples or monitoring the presence of PCMs and NMs all over the world. However, due to a lack of reliable analytical methods for monitoring the presence of MCMs and ACMs, no information is yet available for these synthetic musk fragrances.

3.2. Water samples

Water samples comprise a wide range of environmental matrices with significant differences in terms of their complexity and composition. Wastewater usually contains large amount of surfactants or particulate matter that may interfere with the analysis of synthetic musk fragrances, and they are also where synthetic musk fragrances are found at higher concentrations. However, these compounds are also found in surface waters (rivers, lakes and sea water) and ta water. Regardless of the kind of water analysed (wastewater or surface water), due to the low concentrations at which synthetic musk fragrances are found, the use of a preconcentration technique is required. The methods reported included LLE using organic solvents, such as acetone or hexane, as extraction solvents [50], and SPE with polymeric reverse-phase sorbents, such as divinylbenzen/N-vinylpyrrolidone copolymer (Oasis HLB cartridge) [39,51] or polymerically bonded, octadecyl (17% C), endcapped (ENVI-18 cartridge) [52].

In all cases, any approaches based on LLE or SPE involved the use of large amounts of organic solvents (5-10 mL) and sample volume (wastewater: 0.1-1 L and surface water: 2-1,600 L), which resulted in an increase in analysis time, sample manipulation and cost of analysis [1]. To overcome this, microextraction techniques were recently developed to reduce or eliminate use of organic solvents during the preconcentration step and to obtain more environmentally friendly analytical methods [53,54].

Microextraction techniques based on LLE (e.g., dispersive liquid-liquid microextraction (DLLME), ultrasound-assisted emulsification-microextraction (USAEME) and single drop micro-extraction (SDME)) generally have problems of drop stability (SDME) and risk of water mixture (DLLME or USAEME) that resulted in worse repeatability values than those obtained with LLE. For this reason, use

of a dispersant agent [55] or a membrane [56] to avoid mixture is recommended.

In the particular case of SDME, the instability of the drop when an organic solvent is used as extractant limits the usable volume of the extracting medium and directly affects the precision and the sensitivity of the determinations. To solve the problem of drop volume repeatability, ionic liquids (ILs) [45] were proposed as alternative to organic solvents because of their low vapour pressure and high viscosity, which allow use of larger, more reproducible extracting volumes. The miniaturization version of LLE, known as DLLME, with chloroform as extraction solvent, and with or without methanol as a dispersant agent, was successfully applied to the determination of PCMs and NMs in wastewater and surface water [19,55]. The same solvents can also be used for the extraction of PCMs and NMs by USAEME [26,57]. In both cases, only 50-250 µL of organic solvent (chloroform) and 5-10 mL of water sample are required. Also, assays with membrane-assisted liquid-liquid extraction (MALLE), which involves inserting 500 µL of chloroform into a polyethylene bag, were carried out by Einsle *et al*. [56] for extracting HHCB and AHTN from 500 mL of river water or sewage. ILs were also tested by Vallecillos *et al*. [45] for the determination of PCMs and NMs using IL-HS-SDME. Specifically, a 2 µL drop of 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]) was used to extract the PCMs and the NMs present in 10 mL of wastewater influent/effluent sample diluted 1:2.

Other approaches applied to extract synthetic musk fragrances from aqueous samples include on-line solid-phase extraction (on-line SPE), as well as MEPS, which follow the same principles of SPE but on a miniaturized scale. The lower amount of sorbent used (20 mg for on-line SPE and 1-4 mg for MEPS) allows the reduction of sample volume from hundreds mL to just 1-10 mL while the amount of elution solvent needed to elute the target analytes decrease to µL and allowed the direct injection of the complete elution volume into the chromatograph. In addition, automation of the whole SPE process makes MEPS a promising alternative to SPE for the determination of organic compounds in a broad range of samples. To date, the lack of more specific sorbents commercially available compared with SPE is the main drawback of MEPS and the obstacle to using MEPS in routine analysis.

On-line SPE [23] with Oasis HLB as the extraction sorbent and 100 µL of ethyl acetate as elution solvent was applied for the determination of a group of synthetic musk fragrances, including the most representative PCMs, NMs and MCMs, and the degradation

product HHCB-lactone that were present in 10 mL wastewater samples with method detection limits (MDLs) of 1-30 ng L^{-1} and recovery values of 43-93%, depending of the target compound and the kind of water matrix analysed. A shown in Table 2, MEPS were successfully applied to the determination of synthetic musk fragrances in water samples [24,28,58]. For example, Moeder *et al*. [58] used a MEPS device with a barrel insert and needle (BIN) filled with 4 mg of octadecyl bonded silica (C18) sorbent followed by elution with ethyl acetate (2x50 µL) for extracting HHCB and AHTN from lake water and sewage (0.8 mL), with recovery values of 78%-109%. Meanwhile Cavalheiro *et al.* [24] used a C18 MEPS BIN to extract PCMs and NMs from estuarine water and sewage (5.5 mL) in a procedure different from that developed by Moeder *et al.* [58]. MCMs were also extracted from wastewater influent and effluent samples (4 mL) by using the MEPS procedure developed by Vallecillos *et al.* [28], with MDLs of 5-10 ng L^{-1} and recovery values of 52%-97%.
Moreover, other approaches have been evaluated for extracting PCMs, NMs, MCMs and ACMs in samples from river water and wastewater influents/ effluents, including the use of solvent - free microextraction techniques based on the partitioning of analytes between gaseous or liquid phase and stationary phase, such as SPME [59-61], stir bar sorptive extraction (SBSE) [16,29,36] and needle trap microextraction (NTME) [44] coupled to TD. Extractions by means of SPME were performed in HS [60,61] or direct immersion (DI) [59] mode with sample volumes of 10 mL and 3 mL, respectively. NT extractions were performed in only HS mode (10 mL sample volume poured in 20 mL vials). In DI mode, due to pneumatic restictions on the trap and the formation of bubbles, it is impossible ensure a constant flow rate of the sample inside the needle and the exact sample volume passing through the sorbent, obtaining non-reproducible results [44]. In contrast, SBSE extractions were all performed by DI of the stir bar in 100 mL of water samples in the case of surface waters or 100 mL of wastewater samples diluted 1:5 with ultrapure water [16,29,36]. As shown in Table 2, the use of solvent-free microextraction techniques became significant in recent years, partly because SPME and SBSE (see Table 2) are the microextraction techniques that provide the best MDLs. However, even for the wide variety of commercially available SPME fibres, the recovery values obtained were limited due to the sorbent amount. In SBSE, although the sorbent amount is 50-25 times greater than that in a SPME fibre, the recovery values are limited by the lack of commercially available sorbents. The determination of synthetic musk fragrances present in different kinds of

Table 2. Overview of methods for water samples.

Compounds	Samples	Extraction	GC injector	GC separation column	Detector	MDLs (ng L^{-1})	References
PCMs	Lake water	LLE	Splitless	HP-5MS	MS (quadrupole)	*	[50]
NMs		Acetone:hexane		(30 m x 0.25 mm id,	EI		
		Clean-up:		0.25 μm film thickness)			
		Silica gel column					
		Three sample fractions:					
		Hexane					
		DCM:hexane					
		MeOH					
HHCB	River water	SPE	Splitless	HP-5MS	MS (quadrupole)	*	[51]
AHTN		Oasis HLB		(30 m x 0.25 mm id,	EI		
		ACN:DCM		0.25 μm film thickness)			
		Evaporation					
		Isooctane					
HHCB	Influent/effluent wastewater	SPE	Splitless	HP-5MS	MS (quadrupole)	0.4	[39]
AHTN		Oasis HLB		(30 m x 0.25 mm id,	EI		
		Ethyl acetate		0.25 μm film thickness)			
PCMs	Effluent wastewater	SPE	Splitless	RTX-5MS	MS (quadrupole)	1-2.5	[52]
NMs	Tap water	ENVI-18		(30 m x 0.25 mm id,	EI		
	Groundwater	Hexane:DCM		0.25 μm film thickness)			
	Surface water						
NMs	Sea water	DLLME	Splitless	HP-5MS	MS (quadrupole)	4-33	[19]
	River water	Chloroform		(30 m x 0.25 mm id,	EI		
	Water from a irrigation channel			0.25 μm film thickness)			
	Influent/effluent wastewater						

Acetonitrile=ACN.
Dichloromethane=DCM.
Methanol= MeOH.

Table 2. (Cont.).

Compounds	Samples	Extraction	GC injector	GC separation column	Detector	MDLs ($ng\,L^{-1}$)	References
PCMs	Sea water	DLLME	Splitless	DB-5MS	MS (quadrupole)	28-63	[55]
	River water	MeOH (disperser solvent)		(30 m x 0.25 mm id,	EI		
	Lake water	Chloroform (extraction solvent)		0.25 μm film thickness)			
PCMs	Effluent wastewater	USAEME	Splitless	DB-5MS	MS (quadrupole)	0.2	[26]
	Surface water	Isopropyl alcohol (disperser solvent)		(30 m x 0.25 mm id,	EI		
		Chloroform		0.25 μm film thickness)			
PCMs	Harbour sea water	USAEME	Splitless	VF-5MS	MS (ion trap)	70-2730	[57]
NMs	River water	Chloroform		(30 m x 0.25 mm id,	EI		
	Tap water			0.25 μm film thickness)			
	Bottled water						
	Swimming pool water						
	Influent/effluent wastewater						
HHCB	River water	MALLE	Splitless	HP-5MS	MS (quadrupole)	20	[56]
AHTN	Influent/effluent wastewater	Polyethylene membrane		(30 m x 0.25 mm id,	EI		
		Chloroform		0.25 μm film thickness)			
PCMs	Influent/effluent wastewater	HS-SDME	Splitless	Retention gap	MS/MS (ion trap)	10-24	[45]
NMs		[OMIM][PF_6] (ionic liquid)		(3 m x 0.25 mm id)	EI		
				ZB-50			
				(30 m x 0.25 mm id,			
				0.25 μm film thickness)			

Methanol= MeOH.

Table 2. (Cont.).

Compounds	Samples	Extraction	GC injector	GC separation column	Detector	MDLs ($ng\ L^{-1}$)	References
PCMs	Influent/effluent wastewater	On-line SPE	On-column	Retention gap	MS (quadrupole)	1-30	[23]
NMs		Oasis HLB		(5 m x 0.53 mm id)	EI		
MCMs		Ethyl acetate		Retaining precolumn			
				(2 m x 0.25 mm id,)			
				0.25 μm film thickness)			
				ZB-50			
				(30 m x 0.25 mm id,			
				0.25 μm film thickness)			
HHCB	Lake water	MEPS	PTV	HP-5MS	MS (quadrupole)	37-42	[58]
AHTN	Influent/effluent wastewater	C18		(30 m x 0.25 mm id,	EI		
		Ethyl acetate		0.25 μm film thickness)			
PCMs	Estuarine water	MEPS	PTV	HP-5MS	MS (quadrupole)	5-84	[24]
NMs	Influent/effluent wastewater	C18		(30 m x 0.25 mm id,	EI		
		Ethyl acetate:hexane		0.25 μm film thickness)			
		(50:50, v/v)					
MCMs	Influent/effluent wastewater	MEPS	LVI	ZB-50	MS (ion trap)	5-10	[28]
		C18		(30 m x 0.25 mm id,	EI		
		Ethyl acetate		0.25 μm film thickness)			
PCMs	River water	DI-SPME	Splitless	*	MS (quadrupole)	0.4-9.6	[59]
		PDMS fibre			EI		
		TD					
MCMs	Influent/effluent wastewater	HS-SPME	Splitless	ZB-50	MS (ion trap)	0.75-5	[60]
		PDMS/DVB fibre		(30 m x 0.25 mm id,	EI		
		TD		0.25 μm film thickness)			

*Information not found in the literature.
Polydimethylsiloxane=PDMS.
Divinylbenzene=DVB.

Table 2. (Cont.)

Compounds	Samples	Extraction	GC injector	GC separation column	Detector	MDLs ($ng\,L^{-1}$)	References
HHCB	Influent/effluent wastewater	HS-SPME	Splitless	TG-5SILMS	MS (ion trap)	40	[61]
AHTN		PDMS/DVB fibre		(30 m x 0.25 mm id,	EI		
		TD		0.25 μm film thickness)			
PCMs	Superficial water	SBSE	Splitless	HP-5MS	MS (quadrupole)	5-80	[16]
NMs	Ground water	PDMS		(30 m x 0.25 mm id,	EI		
MCMs	Influent/effluent wastewater	TD		0.20 μm film thickness)			
ACMs							
PCMs	Influent/effluent wastewater	SBSE	Splitless	ZB-50	MS (quadrupole)	0.02-30	[29]
NMs	River water	PDMS		(30 m x 0.25 mm id,	EI		
		TD		0.25 μm film thickness)			
PCMs	Effluent wastewater	SBSE		GC x GC	MS (TOF)	0.02-2.54	[36]
NMs	River water	PDMS	PTV	RTX-5MS			
		TD		(10 m x 0.18 mm id,			
				0.20 μm film thickness)			
				RXI-17MS			
				(1 m x 0.1 mm id,			
				0.10 μm film thickness)			
PCMs	Influent/effluent wastewater	NTME	Splitless	ZB-50	MS/MS (ion trap)	2.5-12	[44]
NMs		HF Bondesil-C18		(30 m x 0.25 mm id,	EI		
		TD		0.25 μm film thickness)			

Polydimethylsiloxane=PDMS.
Divinylbenzene=DVB.

water, such as surface water, including river, lake and sea waters, influent/effluent wastewater, and tap water, confirms that, just as in air samples, the most representative PCMs found in water samples are HHCB and AHTN, with maximum concentrations reported of 818-45,091 ng L^{-1} for HHCB and 82-49,904 ng L^{-1} for AHTN for influent wastewater from Tarragona [23]. In comparison, the concentrations of HHCB and AHTN found in effluent samples decrease to values from 769 ng L^{-1} to 9,000 ng L^{-1} and 391 ng L^{-1} to 7,555 ng L^{-1}, respectively [23].

The presence of HHCB and AHTN was also reported in surface water at lower concentrations of 1-4.7 ng L^{-1} in Lake Michigan (USA) [50] and 13-314 ng L^{-1} in the Somes River (Romania) [51]. NM fragrances such as MX and MK were also found in influent/effluent water samples from Tarragona and Shanghai (China) at concentrations of 8.4-4,110 ng L^{-1} [23,52] and in surface water from Lake Michigan and a campus lake in Shanghai at levels ranging from 0.049-3.5 ng L^{-1} [50,52].

In contrast to PCMs and NMs, only a few studies have focused on the determination of MCMs and ACMs [16,23,28,60]. The concentrations reported by Vallecillos *et al.* [23,28,60] and Arbulu *et al*. [16] showed MCM concentrations of 10-9,740 ng L^{-1} in influent/effluent wastewater samples from Tarragona and Alava (Northern Spain). Also, Arbulu *et al*. [16] reported ACMs concentrations of romandolide and helvetolide of 45-56 ng L^{-1} and 21-70 ng L^{-1} respectively for influent/ effluent samples from Alava. The presence of MCMs and ACMs in surface waters such as lake or river water has not yet been reported.

3.3. Solid samples

Although most research papers focus on the determination of synthetic musk fragrances in aqueous samples, due to the lipophilic properties and slow biodegradation of synthetic musk fragrances, they can be found in environmental solid samples, such as soils, sewage sludge and sediments. Prior to analysis, solid samples are usually dehydrated or lyophilized using a freeze-dry system and crushed and sieved in order to obtain homogeneous particles in the pretreatment step [18,39,62-66]. However, some authors prefer to use centrifugation [67] or filtration [17] and a subsequent drying step with sodium sulphate to remove the water content of the sample. In any case, the concentrations of the target compounds are expressed as the amount of analyte per amount of dry weight (d.w.).

As shown in the analytical methods described in Table 3, different extraction techniques for solid samples have been applied. Soxhlet extraction [67], ultrasound assisted extraction (USAE) [39,62], PLE [63] and

conventional techniques, such as shaking followed by sonication [68] have been applied to the determination of PCMs and NMs found in sewage sludge. PLE has also successfully been applied for the determination of HHCB and AHTN present in soils [64]. However, due to the lack of selectivity of the extraction techniques mentioned above, extracts from sewage sludge or soils have to be subjected to different clean-up steps, usually by SPE [39,62,64] or gel permeation chromatography (GPC) [63,67,68], at the costs of increasing analysis time and solvent consumption. Recoveries of 71-85% and MDLs of 3-10 ng g^{-1} (d.w.) have been reported by Guo *et al.* [63], depending on the synthetic musk fragrance analysed, for the analysis of 1 g (d.w.) of sewage sludge using PLE as the extraction technique and a clean-up step based on GPC (200 µL final volume) followed by GC-MS.

Wang *et al.* [64] working with PLE followed by SPE with an Oasis HLB cartridge (100 µL final volume) and GC-MS, achieved significantly better MDLs of 0.06-0.09 ng g^{-1} (d.w.) and comparable recovery values of 70-132% for the analysis of HHCB and AHTN in 10 g (d.w.) of soil sample.

Recently, Vallecillos *et al.* [18] applied an in-cell clean-up PLE involving the mixture of 1g (d.w.) of sewage sludge with 1 g of Florisil, instead of an inert material, into the PLE cell to perform the extraction and clean-up in one step, followed by IL-HS-SDME (10 mL extract volume) and GC-IT-MS/MS. The results showed comparable MDLs (1-3 ng g^{-1} (d.w.)) and PLE recoveries (63-100%). As such, in-cell clean-up is an attractive alternative to the laborious standard SPE clean-up step, offering reduced sample manipulation and analysis time.

Furthermore, environmentally friendly microextraction techniques such as HS-SPME [17,46,65], HS-SBSE [66] and IL-HS-SDME [18], have successfully been applied for the determination of synthetic musk fragrances in sewage sludge and sediments. As can be seen in Table 3, the direct extraction of the synthetic musk fragrances present in sewage sludge by HS-SPME with a PDMS/DVB fibre followed by GC-IT-MS provides the best results regardless of the kind of musk fragrances analysed, with MDLs 0.04 ng g^{-1} (d.w.) and 0.015 ng g^{-1} (d.w.) for PCMs and sample amounts of 5 g (d.w.) [17] and 0.16 g (d.w.) [17,46], respectively, 0.31-0.61 ng g^{-1} (d.w.) for NMs (0.16 g (d.w.) of sample amount) [46] and 0.005-0.025 ng g^{-1} (d.w.) for MCMs (0.20 g (d.w.) of sample) [65]. The application of HS-SPME directly to the sewage sludge sample without any treatment results in a promising alternative, reducing sample manipulation and treatment time.

In contrast, working with HS-SBSE (0.1 g (d.w.) of sewage sludge) [66] or IL-HS-SDME (1 g (d.w.) of sewage sludge) [18] as the microextraction technique,

followed by TD-GC-Q-MS and GC-IT-MS/MS, respectively, higher MDLs of 1-30 ng g^{-1} (d.w.) were found. For example, as mentioned at Section 3.2, the lack of more specific sorbents commercially available is a possible reason for the increase in the MDLs obtained working with SBSE. In this particular case [66], the application of a high split flow of 15 mL min^{-1} during the TD to avoid trap contamination when working with complex matrix may compromise the sensitivity of the method. Working with IL-HS-SDME followed by GC-IT-MS/MS [18], the high boiling point of the IL and the need to dilute the PLE extract 1:2 may compromise the sensitivity of the method.

Table 3 lets us to conclude that most of the developed methods focused on the determination of PCMs fragrances in solid samples because of the high concentrations of these fragrances reported in solid samples, i.e.:

- 3.4-9,240 ng g^{-1} (d.w.) for HHCB and 5.7-5,040 ng g^{-1} (d.w.) for AHTN in sewage sludge from WWTPs in Tarragona [18,66] and Galicia (NW Spain) [46];
- 1.33-2.55 ng g^{-1} (d.w.) for HHCB and 1.91-3.92 ng g^{-1} (d.w.) for AHTN in soils from Beijing parks (China) [64]; and
- 0.2-4.0 ng g^{-1} (d.w.) for HHCB and 0.1-3.0 ng g^{-1} (d.w.) for AHTN in Shanghai sediments [17].

NM and MCM concentrations were reported in only sewage sludge samples. Concentrations of NMs of 12-1319 ng g^{-1} (d.w.) were reported in studies from Tarragona [18,66], Galicia [46] and Korea [63]. The presence of MCMs has was studied in only sewage sludge from the Tarragona region [65,67] with concentrations of 0.08-1.45 ng g^{-1} (d.w.).

However, as with air and water samples, there is a lack of information about the presence of ACMs fragrances in solid samples. To date, ACMs studies have focused on the synthesis and identification of these compounds, rather than their determination in environmental samples in general and, specifically, in solid samples.

3.4 Biological samples

On account of the widespread use of PCMs and NMs in the cosmetic industry, they are ubiquitous throughout the world and their presence in water and solid samples has been widely reported (Tables 2 and 3). Faced with the possibility of PCMs and NMs reaching the food chain through fish species living in contaminated rivers and estuaries, many analytical methods have been developed to determine synthetic musk fragrances in fish tissue. As MCMs and ACMs are not as widely used as PCM and NMs, their presence in fish samples

Table 3. Overview of methods for sewage sludge samples.

Compounds	Samples	Extraction	Clean-up	GC separation column[a)]	Detector	MDLs ($ng\ g^{-1}$ (d.w.))	References
PCMs	Sludge from urban WWTP	Soxhlet	Silica-alumina column	Retention gap	MS (quadrupole)	*	[67]
NMs		DCM	DCM:pentane (1:1, v/v)	(2 m x 0.25 mm id)	EI		
		Evaporated	Evaporated	HP-5MS			
		Redissolved with: Hexane	Redissolved in Hexane	(30 m x 0.25 mm id, 0.25 μm film thickness)			
HHCB	Sludge from urban WWTP	USAE	SPE	HP-5MS	MS (quadrupole)	HHCB: 200	[39]
AHTN		ultrapure water:MeOH, (3:5, v/v)	Oasis HLB	(30 m x 0.25 mm id,	EI	AHTN: 180	
		Hexane:acetone, (85:15, v/v), triplicate	Ethyl acetate	0.25 μm film thickness)			
HHCB	Sludge from urban WWTP	USAE	SPE	XTI-5	MS (quadrupole)	*	[62]
AHTN	including:	MeOH (three times)	RP-C18	(30 m x 0.25 mm id,	EI		
	Activate sludges	Acetone (twice)	MeOH	0.25 μm film thickness)			
	Anaerobically digested sludges		Silica gel column				
		PLE	*n*-Hexane:acetone,				
		MeOH (three times)	(85:15, v/v)				
PCMs	Sludge from urban WWTP	PLE	Silica gel column	DB-5MS	MS (quadrupole)	3-10	[63]
NMs	Sludge from industrial WWTP	Acetone:hexane, (1:1, v/v)	Acetone:hexane (5.95, v/v)	(30 m x 0.25 mm id,	EI		
	Sludge from livestock WWTP	Evaporated	Evaporated	0.25 μm film thickness)			
		Redissolved with: Hexane	Redissolved in DCM				

[a)] All the authors work with a splitless GC injector.
*MDLs not found in the literature.
Dichloromethane=DCM.
Methanol= MeOH.

Table 3. (Cont.).

Compounds	Samples	Extraction	Clean-up	GC separation column[a)]	Detector	MDLs ($ng\ g^{-1}$ (d.w.))	References
HHCB AHTN	Soil from two public parks	PLE *n*-Hexane:DCM	SPE Oasis HLB Ethyl acetate	HP-5MS (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (quadrupole) EI	HHCB: 0.06 AHTN: 0.09	[64]
PCMs	Sludge from urban WWTP	Shaking + Sonication Ethanol Shaking + Sonication (twice) *n*-Hexane Centrifugation	Aluminum oxide column *n*-Hexane:ethyl acetate (90:10, v/v) Evaporated	DB-5MS (60 m x 0.25 mm id, 0.25 µm film thickness)	MS (quadrupole) EI	5-25	[68]
PCMs NMs	Sludge from urban WWTP	HS-SPME PDMS/DVB fibre TD		VA-5MS (25 m x 0.25 mm id, 0.25 µm film thickness)	MS (ion trap) EI	0.028-0.448	[46]
MCMs	Sludge from urban WWTP	HS-SPME PDMS/DVB fibre TD		ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (ion trap) EI	0.005-0.025	[65]

a) All the authors work with a splitless GC injector.
Dichloromethane=DCM.
Polydimethylsiloxane/Divinylbenzene=PDMS/DVB.

Table 3. (Cont.).

Compounds	Samples	Extraction	Clean-up	GC separation column[a]	Detector	MDLs ($ng\,g^{-1}$ (d.w.))	References
PCMs	Sludge from urban WWTP	MA-HS-SPME		DB-5MS	MS (quadrupole)	0.04-0.1	[17]
	Sediments:	PDMS/DVB fibre		(30 m x 0.25 mm id,	EI		
	Effluent of a women's dormitory	Microwave irradiation		0.25 μm film thickness)			
	Effluent of a cosmetic manufacturer	(80 W, 5 min)					
		TD					
PCMs	Sludge from urban WWTP	HS-SBSE		ZB-50	MS (quadrupole)	5-30	[66]
NMs	Sludge from a DWTP	PDMS stir bar		(30 m x 0.25 mm id,	EI		
MCMs		TD		0.25 μm film thickness)			
PCMs	Sludge from urban WWTP	PLE	In-cell clean-up	Retention gap	MS/MS (ion trap)	1-3	[18]
NMs	Sludge from a DWTP	Ultrapure water:MeOH (1:1, v/v)	Florisil	(3 m x 0.25 mm id)	EI		
				ZB-50			
		HS-SDME		(30 m x 0.25 mm id,			
		[OMIM][PF_6] (ionic liquid)		0.25 μm film thickness)			

[a] All the authors work with a splitless GC injector.
Polydimethylsiloxane/Divinylbenzene=PDMS/DVB.
Methanol= MeOH.

has not yet been studied. As shown in Table 4, most authors used Soxhlet, focused ultrasound assisted liquid extraction (FUSALE) and PLE as extraction techniques, usually followed by a clean-up step to remove all of the lipid content prior to analysis by GC-MS [40,69-71] or GC-MS/MS [22,47,70,72]. However, in contrast of with the analysis of water or solid samples, microextraction techniques have not yet been reported to decrease sample manipulation, analysis time and organic solvent consumption.

The clean-up step mainly involves GPC, a silica gel column, an aluminium oxide column or in-cell clean-up sorbent. However, due to the high lipid content present in this kind of samples, the combination of two or more clean-up steps is sometimes required to decrease the matrix effect as much as possible and to reach low MDLs. For example, Subedi *et al.* [22] proposed a PLE method which used dichloromethane (DCM):ethyl acetate (1:1, v/v) as the extraction solvent and silica as the in-cell clean-up sorbent in combination with a second clean-up step based on GPC with DCM as the mobile phase followed by GC-IT-MS/MS to determine PCMs and NMs present in fish samples. The recoveries obtained with this procedure were 56-76% with MDLs of 1.6-4 ng g^{-1} wet weight (w.w.) and 35-38 ng g^{-1} (w.w.) for PCMs and NMs, respectively. Also, Rüdel *et al.* [47], proposed a PLE method with n-hexane as extraction solvent and two successive clean-up steps consisting of GPC (cyclohexane:DCM (1:1, v/v) as the mobile phase) followed by a silica gel column with cyclohexane:DCM (7:3, v/v) as the elution solvent. The method quantification limits (MQLs) of this methodology were 0.1-0.5 ng g^{-1} (w.w.), while the recovery values were 83-110% for all of the PCMs and NMs studied, except for MX, for which the recovery value was 135%.

QuEChERS, an extraction methodology that has been the subject of growing interest in the field of food analysis over recent years, was recently applied by Vallecillos *et al.* [72] for the determination of synthetic musk fragrances (PCMs and NMs) in fish and mussel samples. To perform the two-phase partitioning, they added 10 mL of ultrapure water to 0.5 g of freeze-dried fish or mussel sample before performing acetonitrile (ACN) extraction and salting out using anhydrous magnesium sulphate to induce liquid-liquid partitioning. The ACN layer was then subjected to a dispersive SPE (dSPE) with 1 g of Florisil and analysed by GC-MS/MS. The recoveries reported ranged from 24-110%, although high matrix effect values (-70% to 16%) were recorded, with MDLs of 0.25-10 ng g^{-1} (d.w.). The authors also compared the QuEChERS procedure with a PLE method that used DCM as the extraction solvent and Florisil as the in-cell clean-up sorbent

[72]. The results showed insignificant differences in terms of method validation and analysis time, with PLE providing the lowest matrix effect (-58% to 5%). Although better results were obtained with PLE, QuEChERS could be used to determine musk fragrances in fish and mussel sample in the absence of PLE equipment.

The PCMs HHCB and AHTN have been detected in eelpout muscles from the North Sea and Baltic Sea [47] at concentrations of 10-164 ng g^{-1} lipid weight (l.w.) and 7-69 ng g^{-1} (l.w.), respectively, in samples containing a lipid content of between 1.4-2.9%. They have also been found at higher concentrations (14-9,750 ng g^{-1} (l.w.) for HHCB and 14-1,460 ng g^{-1} (l.w.) for AHTN, 0.4%-8% lipid content) in bream mussels from Lake Belau and the rivers Danube and Rhine in Germany [47].

Recently, Vallecillos *et al.* [72] reported HHCB and AHTN concentrations of 2.97-8.94 ng g^{-1} (d.w.) and 1.17-5.65 ng g^{-1} (d.w.), respectively, in fish samples, including red mullet, gilt head bream, turbot and mussels from the Mediterranean Sea (Tarragona).

Higher concentrations of HHCB (12.68-18.04 ng g^{-1} (d.w.)) and AHTN (1.38-8.42 ng g^{-1}(d.w.)) were found in fish from the Ebro River (Tarragona), such as perch, sheatfish and carp.

NMs fragrances MX and MK have been detected in fish samples taking from lakes in Switzerland [40] at concentrations of 1.3 ng g^{-1}(l.w.) and 12 ng g^{-1} (l.w.), respectively, in samples containing a lipid content of 1.26-2.93%.

The presence of MX (0.11-0.34 ng g^{-1} (w.w.)) and MK (0.14-0.60 ng g^{-1} (w.w.)) has also been confirmed in mussel samples from the North Sea and Baltic Sea [47].

4. Degradation studies

The first assays on degradation of synthetic musk fragrances date back to 1998-2000 and focused on the photo-degradation of NMs and the study of their amino metabolites [41,73-75]. The reaction kinetics of the photochemical degradation of the NMs compounds MX, MT, MK, MA and MM, and different transformation pathways, were evaluated by Butte *et al.* [75]. However, as the transformation products of NMs have proved to be even more toxic than the parent compounds [73,76], their consumption has decreased significantly, with the consequent increase in the consumption of PCMs.

Over recent years, there was a particular focus on the study of the degradation products of PCMs, mainly HHCB and AHTN. To this end, HHCB and AHTN were subjected to oxidative treatments with ozone (O_3) [34,35], photochemical processes under UV irradiation [34,35] and visible light irradiation [35], and biodegradation assays performed with fungi derived

Table 4. Overview of methods for biological samples.

Compounds	Samples	Extraction	Clean-up	GC injector	GC separation column	Detector	MDLs ($ng\ g^{-1}$ (d.w.))	References
PCMs	River:	PLE	In-cell clean-up	Splitless	HP-5MS	MS/MS (ion trap)	1.6-38	[22]
NMs	Tilapia	DCM:ethyl acetate	Silica		(30 m x 0.25 mm id,	EI		
	Small mouth bass	(1:1, v/v)	GPC		0.25 μm film thickness)			
	Bream		DCM					
			Derivatization					
			MSTFA					
PCMs	Lake:	PLE	GPC	Splitless	DB-Dioxin	MS (quadrupole)	*	[40]
NMs	Brown trout	*n*-Hexane	Cyclohexane:ethyl acetate		(60 m x 0.25 mm id,	EI		
	Brook trout		(50:50, v/v)		0.15 μm film thickness)			
	Alpine char							
	Lake trout							
PCMs	Sea:	PLE	GPC	Splitless	RTX-50	MS/MS (ion trap)	*	[47]
NMs	Bladder wrack	*n*-Hexane	Cyclohexane:DCM, (1:1, v/v)		(30 m x 0.25 mm id,	EI		
	Eel pout		Silica gel column		0.1 μm film thickness)			
	Blue mussels		Cyclohexane:DCM, (7:3, v/v)					
	Herring gull egg							
	River:							
	Zebra mussel							
	Bream							

*Information not found in the literature.
Dichloromethane=DCM.
N-methyl-N-(trimethylsilyl)trifluoroacetamide=MSTFA.

Table 4. (Cont.).

Compounds	Samples	Extraction	Clean-up	GC injector	GC separation column	Detector	MDLs ($ng\,g^{-1}$ (d.w.))	References
PCMs	River:	FUSLE	Silica gel column	Splitless	XTI-5	MS (ion trap)	4-17	[69]
NMs	Bluegill	Acetone	Hexane:Acetone		(30 m x 0.25 mm id,	EI		
	Sonora sucker	Centrifugation	(65:35, v/v)		0.25 μm film thickness)			
		Evapoation	Derivatization		VF-5MS	MS/MS (ion trap)	12-397	
		Hexane:acetone	MSTFA		(30 m x 0.25 mm id,	EI		
		(65:35, v/v)			0.25 μm film thickness)			
PCMs	Sea:	Soxhlet	GPC	Splitless	DB-5MS	MS (quadrupole)	*	[70]
	Atlantic salmon	DCM:hexane, (3:1, v/v)	DCM:hexane, (1:1, v/v)		(30 m x 0.25 mm id,	EI		
	Smallmouth bass		Silica gel cartridge		0.25 μm film thickness)			
PCMs	Sea:	Soxhlet	ACN partitioning	*	*	MS (quadrupole)	0.2-1.3	[71]
NMs	Chinese sturgeon	DCM:methanol, (7:3, v/v)	Hexane			EI		
		Evaporation	Aluminum oxide column					
		Hexane	Hexane:DCM, (3:1, v/v)					
			Evaporation					
			Hexane					
PCMs	Sea:	PLE	In-cell clean-up	LVI	ZB-50	MS/MS (ion trap)	0.25-10	[72]
NMs	Gilt head bream	DCM	Florisil		(30 m x 0.25 mm id,	EI		
	Turbot				0.25 μm film thickness)			
	Red mullet	QuEChERS	Dispersive SPE					
	Mussels	ACN	Florisil					
	River:							
	Perch							
	Sheatfish							
	Carp							

*Information not found in the literature.
Dichloromethane=DCM.
Acetonitrile=ACM.
N-methyl-N-(trimethylsilyl)trifluoroacetamide=MSTFA.

from freshwater environments or commercial enzymes [32,77]. For example, Herrera-López *et al.* [34] studied the transformation products obtained when HHCB was subjected to O_3 and irradiation (UV and visible light). The results showed O_3 as the most efficient degradation technique with over 98% removal after 2 min. However, UV and visible light irradiation can be used for the degradation of HHCB with depletion over 80% after 2 min. In addition, they were also able to identify 18 polar degradation compounds of HHCB using LC/ESI-QTOF-MS by means of mass accuracy and GCxGC-EI-TOF-MS for the identification based on the enhanced separation capacity and screening of unknowns through the acquisition of full-range mass spectra.

In the same way, Santiago-Morales *et al.* [35] evaluated the applicability of different degradation treatments, including O_3 with and without the addition of hydrogen peroxide (O_3, O_3/H_2O_2), UV irradiation and xenon-arc visible light irradiation alone and in combination with O_3 (UV, O_3/UV, Xe, O_3/Xe) and visible light photocatalytic oxidation using Ce-doped titanium dioxide photocatalyst performed under continuous oxygen or O_3 gas bubbling (O_2/Xe/Ce-TiO_2, O_3/Xe/Ce-TiO_2). Independently of the degradation procedure used, AHTN was shown to be more easily removed than HHCB. The best results for the HHCB, more than 75% of removal after 5 min, were obtained for O_3 and O_3/Xe/Ce-TiO_2. Significant removal of both compounds, approximately 60% after 15 min, was also obtained with O_2/Xe/Ce-TiO_2. However, UV was able to deplete more than 90% of AHTN after 15 min but reduced the concentration of HHCB to about half of its initial concentration.

Using a different approach, Martin *et al.* [77] demonstrated the feasibility of two mitosporic fungi derived from freshwater environments, *Myrioconium* sp. Strain UHH 1-13-18-4 and *Clavariopsis aquatic*a, for the biodegradation of AHTN and HHCB via extracellular oxidoreductases, such as laccases. After six days of incubation, the laccases generated by *Myrioconium* sp. cultures were able to remove 30% of HHCB and only 12% of AHTN. In *Clavariopsis aquatica* cultures, the concentration of AHTN remained constant over time and 10% of HHCB was removed. Identification of oxidative coupling degradation products potentially formed upon HHCB and AHTN oxidation by the laccases was impossible with GC-MS covering a range of 50-500 m/z. The application of these enzymes, are regarded to be environmentally friendly and they are relative low-cost. They are considered an attractive alternative in the development of an effective technology for the wastewater treatment. However, the removal percentages obtained by HHCB

with O_3 and UV and visible light irradiation are much higher than those obtained working with fungi as biodegradation agents and the degradation time needed increased from 2 min for O_3 to 6 days for *Myrioconium* sp. cultures.

5. Conclusions

Although the presence of PCMs and NMs in environmental samples has been reported in many research papers, several studies focused on developing more sensitive, more selective analytical methods for their determination. Moreover, the interest in MCMs rose in recent years, as can be seen from the growing number of publications that focus on the determination of MCMs in environmental samples. Information about the presence of ACMs in environmental samples is very limited. There was only one study that determined the presence of ACMs in water samples.

In environmental water and solid samples, there is a current trend towards the development of environmentally friendly methodlogies based on microextraction techniques, thus allowing a decrease in organic solvent consumption and automation of the full analytical method. However, the methods developed for the determination of synthetic musk fragrances in fish samples are still based on PLE or Soxhlet extraction followed by time-consuming clean-up steps.

Regarding instrumental analysis, GC-MS with a Q as the analyzer is the most widely used technique for determining synthetic musk fragrances in environmental samples. HRMS (TOF), IT-MS or MS/MS can also be used when working with complex matrices. GCxGC has been shown to be a powerful tool in screening of transformation products.

The degradation of PCMs by O_3 or UV or visible light irradiation was studied in depth over recent years. However, to date, only a few studies were able to identify the transformation products and there is a lack of information about the transformation pathways. More studies are necessary to assess the occurrence of PCM degradation products in environmental samples and their toxicity.

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1.3 References

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UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
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The main objective of this Doctoral Thesis is the development and application of environmentally friendly analytical methods to determine synthetic musk fragrances in different environmental matrices, such as wastewater, sewage sludge and marine organisms. The fragrances studied belong to the polycyclic musk, nitro musk and macrocyclic musk families. To achieve this objective, cutting-edge microextraction and extraction techniques will be assessed in combination with gas chromatography coupled to mass spectrometry or tandem mass spectrometry detection.

Another objective the evaluation of the biodegradation of the most widespread polycyclic musks via a laccase-mediator system and the subsequent identification of the degradation products generated.

UNIVERSITAT ROVIRA I VIRGILI
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UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
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3. EXPERIMENTAL, RESULTS AND DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
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Dipòsit Legal: T 72-2016

As previously discussed in the Introduction of this Thesis, synthetic musk fragrances are generally not effectively removed during conventional treatments at wastewater treatments plants (WWTPs). Thus, several synthetic musks are still present in the effluent water from WWTPs and reach surface water when these effluents are discharged. Moreover, the reuse of sewage sludge as manure can contribute to the introduction of certain synthetic musks into different environmental compartments because they are, in part, accumulated in sewage sludge during WWTP treatments due to their lipophilic properties.

As mentioned earlier, the research of this Thesis focuses on the study of synthetic musk fragrances in environmental matrices. As such, the target compounds belong to the polycyclic musk (PCM), nitro musk (NM) and macrocyclic musk (MCM) families. This Thesis has been developed in the research group of Chromatography and Environmental Applications at the Universitat Rovira i Virgili, which has extensive experience in the determination of emerging organic compounds (EOCs). This Thesis aims to increase the current knowledge with regard to the applicability of conventional and new microextraction techniques for the determination of synthetic musk fragrances by developing analytical methods for their determination in wastewater, sewage sludge and marine organisms. The results of this Thesis are related to the following projects: (I) General Research Directorate of the Ministry of Science and Technology of Spain (CTM2011-28765-C02-01) and General Research Directorate of the Government of Catalonia (II) (2009SGR223); and the pre-doctoral grant (FI-DGR 2012) provided by the Department of Innovation, Universities and Enterprises and the European Social Fund.

This chapter includes the experimental part and results from the different studies that have been carried out throughout the course of this Thesis. These results have already been published, or are in the process of being published, in international scientific journals. The following sections are organized according to the microextraction technique used (Sections 3.1 and 3.2), the analysis of marine organisms (Section 3.3) and the degradation of synthetic musk fragrances by lignin (Section 3.4). In each section, a brief introduction is included to establish the context of the research, followed by the results presented in paper format and the most notable results are also discussed after the papers. The list of the published papers resulting from this Thesis is included in Appendix II.

In the first section, conventional microextraction techniques (headspace solid-phase microextraction (HS-SPME), HS-stir bar sorptive extraction (SBSE) and on-line solid-phase extraction (SPE)) are successfully applied for the determination of synthetic musk

fragrances in environmental samples. Two methods for the determination of MCMs in environmental waters and sewage sludge were developed. In both studies, the extraction was performed using HS-SPME directly on the sample. Gas chromatography and mass spectrometry detection (GC-MS) with ion trap (IT) analyzer was used in selected ion storage (SIS) mode. Moreover, this section includes the optimization of an on-line SPE based method and an HS-SBSE based method for the determination of a group of synthetic musk fragrances (including PCMs, NMs, MCMs and the degradation product of galaxolide (HHCB)) in wastewater and sewage sludge samples, respectively, prior to analysis by GC-MS. Two different interfaces were applied: an on-column (OC) injector for the on-line SPE and thermal desorption (TD) for the desorption of the stir bar. In both cases, a simple quadrupole (Q) acquiring in SIM mode was used as the analyzer.

In the second section, novel microextraction techniques such as ionic liquid-based headspace single-drop microextraction (IL-HS-SDME), microextraction by packed sorbents (MEPS) and needle trap microextraction (NTME) are evaluated for the determination of synthetic musk fragrances in environmental samples. NTME study was carried out in collaboration with the Department of Chemistry of the Universitat de Girona in Girona (NE Spain). IL-HS-SDME followed by GC-IT-tandem mass spectrometry (MS/MS) was selected for the extraction of PCMs, NMs and the degradation product of HHCB present in wastewater samples. Meanwhile, pressurized liquid extraction (PLE) was used for the extraction of the target compounds present in sludge samples prior to preconcentration by means of IL-HS-SDME. Furthermore, this section includes the development of other two new methodologies for the determination of synthetic musk fragrances in wastewater samples. The first focused on the use of a relatively new microextraction technique known as MEPS for the determination of MCMs in wastewater samples followed by large volume injection (LVI)-GC-IT-MS (SIS), while the second consisted of NTME performed with needle trap (NT) built in-house and exposed in the HS of wastewater samples. The NT was thermally desorbed in the injector port prior to the analysis of the target PCMs and NMs, as well as the degradation product of HHCB by GC-IT-MS/MS.

The methods developed in sections one and two have successfully been applied for the determination of synthetic musk fragrances in wastewater and sludge samples mainly taken from the WWTPs in Tarragona and Reus in Catalonia (NE Spain). Occasionally, wastewater and sewage sludge samples from the WWTP in Girona were analysed, as well as influent and effluent samples from the tertiary treatment of the WWTP in Vila-seca, both of which are located in Catalonia (NE Spain). Water and sludge

samples from the drinking water treatment plant (DWTP) in L'Ampolla (Catalonia, NE Spain) were also analysed.

The third section covers the development of two analytical methods for the simultaneous determination of PCMs, NMs and the degradation product of HHCB in marine organisms (i.e. fish and mussels). Two extraction methodologies were compared and different clean-up strategies were also studied in order to minimize the matrix effect (ME) as much as possible. Firstly, the extraction technique consisted of conventional PLE with an in-cell clean-up with florisil. The second extraction technique involved QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction, a consolidated extraction methodology in the field of food analysis of growing interest in recent years, followed by dispersive solid-phase extraction (dSPE) as the clean-up strategy. LVI followed by GC-IT-MS/MS was chosen as the separation and detection technique. The PLE and QuEChERS extraction methodologies were compared in terms of validation parameters, analysis time and ME when applied to fish and mussels. After that, the PLE/GC-IT-MS/MS method was applied to determine the target musk fragrances in fish samples of red mullet, gilt head bream, turbot and mussels from Tarragona market, and also in perch, sheatfish and carp samples from the River Ebro (NE Spain).

In the last section, the commercial enzyme laccase from *Trametes versicolo*r and the redox mediator 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used to study the biotransformation of the synthetic fragrances 1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethylnaphthalen-yl)ethan-1-one (Iso-E-Super, OTNE), HHCB, tonalide (AHTN) and the degradation product of HHCB (HHCB-lactone) in water. This study was carried out in collaboration with the Department of Environmental Science of the Aarhus University in Roskilde (Denmark), during a research stay conducted during the course of this Thesis. In order to evaluate the effects of the enzyme laccase in the enantioselective degradation of the target compounds, GC with an enantioselective column was used as

the separation technique followed by MS detection (scan mode) with a Q as analyzer. Rate constants were calculated for the degradation of the target fragrances by the enzyme laccase in tap water. In addition, as enantioselective degradation of musk fragrances was observed in wastewater, sewage sludge and fish samples, enantiomeric fractions (EF) of the target compounds were studied during composting of sludge.

UNIVERSITAT ROVIRA I VIRGILI
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3.1. Determination of musk fragrances in environmental samples by conventional microextraction techniques

UNIVERSITAT ROVIRA I VIRGILI
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This section focuses on the determination of MCMs fragrances in wastewater and sewage sludge using HS-SPME/GC-Q-MS in the first study, and the determination of the same compounds in sewage sludge in the second study. As mentioned in previous sections, information about the presence of MCMs fragrances in environmental samples is still very limited [1-3] and, therefore, the main reason for choosing these compounds was to increase the current knowledge about their occurrence in the environment. Later on, in the third and fourth studies, MCM fragrances were included in the group of target fragrances (PCMs and NMs) determined in wastewater samples and sludge samples by on-line SPE/GC-Q-MS and HS-SBSE/GC-Q-MS, respectively.

To date, different analytical methods have been developed to determine MCMs in environmental matrices but most of them are based on the determination of PCMs and NMs and only include one or two MCMs, mainly ambrettolide and musk NN [1-3]. Therefore, specific analytical methods for the determination of MCMs in environmental samples are limited. As it was the first time that HS-SPME has been applied for the determination of MCMs fragrances in wastewater, the first study presented here has a more in-depth focus on the HS-SPME procedure. In this study, five different SPME fibres were tested. Four of these fibres (polydimethylsiloxane (PDMS) 30 µm, PDMS 100 µm, polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 µm and polyacrilate (PA) 85 µm) have previously been used to extract PCMs and NMs from different kinds of water [4,5], while PDMS 7 µm fibre was tested due to its ability to extract non-polar compounds with high molecular weight [6,7]. Moreover, the main parameters that affect the sorption and desorption process in HS-SPME were optimized for each fibre to find the best HS-SPME conditions for the extraction of MCMs fragrances from aqueous matrices.

The second study involved the optimization of HS-SPME for extracting MCMs directly from sewage sludge. It avoids the need to use additional extraction/preconcentration techniques or clean-up procedures and makes the automation of the method easier. In the study, taking into account our previous experience of determining MCMs in aqueous samples, the SPME fibre selected was PDMS/DVB 65 µm. The main parameters affecting the microextraction process were optimized in order to obtain the best extraction efficiency, namely sample amount, water addition, extraction temperature and extraction time.

The last studies in this section focused on the determination of PCMs, NMs and MCMs in wastewater and sewage sludge samples by GC-Q-MS. To do so, two different microextraction techniques were used: on-line SPE for the determination of the target compounds in wastewater, and HS-SBSE for the extraction of the target compounds

from sewage sludge. In reference to on-line SPE extraction, two precolumns were tested: one packed with octadecyl bonded silica (C18) sorbent and the other with divinylbenzene/N-vinylpyrrolidone (Oasis HLB), both with 60 µm particle size, due to their ability to extract the target compounds [8-10]. The parameters affecting the transfer of the analytes from the precolumn to the GC through an on-column interface, were also optimized, as well as SPE parameters. Meanwhile, HS-SBSE was performed with a PDMS stir bar 20 mm long x 0.5 mm film thick, which corresponds to approximately 48 µL of PDMS phase. The parameters related to the HS-SBSE were also optimized, as well as the thermal desorption of the target analytes into the GC-Q-MS.

The proposed methods have mainly been applied to analyse influent and effluent wastewater and sewage sludge samples from two WWTPs located in Tarragona and Reus, because data regarding the presence of these compounds had not previously been reported for these WWTPs. Moreover, sludge samples from the WWTP in Girona and the DWTP in L'Ampolla were also analysed, as well as water samples from the tertiary treatment of the WWTP in Vila-seca.

The results of these studies have been published in *Analytical and Bioanalytical Chemistry 405 (2013) 9547-9554, Journal of Chromatography A 1314 (2013)38-43* and 1364 (2014) 1-11 and *Journal of Separation Science 37 (2014) 1322-1329*.

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AN AUTOMATED HEADSPACE SOLID-PHASE MICROEXTRACTION FOLLOWED BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD TO DETERMINE MACROCYCLIC MUSK FRAGRANCES IN WASTEWATER SAMPLES

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Abstract

A fully automated method has been developed for determining eight macrocyclic musk fragrances in wastewater samples. The method is based on a headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS). Five different fibres (PDMS 7 µm, PDMS 30 µm, PDMS 100 µm, PDMS/DVB 65 µm and PA 85 µm) were tested. The best conditions were achieved when a PDMS/DVB 65 µm fibre was exposed for 45 min in the headspace of 10 mL water samples at 100 °C. Method detection limits were found in the low ng L^{-1} range between 0.75 ng L^{-1} and 5 ng L^{-1} depending on the target analytes. Moreover, under optimized conditions, the method gave good levels of intra-day and inter-day repeatabilities in wastewater samples with relative standard deviations (n=5, 1,000 ng L^{-1}) less than 9% and 14%, respectively. The applicability of the method was tested with influent and effluent urban wastewater samples from different wastewater treatment plants (WWTPs). The analysis of influent urban wastewater revealed the presence of most of the target macrocyclic musks with, most notably, the maximum concentration of ambrettolide being obtained in WWTP A (4,360 ng L^{-1}) and WWTP B (12,291 ng L^{-1}), respectively. The analysis of effluent urban wastewater showed a decrease in target analyte concentrations, with exaltone and ambrettolide being the most abundant compounds with concentrations varying between below method quantification limit (<MQL) and 2,458 ng L^{-1}.

Keywords: *gas chromatography-mass spectrometry; macrocyclic musk fragrances; headspace solid-phase microextraction; urban wastewater.*

1. Introduction

Macrocyclic musk fragrances, which are included in the group known as emerging organic contaminants, have been investigated over recent years because they seem to smell more intensely than polycyclic musks. Therefore, less mass is needed to gain the same performance in perfumery. In addition, they seem to be more easily degradable in the environment [1]. The main shortcoming of these compounds is the cost of their synthesis. As a result, most of the macrocyclic musks studies have focused on the optimization of their synthesis, trying to solve problems with enantio-selectivity that were so significant with respect to their odour strength [2,3], rather than their determination in environmental samples.

After usage, macrocyclic musks enter wastewater treatment plants. Although partial elimination has been reported for polycyclic as well as nitro musks [4,5], little information is yet available for macrocyclic musks. Only two macrocyclic musks, namely ambrettolide and musk NN, have been included in studies where polycyclic and nitro musks have been determined in wastewater and biosolid matrices [6,7]. Recently, a method based on microextraction by packed sorbents followed by gas chromatography-mass spectrometry has been developed by our research group [8] in order to determine eight macrocyclic musks in wastewater samples. Therefore, there is a need to develop more reliable analytical methods to determine macrocyclic musks in environmental samples at trace levels.

Gas chromatography is suitable for separating macrocyclic musks, just as it is for polycyclic and nitro musk fragrances. Nevertheless, prior to gas chromatography (GC) analysis, an extraction/preconcentration step is required due to the low levels expected in environmental samples. Currently, there is a growing analytical trend towards the use of environmentally friendly extraction or microextraction techniques that reduce or eliminate the use of organic solvents. Solid-phase microextraction (SPME) [9,10], single-drop microextraction (SDME) [11,12], dispersive liquid-liquid microextraction (DLLME) [13] and microextraction by packed sorbents (MEPS) [14] have all been successfully applied for the extraction and preconcentration of emerging organic contaminants from wastewater and sewage sludge samples.

SPME is a solventless technique [15] that has been successfully applied to determine a wide range of water pollutants such as phthalates, polychlorinated biphenyls, brominated flame retardants, polycyclic and nitro musk fragrances [9,16-20], among others. The aim of the present study is to develop a fully automated and sensitive method to determine eight macrocyclic musk fragrances in wastewater samples using headspace (HS)-SPME followed by GC-mass spectrometry (MS). To the best of our knowledge, this paper describes the first application of HS-SPME for the determination of macrocyclic musks in wastewater samples. The main experimental parameters affecting the microextraction process were optimized. Accuracy, linear range,

precision, method detection limits (MDLs) and method quantification limits (MQLs) were evaluated in order to assess the performance of the proposed method. Finally, several wastewater samples were analysed in order to demonstrate the applicability of the method.

2. Experimental

2.1. Chemical standards

The standard 9-cycloheptadecen-1-one (civetone) was supplied by Sigma-Aldrich (Steinheim, Germany). Ethylenedodecanedioate (musk MC4), oxacyclohexadecan-2-one (exaltolide), cyclopentadecanone (exaltone), oxa-cyclohexadecen-2-one (habanolide) and oxacycloheptadec-8-en-2-one (ambrettolide) were purchased from Symta (Madrid, Spain). Ethylenetridecane-dioate (musk NN) as 10 μg mL^{-1} solution in cyclohexane, 3-methylcyclopenta-decanone (muscone) as 100 mg L^{-1} solution in cyclohexane and d15-musk xylene (surrogate standard) as 100 mg L^{-1} solution in acetone were also purchased from Symta (Madrid, Spain). Table 1 shows the formula name and the octanol/water partition coefficient of each target compound.
Stock solutions of individual standards were prepared by dissolving each compound in cyclohexane at a concentration of 1,000 mg L^{-1} with the exception of musk NN, muscone and d15-musk xylene. A 10 mg L^{-1} standard solution mixture of all of the target analytes except musk NN was prepared in ethyl acetate. Musk NN standard was provided at a concentration of 10 mg L^{-1} and was used as received. Cyclohexane and ethyl acetate were GC grade with purities of 99.8% (VWR Llinars del Vallès, Barcelona, Spain) and 99.0% (VWR Llinars del Vallès), respectively.
Sodium chloride (ACS reagent ≥ 99%) was supplied by Sigma-Aldrich. Ultrapure water was obtained using an ultrapure water purification system from Veolia waters (Sant Cugat del Vallés, Spain). Helium gas with a purity of 99.999% from Carburos Metálicos (Tarragona, Spain) was used for the chromatographic analysis.

2.2. Samples

Influent and effluent wastewater samples were collected over a period of three months at two urban wastewater treatment plants (WWTP A and WWTP B) located in the area of Tarragona, which have populations of around 140,000 inhabitants (WWTP A) and 107,000 inhabitants (WWTP B). The WWTPs receive urban sewages and some industrial discharges and use activated sludge biological treatment. The biological oxygen demand (BOD_5) for influent water is about 400 mg L^{-1} at both WWTPs and the average flow rate is 30,000 m^3 day^{-1} for WWTP A and 16,000 m^3 day^{-1} for WWTP B. All samples were collected by using pre-cleaned amber glass bottles and filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborought, UK) and a 0.22 μm nylon filter (Scharlab, Barcelona, Spain) and stored at 4 °C until analysis (a maximum of two days in the fridge). Prior to analysis, the samples were allowed to reach room temperature.

Table 1. Formula name, log K_{OW}, retention times (t_R) and quantifier and qualifier ions of the target macrocyclic musk compounds.

No.	Compound	Formula name	Log K_{OW}	t_R (min)	Quantifier ion [a]	Qualifier ions [b]
1	Exaltone	Cyclopentadecanone	2.33	7.83	225 (100)	135 (37) 125 (52)
2	Exaltolide	Oxacyclohexadecan-2-one	6.33	7.86	241 (100)	223 (30) 123 (32)
3	Muscone	3-Methylcyclopentadecan one	2.9	7.91	238 (100)	209 (35) 125 (54)
4	Habanolide	Oxacyclohexadecen-2-one	5.53	7.91	95 (100)	239 (80) 221 (70)
5	d15-MX [c]	d15-Musk xylene	-	8.29	294 (100)	136 (25) 122 (30)
6	Ambrettolide	Oxacycloheptadec-8-en-2-one	6.10	8.58	95 (100)	235 (47) 135 (45)
7	Musk MC4	Ethylenedodecanedioate	5.52	8.99	87 (100)	213 (47) 149 (48)
8	Civetone	9-Cycloheptadecen-1-one	6.31	9.42	250 (100)	251(40) 121 (50)
9	Musk NN	Ethylenetridecanedioate	5.84	9.73	98 (100)	227 (80) 211 (56)

[a] Quantifier ion abundance in % is in brackets.
[b] Qualifier ions abundance in % is in brackets.
[c] Surrogate standard.

2.3. Instrumentation

The GC-MS analyses were performed on a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) connected to a Varian 4000 ion trap mass detector. The GC was equipped with a 1079 programmable temperature vaporizing injector and a 0.8 mm i.d. insert liner (Varian). A ZB-50 analytical column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex (Torrance, California, USA) was utilised for the chromatographic separation. Varian MS Workstation v.6.9 software was used for instrument control and data processing.

A CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) was used for the fully automated HS-SPME. The SPME device and fibres -PDMS 7 µm, PDMS 30 µm, PDMS 100 µm, PDMS/DVB 65 µm and PA 85 µm- were obtained from Supelco (Bellefonte, PA, USA). Before the initial application, fibres were conditioned according to the supplier's instructions by being inserted into the GC injector.

2.4. Analytical method

The HS-SPME procedure was as follows: 10 mL of sample or standard solution was poured into a 20 mL HS vial and immediately sealed tight with a Teflon septum and placed in a tray for SPME. When the temperature of the heat/stir accessory reached 100 °C, the vial was transported there automatically, and the headspace was let equilibrate with the sample at the extraction

temperature for 5 min. The PDMS/DVB 65 µm fibre was then introduced through the septum and kept in the headspace of the vial for 45 min at 100 °C. During the extraction, the sample was magnetically stirred at 750 rpm.

Subsequently, the fibre was withdrawn into the SPME syringe needle, which was then pulled out of the sample vial and immediately inserted into the GC injection port for desorption. The desorption was conducted at 250 °C for 3 min, and the compounds were subsequently analysed by GC-MS.

To prevent carry-over, the PDMS/DVB 65 µm fibres were cleaned by heating to 250 °C for 10 min prior to every extraction, and a blank test was performed to check for possible carry-over. As mentioned above, the entirely automated extractions were performed by a commercial CombiPAL autosampler mounted on the GC-MS system.

For the chromatographic analysis, the injector was operated in splitless mode at 250 °C. The oven temperature programme was as follows: initial temperature 100 °C held for 2.5 min, increased at 50 °C min^{-1} to 220 °C, 5 °C min^{-1} to 260 °C then 20 °C min^{-1} to 280 °C and held for 10 min. All of the compounds were separated within 10 min. Helium was used as the carrier and collision gas at a flow rate of 1 mL min^{-1}. The transfer line, manifold and trap temperatures were set at 280 °C, 50 °C, and 200 °C, respectively. A filament-multiplier delay of 3 min was established in order to prevent instrument damage. The mass spectrometer analysed the substances after electron impact ionization in selected ion storage (SIS) mode.

3. Results and discussion

3.1. GC-MS optimization

A mixed solution of 1 mg L^{-1} of the eight macrocyclic musk fragrances and d15-musk xylene as surrogate standard was prepared in ethyl acetate, and 1 µL of this solution was injected directly into the GC-MS, using an electron ionization and full scan acquisition mode. The target compounds were identified by their molecular weight, and then the chromatographic separation was optimized. The separation was performed in just 10 min as described in the section “Analytical method”. Exaltone and exaltolide co-eluted, as did muscone and habanolide, but as they had different molecular ions and fragments, they could be quantified separately. To achieve maximum sensitivity/selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and then optimizing the ion trap MS/MS parameters (amplitude excitation voltage, CID storage level). However, due to the fact that the excessive fragmentation of the target compounds caused a significant decrease in the analytical signal, SIS mass spectrometry was selected. Table 1 also summarizes the retention time and the qualifier and quantifier ions for each compound.

3.2. HS-SPME optimization

To optimize SPME conditions, five commercially available fibres ("Sample" section) were tested. Four of them, (PDMS 100 µm and 30 µm, PDMS/DVB 65 µm and PA 85 µm) have previously been used to extract polycyclic musks and nitro musks in environmental samples [21,22]. With respect to the PDMS, a 7 µm fibre was tested to extract macrocyclic musk fragrances due to its ability to extract non-polar high molecular compounds (MW 125-600) [23-25].

The main parameters that affect the sorption and desorption process in SPME were optimized for each fibre in order to maximize the chromatographic peak area of the compounds by analysing a standard mixed solution of 1 µg L^{-1} of all of the macrocyclic musk and d15-musk xylene as surrogate standard in 10 mL of ultrapure water. To obtain the best conditions for each fibre, initial conditions such as desorption temperature and time, HS mode, agitation and sample volume were selected based on the previous literature on synthetic musks [21,22,26,27] and the fibre supplier's recommendations, after checking to ensure that these conditions were suitable for work with macrocyclic musks. Finally, such variables as extraction temperature, extraction time and salt concentration were optimized because these factors were expected to be the most influential in the extraction process.

The initial conditions used for each fibre were as follows: HS mode, 10 mL sample volume poured in a 20 mL HS vial, extraction time of 30 min, extraction temperature of 80 °C, 750 rpm, 300 g L^{-1} NaCl addition, desorption time of 3 min and desorption temperature of 250 °C. Among the parameters selected from the literature, the desorption temperature was the only factor that it was necessary to study on the basis of the experimental results. The peak areas obtained at the selected temperature (250 °C) were then compared to those obtained working at the maximum operating temperatures. The results showed that for the PDMS/DVB 65 µm and PDMS (100 µm and 30 µm) fibres, there were no significant differences between the peak areas achieved at 250 °C and those reached at 270 °C and 280 °C, respectively. Therefore, 250 °C was set as the desorption temperature. In the case of the rest of fibres tested, the maximum operating temperature was the optimal desorption temperature 320 °C for PDMS 7µm and 280 °C for PA. The experimental variables expected to be most influential were then subsequently optimized.

Firstly, the extraction temperature was studied by comparison of the peak areas obtained at 45 °C, 65 °C, 80 °C, and 100 °C. Independently of which SPME fibre was applied, working with 10 mL of water sample, exaltolide, exaltone and muscone had the highest peak areas at 80 °C with no significant differences between the peak areas obtained at 80 °C and 100 °C, respectively. In contrast, as can be seen in Fig. 1, with a PDMS/DVB fibre, habanolide reached the highest peak area at 80 °C and a significant decrease in its peak area was observed at 100 °C. The remaining target compounds

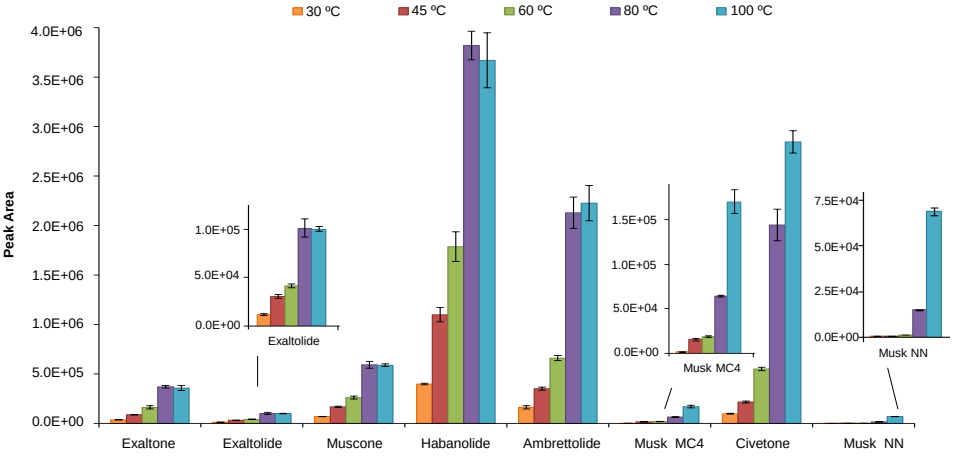

Fig. 1. Effect of the temperature on the chromatographic peak areas obtained working with HS-SPME. Experimental conditions: PDMS/DVB 65 µm fibre in headspace mode, 10 mL sample volume stirred at 750 rpm, 30 min extraction time, no salt addition, 250 °C as desorption temperature and 3 min desorption time (1 µg L^{-1}, *n*=3).

achieved the highest peak areas at 100 °C with a significant decrease in the peak areas when the extraction temperature was 80 °C. Therefore 100 °C was selected as the optimal extraction temperature because, at this temperature, the less intensive analytes, such as musk MC4 and musk NN, achieved the highest peak areas and no significant differences between working at 80 °C or 100 °C extraction temperature were observed for most of the remaining target analytes.

Secondly, the extraction time was evaluated between 15 and 60 min. Working with either PDMS 30 µm or 7 µm fibres, the results suggested that 30 min was the optimal extraction time because the highest peak areas were reached at this point. However, when PDMS 100 µm, PDMS/DVB and PA were used, a progressive increase in peak areas was observed for all of the target analytes up to an optimal time of 45 min, after which there was a decrease in the peak areas of exaltone, exaltolide, musk MC4 and musk NN, when the extraction time was extended up to 60 min. For this reason, 45 min was chosen as the optimal extraction time for PDMS 100 µm, PDMS/DVB and PA fibres.

The last parameter optimized was the salt concentration. To study the influence of adding salt on the efficiency of HS-SPME, the ionic strength of the standard solutions was modified by adding sodium chloride in the range of 0 g L^{-1} to 360 g L^{-1} (salt saturation). Independently of which SPME fibre was used, no NaCl addition was selected as the optimal salt concentration because maximum peak areas were obtained for most of the target analytes. However, musk MC4 and musk NN showed maximum peak areas at 300 g L^{-1}. These two behaviours can be explained by the

differences in polarity between musk MC4 and musk NN and the remaining target analytes. While musk MC4 and musk NN are soluble in water and the increase in the ionic strength promotes their transportation to the headspace and hence to the extraction fibre [28], the rest of target macrocyclic musks are less soluble in water and the migration of these compounds to the headspace was not affected by the presence of NaCl. Table 2 summarizes the optimum conditions for each fibre.

In order to choose the best SPME fibre for the extraction of the target macrocyclic musk present in 10 mL of water sample (spiked at 1 μg L^{-1}) under optimized conditions, a comparison between the five fibres studied was carried out. As can be seen in Fig. 2, PDMS/DVB was the fibre that provided the highest peak areas, followed with a large margin by the PA fibre in the case of musk MC4 and musk NN and the PDMS 100 μm fibre for the rest of target analytes.

Table 2. HS-SPME optimum conditions for each fibre studied.

Process	Variable	PDMS 7μm	PDMS 30μm	PDMS 100μm	PDMS/DVB 65μm	PA 85μm
Sorption	Extraction temperature (°C)	100	100	100	100	100
	Extraction time (min)	30	30	45	45	45
	Salt concentration g L^{-1} (NaCl)	0	0	0	0	0
Desorption	Time (min)	3	3	3	3	3
	Temperature (°C)	320	250	250	250	280

3.3. Method Validation

The method was analytically validated by establishing the linear ranges, MDLs, MQLs and intra-day and inter-day repeatabilities. Procedural blanks of the conditioned PDMS 65 μm fibre were performed before HS-SPME in order to prevent carry-over effects and ensure the repeatability of the analytical method. If no signal of the target analytes was found in the PDMS 65 μm fibre blanks, it could also be reused for several times. As SPME may be strongly influenced by the sample matrix the feasibility of the microextraction procedure must be demonstrated with real samples spiked at different concentrations. To this end, an influent and an effluent sample from a WWTP A were used to validate the method. The WWTP A samples used to validate the method were analysed (*n*=5) and some peaks of exaltone, ambrettolide, musk MC4 and musk NN appeared in the chromatogram of the influent sample while in the effluent sample, only ambrettolide and exaltone were found. The averaged peak area of each detected compound was subtracted from the peak area of each spiked sample.

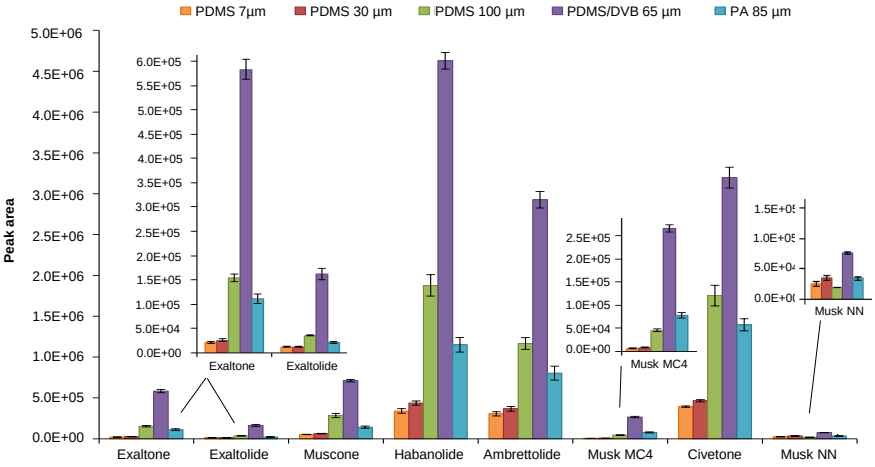

Fig. 2. Comparison of the chromatographic peak areas obtained with the five SPME fibres in the optimal HS-SPME conditions for extracting macrocyclic musks from a 10 mL water sample spiked at 1 µg L^{-1} (*n*=3).

The linear range of the method was obtained by analysing the WWTP A samples spiked with all of the target analytes at concentrations between 2.5 ng L^{-1} and 10,000 ng L^{-1} while the surrogate standard concentration remained constant at 1,000 ng L^{-1}. As can be seen in Table 3, good linear ranges were obtained working with WWTP A influent and effluent samples. In addition, the presence of a surrogate standard enabled us to improve the determination coefficients (r^2) of the calibration curves to values higher than 0.996 for all of the target analytes.

The MDLs were defined as the concentration of analytes in the WWTP A influent or effluent which caused a peak with a signal-to-noise ratio higher than 3 for the compounds that did not appear in the blank. For the compounds that appeared in the blank, the MDLs were estimated as three times the standard deviation of the blank. In all cases, the MQLs were fixed at the lowest calibration level. The MDLs and MQLs ranged from 0.75 ng L^{-1} to 5 ng L^{-1} and from 2.5 ng L^{-1} to 10 ng L^{-1}, respectively, and they are summarized in Table 3.

The intra-day and inter-day repeatabilities were determined by spiking five replicates of the influent and effluent WWTP A samples at 1,000 ng L^{-1}. The results obtained, expressed as relative standard deviation (RSD) percentages, were lower than 9% for intra-day repeatabilities and below 14% for inter-day repeatabilities for all of the compounds analysed.

When the figures of merit obtained in the present study were compared with a fully automated MEPS method [8], slightly lower MDLs were obtained with SPME for all of the target analytes. It should be noted that the MDLs of civetone decreased significantly, from 5 ng L^{-1} working with MEPS to

Table 3. Method linear ranges, determination coefficients, MDLs, MQLs, intra-day repeatabilities and inter-day repeatabilities.

	Influent					Effluent				
Compound	**Linear range [a] ($ng\ L^{-1}$)**	**Determination coefficient (r^2)**	**MDLs ($ng\ L^{-1}$)**	**Intra-day Repeatability (% RSD) [b]**	**Inter-day Repeatability (% RSD) [b]**	**Linear range [a] ($ng\ L^{-1}$)**	**Determination coefficient (r^2)**	**MDLs ($ng\ L^{-1}$)**	**Intra-day Repeatability (% RSD) [b]**	**Inter-day Repeatability (% RSD) [b]**
Exaltone	5-10,000	0.997	1	3	10	5-10,000	0.999	1	3	9
Exaltolide	10-10,000	0.996	5	4	12	5-10,000	0.998	2.5	4	9
Muscone	10-10,000	0.999	5	6	11	10-10,000	0.999	2.5	5	8
Habanolide	10-10,000	0.999	2.5	9	9	5-10,000	0.999	1	7	8
Ambrettolide	10-10,000	0.997	5	6	13	10-10,000	0.999	2.5	4	9
Musk MC4	10-10,000	0.997	5	8	11	10-10,000	0.998	5	5	10
Civetone	5-10,000	0.998	1.5	8	9	2.5-10,000	0.999	0.75	3	7
Musk NN	10-10,000	0.997	5	4	14	10-10,000	0.997	5	3	11

[a] MQL ($ng\ L^{-1}$): were fixed as the lowest calibration level.
[b] n=5, 1,000 $ng\ L^{-1}$.

0.75 ng L^{-1} using SPME as the micro-extraction technique. However, intra-day and inter-day repeatability values were slightly better with MEPS, with RSD percentages varying between 1% and 5% and between 7% and 9%, respectively, compared to between 3% and 9% and between 8% and 14%, respectively, obtained when working with SPME.

3.4. Method application

The method developed was used to determine macrocyclic musk fragrances in influent and effluent samples collected from urban WWTPs (A and B) ("Instrumentation" section). A matrix-matched calibration curve was used for the quantification of analytes in order to obtain more accurate results. Table 4 summarizes the results of the average concentrations of the macrocyclic musk fragrances found in each sample (*n*=8). An analysis of the results shows that all of the macrocyclic musks studied appeared in at least one of the influent WWTP A water matrices, with the most abundant being ambrettolide, with the average concentration ranging between <MQL and 4,360 ng L^{-1} while exaltone, musk MC4 and musk NN, were present in all of the WWTP A influent samples with concentrations ranging between 12 ng L^{-1} and 1,987 ng L^{-1}. Fig. 3 shows a chromatogram of an influent sample from WWTP A. In WWTP B influent water samples, all of the target analytes were present and, as in the case of WWTP A influent, ambrettolide was the most abundant compound with a concentration varying between <MQL and 12,291 ng L^{-1}. Meanwhile the other analytes, such as exaltone, habanolide, musk MC4 and civetone, were present in all of the WWTP B influent samples. As expected, the macrocyclic musks concentrations found in effluent samples were slightly lower than those obtained in influent samples, with exaltone and ambrettolide being the most abundant

Table 4. Concentrations of macrocyclic musks found in wastewater samples (*n*=8) in ng L^{-1}.

Compound	WWTP A		WWTP B	
	Influent	Effluent	Influent	Effluent
Exaltone	394-1,987	249-1,682	389-2,890	<MQL-2,256
Exaltolide	n.d.-410	n.d.-156	n.d.-1,489	n.d.-478
Muscone	n.d.-573	n.d.-370	n.d.-2,460	n.d.-432
Habanolide	n.d.-480	n.d.-159	72-1,569	n.d.-0.53
Ambrettolide	<MQL-4,360	<MQL-1,430	<MQL-12,291	<MQL-2,458
Musk MC4	43-821	n.d.-171	22-614	12-173
Civetone	n.d.-2,210	n.d.-1,790	91-1,341	<MQL-48
Musk NN	12-334	n.d.-136	<MQL-730	<MQL-175

n.d.; not detected.
<MQL; values under the method quantification limit.

compounds with concentrations oscillating between <MQL and 2,458 ng L^{-1}.

Previous work [8] that focused on the determination of macrocyclic musk fragrances in wastewater samples confirms the findings of the present study, i.e. that the most abundant macrocyclic musk is ambrettolide, although other macrocyclic musks such as exaltone, musk MC4 or musk NN may also be present in wastewater samples in minor concentrations. A decrease in the concentrations of all macrocyclic musks was observed when the influent results were compared with the effluent results and some compounds, such as exaltolide, muscone, habanolide or musk NN, were detected at concentrations below the MQL or were not detected at all.

4. Conclusions

The proposed HS-SPME-GC-MS (SIS) method was shown to be completely automated, simple, and environmentally friendly. It also provided low ng L^{-1} MDLs and satisfactory precision (RSD between 3 and 9%) for wastewater samples.

The following main parameters involved in the SPME were evaluated and optimized: SPME fibre, NaCl addition, extraction temperature and extraction time. Five fibres were tested (PDMS 7 µm, PDMS 30 µm, PDMS 100 µm, PDMS/DVB 65 µm and PA 85 µm), and the best conditions were found to be the following PDMS/DVB fibre 65 µm, 10 mL sample volume stirred at 750 rpm, 0% NaCl addition, an extraction temperature of 100 °C for

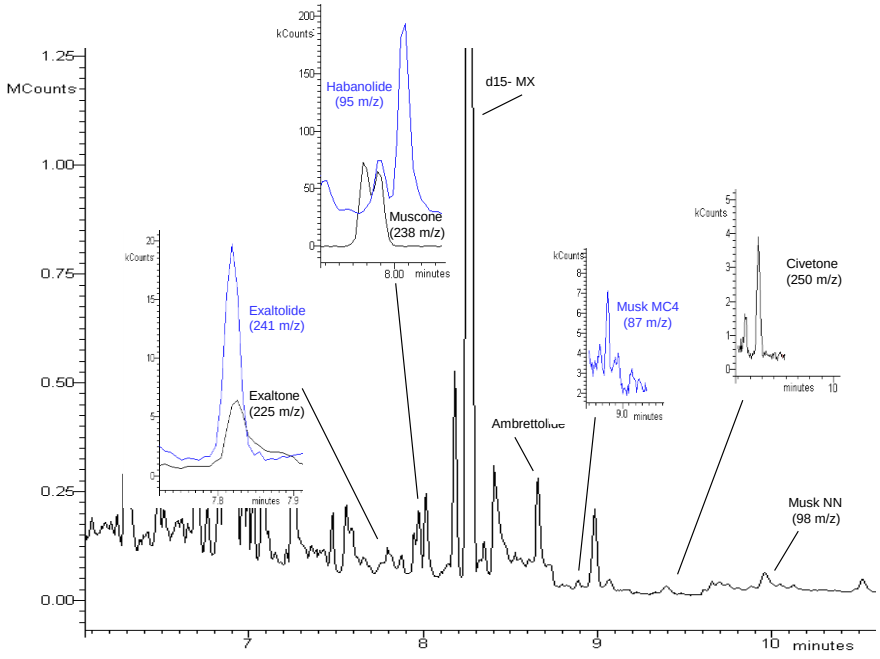

Fig. 3. Chromatogram of an influent water sample from WWTP A.

45 min and 3 min desorption time (250 °C).

The applicability of the method was tested with influent and effluent water samples from two WWTPs. All of the macrocyclic musks studied were present in the influent water analysed with, most notably the highest concentration of ambrettolide of 12,291 ng L^{-1} being recorded in an influent sample from WWTP A. In effluent samples, the macrocyclic musks concentrations found were slightly lower than those obtained in the influent samples and ambrettolide, in addition to exaltone remained the most abundant compounds. Therefore taking into account the presence of most of the target analytes in effluent wastewater samples, WWTPs treatments need to be evolved to increase the efficiency of the removal of macrocyclic musk compounds.

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We would also like to thank the personnel at the wastewater treatment plants for their cooperation with all aspects of this study.

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3.1.2. A simple and automated method to determine macrocyclic musk fragrances in sewage sludge samples by headspace solid-phase microextraction and gas chromatography-mass spectrometry

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

A SIMPLE AND AUTOMATED METHOD TO DETERMINE MACROCYCLIC MUSK FRAGRANCES IN SEWAGE SLUDGE SAMPLES BY HEADSPACE SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

For the first time, headspace solid-phase microextraction (HS-SPME) has shown to be a powerful technique to extract macrocyclic musk fragrances directly from sewage sludge. It avoids the need to use additional extraction/preconcentration techniques or clean-up procedure and facilitates the automation of the method. Thus, a simple and fully automated method based on HS-SPME and GC-MS has been developed which allows the determination of eight macrocyclic musk fragrances at ng g^{-1} dry weight (d.w.) levels. The optimal HS-SPME conditions were achieved when a PDMS/DVB 65 µm fibre was exposed for 45 min in the headspace of 0.25 g sewage sludge samples mixed with 0.5 mL of water stirred at 750 rpm at 80 °C. Optimal desorption conditions were found to be 250 °C for 3 min. Method detection limits were found in the low ng g^{-1} range between 0.01 ng g^{-1} (d.w.) and 0.025 ng g^{-1} (d.w.) depending on the target analytes. In addition, under optimized conditions, the method gave good levels of intra-day and inter-day repeatabilities in sewage sludge with relative standard deviations varying between 1% to 9% and 6% to 15% respectively (n=5, 1 ng g^{-1} (d.w.)). The applicability of the method was tested with sewage sludge from three urban wastewater treatment plants (WWTPs).The analysis revealed the presence of the macrocyclic musks studied in several samples, with concentrations ranging between below MQL (method quantification limit) and 0.89 ng g^{-1} (d.w.).

Keywords: *GC-MS; headspace solid-phase microextraction; macrocyclic musk fragrances; sewage sludge.*

1. Introduction

In recent years, many analytical methods have been developed to determine emergent organic compounds, such as parabens, pharmaceuticals, iodinated contrast media and musk fragrances among others, in solid samples [1-6]. The extraction of these emerging organic compounds from sediment or sewage sludge samples is commonly performed using Soxhlet extraction [4,7], solid-liquid extraction [8], sonication [5,9], pressurized liquid extraction (PLE) [2,10,11] or microwave-assisted extraction (MAE) [12,13]. However, due to the low selectivity of the extraction techniques mentioned above, extracts from complex samples such as sewage sludge have to be subjected to time-consuming clean-up procedures or preconcentration steps prior to chromatographic analysis, resulting in longer analysis times and increasing solvent consumption.

Due to the low levels expected in environmental samples, wastewater or sewage sludge, environmentally friendly microextraction techniques have been developed to reduce or eliminate the use of organic solvents during the preconcentration steps and decrease the detection limits of the analytical methods [14]. Solid-phase microextraction (SPME), single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME) and microextraction by packed sorbents (MEPS) are just a few examples. Moreover, for the best of our knowledge, only SPME and SDME have successfully been applied to the extraction and preconcentration of certain emerging organic contaminants from sewage sludge samples [3,15-18].

Among the microextraction techniques mentioned above, SPME is the most popular technique because it is simple to perform, easy to automate and solvent free. Thus it has successfully been applied to determine a wide range of pollutants such as aliphatic amines and pharmaceutical products, among others, in sewage sludge samples [17,19,20] that had previously undergone PLE. For this reason, this was chosen as the most suitable microextraction technique for developing a fully automated and sensitive method for the analysis of macrocyclic musk fragrances from sludge samples by gas chromatography mass spectrometry. In contrast to most of published papers in which PLE or MAE were applied before SPME, HS-SPME has been directly applied to sewage sludge samples in our case.

Macrocyclic musks are cyclic personal care products (PCPs) that have not widely been used as polycyclic musk because of the cost of their synthesis. However, they are becoming more and more available because of the advances made in synthesis methods over the last few years [6,21,22]. From certain perspectives, macrocyclic musks have advantages in comparison to the polycyclic musks. They seem to smell more intense and so less mass is needed to achieve the same performance in perfumery. They also seem to be more easily degradable in the environment [23]. Thus, there is a need to develop reliable analytical methods that enable their control in complex environmental samples such as sewage sludge. To the best of our knowledge,

this paper describes the first application of HS-SPME for the determination of macrocyclic musks in sewage sludge samples without any previous extraction or post-clean-up procedure. In fact, most of the studies already published have focused on the synthesis of the macrocyclic musks compounds rather than their presence in environmental samples [21,22].

2. Experimental

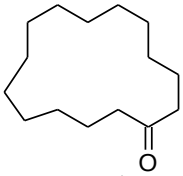
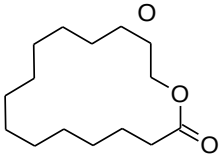
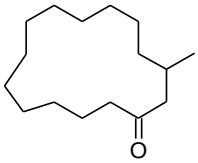
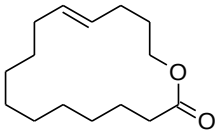
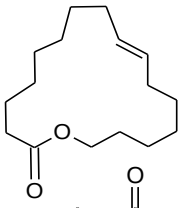
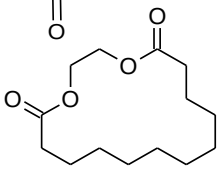
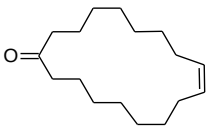
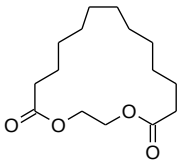
2.1. Chemical standards

The standard 9-cycloheptadecen-1-one (civetone) was supplied by Sigma-Aldrich (Steinheim, Germany). Ethylenedodecanedioate (musk MC4), oxacyclohexadecan-2-one (exaltolide), cyclopentadecanone (exaltone), oxa-cyclohexadecen-2-one (habanolide) and oxacycloheptadec-8-en-2-one (am-brettolide) were purchased from Symta (Madrid, Spain). Ethylenetridecane-dioate (musk NN) as 10 mg L^{-1} solution in cyclohexane, 3-methyl-cyclopentadecanone (muscone) as 100 mg L^{-1} solution in cyclohexane and d15-musk xylene (surrogate standard) as 100 mg L^{-1} solution in acetone were also purchased from Symta (Madrid, Spain). Table 1 shows the main characteristics of the target compounds (formula name, molar mass, octanol/water partition coeffi-cient, boiling point and molecular structure).
Individual 1,000 mg L^{-1} standard solutions of all macrocyclic musks were prepared in cyclohexane with the exception of musk NN, muscone and d15-musk xylene, which were purchased already dissolved. A 10 mg L^{-1} standard solution mixture of all of the target analytes except musk NN was prepared in ethyl acetate. Musk NN standard was supplied at a concentration of 10 mg L^{-1} and was used as received. Cyclohexane and ethyl acetate (VWR Llinars del Vallès, Barcelona, Spain) were GC grade with purities of 99.8% and 99.0%, respectively.
Sodium chloride (ACS reagent ≥ 99%) was supplied by Sigma-Aldrich. Ultrapure water was obtained using a Purelab ultra purification system (Veolia Water, Barcelona, Spain). Helium gas with a purity of 99.999% (Carburos Metálicos, Tarragona, Spain) was used for the chromatographic analysis.

2.2. Sampling and sample preparation

The sewage sludge samples were collected from three urban wastewater treatment plants located in Tarragona (WWTP A), Reus (WWTP B) and Girona (WWTP C). These STPs have a secondary treatment based on activated sludge biological treatment for the removal of dissolved and suspended biological matter [24]. All plants are located in cities with a population greater than 100,000 inhabitants and receive urban wastewater and some industrial discharges. The average flow rates were between 25,000 and 55,000 m^3 day^{-1} and the biological oxygen demands (BOD_5) ranged from 225 to 475 mg L^{-1} for all of the sewage treatment plants. The sewage sludge samples corresponded to a mixture of primary and secondary sewage, which was anaerobically digested and then dehydrated using press filters. After

Table 1. Main characteristics of the target compounds.

Compound	Molar weight (g mol^{-1})	Molecular structure	Boiling point (ºC) 760mmHg	LogK_{ow}
Cyclopentadecanone (Exaltone)	224.39		338.3	5.84
Oxacyclohexadecan-2-one (Exaltolide)	240.39		344.8	6.10
3-Methylcyclopentadecanone (Muscone)	238.41		329.5	6.33
Oxacyclohexadecen-2-one (Habanolide)	238.37		388.4	5.53
Oxacycloheptadec-8-en-2-one (Ambrettolide)	252.39		378.7	5.52
Ethylenedodecanedioate (Musk MC4)	255.33		464.5	2.33
9-Cycloheptadecen-1-one (Civetone)	250.41		371.4	6.31
Ethylenetridecanedioate (Musk NN)	270.36		330.5	2.90

collection, each sludge samples was frozen, lyophilized using the freeze-drying system (Labconco, Kansas City, MO, USA), crushed in a mortar and pestle and sieved through a 125 µm screen to homogenize the diameter of the particles and stored until analysis.
In order to optimize the HS-SPME method, sewage sludge samples were spiked at 1 ng g^{-1} (d.w.) with all of the compounds dissolved in acetone (the required volume to cover the sludge). The acetone was left to evaporate at room temperature in a fume cupboard until the sludge samples were dry (overnight) with frequent homo-genization of the sample.

2.3. Instrumentation

The GC-MS analyses were performed using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) coupled to an ion trap mass spectrometer Varian 4000. The system was controlled by Workstation v.6.9. The GC was equipped with a 1079 programmable temperature vaporizing injector and a 0.8 mm i.d. insert liner (Varian). The chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl/50% dimethylpolysiloxane, 30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex (Torrance, California, USA).
The fully automated HS-SPME experiments were performed with a PDMS/DVB 65µm SPME fibre (Supelco, Bellefonte, PA, USA) on a CTC CombiPal autosampler (CTC Analytics, Zwingen, Switzerland), which was equipped with a sample tray for 32 vials of 20 mL and a temperature-controlled single magnet mixer tray (SMM tray) (Chromtech, Idstein, Germany). The CTC-CombiPal autosampler was controlled and programmed with the Cycle Composer with Macro Editor Software version 2.4.0. Before the initial application and in line with the supplier's instructions, the SPME fibres were conditioned by being inserted into the GC injector.

2.4. Analytical method

The sewage sludge analysis was as follows: 0.25 g (d.w.) of sludge or spiked sludge was placed into 20 mL HS vial with 0.5 mL of ultrapure water and immediately sealed tight with a Teflon septum and placed in a tray for SPME. The vial was then moved to the heating/stirring equipment at 80 °C and after 1 min of stabilization, the PDMS/ DVB 65 µm fibre was then introduced through the septum and kept in the HS of the vial for 45 min, being stirred at 750 rpm. Subsequently, the fibre was withdrawn into the SPME syringe needle and immediately inserted into the GC injection port for desorption. The desorption was conducted at 250 °C for 3 min and the compounds were subsequently analysed by GC-MS.
The PDMS/DVB 65 µm fibres were cleaned by heating to 250 °C for 10 min prior to every extraction, and a blank test was performed to check for possible carry-over problems. As mentioned in the previous section, the whole HS-SPME process (fibre conditioning, microextraction and fibre clean-up) was performed automatically by a commercial CombiPAL auto-

sampler mounted on the GC-MS system.

For the chromatographic analysis, the parameters [25] optimized in previous study were applied. The splitless mode was used for injection and the injector temperature was kept at 250 °C. The GC oven temperature programme was programmed from 100 °C (held for 2.5 min) to 220 °C at 50 °C min^{-1} then 5 °C min^{-1} to 260 °C and 20 °C min^{-1} to 280 °C (held for 10 min). All of the compounds were separated within 10 minutes. Helium was employed as the carrier and collision gas at a flow rate of 1 mL min^{-1}. The ion trap mass spectrometer was operated in the electron impact (EI) ionization mode (70 eV) using an internal ionization configuration. Manifold, ion trap and transfer line temperatures were maintained at 50 °C, 200 °C and 280 °C, respectively. A filament-multiplier delay of 3 min was established in order to prevent instrument damage. The directly coupled mass spectrometer analysed the substances after electron impact ionization in selected ion storage (SIS) mode. Table 2 summarizes the retention time and the qualifier and quantifier ions for each compound.

3. Results and discussion

3.1. HS-SPME optimization

Taking into account our previous experience in the determination of macrocyclic musk in wastewater samples by HS-SPME [25] and previous literature on synthetic musks [15,16], the SPME fibre selected to extract the macrocyclic musks present in sewage sludge samples was PDMS/DVB 65µm. In the same way, the initial conditions selected were as follows: headspace mode, 0.25 g (d.w.) sewage sludge stirred at 750 rpm, extraction time of 45 min, extraction temperature of 100 °C, desorption time of 3 min and desorption temperature of 250 °C [25].

The main parameters affecting the microextraction process, namely sample amount, water addition, extraction temperature and extraction time, were optimized in order to maximize the chromatographic peak area of the compounds by analysing sewage sludge spiked at 1 ng g^{-1} (d.w.) with all of the macrocyclic musk studied.

Firstly, the effect of sample amount on the extracted amount of macrocyclic musks was investigated. A set of experiments was performed using 20 mL vials each containing a different amount of the sewage sludge (0.125, 0.25, 0.5, 0.75 and 1 g (d.w.)), while the analyte concentration remained constant at 1 ng g^{-1}. The results showed that when the sample amount was increased from 0.125 g (d.w.) to 0.25 g (d.w.), a significant increase in the analytical response was observed for all of the target analytes until the maximum peak area was reached (data not shown). However, working at 0.5 g of sample amount, the peak areas decreased to values under those obtained working with 0.125 g (d.w.) of sample amount and at higher sample amounts (0.5-1 g (d.w.)), a slightly decrease in the peak area was observed for most of the target analytes. Only musk MC4 and civetone displayed a different behaviour and an increase in

Table 2. Method parameters. Linear range, determination coefficients, MDLs, MQLs, intra-day and inter-day repeatability.

Compound	Retention time (min)	Ions (SIS) [a]	Determination coefficients (r^2)	linear range [b] (ng g^{-1} (d.w.))	MDLs (ng g^{-1})(d.w.)	Intra-day [c] repeatability (%)	Inter-day [c] repeatability (%)
Exaltone	7.83	**225**, 135, 125	0.999	0.05-10	0.010	1	8
Exaltolide	7.86	**241**, 223, 123	0.998	0.025-10	0.010	9	11
Muscone	7.91	**238**, 209, 125	0.998	0.05-10	0.025	8	10
Habanolide	7.91	239, 221, **95**	0.999	0.05-10	0.025	4	9
Ambrettolide	8.58	235, 135, **95**	0.998	0.025-10	0.010	7	11
Musk MC4	8.99	213, 149, **87**	0.998	0.05-10	0.025	9	13
Civetone	9.42	251, **250**, 121	0.999	0.05-10	0.025	3	6
Musk NN	9.73	227, 211, **98**	0.999	0.025-10	0.010	9	15
d15-MX	8.29	**294**, 136, 122	-	-	-	-	-

[a] Quantifier ions (m/z) are shown in bold type.

[b] MQL (ng g^{-1} (d.w.)): were fixed as the lowest calibration level.

[c] % RSD, n= 5, 1 ng g^{-1} (d.w.).

In the peak areas was observed. Therefore, in order to obtain the highest peak areas for most of the macrocyclic musk studied, 0.25 g (d.w.) was selected as the optimal sample amount and was used to optimize the rest of the HS-SPME variables and to validate the method.

Secondly, ultrapure water was added to the sewage sludge in order to facilitate the desorption and vaporization of the analytes from the sewage sludge to the headspace [15,26,27]. The addition of between 0 mL and 10 mL of water was evaluated. In concordance with the results obtained by Wei [27] and Wu [15] for the determination by microwave-assisted HS-SPME of chlorophenols and polycyclic musks in soils and sewage sludge samples, respectively, the addition of small amounts of water can significantly improve extraction efficiency and, as a result, the peak areas of the target analytes are higher. As can be seen in Fig. 1, a progressive increase in the

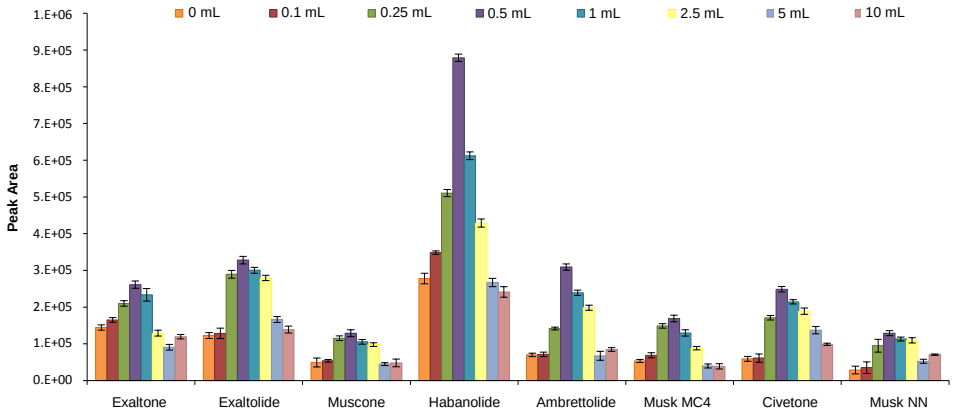


Fig. 1. Comparison of the chromatographic peak areas obtained with a PDMS/DVB 65 µm SPME fibre working with different volumes of water added to 0.25 g (d.w.) of sample under the initial conditions (1 ng g^{-1} (d.w.), *n*=3).

peak areas was observed for the entire range of target analytes up to an optimal water addition of 0.5 mL, after which there was a decrease in the peak areas of all of the target analytes when the addition of water was increased up to 10 mL. Therefore, 0.5 mL was selected as the optimal volume of water to be added to the sewage sludge.

The third parameter optimized was the extraction temperature. Four different extraction temperatures (45 °C, 65 °C, 80 °C, and 100 °C) were chosen to test the effect of this variable on extraction efficiency. Working with 0.25 g (d.w.) of sewage sludge mixed with 0.5 mL of ultrapure water, a successive increase in the peak areas was observed for all of the target analytes up to 80 °C,

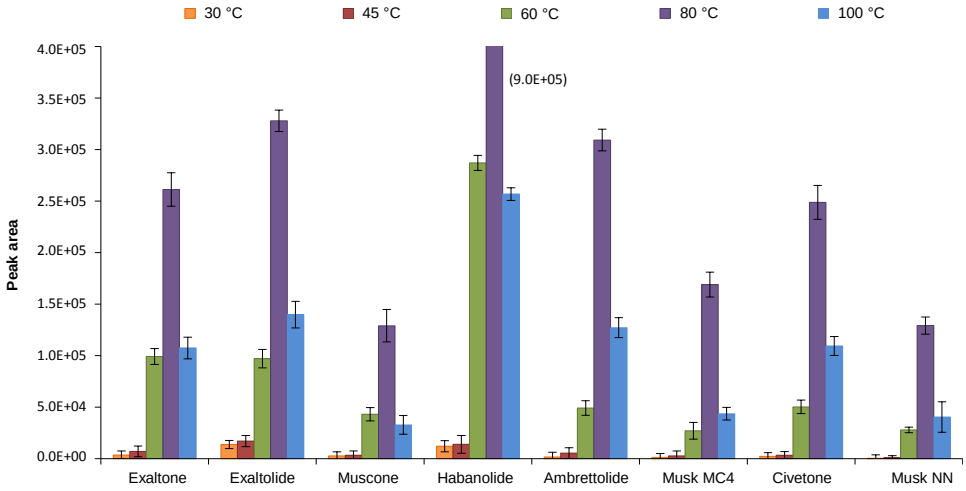


Fig. 2. Comparison of the chromatographic peak areas obtained with PDMS/DVB 65 µm SPME fibre working at different extraction temperatures under the initial conditions (1 ng g^{-1} (d.w.), *n*=3).

because increasing temperature improves the evaporation of the target analytes from the sample to the headspace. While, as can be seen in Fig. 2, working at an extraction temperature of 100 °C, a significant decrease in the peak area of all of the target analytes was observed until reaching values similar to those obtained at an extraction temperature of 60 °C, which could be caused by the increase of interferents in the vapour phase at 100 °C. Eventually, 80 °C was selected as the best extraction temperature because, at this point, all of the macrocyclic musks studied achieved the highest peak areas. Finally, the extraction time was evaluated between 15 and 90 min. A progressive increase (data not shown) in the peak areas was observed for all of the target analytes until reached the equilibrium at 45 min, after which there was not differences between the peak areas obtained at 45 min or higher extraction times as 60 or 90 min. So, 45 min was chosen as the optimal extraction time.

Subsequently, method validation was applied to the simple, fully automated, solvent-free method with minimal pre-treatment developed to determine macrocyclic musks in sewage sludge by HS-SPME under the following optimal conditions: PDMS/DVB 65 µm fibre exposed for 45 min at 80 °C in the headspace of 0.25 g (d.w.) sewage sludge samples mixed with 0.5 mL of water stirred at 750 rpm and a desorption temperature of 250 °C for 3 min.

3.2. Method Validation

The method was analytically validated with a sewage sludge sample from STP A by establishing the linear ranges, method detection limits

(MDLs), method quantification limits (MQLs) and intra-day and inter-day repeatabilities. The sewage sludge sample was analysed (*n*=5) and small peaks of exaltone, muscone, ambrettolide and musk NN appeared in the chromatogram. The average peak areas of these compounds were then subtracted from the corresponding peak areas of each spiked sample.

To evaluate the linear range of the HS-SPME-GC-MS method, a matrix matched-calibration curve was performed by spiking the sewage sludge with all of the target analytes at concentrations between 0.025 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.). As can be seen in Table 2, working with d15-MX (1 ng g^{-1} (d.w.)) as surrogate standard enabled us to obtain determination coefficients (r^2) equal to 0.998 or above for all of the macrocyclic musk studied. Therefore, a directly proportional relationship between the amount of compound extracted and its concentration in the sewage sample was demonstrated.

The method detection limits (MDLs) were defined as the concentration which caused a peak with a signal-to-noise ratio higher than 3 for the compounds that did not appear in the WWTP A sample. MDLs for exaltone, muscone, ambrettolide and musk NN were estimated as the concentration that gave a signal average of plus three times the standard deviation of the WWTP A sample signal. In all cases, the method quantification limits (MQLs) were defined as the lowest point of the calibration curve. The MDLs and MQLs ranged from 0.01 ng g^{-1} (d.w.) to 0.025 ng g^{-1} (d.w.), and from 0.025 ng g^{-1} (d.w.) to 0.05 ng g^{-1} (d.w.), respectively, as shown in Table 2.

Precision were evaluated by calculating intra-day and inter-day repeatabilities. The intra-day repeatabilities were determined by analysing five replicates of the WWTP A sewage sludge sample at 1 ng g^{-1} (d.w.) on the same day. Inter-day repeatabilities were evaluated by determining five replicates of the same sample on five consecutive days (*n*=5). The results obtained, expressed as relative standard deviation (RSD) percentages ranged from 1% to 9% (*n*=5) for intra-day repeatabilities and were lower than 15% for inter-day repeatabilities (Table 2). The validation parameters obtained using HS-SPME for macro-cyclic musk were compared with those obtained when the same micro-extraction technique was used to determine polycyclic musk in sewage sludge samples followed by GC-MS. The results showed that when microwave-assisted HS-SPME was carried out [15], the MDLs for all the polycyclic musks were 0.04 ng g^{-1} (5 g sample amount) with intra-day repeatabilities (RSD %) of 14% or under. And when the same compounds were determined directly by HS-SPME [16] the MDLs were found to be slightly better, ranging between 0.023 ng g^{-1} (d.w.) and 0.052 ng g^{-1} (d.w.)(sample amount: 0.25 g (d.w.)). Our method provides lower MDLs (0.01 ng g^{-1} (d.w.)-0.025 ng g^{-1} (d.w.)) and the automation of the entire HS-SPME results in intra-day repeatability values under 10% for all of the macrocyclic musks studied.

3.3. Method application

The method developed was applied to determine macrocyclic musk fragrances in sewage sludge samples from three urban WWTPs (A, B and C) (Section 2.2). To the best of our knowledge, this is the first time that macrocyclic musks have been determined in sewage sludge samples. Table 3 summarizes the results of the average concentrations of the macrocyclic musk fragrances found in each type of sample (*n*=8).

Analysing sewage sludge from WWTP A, WWTP B or WWTP C, the presence of macrocyclic musks was detected in several samples, with ambrettolide, exaltone and musk NN being the only target analytes found in all of the samples analysed. However, depending on the sewage sludge sample analysed, the most abundant compounds were different. In WWTP A, muscone and ambrettolide were the most abundant compounds, with muscone being detected in the largest quantities with average concentrations between <MQL and 2.0 ng g^{-1} (d.w.). In the case of WWTP B, most of the macrocyclic musks studied were detected at levels below the MQL, with only ambrettolide, musk MC4 and musk NN detected at higher concentrations. In this case, musk NN was the most abundant compound with concentrations between <MQL and 1.45 ng g^{-1} (d.w.). In sludge from WWTP C, all of the compounds studied were present with concentrations ranging between <MQL and 0.89 ng g^{-1} (d.w.), with ambrettolide, habanolide and musk NN being the most abundantly found compounds with maximum concentrations of 0.85 ng g^{-1} (d.w.), 0.50 ng g^{-1} (d.w.) and 0.89 ng g^{-1} (d.w.), respectively. Fig. 3 shows a chromatogram of a sewage sludge sample from WWTP A in which all of the macrocyclic musks studied were determined.

As we have already mentioned, to the best of our knowledge, the presence of

Table 3. Concentrations of macrocyclic musks in sewage sludge samples in ng g^{-1} (d.w.) (*n*=8).

Compound	WWTP A	WWTP B	WWTP C
Exaltone	<MQL-0.08	<MQL	<MQL
Exaltolide	n.d.-0.13	<MQL	<MQL
Muscone	<MQL-2.0	n.d.-<MQL	<MQL
Habanolide	n.d.-0.15	n.d.-<MQL	<MQL-0.50
Ambrettolide	<MQL-0.24	<MQL-0.09	0.09-0.85
Musk MC4	n.d.-0.13	<MQL-0.19	<MQL-0.19
Civetone	n.d.-<MQL	<MQL	<MQL-0.13
Musk NN	<MQL-0.13	<MQL-1.45	<MQL-0.89

n.d.; not detected.
<MQL; values under the method quantification limit.

macrocyclic musk fragrances in sewage sludge has not previously been reported. However, the results are in concordance with previous papers that focus on the determination of macrocyclic musks in wastewater samples [25,28], which indicate the presence of macrocyclic musks in influent and effluent wastewater samples. However, concentrations in effluents were lower than those reported for influents. In addition, these papers [25,28] confirm the findings of the present study, i.e. that ambrettolide is one of the most abundant macrocyclic musks. Although its concentration in sludge is lower than that already reported for wastewater, its presence in sludge suggests that WWTP processes are not effective enough for its complete removal.

4. Conclusions

For the first time, a method which is fully automated, environmentally friendly and without sample pre-treatment has been developed to determine macrocyclic musk fragrances in sewage sludge. The method, which is based on HS-SPME-GC-MS (SIS), provides good linear range, low MDLs and MQLs ranging from 0.01 ng g^{-1} (d.w.) to 0.025 ng g^{-1} (d.w.) and 0.025 ng g^{-1} (d.w.) to 0.05 ng g^{-1} (d.w.), respectively, as well as satisfactory precision, with intra-day and inter-day repeatability values below 9% and 15%

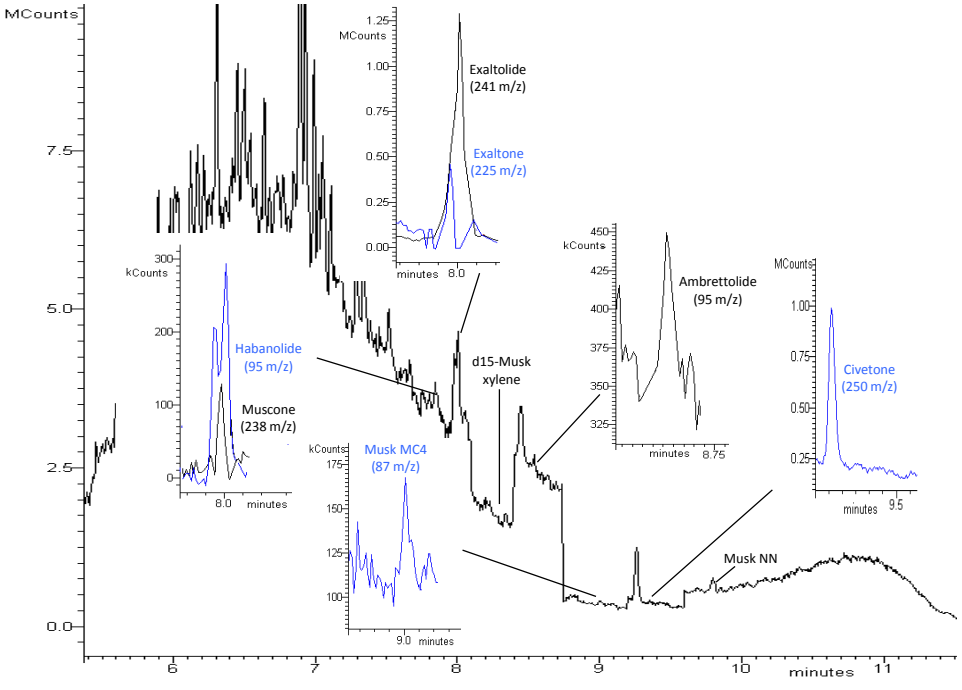


Fig. 3. Chromatogram of a sewage sludge sample from WWTP A.

for all of the target macrocyclic musks.

As well as being an environmentally friendly methodology, SPME is a solventless technique. Meanwhile, the applicability of the HS-SPME directly to the sewage sludge sample instead of the use of an extraction technique, such as accelerated solvent extraction, results in a promising alternative, reducing sample manipulation and treatment time.The main parameters involved in the HS-SPME were evaluated and optimized: sample amount, water addition, extraction temperature and extraction time. The best conditions were found to be the following: PDMS/DVB 65 µm fibre was exposed directly to 0.25 g (d.w.) of lyophilized sewage sludge mixed with 0.5 mL of water for 45 min at 80 °C that was desorbed at 250 °C for 3 min.

The applicability of the method was tested for the first time in sewage sludge from three urban WWTPs. Macrocyclic musks were detected in several samples with concentrations ranging between <MQL and 0.89 ng g^{-1} (d.w.), with ambrettolide, exaltone and musk NN being the only target analytes present in all of the samples analysed.

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3.1.3. On-line coupling of solid-phase extraction to gas chromatography-mass spectrometry to determine musk fragrances in wastewater

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

ON-LINE COUPLING OF SOLID-PHASE EXTRACTION TO GAS CHROMATOGRAPHY-MASS SPECTROMETRY TO DETERMINE MUSK FRAGRANCES IN WASTEWATER

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Abstract

An on-line coupling solid-phase extraction (SPE) has been developed for the first time to preconcentrate trace amounts of 17 musk fragrances extensively used in personal care products from wastewater samples, prior to analysis by gas chromatography and mass spectrometry through an on-column interface. A 10 mm x 2 mm I.D. precolumn packed with Oasis HLB (60µm) or C18 (60µm) was compared for the optimization of the solid-phase extraction process. The parameters affecting the transfer of the analytes from the precolumn to the GC system (e.g. flow-rate, temperature and solvent vapour exit time) as well as SPE parameters (e.g. sample flow, sample volume, elution solvent, etc.) were optimized. An organic modifier such as methanol was added to the sample before the extraction process to avoid adsorption problems. The use of the MS detector under selected ion monitoring acquisition enabled the analytes to be quantified at low ng L^{-1} levels, preconcentrating only 10 mL of sample, and the limits of detection were between 1 and 30 ng L^{-1}. The method was applied for the determination of musk fragrances in wastewater samples from three urban wastewater treatment plants (WWTPs). The analysis of influent urban wastewater revealed that galaxolide, tonalide and ambrettolide were the most abundant musk compounds with concentrations ranging between 818 ng L^{-1} and 45,091 ng L^{-1}, 852 ng L^{-1} and 49,904 ng L^{-1} and 507 ng L^{-1} and 21,528 ng L^{-1} respectively. The remaining musks were present at lower concentrations and two of the macrocyclic musk studied (musk MC4 and civetone) were not detected. The analysis of effluent wastewater showed a decrease in the concentrations of all of the compounds present in influent samples, with the decrease being more significantly in the case of polycyclic and nitro musks than for macrocyclic musks. Only HHCB-lactone remained constant or increased its concentration.

Keywords: gas chromatography-mass spectrometry; musk fragrances; on-line solid-phase extraction; wastewater.

1. Introduction

Among the extensive group of emerging compounds, musk fragrances such as polycyclic musks, nitro musks and macrocyclic musks, which are extensively used in soaps, cosmetics and other personal care products (PCPs) have gained increasing interest due to their presence in environmental waters [1,2]. A vast majority of these products are lipophilic compounds and, due to their use in many PCPs and the lack of effectiveness of procedures for their removal at wastewater treatment plants (WWTPs), they can enter aquatic environments. Therefore, the identification and quantification of these products is important. For this reason, many analytical methods based on gas chromatography (GC) mass spectrometry or tandem mass spectrometry have been developed in the last few years [3,4].

Due to the low concentrations at which musk fragrances are found in environmental water samples, some preconcentration techniques such as liquid-liquid extraction (LLE) [5-7], solid-phase extraction (SPE) [5,8-12], dispersive liquid-liquid microextraction (DLLME) [13-15], solid-phase microextraction (SPME) [16,17] single-drop microextraction (SDME) [18,19] or microextraction by packed sorbents (MEPS) [20,21] have been reported. Of all the extraction techniques mentioned above, SPE is the most widely used in the environmental analytical field because it extracts and preconcentrates in a single step and a great diversity of sorbents is commercially available. Nevertheless, new microextraction techniques have recently been developed to solve some of the drawbacks of SPE and try to reduce or eliminate the use of organic solvents during the preconcentration steps, while also reducing the requirements of large volumes of sample to obtain more environmentally friendly analytical methods [22,23].

On-line SPE coupled to LC or GC appears to solve the disadvantages of off-line SPE, such as an increased chance of losses during sample handling and the requirement of large volume samples. Moreover, the automation of all the SPE procedure minimizes sample losses or contaminations during handling and improves the reproducibility of the analysis. Another advantage is the reduction of both sample volume and analysis time. In addition, it has also been applied for the determination of emerging organic compounds in environmental water samples [24-26]. However, on-line solid-phase extraction coupling to GC requires the injection of relatively large volumes of organic solvents, while conventional GC injectors only permit microliters. Therefore, the use of an injection technique is required, such as partially concurrent solvent evaporation (PCSE) using an on-column interface [27-35]. The aim of this study is the development of an automated method for determining musk fragrances in water samples using an on-line SPE-GC-MS system with an on-column interface. To the best of our knowledge, this on-line combination has never been used before to determine such a wide variety of musk

fragrances that includes the most extensively used polycyclic musks, nitro musks and macrocyclic musks.

2. Experimental

2.1. Standards and reagents

Of the synthetic musk fragrances studied, the following polycyclic musks: 6,7 - dihydro - 1,1,2,3,3 - pentamethyl 4(*5H*)-indanone (cashmeran, DPMI), 4-acetyl-1,1-dimethyl-6-*tert*- butylindane (ADBI, celestolide) 6-acetyl-1,1,2,3,3,5-hexamethylindane (phantolide, AHMI), 5-acetyl- 1,1,2,6- tetramethyl-3-isopropylindane (traseolide, ATII), 1,3,4,6, 7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(*g*)-2-benzopyran (galaxolide, HHCB) and 7-acetyl-1,1,3,4,4, 6-hexa-methyl-1,2,3,4-tetrahydronaphthalene (tonalide, AHTN) were supplied by Promochem Iberia (Barcelona, Spain). 1,3,4,6,7,8-hexahydro-4,6,6,7,8, 8-hexa-methylcyclopenta-(*g*)-2-benzopyran-1-one (galaxolidone, HHCB-lactone) were provided by International Flavors & Fragrances Inc. (Barcelona, Spain). The nitro musk fragrances 2,4, 6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (musk xylene, MX) and 1,1,3,3,5-pentamethyl-4,6-dinitroindane (musk moskene, MM) were purchased as 100 ng μL^{-1} solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Häen (Seelze, Germany), respectively. The standard 4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (musk ketone, MK) was provided by Fluka (Buchs, Switzerland). The macrocyclic musk fragrances ethylenedodecanedioate (musk MC4), oxacyclohexadecan-2-one (exaltolide), cyclopentadecanone (Exaltone) and oxacycloheptadec-8-en-2-one (ambrettolide) were purchased from Symta (Madrid, Spain). 9-cycloheptadecen-1-one (civetone) was supplied by Sigma-Aldrich (Steinheim, Germany). Ethylene-tridecanedioate (musk NN) as 10 ng μL^{-1} solution in cyclohexane, 3-methylcyclopentadecanone (muscone) as 100 ng μL^{-1} solution in cyclohexane and d15-musk xylene (labelled internal standard) as 100 ng μL^{-1} solution in acetone were also purchased from Symta (Madrid, Spain). Table 1 shows the main characteristics (formula name, CAS number, molar mass, boiling point and the octanol/water partition coefficient) of the target compounds [4,21,36,37].

Individual 1,000 ng μL^{-1} standard solutions of all macrocyclic musks were prepared in cyclohexane with the exception of musk NN and muscone which were purchased already dissolved. Individual standard solutions of the polycyclic musks were prepared in acetone at concentrations of 4,000 ng μL^{-1} and 1,000 ng μL^{-1} for musk ketone and HHCB-lactone. A solution of 1 ng μL^{-1} in ethyl acetate was prepared weekly from the individual standard solutions and used to prepare diluted solutions and to spike water samples to the required concentrations.

Trace analysis grade cyclohexane, acetone and ethyl acetate were purchased from VWR (Llinars del Vallès, Barcelona, Spain). The methanol used as organic modifier in the on-line SPE was GC grade with purity > 99.9% (SDS, Peypin, France). The chromic mixture and HPLC grade isopropanol used for cleaning of the glassware were from Sigma Aldrich and VWR, respectively.

Ultrapure water was obtained using a Purelab ultra purification system

(Veolia Water, Barcelona, Spain). Helium and nitrogen were supplied by Carburos Metálicos (Tarragona, Spain) with a quality of 99.999%.

2.2. Instrumentation

Chromatographic analysis were performed using a Hewlett-Packard HP 6890 Series gas chromatograph (Waldbronn, Germany) equipped with an on-column injector and an HP 5973 mass selective detector. In order to inject large volumes and perform the chromatographic separation, a 5 m x 530 µm I.D. retention gap from Micron Phenomenex (Torrance, California, USA), a 2 m x 250 µm I.D, 0.25 µm retaining precolumn and a 30 m x 250 µm I.D., 0.25 µm analytical column, both ZB-50 (50% phenyl/ 50% dimethylpolysiloxane) and from Phenomenex, were installed coupled to a solvent vent valve. The connection between the retention gap and the retaining precolumn was made with an ultimate union from Agilent Technologies (Palo Alto, USA) and a quartz press-fit splitter (Agilent Technologies, Palo Alto USA) was chosen to conduct the excess of solvent injected to the solvent vent valve and the target analytes to the analytical column. Chromatographic data were recorded using a G1701DA MSD ChemStation, which was controlled by Windows (Microsoft).

For the solid-phase extraction, the precolumn (10 x 2 mm I.D.) was hand-packed with 20 mg of C18 (60 µm) or Oasis HLB (60 µm) sorbent from Scharlab (Barcelona, Spain) and Waters (Cerdanyola del Vallès, Barcelona, Spain), respectively. Three six-port Valco valves (Houston, USA) controlled by GC software were used in the SPE process. An HP 1100 pump was used to deliver the sample and the solvents needed to clean and activate the sorbent. The eluent was delivered with a syringe pump (Cole-Parmer, Illinois, USA). The analytes were transferred from the precolumn to the GC system via a 15 cm x 0.5 mm I.D. polyether ether ketone (PEEK) tubing connected to a syringe needle (point style 2). A 100 µm loop of PEEK tubing was used instead of the precolumn for direct injection. The scheme of the equipment described above to perform the on-line SPE-GC-MS method is shown in Fig. 1.

2.3. Sampling

Influent and effluent wastewater samples were collected between October 2012 and February 2013 at three urban wastewater treatment plants (WWTPs) located in Catalonia (NE Spain). The WWTPs receive urban sewages and some industrial discharges. All of the urban wastewater samples from WWTPs (A and B) were taken from the influent and effluent of the activate sludge biological treatment. However, WWTP C samples were taken from the influent and the effluent of the tertiary treatment based on reverse osmosis. Each sample was collected in a 200 mL pre-cleaned glass bottle, filtered through a 0.22 µm nylon filter (Scharlab, Barcelona, Spain) and stored at 4ºC until analysis.

Although a pretreatment step based on filtration with nylon filters (i.e. 0.45 µm or 0.22 µm) is a routine procedure in laboratories when working with wastewater or environmental samples

Table 1. Main characteristics of the target compounds.

nº	Formula name	CAS number	Molar mass (g mol^{-1})	Boiling point (ºC) 760 mmHg	Log K_{ow} [a)]
	Polycyclic musks				
1	6,7-dihydro-1,1,2,3,3-pentamethyl-4(*5H*)-indanone (Cashmean, DPMI)	33704-61-9	206.3	286.1	4.9
2	4-acetyl-1,1-dimethyl-6-*tert*-butylindane (Celestolide, ADBI)	13171-00-1	244.4	309	6.6
3	6-acetyl-1,1,2,3,3,5-hexamethylindane (Phantolide, AHMI)	15323-35-0	244.4	336.6	6.7
4	5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (Traseolide, ATII)	68140-48-7	258.4	350	6.7
5	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran (Galaxolide, HHCB)	1222-05-5	258.4	326	5.9
6	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN)	1506-02-1	258.4	356.8	5.7
7	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran-1-one (Galaxolidone, HHCB-lactone)	*	272.4	*	*
	Nitro musks				
8	2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (Musk xylene, MX)	81-15-2	297.3	392.3	4.8
9	1,1,3,3,5-pentamethyl-4,6-dinitoindane (Musk moskene, MM)	116-66-5	278.3	351.1	5.8
10	4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (Musk ketone, MK)	81-14-1	294.3	369	4.3
	Macrocyclic musks				
11	Cyclopentadecanone (Exaltone)	502-72-7	224.4	338.3	5.8
12	Oxacyclohexadecan-2-one (Exaltolide)	106-02-5	240.4	344.8	6.1
13	3-Methylcyclopentadecanone (Muscone)	541-91-3	238.4	329.5	6.3
14	Oxacycloheptadec-8-en-2-one (Ambrettolide)	123-69-3	252.4	378.7	5.5
15	Ethylenedodecanedioate (Musk MC4)	54982-83-1	255.3	464.5	2.3
16	9-Cycloheptadecen-1-one (Civetone)	542-46-1	250.4	371.4	6.3
17	Ethylenetridecanedioate (Musk NN)	105-95-3	270.4	330.5	2.9

* Information not found at the bibliography.
[a)] Log K_{ow} values predicted from SRC-K_{ow}Win Software.

[38-40], due to the lipophilic properties of the target compounds, the filtration step was checked to assure good recovery values and prevent from significant losses of the target analytes. The results showed recovery values higher than 90% for all the target analytes independently of the kind of water filtered.

The extensive use of synthetic musks as fragrances in a wide range of consumer products means that there is a high risk of samples becoming contaminated. Therefore, special precautions were required for the whole analytical procedure. The glassware used for the sampling and the extraction step, such as vials, bottles and volumetric material, was cleaned overnight with chromic mixture and then rinsed five times with ultrapure water and five times with HPLC grade isopropanol. Furthermore, musk-free gloves were used and the samples were prepared in a fume cupboard.

2.4. On-line trace enrichment and GC-MS procedure

The on-line trace enrichment experiments were performed using three six-port valves connected in series to make the different steps of the preconcentration process possible. Firstly, the Oasis HLB (60 µm) precolumn was conditioned with 3 mL of ethyl acetate and 3 mL of ultrapure water (50% methanol). Then, 10 mL of the sample containing 50% of methanol was preconcentrated. The flow-rate used throughout all of this process was 2 mL min^{-1} and the tubes were purged with the corresponding solution (ethyl acetate, ultrapure water or sample) before it was passed through the precolumn. In the next step, the precolumn was dried with 3 bars nitrogen for 30 min to remove the water. The analytes trapped in the Oasis HLB precolumn were desorbed in the back-flush mode with 100 µL of ethyl acetate, which was pumped at 40 µL min^{-1} with a syringe pump and on-line transferred to the GC system through the transfer line. The solvent vapour exit (SVE) was opened three seconds before the transfer started and closed 1.5 min after the end of the transfer in order to eliminate the ethyl acetate vapours without losing the analytes. The oven temperature was kept at 60 °C during the transfer and the temperature programme started 5 min after the SVE was closed, thus ensuring the elution of the solvent peak. The sequence followed in the extraction and transfer process is described in Table 2 and the scheme of the on-line-SPE-GC-MS system is shown in Fig. 1. The initial temperature of 60 °C that was maintained constant until the analytes had been preconcentrated and transferred was then increased to 180 °C at 40 °C min^{-1}, to 220 °C at 5 °C min^{-1} and finally to 280 °C at 20 °C min^{-1} (2 min). The on-column injector operated in the track oven mode and the carrier gas (helium) was maintained at a flow rate of 1.2 mL min^{-1}. The MS transfer line was kept at 280 °C to prevent analytes from recondensing. The MS-detector acquired in the selected ion monitoring mode (SIM) at electron impact energy of 70 eV SIM detection was carried out by using nine time windows due to the chromato-graphic separation obtained. Three mass fragments were selected for

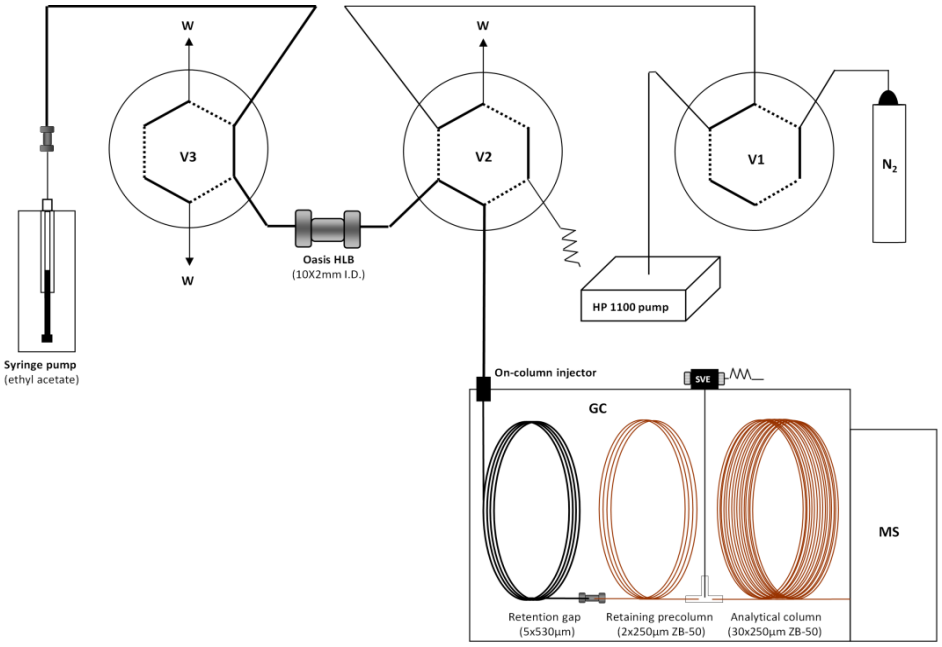

Fig. 1. Set-up of the on-line SPE-GC-MS system. Elution step (V1 = Off, V2 = Off, V3 = Off).

each compound. For the polycyclic and nitro musks studied the most intense ions (relative abundance of 100%), which correspond to the molecular ions were used for quantification and the other ions were used to confirm the presence of the compounds. However, for the macrocyclic musks the ions with a 100% of relative abundance are low m/z ions such as 55, 98 and 67. Due to the potentially presence of interfering compounds (cholesterols, phthalates, …) in wastewater samples and that the ions of these non-targeted compounds may interfere with the signal of the low m/z ions, higher m/z ions with low abundance, which correspond to the molecular ions were selected for the quantification and identification of the macrocyclic musks. The quantifier and qualifier ions are shown in Table 3.

3. Results and discussion

3.1. Chromatographic separation

The chromatographic separation was optimized by GC-MS by manually injecting 1 µL of a standard solution containing 10 ng μL^{-1} of each compound in ethyl acetate into the on-column injector, using an electron ionization fragmentation and full scan acquisition mode. Each of the target compounds were identified by their molecular ion and, afterwards, the chromatographic separation was optimized by testing two analytical columns, a ZB-5 (5% phenyl/95% dimethylpolysiloxane) and

Table 2. Programme for the SPE and transfer processes.

Time (min)	Valve	Event
0.01	SVE off	Wash tubes with ethyl acetate
	V1 off	
	V2 off	
	V3 on	
3	V2 on	Condition precolumn with 3 mL of ethyl acetate
4.50	V2 off	Wash tubes with ultrapure water
7.50	V2 on	Activate precolumn with 3 mL of ultrapure water
9	V2 off	Wash tube with sample (50% MeOH)
12	V2 on	Preconcentrate 10 mL of sample (50%MeOH)
17	V1 on	Dry precolumn with 3bar N_2 for 30 min
46.95	SVE on	
47	V1 off	Transfer analytes with ethyl acetate
	V2 off	(100 µL, 40µL min^{-1})
	V3 off	
50.25	V2 on	End of transfer
	V3 on	
51.75	SVE off	
56.75		Start GC programme

a ZB-50 (50% phenyl/50% dimethyl-polysiloxane), both columns were 30 m x 250 µm I.D., 0.25 µm (Phenomenex). The effects of the helium flow and oven temperature programme on the separation of the target compounds were also evaluated. As a result, the separation was performed in less than 10 min in a ZB-50 analytical column, as described in Section 2.4. The chromatogram (Fig. 2a) also shows the separation of the four HHCB enantiomers (4S,7S; 4S,7R; 4R,7S and 4R,7R). The HHCB was quantified by integrating the 4S and 7R/S isomers peaks. These were chosen because of their high chromatographic signal and the fact that they are the diastereoisomers responsible for the musky odour [37,41]. In order to improve the sensitivity/selectivity of the compounds, selected ion monitoring (SIM) mode in mass spectrometry was selected for the work. Table 3 also summarizes the ions selected for identifying and quantifying the target compounds.

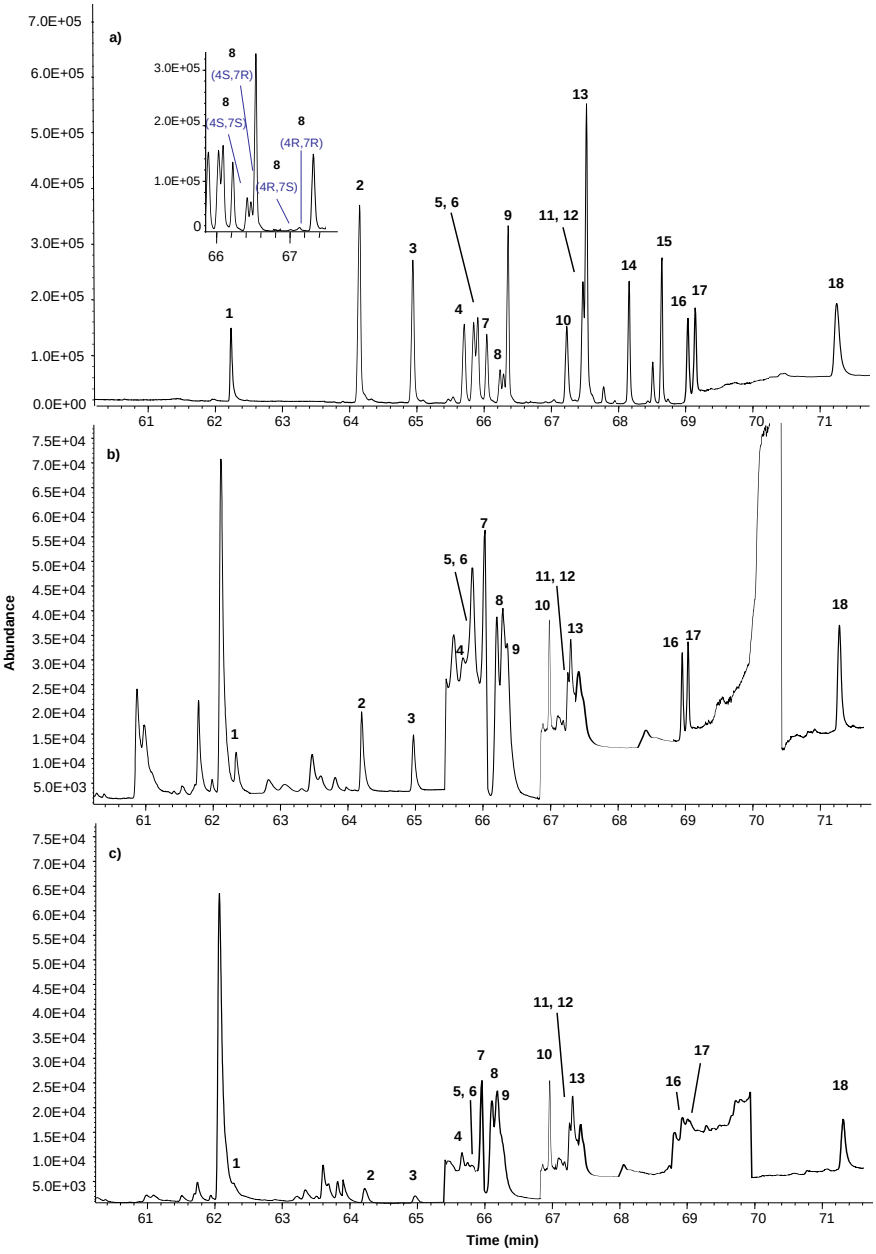

Fig. 2. Chromatogram obtained by on-line SPE-GC-MS: (a) 100µL loop of a 0.1 ng μL^{-1} standard solution (SCAN) and analytical signal enlargements of the HHCB enantiomers; (b) 10 mL of influent sample from WWTP A (SIM); (c) 10 mL of effluent sample from WWTP A (SIM). Peaks: 1, cashmeran; 2, celestolide; 3, phantolide; 4, exaltone; 5, exaltolide; 6, muscone; 7, traseolide; 8, galaxolide; 9, tonalide; 10, d15-MX; 11, MX; 12, MM; 13, ambrettolide; 14, musk MC4; 15, civetone; 16, musk NN; 17, MK; 18, HHCB-lactone.

Table 3. Time window, selected ions and validation data for influent and effluent WWTP B water.

Compound	Time window	Ions (SIM) [a] (m/z)	Influent				Effluent			
			Linear range [b] (ng L^{-1})	MDLs (ng L^{-1})	Intra-day [d] Repeatability (RSD %)	Inter-day [d] Repeatability (RSD %)	Linear range [b] (ng L^{-1})	MDLs (ng L^{-1})	Intra-day [d] Repeatability (RSD %)	Inter-day [d] Repeatability (RSD %)
DPMI	1	135,**191**, 206	10-5,000	5	6	9	7.5-5,000	2.5	4	14
ADBI	2	173, **229**, 244	7.5-5,000	1	7	10	5-5,000	1	5	11
AHMI	2	187, **229**, 244	7.5-5,000	3	6	10	5-5,000	1	5	13
Exaltone	3	125, 135, **224**	25-5,000	15	3	8	20-5,000	12	2	9
Exaltolide	3	124, **222**, 240	40-5,000	30	5	8	35-5,000	25	5	9
Muscone	3	125, 209, **238**	35-5,000	20	3	7	30-5,000	18	3	6
ATII	3	173, **215**, 258	7.5-5,000	2.5	5	11	5-5,000	1	5	10
HHCB	4	213, **243**, 258	5-5,000	2	6	8	5-5,000	1.5	6	8
AHTN	4	**243**, 244, 258	5-5,000	2.5	4	12	5-5,000	2	3	9
MX	5	253, **282**, 283	7.5-5,000	3	4	10	7.5-5,000	2.5	2	8
MM	5	253, **263**, 264	7.5-5,000	5	5	10	5-5,000	1	4	4
Ambrettolide	5	135, 195, **235**	25-5,000	15	4	8	25-5,000	15	5	8
Musk MC4	6	149, 197, **213**	25-5,000	10	5	11	20-5,000	10	7	9
Civetone	7	121, **250**, 251	30-5,000	15	6	8	25-5,000	12	4	6
Musk NN	7	197, 211, **227**	35-5,000	18	8	8	30-5,000	15	7	7
MK	8	253, **279**, 294	7.5-5,000	2.5	5	7	5-5,000	1	4	7
HHCB-Lactone	9	253, 197, **257**	7.5-5,000	7	4	7	7.5-5,000	6	3	6
d15-MX [c]	5	285, 218, **294**								

[a] Quantifier ions (m/z) are shown in bold type.
[b] MQLs (ng L^{-1}); were fixed as the lowest calibration level.
[c] d15-MX is the labelled internal standard.
[d] n=3, 1,000 ng L^{-1}.

3.2. Optimization of transfer conditions

The transfer conditions were optimized in order to operate under partially concurrent solvent evaporation (PCSE) conditions using an on-column interface. The transfer conditions, such as transfer flow-rate, transfer temperature and SVE open time, were the parameters optimized. They were all optimized by injecting 100 µL of a standard solution containing 0.1 ng μL^{-1} of all of the target analytes in ethyl acetate. A 100 µL loop which was filled with the standard solution using a syringe was used for these injections instead of the SPE precolumn. The ethyl acetate from the syringe pump then pushed the standard solution into the retention gap.

The transfer flow-rate was increased step-by-step from 35 µL min^{-1} to 50 µL min^{-1}, the value that caused the distortion of the analyte peaks, which suggests that the retaining precolumn had been flooded with ethyl acetate. The transfer temperature was also varied from 60 °C to 70°C. Higher temperatures were not tested so as not to exceed the boiling point of the solvent (ethyl acetate), which would remove the solvent film created in the retention gap. The SVE was opened 3 s before the transfer started and closed 1 min after the transfer finished for eliminating most of the vapour generated during the injection of 100 µL of standard solution. The optimal temperature and flow-rate were 60 °C and 40 µL min^{-1}, respectively. This flow-rate was 5 µL min^{-1} below the rate which caused flooding and it was chosen to prevent peak distortion.

Finally, the SVE open time was optimized. The SVE was opened 3 s before the transfer started and closed at different times, between 1 and 2 min, after the transfer finished. A time of 1.5 min was selected as optimal because most of the ethyl acetate vapours generated were eliminated and no decrease in peak areas of the target analytes was observed. Fig. 2a showed a chromatogram obtained in SCAN mode under optimal transfer conditions (injection of a 100 µL standard solution containing 0.1 ng μL^{-1} of all of the target analytes in ethyl acetate).

3.3. Optimization of the SPE process

First of all, based on our previous experience in this field and the literature, certain parameters that affect SPE were established. Two types of adsorbents were selected as the sorbents for the SPE precolumn, octadecyl bonded silica (C18) and divinylbenzene/N-vinylpyrrolidone co-polymer (Oasis HLB), both with 60 µm particle size, because of their ability to extract macrocyclic musk and polycyclic and nitro musk, respectively [3,20,21,42-45]. Ethyl acetate was chosen as the desorption solvent because it desorbs analytes with a wide range of polarities from C18 or Oasis HLB packed SPE precolumns and can also be used under partially concurrent solvent evaporation (PCSE) conditions. Before the elution step, the sorbent was dried with nitrogen to remove all water from the precolumn. Although in

some papers, such as Koning *et al.* [46] and Brossa *et al*. [25], only 15 min were used to dry the sorbent, the most common conditions [47], 3 bar N_2 for 30 min, were applied to ensure the elimination of the water. This is a critical point in the on-line SPE coupling to GC because traces of water can destroy the deactivation of the retention gap.

After selecting these parameters, sample flow, elution volume, sample pretreatment and sample volume were optimized for maximum recoveries. Standards of 2 mL in ultra-pure water spiked at 5 ng mL^{-1} with all of the target analytes were used for the optimization of the on-line SPE. Recoveries were calculated by comparing the concentration found with a calibration curve obtained by 100 µL loop direct injections (0.05-0.5 ng μL^{-1}) with the expected concentration.

The initial conditions used for each sorbent were as follows: sample flow 2 mL min^{-1}, 2 mL sample volume, 3 bars N_2 for 30 min, 100 µL of ethyl acetate as the elution solvent injected at a flow rate of 40 µL min^{-1}.

Firstly, the sample flow was studied by comparing the recoveries obtained at 1 mL min^{-1}, 1.5 mL min^{-1}, 2 mL min^{-1}, 2.5 mL min^{-1} and 3 mL min^{-1}. Working with C18 sorbent, all of the compounds achieved the highest recoveries at 1.5 mL min^{-1} and a significant decrease in its recoveries was observed at higher flow rates. Meanwhile, when an Oasis HLB was used as the extraction sorbent, all of the compounds achieved the highest recoveries working at 2 mL min^{-1} and the increase in the sample flow turned into a gradual decrease of the recoveries. Therefore, 1.5 and 2 mL min^{-1} were the sample flow rates selected for working with C18 and Oasis HLB, respectively. Secondly, the elution volume was optimized by preconcentrating 2 mL of working solution (5 ng mL^{-1}) and eluting with volumes of ethyl acetate between 100 and 400 µL. Regardless of which extraction sorbent was used, 100 µL of ethyl acetate was chosen as the optimal elution volume because recoveries were no greater when the elution volume was higher.

In order to increase the low recoveries caused by the adsorption of analytes in the system [25,47], different quantities of methanol (0-80%) were then added to the sample, which was prepared with ultrapure water spiked at 5 ng mL^{-1} of each musk fragrance. The results showed that the presence of methanol increased the recoveries of all of the target analytes until reached the highest recovery values working with 50% of methanol, independently of the extraction sorbent used (data not shown). Higher methanol percentages (60, 70 or 80%) were studied but this meant that recoveries of cashmeran would decrease significantly. Taking into account the recoveries of all of the target analytes, 50% was chosen as the best condition.

Finally, the influence of the sample volume on the efficiency of the on-line SPE was tested by preconcentrating volumes between 2 and 50 mL of working solution (50% methanol) at different concentrations, so that theoretical final amount preconcentrated (10 ng) would remain constant. Working with C18 sorbent, a significant decrease in the recoveries of

all of the target analytes was observed when the sample volume was extended to values higher than 2 mL. However, when Oasis HLB was used as the extraction sorbent, no significant differences were observed between the recoveries obtained working at samples volumes of 2 mL, 5 mL or 10 mL. At higher sample volumes (i.e. 25 mL and 50 mL) a significant decrease in the target analytes was observed (data not shown). Therefore, taking into account the recovery values, 2 mL and 10 mL were selected as the optimal sample volumes for C18 and Oasis HLB, respectively.

In order to choose the most appropriate sorbent to preconcentrate trace amounts of the target analytes presents in the water samples, both C18 and Oasis HLB sorbents were compared in terms of overall extraction efficiency and selectivity when applied to a wastewater sample.

As can be seen in Fig. 3, recovery values obtained with Oasis HLB were higher than 80% for the vast majority of the

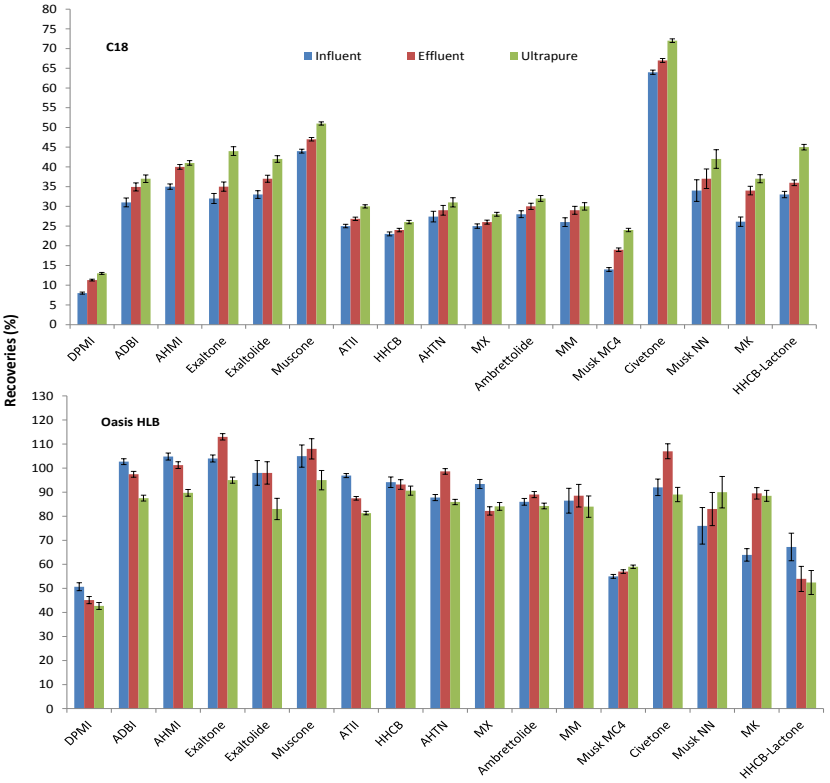

Fig. 3. Recovery values obtained with C18 and Oasis HLB sorbent under optimal conditions. C18: 2mL sample volume at 1.5 mL min^{-1}, dried with 3 bar N_2 for 30 min and eluted with 100 µL of ethyl acetate. Oasis HLB: 10 mL sample volume at 2 mL min^{-1}, dried with 3 bar N_2 and eluted with 100 µL of ethyl acetate.

target analytes even in influent waters, except DPMI (43-51%), musk MC4 (54-60%) and HHCB-lactone (52-67%). In contrast, with a C18 sorbent, no quantitatively representative recovery values were obtained, regardless of the kind of water analysed, with values under 50% for all of the target analytes, except civetone (64-72%). In addition, the analytical signals obtained in the chromatogram were studied to select the sorbent that provided the best selectivity. Independently of the matrix analysed (influent wastewater, effluent wastewater or ultrapure water), Oasis HLB sorbent proved to be the most selective sorbent with cleaner chromatograms and lower base lines. Therefore Oasis HLB was chosen as the optimal extraction sorbent for the extraction of the musk fragrances present in wastewater samples.

3.4. Method validation

The method was analytically validated working with an Oasis HLB sorbent, 10 mL sample (50% methanol) at 2 mL min^{-1} flow rate, dried with 3 bars N_2 for 30 min and eluted with 100 µL of ethyl acetate at 40 µL min^{-1}. Procedural blanks of the conditioned Oasis HLB (60 µm) precolumn were performed before on-line SPE each five analysis in order to prevent carry over effects and ensure the repeatability of the analytical method. If no signal of the target analytes was found in the Oasis HLB precolumn blanks, it could also be reused for several times (40 times). In addition blank of the system were conducted daily to avoid memory effects. None of the blanks contained detectable traces of the target analytes.

Before validating the method, the matrix effect was studied by statistically comparing the slopes of the calibration curves for influent and effluent WWTPs samples with those obtained using ultrapure water. As expected, the matrix effect was observed in both kinds of water, especially influent water. In order to correct the matrix effect, a labelled internal standard (LIS) d15-MX was used. However, the matrix effect observed could only be partially corrected by the presence of the LIS. To this end, both an influent and an effluent sample from WWTP B were used to validate the method by establishing the linear ranges, method detection limits (MDLs), method quantification limits (MQLs), and intra-day and inter-day repeatabilities.

The WWTP B samples used to validate the method were analysed (n=5) and some peaks of AHMI, HHCB, AHTN, ambrettolide, and HHCB-lactone appeared in the chromatogram of the influent sample while, in the effluent sample, only HHCB, AHTN and HHCB-lactone were found. The average peak area (n=5) of each detected compound was subtracted from the peak area of each spiked sample. The concentrations found in the WWTP B samples used to validate the method were placed in Table 4 and corresponding to the lowest level of concentrations found in the WWTP B. The linear range of the method was obtained by analysing the WWTP B samples spiked with all of the target analytes at concentrations between 5 ng L^{-1} and 5,000 ng L^{-1} while

the LIS concentration remained constant at 1,000 ng L^{-1}. As can be seen in Table 3, good linear ranges were obtained working with both WWTP B influent and effluent samples. In addition, the presence of a LIS enabled us to improve the determination coefficients (r^2) of the calibration curves to values higher than 0.993 for all of the target analytes (data not shown).

The MDLs were defined as the concentration of analytes in the WWTP B influent or effluent which caused a peak with a signal-to-noise ratio higher than three for the compounds that did not appear in the blank. For the compounds that appeared in the blank, the MDLs were estimated as three times the standard deviation of the blank. In all cases, the MQLs were fixed at the lowest calibration level. The MDLs and MQLs ranged from 1 ng L^{-1} to 30 ng L^{-1} and from 5 ng L^{-1} to 40 ng L^{-1}, respectively, and they are summarized in Table 3.

The intra-day and inter-day repeatabilities were determined by spiking three replicates of the influent and effluent WWTP B samples at 1,000 ng L^{-1}. The presence of the LIS (d15-MX) improved the method repeatabilities, expressed as relative standard deviation (RSD) percentages, obtaining intra-day repeatability values lower than 8% and inter-day repeatability values below 14% for all of the compounds analysed (Table 3).

The figures of merit obtained using on-line SPE and GC-MS were compared with those obtained using other extraction techniques such as SPE [8-10], MEPS [20,21], SPME [16,17,48], SDME [19] or DLLME [15] followed by GC-MS. Of these extraction techniques, on-line SPE (10 mL sample volume) is the microextraction technique with the lowest MDLs, with values ranging between 1 ng L^{-1} and 30 ng L^{-1}, which is only beaten by SPME (10 mL sample volume) with slightly better MDLs (0.1 ng L^{-1} and 5 ng L^{-1}). Apart from that, the automation of the entire on-line SPE provided intra-day and inter-day repeatabilities comparable with those obtained with the other microextraction techniques, with values of relative standard deviation percentages ranging between 2%-8% and 6%-14%, respectively. In addition on-line SPE is a promising alternative to laborious and time consuming standard SPE enrichment. Moreover the sample volume needed for the extraction and the volume of organic solvent used decrease significantly.

3.5. Method application

The method developed was applied to determine the presence of musk fragrances in different kinds of water: influent and effluent samples collected from the secondary treatment in urban WWTPs A and B and influent and effluent samples from the tertiary treatment based on reverse osmosis (RO) membranes in WWTP C between October 2012 and February 2013 (Section 2.3). As mentioned above, in Section 3.4, a matrix-matched calibration curve was used for the quantification of analytes in order to obtain more accurate results. Table 4 summarizes the results of the average concentrations of the target musk fragrances found in each type of sample (*n*=8).

An analysis of the results shows that AHMI, HHCB, AHTN, ambrettolide and HHCB-lactone were present in all WWTP A and B influent samples with a maximum concentration of 34,674 ng L^{-1}, 45,091 ng L^{-1}, 49,904 ng L^{-1}, 9,744 ng L^{-1} and 4,178 ng L^{-1}, respectively. Meanwhile, all of the remaining musks studied, were present in some of the WWTP A and B influent samples analysed, except civetone and musk MC4, with concentrations ranging between lower than the method quantification limit (<MQL) and 44,319 ng L^{-1}. In WWTP C influent samples, exaltolide, HHCB, AHTN and ambrettolide were the most abundant compounds and were present in all of the samples analysed, with the highest concentrations being 10,79 ng L^{-1}, 22,524 ng L^{-1}, 14,300 ng L^{-1} and 21,528 ng L^{-1}, respectively. ATII, MX, MM, musk MC4, and civetone were not detected in influent WWTP C samples. Fig. 2b shows a chromatogram of a non-spiked influent WWTP A water sample in which all the target analytes except musk MC4 and civetone were detected.

In WWTP A and B effluent waters, only HHCB, AHTN and HHCB-lactone were present in all the samples analysed and were detected at values between <MQL and 11,007 ng L^{-1}. However, while HHCB and AHTN concentrations were significantly lower in comparison with those in influent samples, HHCB-lactone concentration remained constant as a result of the degradation of the HHCB to HHCB-lactone during the WWTP A and B treatments [36,49]. The rest of the target musk fragrances, with the exception of musk MC4 and civetone, were present in some of the effluent samples analysed at concentrations varying between <MQL and 9,930 ng L^{-1}. Fig. 2c shows a chromatogram of a non-spiked effluent WWTP A water sample. In effluent WWTP C samples, due to the tertiary treatment based on RO, only HHCB, AHTN, musk NN, MK and HHCB-lactone were detected at values higher than the MQL and at concentrations significantly lower than the influent samples.

Previous works [8,50-52] that have focused on the determination of musk fragrances in wastewater samples confirm the findings of the present study, namely that the most abundant polycyclic musks are HHCB and AHTN, although other polycyclic musks such as ATII, AHMI or ADBI may also be present in water samples in minor concentrations. A decrease in the concentrations of all polycyclic musks was observed when these results were compared with those obtained in influent and effluent WWTP water samples after the wastewater treatment plant depuration process. Only the HHCB-lactone concentration remained constant. Other works [17,21] that focus on the determination of macrocyclic musk fragrances in wastewater also confirm the findings of the present study, namely that the most abundant macrocyclic musk is ambrettolide, although other macrocyclic musks such as exaltone, exaltolide or musk NN may also be present in wastewater samples in minor concentrations. However, musk MC4 and civetone show significant differences in their concentrations, while in previous studies [17,21] were both detected at ng L^{-1} levels in influent

Table 4. Concentrations of the target musks found in the wastewater samples (*n*=8), expressed in ng L^{-1}.

Compound	MQLs		WWTP A[a)]		WWTP B[a)]		WWTP C	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent RO	Effluent RO
DPMI	10	7.5	690-3,410	91-478	n.d.-32,933	n.d.-9,437	409-4,342	n.d.
ADBI	7.5	5	232-1,133	34-277	n.d.-44,319	n.d.-9,930	<MQL-10	<MQL
AHMI	7.5	5	238-733	<MQL-113	<MQL-34,674	n.d.-3,738	<MQL-54	<MQL
Exaltone	25	20	338-1,579	184-480	n.d.-1,741	n.d.-415	95-199	n.d.
Exaltolide	40	35	515-1,249	291-791	n.d.-3,487	n.d.-1,276	520-10,797	n.d.
Muscone	35	30	69-2,865	<MQL-651	n.d.-3,022	n.d.-1,748	n.d.-258	n.d.
ATII	7.5	5	214-963	<MQL-185	n.d.-28,377	n.d.-4,210	n.d.	n.d.
HHCB	5	5	2,398-5,516	769-895	818-45,091	<MQL-900	1,820-22,524	0.01-0.4
AHTN	5	5	852-2,575	391-878	990-49,904	<MQL-7,555	2,212-14,300	42-138
MX	7.5	7.5	208-632	29-84	n.d.-321	n.d.	n.d.	n.d.
MM	7.5	5	159-639	137-221	n.d.-1,182	n.d.	n.d.	n.d.
Ambrettolide	25	25	807-5,543	215-4,021	507-9,744	n.d.-2,175	3,634-21,528	n.d.-86
Musk MC4	25	20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Civetone	30	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Musk NN	35	30	2,176-11,339	130-5,607	n.d.-11,758	n.d.-8,939	1,322-4,932	99-194
MK	7.5	5	987-4,110	218-457	n.d.-1,677	n.d.-465	348-500	104-689
HHCB-Lactone	7.5	7.5	1,188-1,791	1,160-2,423	3,461-4,119	5.95-11,007	1,315-4,178	21-161

n.d.; not detected.
<MQL; values under the method quantification limit.
[a)] WWTP A and B; urban wastewater treatment plants A and B. Samples were taken from the influent and effluent of the active sludge biological treatment.
[b)] WWTP C; urban wastewater treatment plant C. Samples were taken from the influent and effluent of the tertiary treatment based on reverse osmosis.

samples in this study they were not detected in any sample. A decrease in the concentrations of all of the macrocyclic musks studied was observed when the influent results were compared with those for the effluent.

4. Conclusions

For the first time, SPE was successfully on-line coupled to GC-MS through an on-column interface to determine a group of musk fragrances extensively used in personal care products that includes polycyclic musks, nitro musks and macrocyclic musks in several water samples. Transfer conditions such as flow-rate, temperature and SVE open time were optimized.

As well as transfer conditions, parameters affecting the SPE process were optimized. The best recovery values, higher than 80% for most of the target analytes, were achieved working with an Oasis HLB sorbent instead of C18 and adding 50% of methanol to the samples before the preconcentration step in order to minimize adsorption problems. The developed method also provided good linearity, low ng L^{-1} MDLs and intra-day and inter-day repeatability values below 10% for most of the target musks. The analysis of wastewater samples from three different wastewater treatment plants (WWTP A, B and C) revealed that all of the target analytes, except musk MC4 and civetone, were present in several influent samples while a decrease in the target analyte concentrations was observed in effluent samples. Only HHCB-lactone remained, due to the degradation of HHCB during the wastewater treatment process. In addition, the analysis of effluent samples from WWTP C demonstrates the effectiveness of tertiary treatments based on RO for the removal of musk fragrances, bearing in mind that the musk concentrations were significantly lower than those obtained at WWTP A and B, and most of the compounds were not detected or detected at levels <MQL.

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3.1.4. Headspace stir bar sorptive extraction followed by thermal desorption and gas chromatography with mass spectrometry to determine musk fragrances in sludge samples without sample pretreatment

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HEADSPACE STIR BAR SORPTIVE EXTRACTION FOLLOWED BY THERMAL DESORPTION AND GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY TO DETERMINE MUSK FRAGRANCES IN SLUDGE SAMPLES WITHOUT SAMPLE PRETREATMENT

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Abstract

A direct, simple and solvent-free method based on headspace stir bar sorptive extraction and thermal desorption-gas chromatography-mass spectrometry was developed to determine thirteen musk fragrances (six polycyclic musks, three nitro musks and four macrocyclic musks) in sludge without sample treatment. The optimal headspace stir bar sorptive extraction conditions were achieved when a polydimethylsiloxane stir bar was exposed for 45 min in the headspace of a 10 mL vial filled with 100 mg of sludge mixed with 0.2 mL of water stirred at 750 rpm at 80 °C. The stir bar was then desorbed in the thermal desorption-gas chromatography-mass spectrometry system, obtaining method detection limits between 5 and 30 ng g^{-1} dry weight (d.w.). The method applicability was tested with sewage sludge from two urban wastewater treatment plants and from a drinking water treatment plant. Results showed galaxolide and tonalide to be the most abundant musk fragrances found in wastewater treatment plants with maximal concentrations of 9,240 ng g^{-1} (d.w.) and 7,500 ng g^{-1} (d.w.), respectively. Maximum concentration levels between 35 and 635 ng g^{-1} (d.w.) were found for musk ketone, musk moskene, traseolide, phantolide and celestolide in this kind of samples. Concentrations below the limits of quantification of phantolide, galaxolide, tonalide and musk ketone were found in sludge from a potable water treatment plant.

Keywords: *stir bar sorptive extraction; musk fragrances; sewage sludge; thermal desorption; gas chromatography- mass spectrometry.*

1. Introduction

Musk fragrances are widely used as ingredients in personal care products, shampoos, lotions, and household cleaning agents [1-4]. These fragrances compromise a broad range of different compounds, including polycyclic, nitro and macrocyclic musks. Since 1990, the polycyclic musk fragrances galaxolide and tonalide have been increasingly used to replace nitro musk fragrances, having been included on the environmental protection agency's high production list (http://www.epa.gov/chemrtk/index.htm). Despite macrocyclic musk fragrances not being as widely used, some authors have shown interest in their presence in the environment due to their increasing use in recent years [5-7].

The scientific community is concerned about the presence of musk fragrances in the environment and the food chain. Although their persistence and potency are not well understood, which means that the long-term effects of these compounds on human and the environment remain unknown, they are being studied extensively. Several studies have reported their presence in wastewaters and surface waters [8-16], suspended particulate matter [17,18], aquatic biota [19,20], and even human adipose tissue and breast milk [21]. Only a few studies of musk fragrances have been reported in sewage sludge [22-24], but they confirm their presence at low milligram per kilogram levels.

A sample preparation step normally involving Soxhlet extraction [25,26], ultrasonic extraction [12] or pressurized liquid extraction [22,27] is mandatory to determine musk fragrances in sludge samples as it has been reviewed by Zuloaga *et al.* [28]. Therefore, due to the low selectivity of the extraction techniques mentioned above, extracts from complex matrices as sewage sludge have to be subjected to a clean-up step, to the detriment of analyses time and increasing solvent consumption. Sulphur elimination, SPE, gel permeation chromatography or selective pressurized liquid extraction are the major clean-up techniques. In the last decade, the trend in modern sample enrichment techniques has been towards greater simplification, miniaturization, easy manipulation, strong reduction or absence of organic solvents and low sample volume [29]. For example, headspace solid-phase microextraction, a solvent-free enrichment procedure, which allows the direct extraction of the analytes, implying the minimal manipulation of the sample and avoiding the use of organic solvents, with enhanced sensitivity. Furthermore, the combination of the extraction and the concentration of the analytes in one step also reduce the time of sample preparation.

Another solvent-free sampling technique is stir bar sorptive extraction (SBSE), a more powerful extraction technique, with higher preconcentration capacity as the amount of sorbent is 50-250 times higher than in a solid-phase micro-extraction fibre. Since the development of SBSE in 1999 [30], this extraction technique has been successfully applied for the analysis of trace environmental pollutants in different matrices [31-33]. Typical analytical techniques for the

identification and quantification of musk fragrances include GC-MS [11,34]. Moreover, thermal desorption (TD) methods have recently been successfully used to determine semi-volatile compounds, such as musk fragrances, in different matrices [17,35]. A headspace stir bar sorptive extraction (HS-SBSE) followed by TD-GC-MS analysis requires the extraction of the analytes from the matrix and subsequent desorption of these analytes to the GC system. To the best of our knowledge, this study describes the first application of HS-SBSE TD-GC-MS for the determination of a mixture of the most extensively used musk fragrances in sludge, including the most representative polycyclic, nitro and macrocyclic musks. The method is environmentally friendly and no treatment of the sample is necessary. The applicability of the method was also tested to determine thirteen musk fragrances in sludge from wastewater treatment plants (WWTPs) and a drinking water treatment plant (DWTP).

2. Materials and methods

2.1. Chemical standards

Promochem Iberia (Barcelona, Spain) supplied the six polycyclic musk fragrances: 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6- *tert*-butylindane (ADBI, celestolide), 6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI, phantolide), 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (ATII, traseolide), 1,3,4,6,7,8,-hexahydro-4,6,6,7,8,8- hexamethylcyclopenta-(*g*)- 2-benzopyran (HHCB, galaxolide) and 7-acetyl -1,1,3,4,4,6- hexamethyl- 1,2,3,4 tetrahydronaphthalene (AHTN, tonalide). The three nitro musk fragrances 2,4,6- trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX, musk xylene) and 1,1,3,3,5 -pentamethyl- 4,6-dinitroindane (MM, musk moskene) were purchased as 100 mg L^{-1} solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The standard 4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (MK, musk ketone) was sourced from Fluka (Buchs, Switzerland). The four macrocyclic musk fragrances: oxacyclohexadecan-2-one (exaltolide), cyclopentadecanone (exaltone), oxacycloheptadec-8-en-2-one (ambrettolide) and ethylene-dodecanedioate (musk MC4) were supplied by Symta (Madrid, Spain). Suppl. Table 1 shows the main characteristics (formula name, molecular structure, CAS number, molar mass and boiling point) of the target compounds.

Individual 4,000 mg L^{-1} standard solutions of the polycyclic musks were prepared in acetone. The nitro musk fragrances musk xylene and musk moskene were used as received while a 1,000 mg L^{-1} standard solution of musk ketone was prepared in acetone. Individual 1,000 mg L^{-1} standard solutions of macrocyclic musks were prepared in cyclohexane. A 10 mg L^{-1} standard solution mixture of all of the target analytes in ethyl acetate was prepared weekly from the individual standard solutions and used to prepare diluted solutions and to fortify sludge samples to the required concentrations. Acetone, cyclohexane and ethyl acetate were GC grade with

purities of 99.0% (VWR, Llinars del Vallès, Barcelona, Spain).
Ultrapure water was obtained using a Purelab ultra-purification system (Veolia Water, Barcelona, Spain). Nitrogen gas and helium gas of purity 99.999% (Carburos Metálicos, Tarragona, Spain) were used for the TD and the chromatographic analysis, respectively.

2.2. Sample collection and preparation

Sludge samples from different WWTPs in Catalonia (NE Spain) were analysed. Eight sewage sludge samples were collected from two urban WWTPs located in two cities with populations of around 120,000 inhabitants. These WWTPs receive urban sewages and some industrial discharges and they have a secondary treatment based on conventional activated sludge for the removal of dissolved and suspended biological matter (http://aca-web.gencat.cat/aca/appmanager/aca/aca).
The sewage sludge samples corresponded to a mixture of primary and secondary sewage, which was anaerobically digested and then dehydrated using press filters. Moreover, eight sludge samples from a DWTP were analysed. This DWTP receive water from the Ebro River and irrigation canals and is a conventional activated sludge treatment plant that uses carbon filters in the last process to obtain a high quality effluent (http://www.ccaait.com/cat/qualitat_de_l_aigua_processos.htm).
Anaerobically digested and dehydrated sewage sludge samples were taken from dry bulks of sludge accumulated in an established area throughout time. All samples were frozen, lyophilized using a freeze dry system (Labconco, Kansas City, MO, USA) and then crushed in a mortar and pestle and sieved (125 µm). Spiked samples were prepared by adding the stock mixture of standards in acetone (the volume required to cover the sludge). After spiking, the samples were stirred intensively so that there would be sufficient contact between the compounds and the matrix. The acetone was left to evaporate at room temperature in a fume cupboard with frequent homogenization of the sample.

2.3. Stir bar sorptive extraction

HS-SBSE extractions of the target musk fragrances were carried out with polydimethylsiloxane (PDMS) coated stir bars (20 mm long x 0.5 mm film thick, from Gerstel, Mülheim an der Ruhr, Germany), which correspond to approximately 48 µL of PDMS phase. To prevent carry-over, stir bars were thermally conditioned at 300 °C for 3 hours in pure helium flow of 100 mL min^{-1} and stored in cleaned 2 mL vials until use. In addition, to assess possible contamination, procedural blanks of the thermally cleaned stir bars were performed between analyses. No signal of the target analytes was found in the stir bar blanks, they could also be reused for 25 times with this kind of sample.
SBSE extraction of the target compounds was carried out by holding a clean stir bar suspended in the headspace, which was then thermal desorbed. The extraction was performed in a 10 mL borosilicate

glass vial containing 100 mg of sample with 0.2 mL of water and the vial was immediately closed with magnetic screw cap with polytetra-fluoroethylene-coated silicone septa and stirred at 750 rpm for 45 min at 80 °C. After extraction, the stir bars were rinsed with water, dried with a lint-free tissue and placed inside a thermally cleaned stainless-steel tube for TD. The scheme of the HS-SBSE performance is shown in Suppl. Fig. 1.

2.4. TD GC-MS analysis

TD of the musks retained on the stir bars was performed in a Unity 2 TD system (Markes International, Llantrisant, UK). Stir bars were placed in empty stainless-steel tubes (9 cm long x 6.35 mm o.d. x 5 mm i.d. also from Markes International) for TD. Prior to the analysis, the empty tubes were thermally cleaned at 300 °C for 15 min and then stored in a hermetically sealed glass jar under nitrogen atmosphere. The optimized TD conditions for the stir bar were as follows: pre-purge for 1 min at room temperature, stir bar desorption at 320 °C for 10 min using helium carrier gas at 100 mL min^{-1} in splitless mode, trapping at 0 °C in a general purpose hydrophobic trap (filled with Tenax TA and Carbograph 1TD, Agilent Technologies, Palo Alto, CA, USA). Finally, the trap was desorbed at 320 °C for 10 min with a split of 15 mL min^{-1}.
Separation and detection were performed in a 6890N gas chro-matograph and 5973 *inert* mass spectrometer (Agilent Technologies) using a ZB-50 capillary column (30 m x 0.25 mm x 0.25 µm) provided by Phenomenex (Torrance, CA, USA). For the GC-MS analysis, the helium carrier gas flow was set at 1.2 mL min^{-1}. The oven temperature programme began at 70 °C, then increased to 150 °C at 40 °C min^{-1}, then to 200 °C at 5 °C min^{-1} and finally to 280 °C at 20 °C min^{-1} for 5 min. The GC interface was set at 280 °C. The MS-detector acquired in the selective ion monitoring (SIM) mode operated at electron impact energy of 70 eV. Table 1 shows the retention time and the quantifier and qualifier ions used for the identification of each target analyte in SIM mode.

3. Results and discussion

3.1. HS-SBSE optimization

The main parameters affecting the HS-SBSE process, namely sample amount and water addition, extraction temperature and extraction time, were optimized in order to maximize the chromatographic peak area of the compounds by analysing sewage sludge from DWTP. The sludge samples used to optimize the HS-SBSE procedure were analysed (n=5) and the presence of HHCB and AHTN was detected. The average peak area of each detected compound was subtracted from the corresponding peak area of each spiked sample. Sludge samples were then spiked at 500 ng g^{-1} (d.w.) with all of the target analytes to optimize the HS-SBSE variables.
Based on our previous experience [36], the initial experimental sorption conditions were as follows: headspace mode, 250 mg (d.w.) sewage sludge

Table 1. Method parameters. MDLs, MQLs, linear range, intra-day and inter-day repeatability.

Compound	t_R (min)	Ions [a] (m/z)	MDLs (ng g^{-1} (d.w.))	Linear range [b,c] (ng g^{-1} (d.w.))	Intra-day repeatability [d] (% RSD)	Inter-day repeatability [d] (% RSD)
DPMI	6.8	135, **191**, 206	15	25-1,000	8	11
ADBI	9.8	173, **229**, 244	5	25-1,000	5	8
AHMI	10.8	187, **229**, 244	10	25-1,000	1	11
Exaltone	11.8	125, 135, **224**	15	25-1,000	5	9
Exaltolide	11.9	124, 222, **240**	30	100-1,000	3	10
ATII	12.1	173, **215**, 258	15	50-1,000	10	15
HHCB	12.4	213, **243**, 258	25	50-1,000	7	7
AHTN	12.6	**243**, 244, 258	25	50-1,000	1	3
MX	14	197, **282**, 264	20	100-1,000	5	10
MM	14.1	252, **263**, 264	10	50-1,000	1	9
Ambrettolide	14.6	135, 195, **235**	15	50-1,000	9	14
Musk MC4	15.5	149, 197, **213**	10	100-1,000	6	7
MK	17.6	**279**, 280, 294	5	25-1,000	2	11

[a] Quantifier ions are shown in bold type.
[b] MQLs (ng g^{-1} (d.w.)) were fixed as the lowest calibration level.
[c] r^2 >0.992.
[d] % RSD, n=5, 100 ng g^{-1} (d.w.).

stirred at 750 rpm, 0.5 mL of water, extraction temperature of 80 °C and extraction time of 45 min.

Firstly, the effect of sample amount and water addition on the extraction process was evaluated simultaneously since these parameters are clearly correlated. A set of experiments wasperformed using 10 mL vials each containing a different amount of the sewage sludge from DWTPs (50, 100, 250 and 500 mg (d.w.)), while the analyte concentration remained constant at 500 ng g^{-1} (d.w.). Taking into account the results obtained by Wei and Jen [37] and Wu and Ding. [38], the addition of between 0 mL and 5 mL of water was evaluated to facilitate the desorption and vaporization of the analytes from the sewage sludge to the headspace. The results clearly demonstrate that the addition of water to the samples is necessary to release the semi-volatiles into the gas phase. Thus, the highest peak areas were obtained working at 50 mg (d.w.) sludge/0.1 mL water, 100 mg (d.w.) sludge/0.2 mL water, 250 mg (d.w.) sludge/1 mL water and 500 mg (d.w.) sludge/2.5 mL water. As can be seen in Fig. 1, when the sample amount was increased from 50 to

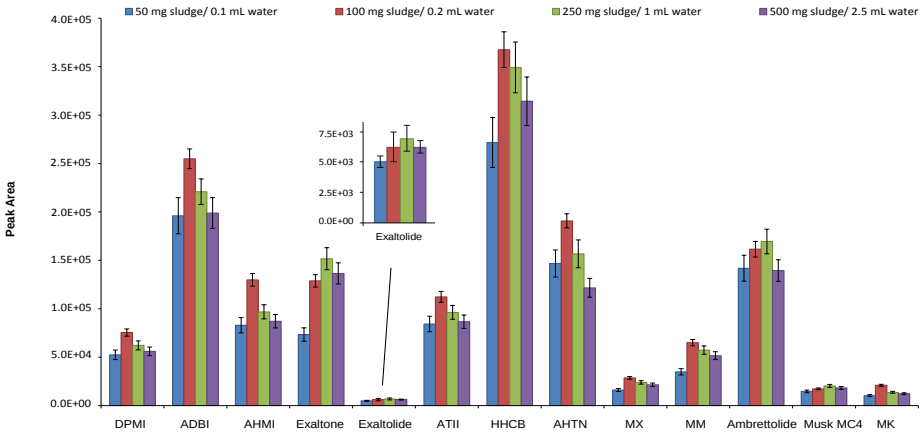

Fig. 1. Effect of sample amount and water addition on the HS-SBSE procedure. Experimental conditions: 80 °C, 750 rpm, 45 min (500 ng g^{-1}(d.w.), *n*=5).

100 mg (d.w.) a significant increase in the peak areas was observed for all of the target polycyclic and nitro musks until the maximum peak areas were reached. While, working at higher samples amounts, i.e. 250 and 500 mg (d.w.), a significant decrease in the analytical response of all of the polycyclic and nitro musks was observed. Macrocyclic musks reached the highest peak areas working with 250 mg (d.w.) of sample amount with slightly better peak areas than those obtained with 100 mg (d.w.) of sample. Therefore 100 mg (d.w.) of sample amount and 0.2 mL of water to be

added to the sewage sludge were chosen as a compromise.

Then, to test the effect of the extraction temperature on the extraction efficiency four different extraction temperatures (45, 60, 80 and 100 °C) were chosen. The other HS-SBSE variables were fixed at: headspace mode, 100 (d.w.) mg sewage sludge stirred at 750 rpm, 0.2 mL of water and 45 min as extraction time. As increasing temperature improves the evaporation of the target analytes from the sludge to the headspace, a progressive increase in the peak areas was observed for all of the target analytes until reach the equilibrium at 80 °C. Meanwhile, at 100 °C as extraction temperature, the equilibrium has been broken and temperature disfavours the extraction of the target compounds. Thus, a slightly decrease in the peak areas of most of the target analytes was observed. DPMI, ADBI, exaltolide and especially ATII, decreased their peak areas down to values comparable with those obtained working at 60 °C. The extraction of these compounds may be more sensitive with the changes in the vapour phase than the extraction of the rest of the musk fragrances studied. Therefore, 80 °C was selected as the extraction temperature.

Finally, the extraction time was evaluated between 45 min and 180 min. A progressive increase in the peak area was observed as the extraction time increased. However, some of the compounds showed slight differences between 45 min and 180 min. A high extraction time was only more favourable in the case of HHCB. To achieve a compromise and not to lengthen the time of the process, 45 min was chosen as the optimal extraction time.

3.2. TD-GC-MS optimization

The GC-MS method of the thirteen musk fragrances was optimized according to data of previous studies [39,40]. The chromatographic separation was performed by injecting the standards into the TD system using Tenax TA tubes (9 cm long x 6.35 mm o.d. x 5 mm i.d. also from Markes International) to optimize the desorption process. The tubes were attached to a Calibration Solution Loading Rig (Markes International), into which the analytes were loaded using a conventional syringe. Afterwards, they were purged for 5 min with a helium stream of 100 mL min^{-1} at room temperature to evaporate the solvent and to ensure the repeatability of the method. After this, the tubes were sealed and then immediately analysed.

The desorption parameters were adapted based on our previous experience of semi-volatile compounds [5,36]. Musk fragrances are semi-volatile compounds with relatively high boiling points (between 286 °C and 464 °C). Therefore, high desorption temperatures and flow should be applied to the PDMS stir bars for the quantitative desorption of the analytes. Carry-over experiments were under 5% when the maximum temperature (320 °C) and flow (100 mL min^{-1}) were applied for 10 min. Longer times did not improve the extraction efficiencies. The analytes were then trapped in a Tenax TA/Carbograph 1TD trap set at 0 °C during stir bar desorption and then

desorbed at 320 °C for 10 min with a split flow of 15 mL min^{-1}. The high split may compromise the sensitivity but it was needed due to the complex matrix studied in order to avoid trap contamination.

Taking into account the fact that TD of semi-volatile compounds can cause memory effects in the instrumentation, either in the cryogenic trap or in the transfer lines [35,39], blanks of the system were conducted daily. None of the blanks contained detectable traces of the target analytes.

Subsequently, method validation was applied to the method developed to determine musk fragrances in sewage sludge by HS-SBSE under the extraction and desorption optimal conditions summarized in Table 2.

3.3. Method Validation

The method was analytically validated with a sludge sample from a DWTP by establishing linear ranges, method detection limits (MDLs), method quantification limits (MQLs) and intra-day and inter-day repeatabilities. Before starting the validation, the sludge sample mentioned above was analysed (*n*=5) and small peaks of HHCB and AHTN were found. The average peak area of each compound was then subtracted from the corresponding peak area of each spiked sample.

Table 2. Optimized conditions for the stir bar extraction and thermal desorption.

Proces	Variables	
Stir bar extraction	Sample amount (mg)	100
	Stirring speed (rpm)	750
	Temperature (°C)	80
	Water addition (mL)	0.2
	Time (min)	45
Stir bar thermal desorption	Temperature (°C)	320
	Time (min)	10
	Flow (mL min^{-1})	100
	Trap temperature (°C)	0
	Split (mL min^{-1})	splitless

The linear range of the method was obtained by analysing the sludge sample spiked at eight calibration levels with concentrations between 25 ng g^{-1} (d.w.) and 1,000 ng g^{-1} (d.w.) (Table 1). Determination coefficients (r^2) higher 0.992 were obtained for all of the target compounds analysed. The MDLs were defined as the concentration which caused a peak with a S/N higher than 3, except for HHCB and AHTN, which were present in the sludge sample. For HHCB and AHTN, the MDLs were estimated as the concentration

that gave a signal average of plus three times the standard deviation of the sample signal. In all cases, the MQLs were defined as the lowest point of the calibration curve.

The MDLs and MQLs ranged from 5-30 ng g^{-1} (d.w.), and 25-100 ng g^{-1} (d.w.), respectively, as shown in Table 2. It is worth mentioning that these MDLs are better than those obtained in the literature when 0.2 g (d.w.) and 1 g (d.w.) of sewage sludge are used with pressurized liquid extraction followed by SPE [41] and gel permeation chromatography [27], respectively previous to the GC-MS analysis. On the other hand, working with microwave-assisted headspace solid-phase microextraction and 5 g (d.w.) of sewage sludge slightly lower MDLs were obtained by Wu and Ding [38]. Apart from that, our method allows us to work directly with low amounts of sample and that imply the reduction of the base line, obtaining cleaner chromatograms. Moreover, in contrast to the consulted literature, the HS-SBSE method developed was optimized to work with a mixture of the most representative musk fragrances, including polycyclic, nitro and macrocyclic musks instead of focusing attention in just one of the musk fragrances families.

The intra and inter-day repeatabilities were determined by spiking fives replicates of a DWTP sample at 100 ng g^{-1} (d.w.). The results obtained, expressed as RSD % were lower than 10% for intra-day repeatabilities and lower than 15% for inter-day repeatabilities. Values were comparable with those obtained by Wu and Ding [38] when microwave-assisted headspace solid-phase microextraction was applied for the determination of polycyclic musk fragrances in sewage sludge.

3.4. Method Application

The method developed was applied to determine the presence of a mixture of musk fragrances (polycyclic, nitro and macrocyclic musk) in eight sewage sludge samples from two urban WWTPs and eight samples from a DWTP. Table 3 summarizes the results of the average concentrations of the musk fragrances found in each type of sample.

As expected, the sewage sludge from WWTPs showed the highest concentrations. In these kinds of samples, the highest values were found for HHCB and AHTN with maximum concentrations of 9,240 ng g^{-1} (d.w.) and 7,500 ng g^{-1} (d.w.), respectively. These results are in agreement with the literature [12,42], in which all of the studies show both these compounds to be the most abundant.

Particularly high levels of HHCB (82,100 ng g^{-1} (d.w.)) and AHTN (26,000 ng g^{-1} (d.w.)) were detected in sludge samples from WWTPs in Hong Kong and Korea [27], suggesting the extensive use of these two polycyclic musk fragrances around the world. Not only are these polycyclic musk fragrances present in WWTPs, but also in sediments from lakes or rivers [12,34]. For example, Che *et al.* [34] revealed concentrations ranging between 0.3-3.1 ng g^{-1} (d.w.) of HHCB and 0.1-1.2 ng g^{-1} (d.w.) of AHTN in sediments from a lake in China

Table 3. Concentrations of the target musks found in sludge samples from two WWTPs and a DWTP (*n*=8).

Compounds	WWTP (ng g^{-1} (d.w.))	DWTP (ng g^{-1} (d.w.))
DPMI	n.d.	n.d.
ADBI	n.d.-35	n.d.
AHMI	85-143	<MQL
Exaltone	n.d.	n.d.
Exaltolide	n.d.	n.d.
ATII	n.d.-127	n.d.
HHCB	7,890-9,240	<MQL
AHTN	5,040-7,500	<MQL
MX	n.d.-1,319	n.d.
MM	n.d.-341	n.d.
Ambrettolide	n.d.	n.d.
Musk MC4	n.d.	n.d.
MK	136-635	<MQL

n.d.; not detected.
<MQL; values under the method of quantification.

Our study also revealed maximum levels of MX and MK in WWTPs sewage sludge samples, with totals of 1,319 ng g^{-1} (d.w.) and 635 ng g^{-1} (d.w.), respectively. Results were in agreement with Guo *et al.* [27], who studied WWTPs sludge from cities and rural areas. By way of an example, MK showed similar values (200 ng g^{-1} (d.w.)) in all of the WWTPs studied.

As can be seen in Table 3, the other musk fragrances studied were only present in significantly lower concentrations, implying lower usage. Levels <LOQ of AHMI, HHCB, AHTN and MK were found in DWTP while the remaining musks studied were not detected. Fig. 2 shows a chromatogram of a non-spiked sample from WWTP where AHMI, HHCB, AHTN and MK were detected.

4. Concluding remarks

This study successfully developed a method for determining a mixture of thirteen musk fragrances, including polycyclic, nitro and macrocyclic musks, in sewage sludge from WWTPs and DWTPs. The optimized method was based on direct HS-SBSE of sewage sludge and analysis by means of TD-GC-MS. As well as being an environmentally friendly methodology HS-SBSE is a simple and solvent-free technique that allowed us to work directly on the sewage sludge without any previous extraction process avoiding the risks of background contamination, reducing sample manipulation and treatment time. The method also provides good linearity, intra-day and inter-day repeatability and MDLs and MQLs at nanogram per gram levels.

The proposed method was used to analyse sewage sludge samples from WWTPs and DWTPs. As expected, significantly higher musk concentrations were detected in WWTPs than in DWTPs, with HHCB and AHTN being the most abundant. Meanwhile, the remaining musks studied were only found at some of WWTPs sewage sludge samples analysed at lower

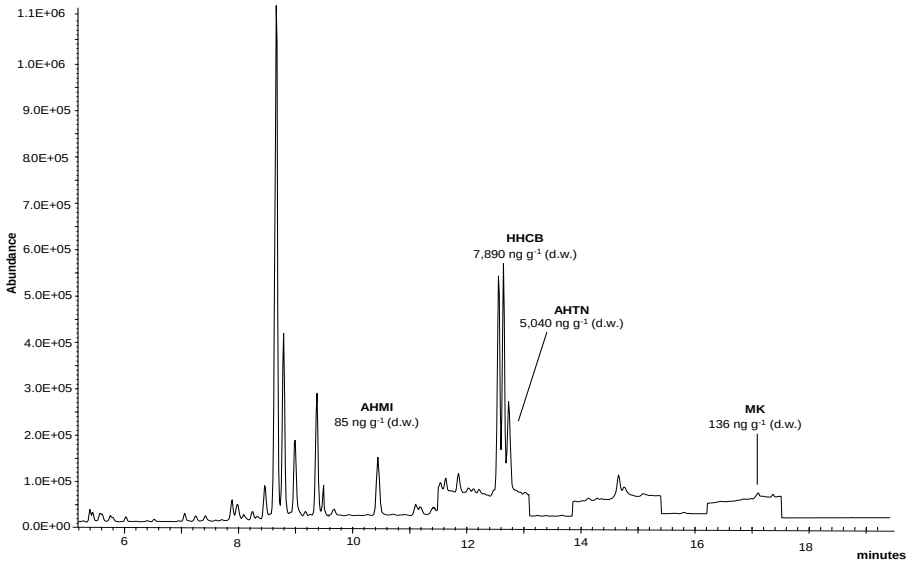

Fig. 2. Chromatogram of a sludge sample from a WWTP, indicating peaks of target musk fragrances and their concentrations at ng g^{-1} (d.w.).

concentrations. The high levels of musk fragrances found in the sewage sludge from the WWTPs, as well as their presence in DWTPs, confirm the extensive use of these compounds and the importance of determining organic contaminants in sludge from treatment plants.

Acknowledgments

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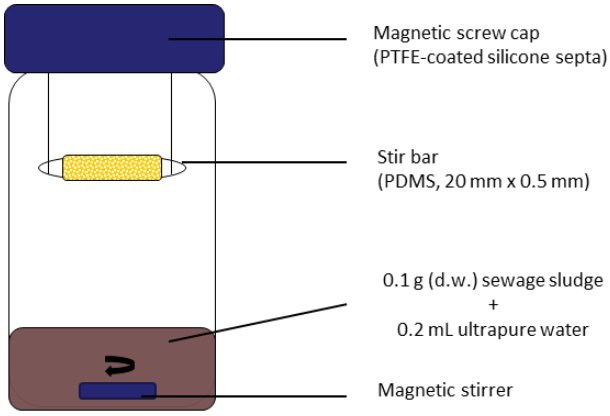
Supplementary material

Suppl. Table 1. Main characteristics of the target compounds.

nº	Formula name	CAS number	Molar mass (g/mol)	Boiling point (ºC) 760 mmHg	Log K_{ow} [a)]
	Polycyclic musk				
1	6,7-dihydro-1,1,2,3,3-pentamethyl-4(*5H*)-indanone (Cashmeran, DPMI)	33704-61-9	206.32	286.1	4.9
2	4-acetyl-1,1dimethyl-6-*tert*-butylindane (Celestolide, ADBI)	13171-00-1	244.38	309	6.6
3	6-acetyl-1,1,2,3,3,5-hexamethylindane (Phantolide, AHMI)	15323-35-0	244.37	393	6.7
4	5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (Traseolide, ATII)	68140-48-7	258.4	350	6.7
5	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran (Galaxolide, HHCB)	1222-05-5	258.4	326	5.9
6	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN)	1506-02-1	258.4	393	5.7
	Nitro musk				
7	2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (Musk xylene, MX)	81-15-2	297.26	392.3	4.8
8	1,1,3,3,5-pentamethyl-4,6-dinitoindane (Musk moskene, MM)	116-66-5	280.32	351.1	5.8
9	4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (Musk ketone, MK)	81-14-1	294.3	369	4.3
	Macrocyclic musk				
10	Cyclopentadecanone (Exaltone)	502-72-7	224.39	338.3	5.8
11	Oxacyclohexadecan-2-one (Exaltolide)	106-02-5	240.39	344.8	6.1
12	Oxacycloheptadec-8-en-2-one (Ambrettolide)	123-69-3	252.39	378.7	5.5
13	Ethylenedodecanedioate (Musk MC4)	54982-83-1	255.33	464.5	2.3

[a)] Log K_{ow} values predicted from SRC-K_{ow}Win Software.

Suppl. Fig. 1. Illustration of the extraction vial and the stir bar placed in the headspace.



3.1.5. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

Although the results of the experimental part of the studies included in this section have been already discussed in their respective papers, the current section presents and discusses the most important aspects of these results.

In the first two studies, HS-SPME followed by GC-IT-MS was successfully applied for the determination of MCMs fragrances in wastewater and sewage sludge. Both HS-SPME based methods were shown to be completely automated, simple and environmentally friendly. Moreover, the applicability of the HS-SPME directly on the sewage sludge samples, instead of the use of an extraction technique, such as PLE, results in a promising alternative, reducing sample manipulation and treatment time. They also provided low ng L^{-1} MDLs in the case of wastewater samples (0.75 ng L^{-1} and 5 ng L^{-1}), while, for sewage sludge MDLs were found in the low ng g^{-1} (d.w.) range between 0.01 ng g^{-1} (d.w.) and 0.025 ng g^{-1} (d.w.).

The most relevant aspect observed during the optimization of the chromatographic separation was that, even under optimized conditions, exaltone and exaltolide co-eluted, as well as muscone and habanolide. However, they could be quantified separately, as they had different molecular ions and fragments. As regards MS, in order to achieve better selectivity/sensitivity for the target compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and then optimizing IT MS/MS parameters, such as amplitude excitation voltage and CID storage level. Two kinds of fragmentation voltages were tested: resonant mode (intense voltages) and non-resonant mode (weak voltages). However, the excessive fragmentation of the target MCMs observed in MS/MS mode caused a significant decrease in the analytical signal and SIS (selected ion storage) MS was selected for successive analysis.

In the optimization of the HS-SPME process for aqueous samples, five different SPME fibres were tested, PDMS 7 µm, 30 µm and 100 µm, PDMS/DVB 65 µm and PA 85 µm, with PDMS/DVB 65 µm fibre providing the best results. The main parameters affecting the sorption and desorption process in SPME were optimized as well as variables such as extraction temperature and time, and salt concentration, because these factors were expected to be the most influential in the extraction process [1,2]. For aqueous samples, the best conditions were found to be the following: PDMS/DVB 65 µm fibre, 10 mL sample volume stirred at 750 rpm, 0% NaCl, an extraction temperature of 100 °C for 45 min and 3 min of desorption time (250 °C). In the case of sewage sludge, the variables optimized were sample amount, extraction temperature and time and the addition of water to increase the efficiency of the extraction [3]. The optimal conditions were found to be the following: PDMS/DVB 65 µm fibre exposed to 0.25 g (d.w.) mixed with 0.5 mL

of water for 45 min at 80 °C that was desorbed at 250 °C for 3 min. In agreement with the results obtained by Wu *et al.* [3], the addition of small amounts of water can significantly improve the extraction efficiency.

Regarding on-line SPE study, this was the first time that SPE was successfully coupled on-line to GC-(Q)-MS through an on-column interface for determining a group of synthetic musk fragrances including PCMs, NMs and MCMs in water samples. Under optimized conditions, the developed method provided good linearity, MDLs at ng L^{-1} levels (between 1 ng L^{-1} and 30 ng L^{-1}) and repeatability values below 10% for the vast majority of the target musk fragrances.

Apart from the evaluation of the effects of helium flow and oven temperature programme on the chromatographic separation, two analytical columns were tested: the ZB-50 (50% phenyl/50%dimethylpolysiloxane), previously used for the determination of MCMs by HS-SPME, and a ZB-5 (5%phenyl/95% dimethylpolysiloxane). Both columns were 30 m × 250 µm I.D., 0.25 µm. The best chromatographic separation was performed in less than 10 min in a ZB-50 analytical column. The chromatogram also showed the separation of the four enantiomers of galaxolide (4S,7S; 4S,7R; 4R,7S and 4R,7R). However, for the quantification, only 4S and 7R/S were integrated because these are the diastereoisomers responsible for the musky odour [4]. An MS detector acquiring in selected ion mode (SIM) and three mass fragments were selected for the identification and quantification of the target fragrances. The quantifier ions (relative abundance 100%) corresponded to the molecular ions for PCMs and NMs, while for MCMs were low m/z ions such as 55, 98 and 67. Due to the potential presence of interfering compounds in wastewater samples, which could contribute to the signal of the low ms/z ions, higher m/z ions corresponding with the molecular ions of MCMs were selected for the identification and quantification of the MCMs. However, the MDLs obtained for MCM fragrances were compromised by this loss of signal and, as a consequence, the MDLs obtained with on-line SPE followed by GC-Q-MS (10-30 ng L^{-1}, 10 mL) were slightly higher than those obtained with HS-SPME followed by GC-IT-MS (1-5 ng L^{-1}, 10 mL sample volume).

The parameters affecting the transfer of the fragrances from the precolumn to the GC system, such as flow-rate, temperature and solvent vapour exit (SVE) time, were optimized in order to minimize losses of the analytes and obtain well defined chromatographic peaks [5]. The best transfer conditions were found to be a flow-rate of 40 µL min^{-1}, a temperature of 60 °C and a SVE open time of 1.5 min after the transfer started. Not only transfer conditions were optimized, but also parameters affecting the

SPE procedure. The precolumn was filled with two extraction sorbents extensively used for the determination of musk fragrances: Oasis HLB and C18 both of 60 µm [6,7]. Oasis HLB was the sorbent that provided the best recovery values, higher than 80% for all the target fragrances, except cashmeran (50%), musk MC4 (60%) and HHCB-lactone (50-70%). The addition of 50% of methanol to the water sample before the SPE procedure was required in order to minimize adsorption problems.

In the last study in this section, an HS-SBSE extraction followed by TD-GC-Q-MS method was developed for determining synthetic musk fragrances (PCMs, NMs and MCMs) in sewage sludge. As in the case of HS-SPME (second study of this section), HS-SBSE is a simple and solvent-free technique that allowed us to work directly on the sewage sludge without any extraction process, thereby avoiding risks of background contamination, and reducing sample manipulation and treatment time. Moreover, HS-SBSE is a more powerful extraction technique, with higher preconcentration capacity, as the amount of sorbent is 50-250 times higher than in an SPME fibre. The developed method also provided good linearity, repeatabilities and MDLs at ng g^{-1} (d.w.) levels between 5 and 30 ng g^{-1} (d.w.).

The main parameters affecting HS-SBSE were optimized: sample amount, water addition, extraction temperature and extraction time. Specifically, the effect of sample amount and water addition on the extraction process was evaluated simultaneously since these parameters are clearly correlated. As in the case of HS-SPME, the results clearly demonstrate that the addition of water to the sample is necessary to release the musk fragrances into the gas phase. Optimal HS-SBSE conditions were found to be 0.1 g (d.w.) of sample amount mixed with 0.2 mL of water, stirred at 750 rpm for 45 min at 80 °C.

With respect to the TD-GC-Q-MS method, it was optimized in accordance with data from previous studies [4,8]. It should be noted that, under optimal conditions, high desorption temperatures (320 °C) and flow (100 mL min^{-1}) should be applied to the PDMS stir bar for the quantitative desorption of the target fragrances. The analytes were then trapped in a Tenax/Carbograph trap (0° C) during stir bar desorption and then desorbed at 320 °C for 10 min with a split flow of 15 mL min^{-1}. The sensitivity of the method was compromised by the high split. However, it was required to avoid trap contamination due to the complexity of the matrix analysed. The chromatographic separation was performed in a ZB-50 which, as mentioned earlier, allows the separation of the four enantiomers of galaxolide and the MS detector acquired in SIM. A comparison of the MDLs obtained with HS-SPME and HS-SBSE for the determination of

MCMs in sludge samples showed that significantly lower MDLs were achieved with HS-SPME, ranging between 0.01 and 0.025 ng g^{-1} (d.w.) (0.25 g (d.w.) sample amount), while with HS-SBSE, the MDLs increased to 10-30 ng g^{-1} (d.w.) (0.2 g (d.w.) sample amount). The use of a PDMS/DVB 65 µm fibre instead of a PDMS fibre significantly increased the extraction efficiency which, together with the application of a high split during the thermal desorption of the PDMS stir bar, were the factors that primarily explain the differences observed in terms of method sensitivity.

Of the conventional microextraction techniques applied in this section, as shown in Table 3.3.1 (page 373), HS-SPME provided the best MDLs with both wastewater and sewage sludge samples. Moreover, HS-SPME methodology developed for the determination of MCMs in wastewater is fully automated and does not require additional instrumentation as in the case of on-line SPE. HS performance of SPME also minimizes the presence of interfering compounds (cholesterols, phthalates, etc.) in wastewater chromatograms. In the case of sewage sludge samples, SBSE methodology is not fully automated and the sensitivity of the method is limited by the high split applied during the thermal desorption of the stir bar.

In any case, regardless of the microextraction technique used with all of the methods developed for aqueous and sewage sludge matrices, MDLs at few ng L^{-1} and ng g^{-1} (d.w.) were obtained, which make them suitable for application to environmental samples. In this respect, PCMS, NMs and MCMs fragrances were determined in influent and effluent samples from WWTP A (Tarragona) and WWTP B (Reus) and also in influent and effluent samples from the tertiary treatment of WWTP C (Vila-seca). In addition, sewage sludge samples from the aforementioned WWTPs and WWTP D (Girona) and the DWTP E (L'Ampolla) were also analysed.

All of the MCM fragrances studied were present in the influent water analysed, with ambrettolide present in all the influent samples analysed at concentrations varying between <MQL and 21,528 ng L^{-1}. Another point worth noting is the highest concentration of musk NN found in influent samples, at 11,758 ng L^{-1}. The rest of the MCMs were found in influent samples at lower concentrations between 34 ng L^{-1} and 3,487 ng L^{-1}. In effluent samples, the MCMs concentrations found were slightly lower than those obtained in the influent samples, and ambrettolide remained the most abundant compound, along with exaltone and musk NN. In effluent samples from the tertiary treatment of WWTP C, only ambrettolide (86 ng L^{-1}) and musk NN (99-194 ng L^{-1}) were found. In line with the concentrations found in wastewater samples, MCMs were detected in several sewage sludge samples from WWTPs A, B and D with

concentrations ranging between <MQL and 0.89 ng g^{-1} (d.w.), with ambrettolide, exaltone and musk NN as the most abundant compounds. Their presence was not detected in sewage sludge from DWTP E.

With respect to PCMs and NMs, phantolide, galaxolide, tonalide and HHCB-lactone were present in all influent samples, with maximum concentrations of 34,674 ng L^{-1}, 45,091 ng L^{-1}, 49,904 ng L^{-1} and 4,178 ng L^{-1}, respectively. Meanwhile, the rest of the PCMs and NMs studied were found in influent samples at concentrations between <MQL and 44,319 ng L^{-1}. In WWTP A and B effluent samples, the concentrations of PCMs and NMs decrease significantly to values between <MQL and 11,007 ng L^{-1}. In contrast, HHCB-lactone concentration remained constant because of the degradation of galaxolide during WWTP treatments. Moreover, due to the effectiveness of the tertiary treatment based on reverse osmosis (RO), the concentrations found in effluent samples from WWTP C were significantly lower than those obtained at WWTP A and B, and most of the fragrances were not detected or detected at levels <MQL. The presence of PCMs and NMs in sewage sludge from WWTP A and B and the DWTP E were also evaluated. As expected, the sewage sludge from WWTPs showed the highest concentrations, with galaxolide and tonalide being the most common compounds, with maximum concentrations of 9,240 ng g^{-1} (d.w.) and 7,500 ng g^{-1} (d.w.), respectively. These results are in agreement with the literature [9,10], in which all of the studies show both these fragrances to be the most abundant.

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3.2. Determination of musk fragrances in environmental samples by novel microextraction techniques

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

Aside from the conventional microextraction techniques used in Section 3.1, over the last few decades, there has been an increasing interest in novel microextraction techniques such as dispersive liquid-liquid microextraction (DLLME), single-drop microextraction (SDME), needle trap microextraction (NTME) and microextraction by packed sorbents (MEPS). These microextraction techniques are based on the miniaturization of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) and they represent a promising tool for the development of environmentally friendly methodologies as they allow us to reduce or eliminate the use of organic solvents during the preconcentration step, the sample amount required and, consequently, the analysis times. In this section, the studies focus on the development of sensitive GC-MS methods for the determination of the synthetic musk fragrances in environmental samples by using novel microextraction techniques.

In line with the above, in the first two studies, a miniaturization of LLE such as SDME was chosen for the development of a fully automated method to determine PCMs and NMs in environmental samples followed by GC-IT-MS/MS. Specifically, in the first study, the variables affecting SDME were studied in depth. Preliminary assays were performed to study the drop stability and to take into account four correlated variables: extraction solvent, drop volume, stirring rate and extraction temperature. It should be highlighted that not only organic solvents were used as the extraction solvent, but also two ionic liquids (ILs) previously applied for the determination of EOCs in aqueous samples [1,2], namely 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF_6]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]). They were evaluated due to their extraction capacity and chemical properties, such as hydrophobicity and viscosity. Due to the low volatility of the ILs, the combination of IL-SDME and GC has been reported before with some modifications in the injector port [3], modifying the liner [4] and using thermal desorption tubes [5]. In our case, a liner with a high internal diameter (3.4 mm) was used filled with glass wool, without any modification in the injector port, thus allowing the full automation of the IL-HS-SDME method.

The second study focuses on the development of a method for the quantitative determination of PCMs and NMs in sewage sludge samples by pressurized liquid extraction (PLE) followed by IL-HS-SDME and GC-IT-MS/MS. With regard to the optimization of PLE, initially, the extraction solvent was water. However, due to the low recovery values obtained, different percentages of methanol were added to increase the efficiency of the extraction. Moreover, to reduce the presence of fatty precipitates in the extract and obtain a clear solution for the IL-HS-SDME, an in-cell clean-up sorbent

was used. Three different sorbents were tested for this purpose: diatomaceous earth, florisil and silica.

With regard to the third study, a fully automated method for the determination of MCM fragrances in wastewater was developed. The methodology includes the enrichment of the analytes by MEPS followed by LVI-GC-IT-MS. MEPS, which was described by Adbdel-Rehim in 2004 [6], follows the same principles of SPE but on a miniaturized scale. It also presents some interesting features, such as the reduction of the sorbent amount to 1-4 mg with the consequent reduction of the sample volume needed to 1-4 mL, while the amount of elution solvent needed to elute the target analytes decreases significantly to µL levels. In the present study, two extraction sorbents were evaluated that hade been successfully applied by Moeder *et al.* [7] and Cavalheiro *et al.* [8] for determining PCMS and NMs in wastewater samples by MEPS: the silica gel sorbents modified with octyl (C8) or octadecyl (C18). As in SPE, the extraction, clean-up and elution steps were optimized. Moreover, in an automated MEPS procedure, additional steps for post-cleaning, re-conditioning and the extraction regime were optimized in order to enable multiple use of the MEPS-BIN. Thus, the following MEPS variables were optimized for each extraction sorbent: extraction regime, carry-over, fill and ejection speed, injection speed and elution solvent.

With respect to the fourth study, this focused on the determination of PCMs and NMs fragrances in wastewater using a dynamic HS-NTME followed by GC-IT-MS/MS. The first device, based on a needle filled with sorbent, was developed by Raschdorf [9] in the 1970s. However, it was not until a few years ago that NTME become popular. NTME is a robust and reproducible sample preparation technique, combining the advantages of SPME and SPE [10,11]. Like SPME, NTME only requires small sample volumes. Nevertheless, sensitivity of the analytical method can be increased by increasing the samples volume as it is an exhaustive technique like SPE. In the present study, the needle trap (NT) was filled with HF Bondesil-C18 as the extraction sorbent and different parameters affecting the adsorption capacity of the sorbent were studied, including extraction mode, extraction temperature, salt concentration, pre-incubation time, fill and ejection speed and fill volume. The parameters involved in the desorption and transfer of the target compounds into the GC (e.g. desorption mode, desorption temperature and time) were also evaluated.

Lastly, in the first and third study, influent and effluent wastewater samples were analysed from WWTPs located in Tarragona and Reus. In the second study, the sewage sludge samples analysed were also from WWTPs in Tarragona and Reus. Meanwhile, the

applicability of the last study was proven by the analysis of influent and effluent wastewater samples from WWTPs in Tarragona and Reus, as well as influent and effluent samples from the tertiary treatment of the WWTP in Vila-seca.

The results of these studies have been published in Talanta *99 (2012) 824-832 and 132 (2015) 548-556, Journal of Separation Science 35 (2012) 2735-2742 and Journal of Chromatography A 1264 (2012) 87-94*.

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UNIVERSITAT ROVIRA I VIRGILI
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3.2.1. Fully automated ionic liquid-based headspace single-drop microextraction coupled to GC-MS/MS to determine musk fragrances in environmental water samples

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FULLY AUTOMATED IONIC LIQUID-BASED HEADSPACE SINGLE-DROP MICROEXTRACTION COUPLED TO GC-MS/MS TO DETERMINE MUSK FRAGRANCES IN ENVIRONMENTAL WATER SAMPLES

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Abstract

A fully automated ionic liquid-based headspace single-drop microextraction (IL-HS-SDME) procedure has been developed for the first time to preconcentrate trace amounts of ten musk fragrances extensively used in personal care products (six polycyclic musks, three nitro musks and one polycyclic musk degradation product) from wastewater samples prior to analysis by gas chromatography and ion trap tandem mass spectrometry (GC-IT-MS/MS). Due to the low volatility of the ionic liquids (ILs), a large internal diameter liner (3.4 mm i.d.) was used to improve the ILs evaporation. Furthermore, a piece of glass wool was introduced into the liner to avoid the entrance of the ILs in the GC column and a guard column was used to prevent analytical column damages. The main factors influencing the IL-HS-SDME were optimized. For all species, the highest enrichments factors were achieved using 1µL of 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]) ionic liquid exposed in the headspace of 10 mL water samples containing 300 g L^{-1} of NaCl and stirred at 750 rpm and 60 °C for 45 min. All compounds were determined by direct injection GC-IT-MS/MS with a chromatographic time of 19 min. Method detection limits were found at ng L^{-1} range between 10 ng L^{-1} and 30 ng L^{-1} depending on the target analytes. Also, under optimized conditions, the method gave good levels of intra-day and inter-day repeatabilities in wastewater samples with relative standard deviations varying between 3% and 6 % and 5% and 11 % respectively (*n*=3, 1,000 ng L^{-1}). The applicability of the method was tested with different wastewater samples from influent and effluent urban wastewater treatment plants (WWTPs) and one drinking water treatment plant (DWTP). The analysis of influent urban wastewater revealed the presence of galaxolide and tonalide at concentrations of between 290 ng L^{-1} and 2,060 ng L^{-1} and <MQL (Method Quantification Limit) and 321 ng L^{-1} respectively; while the remaining polycyclic musks concentrations were below the method quantification limits and two of the nitro musks (musk xylene and musk moskene) were not detected. The analysis of effluent urban wastewater showed a decrease in galaxolide and tonalide concentrations while the other target analytes were not detected. In waters from DWTP only galaxolide was found at a concentration higher than MQL.

Keywords: *GC-IT-MS-MS; IL-HS-SDME; ionic liquids; musk fragrances; single drop microextraction; urban wastewater.*

1. Introduction

Personal care products (PCPs) include a broad range of compounds widely used as additives in cosmetics, flavourings, body oils, soaps, foods and drinks: in short, in a broad range of daily products. They are included in the so-called emerging organic contaminants, which have been of increasing interest to scientists in recent years [1-8].

The musk fragrances are a family of cyclic PCPs which include polycyclic musks, nitro musks and macrocyclic musks. Discussions on the toxicology of nitro musks emerged very early on because of the presence of a nitro-aromatic compound in their structure, and it has been demonstrated, that these compounds can be transformed in both wastewater treatment and vertebrate physiology into aniline transformation products [9,10]. These transformation products can be even more problematic than the parent compounds and this has led to a significant decrease in the use of these compounds and an increase in the production of polycyclic and macrocyclic musks. Nowadays poly-cyclic musks have a greater presence in environmental matrices than do nitro musks and two of them, galaxolide and tonalide, are included in the US Environmental Protection Agency's (EPA) High Production Volume (HPV) list [11]. In contrast, macrocyclic musks are not as widely used as polycyclic musks because of they are more expensive to synthesize, although they are becoming more readily available [2]. Macrocyclic musks seem to have a more intense smell and so less mass is needed to gain the same performance in perfumery**.** Also, these compounds seem to be more easily degradable in the environment [4,12].

Several analytical methods have been developed for identifying and quantifying of musk compounds in a variety of environmental sample. Available methods are based on gas chromatography (GC) using electron capture detection [13], or GC coupled to mass spectrometry (MS), in either the electron ionization mode [14,15] or in the negative chemical ionization mode [16], and tandem MS [16].

Due to the low concentrations at which musk fragrances are found in environmental water samples, some preconcentration techniques such as liquid-liquid extraction [17], solid-phase extraction [18-20] and semipermeable membrane devices [21] have been reported. In any case, any approaches based on liquid-liquid extraction and solid-phase extraction involves the use of organic solvents, which constitutes a pollution problem in itself. To solve this, new microextraction techniques have recently been developed to reduce or eliminate the use of organic solvents during the preconcentration steps and to obtain more environmentally friendly analytical methods [22,23]. Dispersive liquid-liquid microextraction (DLLME) [24-26], ultrasound-assisted emulsification-microextraction (USAEME) [27], solid-phase microextraction (SPME) [28], single-drop microextraction (SDME) [29], microextraction by packed sorbents (MEPS) [15] and membrane-assisted liquid-liquid extraction (MALLE) [30], are only a few examples. However, although fully automated SDME have been used previously for

the determination of alkaloids with micellar electrokinetics chromatography [31] or for the determination of phenols with capillary electrophoresis [32], not reports were found with fully automated SDME applied to the determination of musk fragrances.

The main shortcoming of SDME is the instability of the drop when an organic solvent is used as extractant. This limits the usable volume of the extracting medium and directly affects the precision and also the sensitivity of the determinations. This limitation is more marked when headspace single-drop microextraction (HS-SDME) is performed at high temperature because of the evaporation of the organic solvent during the extraction [29,33]. To solve the problem of drop volume repeatability, ionic liquids (ILs) have been proposed as an alternative to organic solvents because their low vapour pressure and high viscosity, which allows the use of larger and more reproducible extracting volumes [34,35].

Ionic liquids, which are ionic media resulting from the combination of organic cations and various anions, are gaining an important recognition as novel solvents in chemistry due to some unique properties, such as dual natural polarity, good thermal stability even at high temperatures and miscibility with water and organic solvents. Additionally, they are easily synthesized and commercially available [36]. These characteristics have led to an extensive range of applications in analytical chemistry as recently reviewed [37-39], which supports their consideration as very potential extractants for liquid phase microextraction (LPME). However, when ILs are employed as extractants in SDME, liquid chromatography [35,40-42] is preferred to GC as separation technique since the low volatility of the ILs. Thus, to the best of our knowledge, the combination of IL-SDME and GC has been described before with some modifications in the injector port [43], modifying the liner [44] and using thermal desorption tubs [45] but not reports were found by direct injection in the GC injector port.

The aim of this study is to develop for the first time a sensitive, environmental friendly and fully automated method to determine ten synthetic musks (polycyclic and nitro musks) in wastewater samples using ionic liquid-based headspace SDME followed by GC-IT-MS/MS.

2. Experimental

2.1. Chemical Standards

The six polycyclic musks were supplied by Promochem Iberia (Barcelona, Spain) and were the following: 6,7-dihydro-1,1,2,3,3-pentamethyl-4 (*5H*)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-*tert*-butylindane (ADBI, celestolide), 6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI, phantolide), 5-acetyl-1,1,2,6-tetramethyl-3 isopropylindane (ATII, traseolide), 1,3,4,6,7,8-hexahydro- 4,6,6,7,8,8- hexamethyl cyclopenta-*(g)*-2-benzopyran (HHCB, galaxolide), 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4- tetrahydronaphthalene (AHTN, tonalide). International Flavors & Fragances Inc. (Barcelona, Spain) provided 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8- hexamethylcyclopenta-*(g)*-2-ben-

zopyran-1-one (HHCB-lactone, galaxolidone). The nitro musk fragrances 2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX, musk xylene) and 1,1,3,3,5- pentamethyl-4,6- dinitroindane (MM, musk moskene) were purchased as 100 mg L^{-1} solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The standard 4-aceto-3,5- dimethyl-2,6-dinitro-*tert*-butylbenzene (MK, musk ketone) was provided by Fluka (Buchs, Switzerland) and d15-Musk xylene (internal standard) was supplied as a 100 mg L^{-1} solution in acetone by Symta (Madrid, Spain). Table 1 shows the main characteristics (formula name, boiling point, vapour pressure and molecular structure) of the target compounds.

Individual standard solutions of the synthetic musks were prepared in acetone at concentrations of 4,000 mg L^{-1} for polycyclic musks and 1,000 mg L^{-1} for musk ketone and HHCB-lactone. A standard mixture solution of 100 mg L^{-1} was prepared in methanol. MX and MM standards were supplied directly at a concentration of 100 mg L^{-1} and used as received. Acetone and metanol were GC grade with purity >99.9 % (SDS, Peypin, France).

The extraction solvents, toluene and *n*-heptane (both with >99.9 % purity) were purchased from SDS and ionic liquids, 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF_6]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]) with purities of 99% and 98% respectively were provided by ACROS Organics (Geel, Belgium). Sodium chloride (ACS reagent ≥ 99 %) was supplied by Sigma-Aldrich. Ultrapure water was obtained using a purelab ultra purification system (Veolia Water, Barcelona, Spain). Helium gas with a purity of 99.999% was used for the chromatographic analysis (Carburos Metálicos, Tarragona, Spain).

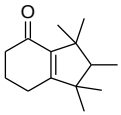
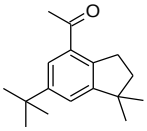
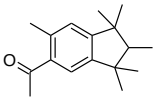
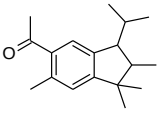
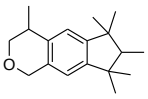
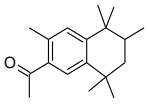
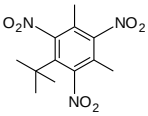
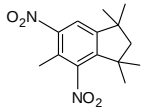
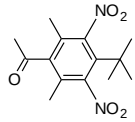
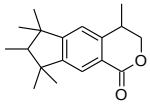
2.2. Instrumentation

The GC-IT-MS/MS analyses were performed using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) connected to a Varian 4000 ion trap mass detector. The GC was equipped with a 1079 programmable vaporizing temperature injector and a 3.4 mm i.d. insert liner (Varian) with a piece of glass wool. A fused silica capillary column (3 m x 0.25 mm i.d.) from Micron Phenomenex (Torrance, California, USA) was used as a guard column connected to a ZB-50 analytical column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex. Helium was used as a carrier and collision gas at a flow rate of 1 mL min^{-1}. Varian MS Workstation software v.6.9 was used for instrument control and data processing.

A CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). equipped with a 10 µL, fixed needle, 26 gauge bevel tip syringe (Agilent Technologies, San Jose, CA,USA) and a single Magnet Mixer and controlled by the Cyclo Composer Macro Editor 1.4 Software was used for the fully automated IL-HS-SDME.

Due to the high viscosity of the ILs, to make easy take 1 µL of ILs, the fill and ejection speed of the syringe during all the HS-SDME procedure was 1 µL s^{-1}.

Table 1. Main characteristics of the target compounds.

Compound	Formula name	Boiling point (°C)	Vapour pressure (mmHg)	Molecular structure
Cashmeran (DPMI)	6,7-dihydro-1,1,2,3,3-pentamethyl-4(*5H*)-indanone	286.1	2.69×10^{-3}	
Celestolide (ADBI)	4-acetyl-1,1-dimethyl-6-*tert*-butylindane	309	6.5×10^{-4}	
Phantolide (AHMI)	6-acetyl-1,1,2,3,3,5 hexamethylindane	336.6	1.11×10^{-4}	
Traseolide (ATII)	5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane	350	4.56×10^{-5}	
Galaxolide (HHCB)	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl cyclopenta-(*g*)-2-benzopyran	326	4.14×10^{-4}	
Tonalide (AHTN)	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene	356.8	2.86×10^{-5}	
Musk xylene (MX)	2,4,6-trinitro-1,3-dimethy 1,5-*tert*-butylbenzene	392.3	5.23×10^{-6}	
Musk Moskene (MM)	1,1,3,3,5-pentamethyl-4,6-dinitroindane	351.1	8.49×10^{-5}	
Musk ketone (MK)	4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene	369	1.22×10^{-5}	
HHCB-Lactone	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-Hexamethyl-cyclopenta-(*g*)-2-benzopyran-1-one	*	*	

* Information not found at the bibliography.

2.3. Sampling

All samples were collected from treatments plants located in Catalonia (NE Spain). The urban WWTPs (A and B) are located in two cities with populations of about 120,000 inhabitants and the potable water plant (C) is situated near the Ebro River. For each sample 200 mL was collected in a glass bottle, filtered through a 0.45 µm nylon filter (Scharlab, Barcelona, Spain) and stored at 4 °C until analysis.

2.4. Control of blanks

The extensive use of synthetic musks as fragrances in a wide range of consumer products means there is a high risk of sample becoming contaminated. Therefore, special precautions were required through the whole analytical procedure. Even so, further precautions were taken in this regard. All the glassware used for the sampling and the extraction steps and the stir bars used for stirring the solution during single drop microextraction were cleaned overnight with chromic mixture and then rinsed five times with ultrapure water and five times with HPLC grade isopropanol. Furthermore, musk-free gloves were used and the samples were prepared in a fume cupboard. In the same way, the piece of glass wool which was placed inside the GC liner was removed each ten analysis and then the liner was cleaned with acetone and methanol to eliminate ionic liquid residues and to prevent blank signals.

2.5. IL-HS-SDME and GC-IT-MS/MS analysis

The fully automated headspace single-drop microextraction was optimized to work with the ionic liquid [OMIM][PF_6]. The general HS-SDME procedure was as follows: 10 mL of 1:2 diluted sample or standard solution containing sodium chloride (at a final concentration of 300 g L^{-1}) was placed in a 20 mL glass vial which was tightly sealed with a teflon septum. When the temperature of the Magnet Mixer reached 60 °C, the vial was automatically transported there and stabilised for 1 min. Later on the GC syringe, previously filled with 1µL of [OMIM][PF_6], was inserted in the vial through the septum until its needle tip was located about 1 cm above the surface of the stirred solution. The plunger was depressed and a microdrop of the acceptor phase was exposed on the headspace above the aqueous solution at 60 °C for 45 min. After the extraction, the drop was retracted and injected into the GC. Both procedures, SDME extraction and GC injection, were performed by the CombiPal autosampler.

For the chromatographic analysis, the injector was operated in splitless mode at 280 °C. The oven temperature programme was as follows: initial temperature 100 °C, 30 °C min^{-1} to 170 °C, 5 °C min^{-1} to 210 °C then 20 °C min^{-1} to 290 °C and held for 4 min. All the compounds were separated within 19 minutes. The transfer line, manifold and trap temperatures were 280 °C, 50 °C, and 200 °C, respectively.

A filament-multiplier delay of 3 min was established in order to increase filament's service life. The analytes were ionized by EI. The EI-MS/MS process was carried out by CID using a resonant waveform type. Table 2 summarizes the retention time and the optimal MS parameters for each compound.

3. Results and discussion

3.1. GC-IT-MS/MS

A mixed solution of 10 mg L^{-1} of ten musk fragrances and 1 mg L^{-1} of d15-musk xylene as internal standard was prepared in methanol and 1 µL of this solution was directly injected into the GC-MS, using electron ionization fragmentation in full scan mode. All the compounds were identified by their molecular ion and afterwards the chromatographic separation was optimized by testing several oven temperature programmes. All compounds were separated in just 19 min using the chromatographic conditions described in Section 2.5. In order to achieve maximum sensitivity/selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and then optimizing the IT-MS/MS parameters. Table 2 summarizes the parent ion selected for each compound. All the parent ions were submitted to a CID with a resonant excitation waveform and the isolation window was 3 m/z for all of the compounds. The EI-MS/MS fragmentations were optimized for each compound by selecting an amplitude excitation voltage that gave the maximum abundance of one of the product ions (100%) and a relative abundance of the parent ion between 10% and 20%. The range of the CID amplitude voltage tested for each compound was between 0 V and 1.11 V. Table 2 also summarizes, for each compound, the optimum amplitude excitation voltage, the CID storage level, the product ions (quantifiers and qualifiers), the m/z range of ions analysed by EI-MS/MS, and the scan time. Each compound was acquired separately in one segment, except ATII, HHCB and AHTN (segment 4), and d15-MX, MX and MM, (segment 5); because of this, the scan time of these compounds was shorter than the others.

3.2 HS-SDME

The performance of HS-SDME is influenced by several parameters such as extraction solvent, drop volume, stirring rate, extraction temperature, salt concentration, sample volume and extraction time. Of these, the first four variables are strongly correlated and affect drop stability. Thus, they were optimized first.

The HS-SDME was optimized using standard solutions containing all the analytes at a concentration of 1 µg L^{-1} in ultrapure water (n=3). The best extraction conditions were those that provided the highest analyte signal.

3.2.1. Drop stability

Extraction solvent, drop volume, stirring rate and extraction temperature were the four variables studied to obtain the best drop stability. Four extractants were provided to

Table 2. Retention times and MS conditions.

Compound	Retention time (min)	Parent ion (m/z)	CID Amplitude (V)	CID Storage level (m/z)	Products ions [c] (m/z)	m/z range	Scan time (s/scans)
DPMI	5.39	191	0.82	84.1	**135**, 107, 173	94-201	1.08
ADBI	7.63	229	0.92	100	**173**, 187, 131	110-239	1.01
AHMI	8.48	229	0.92	100	145,131, **187**	110-239	1.01
ATII	9.64	215	0.88	94.7	131,171, **173**	104-225	1.01
HHCB [a]	9.87	243	0.96	122	171, **213**	132-253	0.53
AHTN [a]	10.02	243	0.96	103	159, **145**, 187	113-253	0.53
MX [b]	11.10	282	1.08	124.2	**265**, 281, 266	134-292	0.59
MM [b]	11.18	263	1.02	115.9	**211**, 187, 201	125-273	0.59
MK	13.34	279	1.07	122.9	**191**, 247, 280	132-289	1.05
HHCB-Lactone	14.16	257	1.00	113.2	201, 183, **239**	123-267	1.03
d15-MX (IS) [b]	10.69	294	1.11	129.5	170, **276**,295	139-304	1.04

[a], [b] Compounds were separated using a Multiple Reaction Monitoring.

[c] Quantification ions (m/z) are shown in bold type.

optimize the single drop micro-extraction; two conventional organic solvents (toluene and *n*-heptane) and two ionic liquids ([OMIM][PF_6] and [HMIM][PF_6]). The organic solvents were selected on the basis of two requirements: that they should have low volatility in order to be stable during the extraction period; and that they should have affinity with the analyte so as to facilitate the extraction. The ionic liquids (ILs) were selected on the basis of their extraction capacity and their chemical properties such as hydrophobicity and viscosity. ILs that had high miscibility with water were not used because they can introduce moisture into the GC system and increase the drop volume, thus causing excessive humidification of the drop. Furthermore, ILs with a low viscosity were excluded since they would be easily removed from the glass wool piece of the liner damaging the GC column. To work with ILs some preventive steps needed to be taken regarding the GC. A liner with a 3.4 mm i.d. was chosen to improve the ILs evaporation into which a piece of glass wool was introduced to avoid the entrance of the ILs in the GC column and a guard column was installed before the analytical column to ensure column protection.

In the HS-SDME process, a large microdrop volume may affect both the precision for sampling and the stability of the microdop suspended in the needle. To optimize the drop volume a test was carried out that consisted of exposing 1 µL, 2 µL or 3 µL drops of the extractants in the headspace above 10 mL of water at different agitation intensities between 0 and 750 rpm and at a room temperature for 15 min. The results showed that drops stability was independent of agitation intensity for both kinds of solvents. For organic solvents drops were stable under all the conditions tested while the ionic liquid drop was only stable up to 2 µL. Nevertheless, it was decided to handle only 1 µL of ionic liquid because a non-modified GC injector port was used. Recently some scientists have used laboratory modified injectors to improve the evaporation of the ionic liquid and thus be able to inject larger volumes [34,46]. However, our aim was to use non-modified equipment to make compatible the use of the CombiPal autosampler with the GC to be able to do all the extraction and injection steps automatically.

The drop stability was then tested at temperatures between 25 °C and 80 °C, with the other variables fixed at 15 min extraction time, 750 rpm and 3 µL and 1 µL drop sizes for organic solvents and ILs, respectively. The results showed that the drop volume of the organic solvents decreased when the extraction temperature was higher than 30 °C. On the basis of this, 30 °C was chosen as the optimum extraction temperature for organic solvents. However, when the ILs were used musks peak areas increased at temperatures higher than 45 °C because an increase in temperature improves the evaporation of the target compounds from the sample to the headspace. Fig. 1 therefore shows that the polycyclic musk extraction for [OMIM][PF_6] ionic liquid were more sensitive to extraction temperature than nitro musks and HHCB-lactone because of the polycyclic musk's higher slopes. Although higher

peaks areas were obtained at 80 °C for all the analytes, 60 °C was selected as the optimum extraction temperature to avoid introducing trace levels of water into the MS detector.

Under the conditions selected above, peaks areas were compared to select the best extractant. The results shown that [OMIM][PF_6] and toluene gave the highest peak areas. However, [OMIM][PF_6] was chosen as the extracting solvent because its low volatility compared with toluene permitted the extraction temperature to be increased from 30 °C to 60 °C, thus stimulating the presence of the target analytes in the vapour phase and increasing extraction efficiency. At the same time, intra-day repeatability values for toluene and [OMIM][PF_6] were calculated and RSD (1 μg L^{-1}, *n*=3) decreased significantly from 7% and 24% to 4% and 9% respectively, which confirmed that the ILs were more stable than the organic solvents. Thus the optimal conditions selected were 1 μL of [OMIM][PF_6] as the extraction solvent, 60 °C and 750 rpm.

3.2.2. Salt concentration

To study the influence of adding salt on the efficiency of HS-SDME, the ionic strength of the standard solutions was modified by adding sodium chloride in the range 0 g L^{-1} to 360 g L^{-1}. The other experimental conditions were the same as before: 10 mL volume of the standard solution and the HS-SDME was tested at 60 °C, during 15 min., 1 μL [OMIM][PF_6] and at a 750 rpm stirring rate. Plots of the peak area versus NaCl concentration are shown in Fig. 2. 300 g L^{-1} was selected as the optimal salt concentration because maximal peak areas were obtained for most of the analytes. ADBI, HHCB-lactone, MK and DPMI showed maximal peak areas at 200 g L^{-1} NaCl but only a slight decrease in those analytical signals was observed at 300 g L^{-1} NaCl.

It is clear that the addition of NaCl increased the ionic strength and thus promotes the transport of the analytes to the headspace and hence to the extracting drop. This tendency can be explained by the engagement of more water molecules in the hydration sphere around the ionic salt. These hydration spheres reduce the concentration of water available to dissolve the analyte molecules [47]. Hence, it is to be expected that this will drive additional analytes into the headspace or gaseous phase and extractant.

3.2.3. Sample volume

Sample volume plays an important role in HS-SDME analysis. According to the Pawliszyn equation [48], which explain the steady-state mass transfer that is established in the HS-SDME analysis, an increase in sample volume and consequently a decrease in headspace volume, enhances the extracted amount of analyte, and thus improves the sensitivity [49,50].

The effect of sample volume on the extracted amount of musks was investigated as follows; a set of experiments were performed using three 20 mL vials each containing a different volume of the aqueous phase,

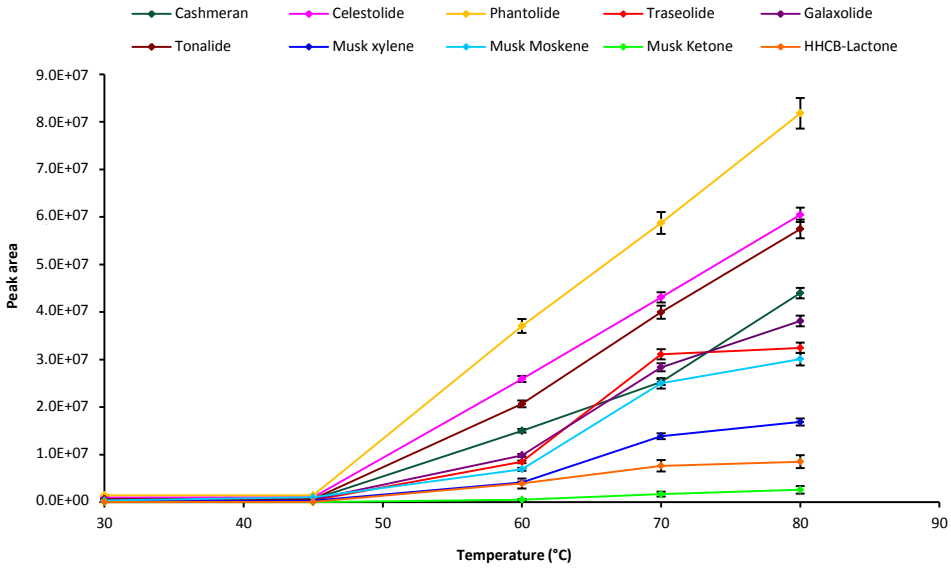


Fig. 1. Effect of temperature on the extraction efficiency of the HS-SDME used to determine musks. Experimental conditions: 1 µg L^{-1} concentration level, 10 mL standard solution in 20 mL glass vial; 1 µL [OMIM][PF_6]; 750 rpm stirring rate, 15 min sampling time and not salt addition.

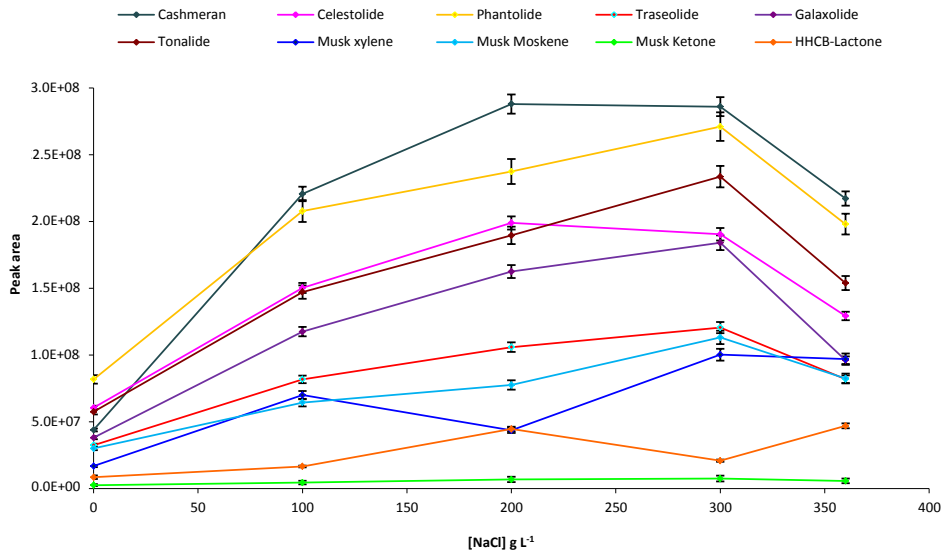


Fig. 2. Effect of salt concentration on the extraction efficiency of the HS-SDME used to determine musks. Experimental conditions: 1 µg L^{-1} concentration level, 10 mL standard solution in 20 mL glass vial; 1 µL [OMIM][PF_6]; 750 rpm stirring rate, 15 min sampling time and 60 °C temperature.

these being 5 mL, 10 mL and 15 mL while the analytes concentration remain constant at 1 μg L^{-1}. When the sample volume was increased from 5 mL to 10 mL a nearly linear increase in response was observed for all these compounds except for DPMI, which presented the highest peak area at 5 mL. At higher volumes (15 mL) a slightly decrease in the signal was observed. This can be explained by the fact that the convection is not as good in the aqueous phase when the solution is stirred at a fixed rate with larger volume which in turn results in less extraction. Therefore, 10 mL was chosen as the optimum sample volume.

3.2.4. Extraction time

In the HS-SDME method, the amount of extracted analyte is expected to increase in line with the amount of time that the stirred sample solution is exposed to the microdrop in the headspace. However, the HS-SDME is not an exhaustive extraction method, so for optimum repeatability of the extraction, it is necessary to choose a time in which equilibrium between the extracting microdrop, the headspace and the sample solution is reached: that is, the equilibrium time.

To test the effect of extraction time on extraction efficiency, we worked at the following different extraction times: 15 min, 30 min, 45 min and 60 min. The other HS-SDME variables were fixed at the values already mentioned above. A progressive increase in peak areas was observed for all the analytes up to an optimum time of 45 min after which there was a decrease when the extraction was extended up to 60 min. The probable reason is that at 45 min the analytes achieved the equilibrium between gas phase and liquid phase while the increasing of extraction time at the given extraction temperature, more water vapour will present in the headspace and the amount of analytes in IL drop decreased due to the distribution of analytes between the IL and the water vapour phase.

To summarize, the optimal conditions for working with HS-SDME were: 1 μL [OMIM][PF_6] used as the extraction solvent suspended in the headspace of a 20 mL vial filled with a 10 mL sample containing 300 g L^{-1} NaCl and stirred at 750 rpm for 45 min at 60 °C.

3.3. Method Validation

Before validating the method with a sample from a WWTP, the matrix effect was studied by statistically comparing the slopes of the calibration curves for influent and effluent WWTPs samples with that obtained using ultrapure water. As expected, the matrix effect was observed in both kinds of water, especially influent water. In order to reduce the matrix effect, an internal standard (IS) d15-MX was used but no differences were observed between the external calibration curve and that obtained with IS. Then, the sample dilution was tested and it was found that a 1:2 dilution with ultrapure water eliminated the matrix effect in both matrices. Nevertheless d15-MX was added to the ionic liquid (1 mg L^{-1}) to validate the method because its presence reduces the RSD and gives better values of intra-day and inter-day repeatabilities.

The method was then analytically validated with a diluted effluent water sample from WWTP A by establishing the linear range, method detection limits (MDLs), method quantification limits (MQLs), intra-day and inter-day repeatabilities.

Diluted samples from the effluent WWTP A mentioned above were analysed in triplicate to determine if any analyte was present, and the results revealed peaks of HHCB and AHTN in the chromatogram. The averaged peak area of each detected compound was subtracted from the peak area of each spiked sample.

The linear range of the method was obtained by analysing the WWTP A effluent sample spiked with musks at concentrations of between 25 ng L^{-1} and 10,000 g L^{-1}. The compounds showed a good linear range between 50 ng L^{-1} and 10,000 ng L^{-1} (polycyclic musks) or between 100 ng L^{-1} and 10 ng L^{-1} (nitro musks and HHCB-lactone) with determination coefficients (r^2) higher than 0.994 (Table 3).

The method detection limits (MDLs) for each compound were calculated depending on their presence in the blank analysis. For target compounds without blank signals, the MDLs were calculated as concentrations that give a signal of the quantifier ion three times higher than the noise signal, whereas for target compounds with a blank signal (HHCB and AHTN), the MDLs were estimated as three times the standard deviation of the blank signal of each target compound (*n*=3). In all cases the method quantification limits (MQLs) were fixed at the lowest calibration level. As can be seen in Table 3, the MDLs and MQLs ranged from 10 ng L^{-1} to 30 ng L^{-1} and 50 ng L^{-1} to 100 ng L^{-1} respectively.

The intra-day and inter-day repeatabilities were determined by spiking three replicates of the effluent

Table 3. Method liner ranges, determination coefficients, MDLs, MQLs, intra-day and inter-day repeatabilities (% RSD, *n*=3, 1,000 ng L^{-1}) for effluent WWTP A.

Compound	Linear range (ng L^{-1}) *	Determination coefficient (r^2)	MDLs (ng L^{-1})	Intra-day repeatability (%RSD)	Inter-day repeatability (%RSD)
DPMI	50-10,000	0.999	10	3	7
ADBI	50-10,000	0.994	10	2	5
AHMI	50-10,000	0.995	10	5	7
ATII	50-10,000	0.997	10	3	7
HHCB	70-10,000	0.998	24	4	8
AHTN	70-10,000	0.996	21	6	7
MX	100-10,000	0.997	30	5	9
MM	100-10,000	0.997	30	6	9
MK	100-10,000	0.996	30	3	9
HHCB-Lactone	100-10,000	0.994	30	4	11

* MQLs (ng L^{-1}) = were fixed as the lowest calibration level.

WWTP A at 1,000 ng L^{-1}. The results obtained (Table 3), expressed as % RSD, were lower than 7% for intra-day repeatability and 12% for inter-day repeatability.

The validation parameters obtained using IL-SDME for HHCB and AHTN were compared with those obtained using other microextraction techniques such as MEPS [15], SPME [28] or DLLME [25] followed by GC-MS. Similar MDLs values for HHCB and AHTN were obtained with IL-SDME (5 mL sample volume), DLLME (5 mL sample volume) and MEPS (800 µL), with values ranging between 24 ng L^{-1} and 53 ng L^{-1} for HHCB and between 21 ng L^{-1} and 49 ng L^{-1} for AHTN. On the other hand, working with SPME significantly lower MDLs, 0.4 ng L^{-1} for HHCB and 0.5 ng L^{-1} for AHTN, were achieved. Apart from that, the automation of the entire IL-SDME avoid all the repeatability problems associated with the SDME microextraction technique with intra-day and inter-day repeatability values slightly better that those obtained with the compared microextraction techniques.

Other parameters related to promoting green chemistry play an important role in this method; for example, it uses ionic liquids whereas the MEPS and USAEME use organic solvents. Also the IL-SDME reduces the extractant solvent from millilitres to 1 microliter.

3.4 Method Application

The method developed was applied to determine the presence of musk fragrances in different kinds of water: influent and effluent samples collected from urban WWTPs and influent and effluent samples taken from a DWTP over a period of six months (March-August) (Section 2.3).

Table 4 summarizes the results of the average concentrations of the ten musk fragrances found in each type of sample (*n*=8). As expected, the influent musk concentrations were higher than the effluent ones.

An analysis of the results shows that HHCB was the most abundant compound and appeared in all influent water matrices and at the highest concentrations, ranging from 290 ng L^{-1} in the DWTP to 2,060 ng L^{-1} in the WWTP A. AHTN was also present in all the influent samples with a maximum concentration of 321 ng L^{-1} in the WWTP B. The remaining musk fragrances, except MX and MM, were detected in influent samples but the average concentrations were lower than the quantification limit. Fig. 3 shows a chromatogram of a non-spiked influent WWTP B water sample.

In effluent waters only HHCB was detected at values higher than the quantification limit and ranged from 94 ng L^{-1} in the DWTP to 689 ng L^{-1} in the WWTP A. The remaining compounds detected in the influent waters were eliminated during the WWTP process, only HHCB-lactone remained as a result of the degradation of the HHCB to HHCB-lactone during the WWTP treatment [51,52].

Previous works [18,53-55] that have focused on the determination of musk fragrances in wastewater samples confirm the findings of the present study, i.e. that the most abundant polycyclic musks are HHCB and AHTN, although other polycyclic musks such as ADI, AHMI or DPMI can also be present

Table 4. Concentrations of the target musks found in the water samples (*n*=8), expressed in ng L^{-1}.

Compound	WWTP A		WWTP B		DWTP C	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
DPMI	<MQL	n.d.-<MQL	<MQL	<MQL	n.d.	n.d.
ADBI	<MQL	n.d.	<MQL	n.d.-<MQL	<MQL	n.d.
AHMI	<MQL	n.d.	<MQL	n.d.-<MQL	<MQL	n.d.
ATII	n.d.	n.d.	n.d.-650	n.d.-<MQL	n.d.	n.d.
HHCB	330-2,060	130-689	392-1,030	92-110	290	94
AHTN	<MQL-100	n.d.-<MQL	<MQL-321	<MQL-143	<MQL	n.d.
MX	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MK	n.d.-<MQL	n.d.	n.d.-<MQL	n.d.	n.d.	n.d.
HHCB-Lactone	<MQL	<MQL	n.d.-<MQL	<MQL	<MQL	n.d.

n.d.; values <MDL.
<MQL; values under the quantification limit.

in water samples in minor concentrations. A decrease in the concentrations of all polycyclic musks was observed when these results were compared with those obtained in influent and effluent WWTP water samples after the wastewater treatment plant depuration process. Only the HHCB-lactone concentration remains constant. On the other hand, the nitro musks (MX, MM or MK) show important differences in their concentrations depending on the location of the water sample and on if the study was done before they became subject to regulation [18,53-55].

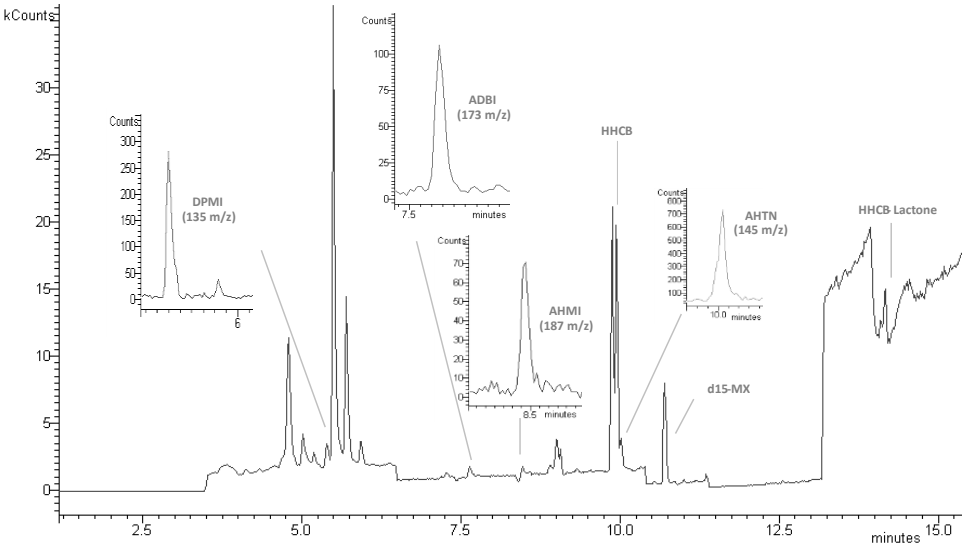


Fig. 3. Chromatogram of a non-spiked influent water sample from WWTP B and analytical signals enlargements.

4. Conclusions

In this study, an automated IL-HS-SDME followed by a GC-IT-MS/MS procedure was developed for determining 10 musk fragrances in water samples.

To adapt the IL (viscous solvent) to the GC a large internal diameter (3.4 mm) liner was used to improve the ILs evaporation into which a piece of glass wool was introduced to avoid the entrance of the ILs in the GC column and a guard column was used to prevent analytical column damages. The non-modification of the GC injector permitted the development of a completely automated, simple, and environmentally friendly method. Under optimized conditions the method also provide good linearity, acceptable MDLs and MQLs ranging between 10 ng L^{-1} and 30 ng L^{-1} and between 50 ng L^{-1} and 100 ng L^{-1}, respectively, and intra-day and inter-day repeatability values below 10% for most of the target musks. Also the

applicability of the method was tested with water samples from influent and effluent WWTPs and a DWTP. The most abundant musk fragrances in the samples were HHCB and AHTN.

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UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
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Dipòsit Legal: T 72-2016

3.2.2. Determination of musk fragrances in sewage sludge by pressurized liquid extraction coupled to automated ionic liquid-based headspace single-drop microextraction followed by GC-MS/MS

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DETERMINATION OF MUSK FRAGRANCES IN SEWAGE SLUDGE BY PRESSURIZED LIQUID EXTRACTION COUPLED TO AUTOMATED IONIC LIQUID-BASED HEADSPACE SINGLE-DROP MICROEXTRACTION FOLLOWED BY GC-MS/MS

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Abstract

A method for the quantitative determination of ten musk fragrances extensively used in personal care products from sewage sludge was developed by using a pressurized liquid extraction (PLE) followed by an automated ionic liquid-based headspace single-drop microextraction and gas chromatography-tandem mass spectrometry. The influence of main factors on the efficiency of PLE was studied. For all musks, the highest recovery values were achieved using 1 g of pre-treated sewage sludge, H_2O/methanol (1:1) as an extraction solvent, a temperature of 80 °C, a pressure of 1,500 psi, an extraction time of 5 min, 2 cycles, a 100% flush volume, a purge time of 120 s, and 1 g of florisil as in-cell clean-up extraction sorbent. The use and optimization of an in-cell clean-up sorbent was necessary to remove fatty interferents of the PLE extract that make the subsequent ionic liquid-based headspace single-drop microextraction difficult. Validation parameters, namely method detection limits (MDLs) and method quantification limits (MQLs), ranged from 0.5-1.5 to 2.5-5 ng g^{-1} dry weight (d.w.), respectively. Good levels of intra- and inter-day repeatabilities were obtained analysing sewage sludge samples spiked at 10 ng g^{-1} (d.w.) (n=3, RSDs <10%). The method applicability was tested with sewage sludge from different wastewater treatment plants (WWTPs). The analysis revealed the presence of all the polycyclic musks studied at concentrations higher than the MQLs, ranging from 6 to 530 ng g^{-1} (d.w.). However, nitro musk concentrations were below the MQLs or, in the case of musk xylene, was not detected.

Keywords: *GC-MS/MS; ionic liquid-based headspace single-drop microextraction; musk fragrances; pressurized liquid extraction; sewage sludge.*

1. Introduction

Currently, large quantities of synthetic musks are manufactured and used in a wide variety of personal care products, such as perfumes, skin creams, deodorants, and soaps, which are used in a broad group of everyday products. Musk fragrances, as all analytes classified as emerging organic compounds (e.g., X-ray contrast agents, pharmaceutical products, UV filters, and among others), have been investigated in recent years to detect their presence in environmental matrices such as river water, sediments, or urban air [1-8].

The most common synthetic musk compounds are nitro musks (NMs) and polycyclic musks (PCMs). Musk xylene (MX), an NM, acts as cogenotoxicant and, due to its intrinsic properties, the substance is the cause of a lot of concern in Europe and subject to the authorization processes under REACH [9]. PCMs were the major substitutes for NMs, due to the latter's toxicity and molecular instability. Nowadays, PCMs are present in environmental matrices at higher concentrations than NMs. Galaxolide and tonalide are two of the most commonly used PCMs and both feature on the US Environmental Protection Agency's (EPA) High Production Volume (HPV) list [10]. At the same time, another type of musk fragrances, not as widespread as PCMs and NMs, is the group of macrocyclic musks. Despite the cost of their synthesis, they are becoming more and more available [2]. They seem to give a more intense aroma and so less mass is needed to gain the same performance in perfumery. Additionally, these compounds seem to be more easily degradable in the environment [4,11].

The methods available for the identification and quantification of musk compounds comprise a sample preparation step followed by gas chromatography using electron capture detection [12] or mass spectrometry (MS), in the electron ionization mode [13,14] or in the negative chemical ionization mode [14], and tandem MS [15].

Sample preparation step normally involves the extraction of musk fragrances from sewage sludge using Soxhlet extraction [16,17], ultrasonic bathing [18], or pressurized liquid extraction (PLE) [19,20]. However, due to the low selectivity of the extraction techniques mentioned above, extracts from complex samples such as sewage sludge have to be subjected to clean-up steps, usually by solid-phase extraction (SPE), to the detriment of analysis times and increasing solvent consumption. To solve this problem, in the recent years, an evolution of the PLE, named in-cell clean-up PLE, has been applied to the determination of emerging organic compounds in environmental samples [21,22]. It consists of adding a sorbent, instead of an inert material, into the PLE cell to perform extraction and clean-up in one step, reducing solvent amount and analysis time required to extract the compounds from the sample.

Apart from that, environment-friendly microextraction techniques have been developed to reduce or eliminate the use of organic solvents during the preconcentration steps and decrease the detection limits of the analytical

methods [23]. Dispersive liquid-liquid microextraction (DLLME), solid-phase microextraction (SPME), single-drop microextraction (SDME), and hollow fibre membrane solid-phase micro-extraction (HF-SPME) are just a few examples. Furthermore, only solid-phase microextraction has been applied to the determination of synthetic musks in sewage sludge [24,25].

The aim of this study is to develop a sensitive and environment-friendly method based on PLE with in-cell clean-up extraction followed by ionic liquid-based headspace single-drop micro-extraction (IL-HS-SDME) and GC-MS/MS to determine ten synthetic musk fragrances in sewage sludge. Tandem MS has been selected to improve selectivity and sensitivity of the method.

2. Experimental

2.1. Materials and reagents

The six PCMs such as 6,7-dihydro-1,1,2,3,3-pentamethyl- 4(5*H*)-indanone (DPMI, Cashmeran), 4-acetyl-1,1-dimethyl-6-*tert*-butylindane (ADBI, Celestolide), 6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI, Phantolide), 5-acetyl-1,1,2,6-tetramethyl-3-isopropyl-indane (ATII, Traseolide), 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(*g*)-2-benzopyran (HHCB, Galaxolide), and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN, Tonalide) were supplied by Promochem Iberia (Barcelona, Spain). 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran-1-one (HHCB-lactone, Galaxolidone) was provided by International Flavors & Fragances Inc. (Barcelona, Spain). The NMs fragrances 2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX) and 1,1,3,3,5-pentamethyl-4,6-dinitro-indane (MM, musk moskene) were purchased as 100 mg L^{-1} solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Häen (Seelze, Germany), respectively. The standard 4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (MK, musk ketone) was provided by Fluka (Buchs, Switzerland) and d15-MX (internal standard) was supplied as 100 mg L^{-1} solution in acetone by Symta (Madrid, Spain).

Individual standard solutions of the synthetic musks were prepared in acetone at concentrations of 4,000 mg L^{-1} for PCMs and 1,000 mg L^{-1} for musk ketone and HHCB-lactone.

A standard mixture solution of 100 mg L^{-1} was prepared in methanol, except for MX and MM that were purchased directly at 100 mg L^{-1}. Working solutions were prepared daily in methanol or acetone GC grade with purity >99.9% (SDS, Peypin, France).

The IL needed to work with SDME, 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF_6]) with purity 98% was provided by ACROS Organics (Geel, Belgium).

Sodium chloride (ACS reagent, ≥99%) and florisil were supplied by Sigma-Aldrich. Ultrapure water was obtained using a Purelab ultra purification system (Veolia Water, Barcelona, Spain).Helium gas 99.999% (Carburos Metálicos, Tarragona, Spain) was used for the chromatographic analysis.

2.2. Control of blanks

The widespread use of musk fragrances in many consumer products means that special precautions are required throughout the analytical procedure. In order to prevent musks contamination, all the glassware used for the sampling and the extraction steps was cleaned overnight with chromic mixture and then rinsed five times with ultrapure water and five times with HPLC-grade isopropanol. Moreover, musk-free gloves were used and the samples were prepared in a fume hood.
Furthermore, the GC liner was cleaned after every ten analyses to eliminate ILs residues and prevent blank signals. The clean-up consisted of washing the liner with acetone and methanol and removing the piece of glass wool.

2.3. Sample pretreatment

The sewage sludge samples were obtained from two wastewater treatment plants (WWTPs) located in the area of Tarragona. These WWTPs receive mostly urban wastewaters and some industrial discharges.
Each sample was stored in glass bottles and stored in the freezer until analysis. They were lyophilized before analysis using the freeze dry system (Labconco, Kansas City, MO, USA) and sieved through a 125 µm screen.
To optimize the method, sludge samples were spiked with 1 µg g^{-1} (d.w.) of each compound, which were dissolved in acetone. After spiking, the samples were stirred intensively so that the compounds would be in sufficient contact with the matrix.
The acetone was left to evaporate at room temperature until the sludge samples were dry (overnight).

2.4. PLE extraction

Sludge samples were extracted using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) equipped with a 11 mL capacity stainless steel extraction vessel. One cellulose filter followed by 1 g florisil previously conditioned at 400 °C overnight (in-cell clean-up sorbent) was placed at the bottom of each cell. Then 1 g of the dried sample was accurately weighed into a glass container and mixed with 1 g of diatomaceous earth (conditioned at 400 °C for 8 h) until the mixture became homogeneous. The mixture was then introduced into the cell, which was refilled with diatomaceous earth, and was tightly closed.
During the PLE optimization, the extract obtained was processed by SPE to eliminate interferences. For this clean-up step, a vacuum system (Teknokroma, Barcelona, Spain) and Oasis HLB cartridges (150 mg, 6 cc, 30 µm) from Waters Corporation (Milford, MA, USA) previously conditioned with 5 mL of methanol followed by 5 mL of ultrapure water were used. The elution was performed two times with 4 mL of methanol at a flow rate of 1 mL min^{-1} [26]. The extracts were then evaporated with a rotary evaporator (R-114, Büchi, Switzerland) fixed at 35 °C and the residue was reconstituted to 2 mL with methanol (d15-MX 1 mg L^{-1}) and finally 1 µL was injected directly to the GC.

The best recovery values were set working with a 50% H_2O/MeOH solvent mixture as an extraction solvent. The operating conditions were as follows: extraction temperature, 80 °C; extraction pressure, 1,500 psi; extraction time, 5 min; number of cycles, 2; flush volume, 100% of extraction cell volume; and nitrogen purge, 120 s. For the validation of the method, after extraction, a half of the extract volume was taken to the rotary evaporator to evaporate methanol, was then filtered with a 0.22 µm membrane nylon filter (Scharlab, Barcelona, Spain) and diluted up to a volume of 10 mL with ultrapure water previously to IL-HS-SDME preconcentration step.

2.5. IL-HS-SDME GC-MS/MS analysis

The IL-HS-SDME procedure, previously developed for the determination of the same musk fragrances in environmental water samples [27], was as follows: 1 µL of [OMIM][PF_6] as an extraction solvent, with 1 mg L^{-1} of d15-MX as internal standard, was filled with a GC syringe and then inserted into the 20 mL glass vial through the silicone septum until the needle tip was situated 1 cm above the surface of 10 mL of PLE extract or standard solution. Then, the plunger was depressed and a microdrop of the extraction solvent was exposed on the headspace of the working solution, which contains 300 g L^{-1} of sodium chloride and was stirred at 750 rpm, at 60 °C for 45 min. After that, the drop was retracted and injected automatically into the GC.
The chromatographic instrument was a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) connected to a Varian 4000 ion trap mass detector. The GC was equipped with a 1079 programmable temperature vaporizing injector and a 3.4 mm id insert liner (Varian) with a piece of glass wool. A ZB-50 analytical column (30 m × 0.25 mm id; 0.25 µm film thickness) from Micron Phenomenex (Torrance, CA, USA) was used for the chromatographic separation connected to a fused silica capillary column (3 m × 0.25 mm id) from Micron Phenomenex as a guard column. Helium was used as a carrier and collision gas at a flow rate of 1 mL min^{-1} and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) was utilized for the fully automated IL-HS-SDME.
The GC injector was set in splitless mode at 280 °C, and the oven temperature programme was as follows: initial temperature 100 °C, 30 °C min^{-1} to 170 °C, 5 °C min^{-1} to 210 °C then 20 °C min^{-1} to 290 °C and held for 4 min, with a total run time of 19 min. The transfer line, manifold, and trap temperatures were 280 °C, 50 °C, and 200 °C, respectively. A filament multiplier delay of 3 min was established in order to prevent instrument damage. The analytes were ionized by EI and the EI-MS/MS process was carried out by collision-induced dissociation (CID) using a resonant waveform mode. Table 1 summarizes the retention time, the precursor and product ions, and the CID amplitude and CID storage level for each compound in GC-MS/MS.

3. Results and discussion

3.1. PLE optimization

In the pressurized solvent extraction, six parameters were optimized such as temperature, extraction solvent, number of cycles, static time, flush volume, and in-cell clean-up sorbent.

Purge time and pressure were fixed at 120 s and 1,500 psi, respectively. A spiked sludge that contained 1 μg g^{-1} (d.w.) of all the analytes was used for the optimization and the best extraction conditions were those providing the highest recovery values. The initial experimental conditions were 80 °C, 3 cycles, 10 min of static time, 120 s of purge time, 1,500 psi, 1 g (d.w.) of sample, 100% flush volume, and 1 g of diatomaceous earth previously conditioned at 400 °C for 8 h as an in-cell clean-up sorbent.

First, the extraction solvent was optimized. In order to develop an environment-friendly method for the determination of musk fragrances in sewage sludge, hot water was chosen as the initial extraction solvent. However, due to the low recovery values obtained, different amounts of methanol were added (0-100% v/v) to study the influence of the extraction solvent on the efficiency of PLE. As can be seen in Fig. 1, hot water gave recoveries lower than 45% for all the analytes and most of them were not recovered. Subsequently, when the percentage of methanol was increased from 0 to 50%, the recoveries for all of the compounds were improved obtaining the best recovery values at 50% H_2O/MeOH. A decrease in the recoveries was observed due to the presence of fatty precipitates when the methanol percentage was set at 75 or 100%. The lipophilic power of musk fragrances increases their tendency to be retained in the fatty precipitates that appear at higher

Table 1. Retention times and MS conditions.

Compound	Retention time (min)	Parent ion (m/z)	Product ions [c] (m/z)	CID Amplitude (V)	CID Storage level (m/z)
DPMI	4.98	191	**135**, 107, 173	0.82	84.1
ADBI	5.88	229	**173**, 187, 131	0.92	100
AHMI	6.46	229	145, 131, **187**	0.92	100
ATII	7.56	215	131, 171, **173**	0.88	94.7
HHCB [a]	7.62	243	171, **213**	0.96	122
AHTN [a]	7.73	243	159, **145**, 187	0.96	103
MX [b]	8.32	282	**265,** 281, 266	1.08	124.2
MM [b]	8.53	263	**211**, 187, 201	1.02	115.9
MK	10.78	279	**191**, 247, 280	1.07	122.9
HHCB-Lactone	12.72	257	201, 183, **239**	1.00	113.2
d15-MX (IS)[b]	8.19	294	170, **276**, 295	1.11	129.5

[a), b)] Compounds were separated using a Multiple Reaction Monitoring.

[c)] Quantification ions (m/z) are shown in bold type.

methanol percentages. Thus, 50% H_2O /MeOH mixture was selected as an extraction solvent. Second, the extraction temperature was studied by comparison of the recoveries obtained at 60, 80, and 100 °C. When the PLE extraction temperature rose from 60 to 80 °C, a slight recovery increase was obtained. However, as can be seen in Table 2, a change in this tendency was observed at 100 °C. Working at the highest temperature, recoveries for all the analytes decreased to percentages below those obtained at 60 °C. The explanation for this is the increase of the presence of fatty compounds in the PLE extractant with the temperature that makes the SPE clean-up step difficult and saturates the SPE cartridge. Finally, the extraction temperature chosen was 80 °C with recovery values between 63 and 95%.

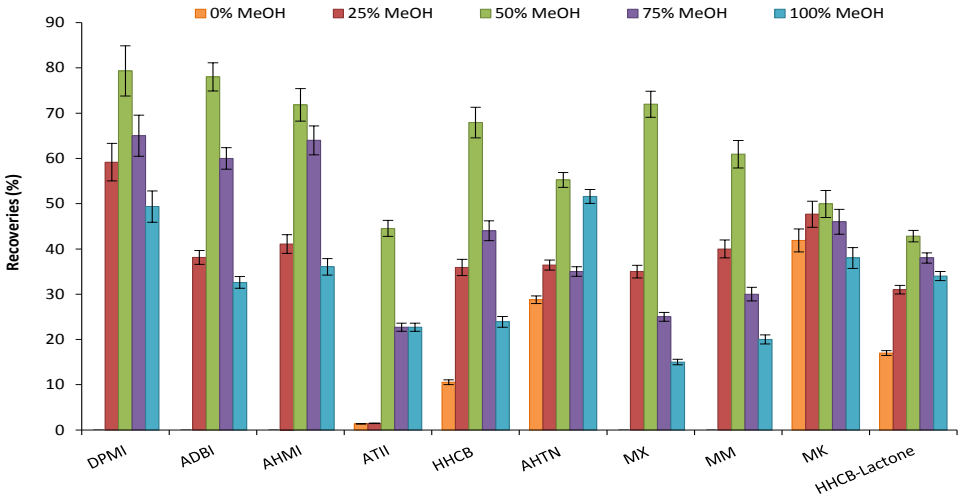

Fig. 1. Effect of the extraction solvent solution on the recoveries. Experimental conditions: 80 °C, 1,500 psi, 5 min, 2 cycles, 120 s purge time, 100% flush volume; 1 g (d.w.)of sample and 1 g diatomaceous earth as in-cell clean-up sorbent (1 µg g^{-1} (d.w.), *n*=3).

The third parameter optimized was the static time. Three different extraction times 5, 10, and 15 min were chosen to test the effect of this variable on the extraction efficiency. The best results were obtained working with 5 min of static time with recovery values between 71 and 98% for all the musk fragrances. Contrary to what may be expected the increase of the extraction time did not result in the increase of the recoveries. Because of the extraction of fatty compounds present in the sewage sludge was better with the extension of the static time.

The fourth variable optimized was the number of cycles and three consecutive simple extractions to the sample were done. As can be seen in Fig. 2, recovery values obtained working with one cycle

Table 2. Influence of the extraction temperature on recovery (%) of target compounds.

Compound	60 °C	80 °C	100 °C
DPMI	86	88	79
ADBI	86	88	78
AHMI	82	81	72
ATII	57	63	45
HHCB	71	75	68
AHTN	81	90	55
MX	83	95	72
MM	70	68	61
MK	53	68	50
HHCB-Lactone	64	63	43

Sludge spiked at 1 μg g^{-1} (d.w.).
% RSD ≤6 (*n*=3).

were between 60 and 80% for all the target musks whereas with two cycles, all recoveries were higher than 90%. On the other hand, when three cycles were applied, the appearance of precipitates in the PLE extract as well as the lipophilic properties of the musk fragrances made their extraction from the sludge difficult causing a decrease in the recovery values. Therefore, two cycles were chosen as optimal because of the better recovery values.

To increase the preconcentration factor, the flush value was optimized. Two percentages of flush volume were tested 60 and 100%. The results found that at 100% flush volume, recovery values between 72% and 98% were recorded for all the musk fragrances, while at 60% flush volume, the percentages decreased to 50 and 80%. Due to the recovery percentages, 100% was chosen as optimal flush volume to extract musk fragrances.

During the PLE optimization, the problem that persists is the precipitation of some fatty elements that act as interferents in the extract vial. These elements directly affect the recovery values of the musk fragrances. Due to the optimization of PLE parameters, these fatty precipitates were considerably reduced until turbidity. However, in order to apply the IL-HS-SDME, a clear solution was needed and so the in-cell clean-up sorbent was used.

To optimize the in-cell clean-up sorbent, three sorbents were tested - diatomaceous earth, florisil, and silica, all conditioned at 400 °C overnight. As can be seen in Table 3, although diatomaceous earth presented the best recovery results, florisil was selected as the in-cell clean-up sorbent because its recovery values and its efficiency in the removal of interferents found in the PLE extract.

3.2. Method validation

A sewage sludge sample from WWTP 1 was taken to validate the developed method. Table 4 shows the following validation parameters: linear ranges, method detection limits (MDLs), method quantification limits (MQLs), intra-, and inte-rday repeatabilities for the musk fragrances studied under the optimized experimental conditions.

Due to the matrix effect found when IL-HS-SDME followed by GC-MS/MS was applied to determine the same musks in wastewater samples [27], the matrix effect was checked with a sewage sludge sample. The calibration curves obtained with ultrapure water were statistically compared with those obtained with the PLE extract of

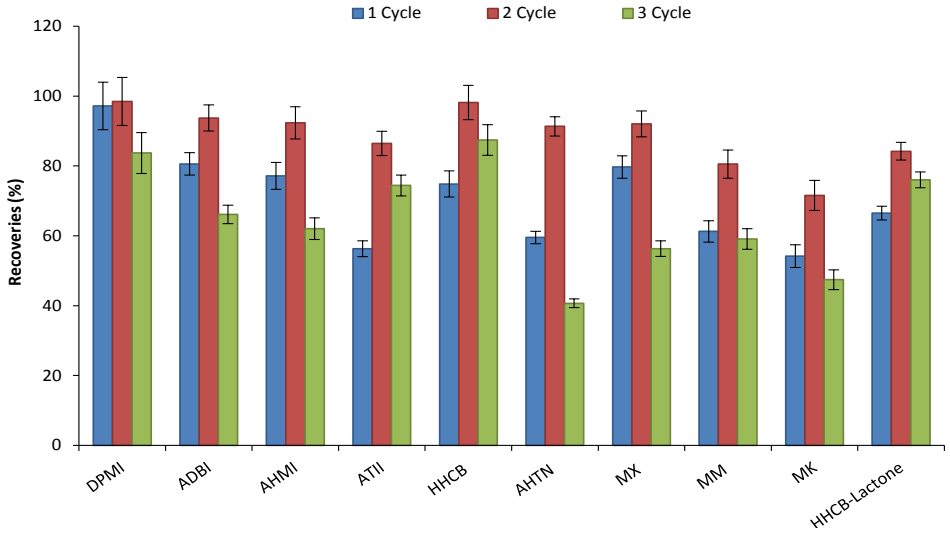

Fig. 2. Recoveries versus number of cycles. Experimental conditions: 50% MeOH/ H_2O mixture, 80 °C, 1,500 psi, 5 min, 120 s purge time, 100% flush volume, 1 g (d.w.)of sample and 1 g diatomaceous earth as in-cell clean-up sorbent (1 µg g^{-1} (d.w.), *n*=3).

sewage sludge from WWTP 1. The test t results, with 0.05 significance level showed that the calibration curves were not comparable because the slopes of the PLE extract were much lower than ultrapure ones. In order to reduce the matrix effect, an internal standard d15-MX was used but no differences between the external calibration curve and that obtained with IS were detected. Finally, after PLE extraction, a half of the extract volume was taken to the rotary evaporator to evaporate the methanol and then applying a dilution 1:2 with ultrapure water the matrix effect was eliminated. However d15-MX was added to the IL (1 mg L^{-1}) to validate the method because its presence reduced RSDs percentages and better values of intra- and inter-day repeatabilities were obtained.

Before starting the validation, the WWTP1 sample mentioned above was analysed in triplicate and the peaks of HHCB, AHTN, and HHCB-lactone were detected. The averaged peak area of each detected compound was subtracted from the peak area of each spiked sample.

The linear range of the method was obtained by analysing the WWTP1 sample spiked at concentrations between 2.5 and 100 ng g^{-1} (d.w.). As can be seen in Table 4, compounds have a good linear range between 3 and 100 ng g^{-1} (d.w.) (PCMs) or between 5 and 100 ng g^{-1} (d.w.) (NMs and HHCB-lactone) with determination coefficients (r^2) higher than 0.996.

For target compounds without blank signals, the MDLs were calculated as the concentrations corresponding to three times the noise signal. For target

Table 3. Effect of the in-cell clean-up sorbents on recovery (%).

Compound	Diatomaceous earth	Silica	Florisil
DPMI	98	77	83
ADBI	94	99	100
AHMI	92	88	92
ATII	86	91	90
HHCB	98	72	84
AHTN	91	56	63
MX	92	83	77
MM	81	63	69
MK	72	62	74
HHCB-Lactone	92	68	75

Sludge spiked at 1 μg g^{-1} (d.w.), % RSD ≤6 (*n*=3).

Table 4. MDLs, MQLs, intra-day and inter-day repeatabilities (% RSD, *n*=3, 10 ng g^{-1} (d.w.)) for sewage sludge.

Compound	MDLs (ng g^{-1} (d.w.))	Linear range [a] (ng g^{-1} (d.w.))	Intra-day repeatability (% RSD)	Inter-day repeatability (%RSD)
DPMI	1	3-100	7	8
ADBI	1	3-100	4	6
AHMI	1	3-100	5	7
ATII	1	3-100	4	6
HHCB	1	3-100	5	10
AHTN	1	3-100	3	8
MX	3	5-100	4	10
MM	3	5-100	5	7
MK	3	5-100	6	8
HHCB-Lactone	3	5-100	3	8

[a] MQLs (ng g^{-1} (d.w.)) = were fixed as the lowest calibration level.

compounds present in the blank samples, the MDLs were estimated by comparing the signal of the compound in the blank sample with that obtained when a low concentration standard was analysed. In all cases, the MQLs were fixed as the lowest calibration level. The MDLs and MQLs, as can be seen in Table 4, ranged from 0.5 to 1.5 ng g^{-1} (d.w.) and from 2.5 to 5 ng g^{-1} (d.w.), respectively, depending on the musk fragrances.

The intra- and inter-day repeatabilities were determined by spiking three replicates of a WWTP1 sample at 10 ng g^{-1} (d.w.). The results obtained,

expressed as % RSD, were lower than 7% for intra-day repeatabilities and 10% for inter-day repeatabilities (Table 4).

Comparing the validation parameters obtained applying IL-HS-SDME after the PLE with those obtained using SPE or gel permeation column followed the PLE [19,20] to determine some of the target musks, better MDLs and MQLs were provided with the use of a microextraction technique. With MQLs values between 2.5 and 5 ng g^{-1} (d.w.) applying IL-HS-SDME, while using SPE or gel permeation column after the PLE extraction MQLs values increase to 250 ng g^{-1} (d.w.) or 10-30 ng g^{-1} (d.w.), respectively. On the other hand, the automation of the entire IL-HS-SDME process led to the best intra- and inter-day repeatability values. And the application of the in-cell clean-up sorbent instead of the SPE step of clean-up decreases significantly the work time and the sample manipulation.

3.3. Method application

The method developed was applied in order to determine the presence of musk fragrances in sewage sludge of two WWTPs (WWTP1 and WWTP2) during a period of time of 8 months (from March to October 2011). Table 5 summarizes the results of the average concentrations of the ten musk fragrances found in each type of sample (*n*=8).

Analysing sludge of WWTP1 and WWTP2, the presence of all the PCMs was detected. However, differences in concentration were observed between both matrices. While in WWTP1, the concentrations ranged from below MQLs to 6.1 ng g^{-1} (d.w.), in WWTP2 the values fluctuated between below MQLs and 90.8 ng g^{-1} (d.w.). Fig. 3 shows a chromatogram of a non-spiked sludge sample from WWTP2. The NMs fragrances were detected in some sewage sludge but the average

Table 5. Concentrations of the target musks found in the sewage sludge (*n*=8), expressed in ng g^{-1} (d.w.).

Compounds	WWTP1	WWTP2
DPMI	<MQL	<MQL-90.8
ADBI	<MQL-6.1	<MQL-26.9
AHMI	<MQL-4.2	<MQL-25.4
ATII	<MQL-3.2	8.0-34.9
HHCB	3.6-5.1	3.4-48.1
AHTN	<MQL-5.7	n.d.-14.5
MX	n.d.	n.d.
MM	n.d.-<MQL	n.d.-<MQL
MK	n.d.-<MQL	n.d.-<MQL
HHCB-Lactone	<MQL-42.5	97.4-530.5

n.d.; not detected.
< MQL; values under the method quantification limit.

concentration was lower than the quantification limit. Musk xylene was not detected in any of the sludge samples analysed.

The degradation product of HHCB, HHCB-lactone, appeared in all the samples and, as for the PCMs, the concentration ranges were very different depending on the origin of the sludge. In sludge from WWTP1, the concentration ranged from below MQLs to 42.5 ng g^{-1} (d.w.); while in the samples from WWTP2, the concentration varied between 97.4 ng g^{-1} (d.w.) and 530.5 ng g^{-1} (d.w.).

Previous works [17,18,24,25,28] that focus on the determination of musk fragrances in sewage sludge confirm findings of this study, i.e., that all six investigated PCMs were present in the sewage sludge. However, in contrast to our study, in these reports the most abundant PCMs were HHCB and AHTN while the other PCMs studied (DPMI, ADBI, AHMI, and ATII) were only present in significantly lower concentrations in sewage sludge. Furthermore, the concentrations of NMs were more unstable than those of the PCMs and their concentrations were below MQLs, according on the location of the sludge.

4. Concluding remarks

In this study, an IL-HS-SDME followed by GC-MS/MS procedure was developed to determine ten musk fragrances present at ng g^{-1} levels in sewage sludge that had previously undergone PLE. It also provides good linearity, acceptable MDLs and MQLs ranging between 1-3 ng g^{-1} (d.w.) and

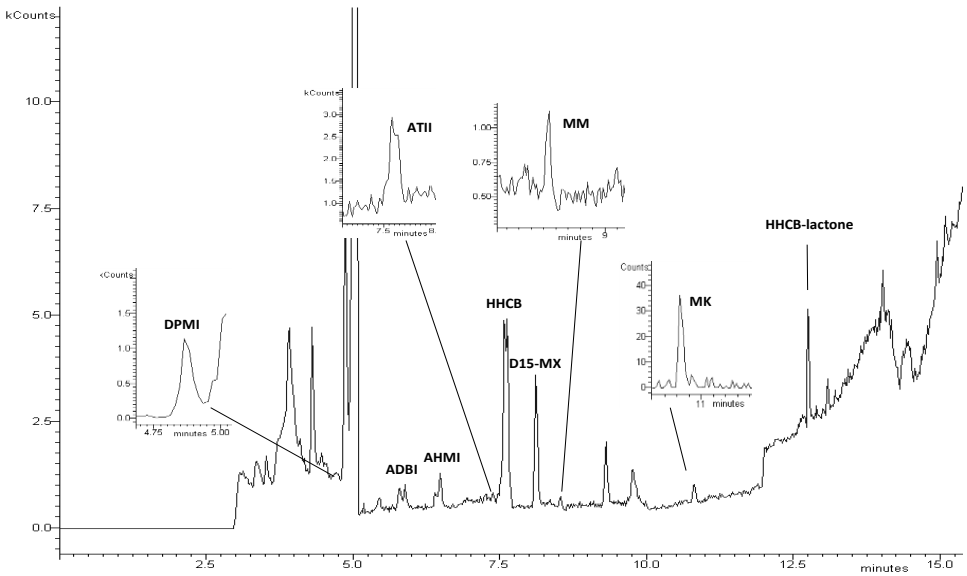

Fig. 3. MS/MS chromatogram of a non-spiked STP 1 sewage sludge.

3-100 ng g^{-1} (d.w.), respectively, and intra- and inter-day repeatability values below 10% for most of the target musks.

Moreover, the use of MS/MS instead of MS detection provided high selectivity and sensibility for the determination of the PCMs and NMs in such highly complex environmental samples as sewage sludge.

Apart from that, working with a 50% H_2O/MeOH extraction solvent, an in-cell clean-up sorbent and IL-HS-SDME, the volume of organic solvent used decreases significantly. At the same time, the use of environment-friendly solvents as ultrapure water or ILs was favoured. As well as being an environment-friendly technique, the use of in-cell clean-up sorbent results in an attractive alternative to laborious standard SPE clean-up step reducing the sample manipulation and treatment time.

Also, the applicability of the method was tested with sewage sludge and the most abundant musk fragrances in the samples were PCMs and HHCB-lactone, the degradation product of HHCB.

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The authors have declared no conflict of interest.

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3.2.3. Fully automated determination of macrocyclic musk fragrances in wastewater by microextraction by packed sorbents and large volume injection gas chromatography-mass spectrometry

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

FULLY AUTOMATED DETERMINATION OF MACROCYCLIC MUSK FRAGRANCES IN WASTEWATER BY MICROEXTRACTION BY PACKED SORBENTS AND LARGE VOLUME INJECTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

A fully automated method has been developed for the determination of eight macrocyclic musk fragrances in wastewater. The procedure includes the enrichment of the analytes by microextraction by packed sorbents (MEPS) followed by large volume injection-gas chromatography-mass spectrometry (LVI-GC-MS). The main factors in the MEPS technique were optimized. For all of the analytes, the highest enrichment factors were achieved when 4 mL samples were extracted by using C18 MEPS-sorbent and 50 µL of ethyl acetate were used for desorption. The eluate was directly analysed by GC-MS. Detection limits were found between 5 ng L^{-1} and 10 ng L^{-1}, depending on the target analytes. In addition, under optimized conditions, the method gave good levels of intra-day and inter-day repeatability in wastewater samples with relative standard deviation (RSD) (*n*=3, 1,000 ng L^{-1}) less than 5% and 9%, respectively. The applicability of the method was tested with wastewater samples from two influent and effluent urban wastewater treatment plants (WWTPs). The analysis of influent urban wastewater revealed the presence of most of the macrocylic musks at concentrations higher than the method quantification limits (MQLs), being the most abundant analyte ambrettolide at 9,289 ng L^{-1}. In addition, the analyses of effluent urban wastewater showed a decrease in the concentrations with macrocyclic musk concentrations between not detected (n.d.) and 2,263 ng L^{-1} being detected.

Keywords: *GC-MS; large volume injection; macrocyclic musk fragrances, microextraction by packed sorbents; urban wastewater.*

1. Introduction

Emerging organic contaminants (EOCs), which have been of increasing interest to scientists in recent decades [12-19], are a group that includes a broad variety of compounds. One subgroup of EOCs is personal care products (PCPs), which include a broad range of compounds widely used as additives in flavourings, body oils, soaps, cosmetics and other such as every day products.

Musk fragrances are cyclic PCPs which can be classified into three types: polycyclic musks, nitro musks and macrocyclic musks. Discussions on the toxicology of nitro musk soon emerged because of the presence of a nitro aromatic compound in their structure. These toxicological problems have led in a decrease in the use of nitro musks, while polycyclic musks production has increased significantly. Nowadays, polycyclic musks are the musk fragrances which are dominating the global market and two of them, galaxolide and tonalide, have been included on the EPA's high production list [20]. In contrast, macrocyclic musks are not as widely used as polycyclic musks because of the cost of their synthesis. However, they are becoming more and more available because of the advances made in synthesis methods over the last few years [13,21,22]. From certain perspectives, macrocyclic musks have advantages in comparison to the polycyclic musks. They seem to smell more intense and so less mass is needed to achieve the same performance in perfumery. They also seem to be more easily degradable in the environment [23]. It is expected that in the coming years the decrease on the synthesis price of macrocyclic musks and their environmentally friendly properties favour the replacement of the polycyclic musks by the macrocyclic musks in the market.

After usage, macrocyclic musks enter wastewater treatment plants. Although partial elimination has been reported for polycyclic as well as nitro musks [24-26], no information is yet available for macrocyclic musks. In fact, most of the studies carried out to date have focused on the optimization of their synthesis, in an attempt to solve problems with enantioselectivity that were so significant to the odour strength of macrocyclic musks [21,22]. The aim was to reduce the price rather than the development of analytical methods to control them. Only two macrocyclic musks have been included in studies, namely ambrettolide and musk NN, in places where polycyclic and nitro musks have been determined in wastewater and biosolid matrices [15,16]. Therefore, there is a need to develop reliable analytical methods to determine macrocyclic musks in environmental waters.

Gas chromatography (GC) is suitable for separating macrocyclic musks, as is the case with the other musk fragrances. However, prior to GC analysis, an extraction/preconcentration step is required due to the low levels expected in environmental water. One current analytical trend is the use of environmentally friendly techniques that reduce the use of organic solvents. Solid-phase microextraction (SPME) [27], single-drop microextraction (SDME) [28], dispersive liquid-liquid

microextraction (DLLME) [29] or microextraction by packed sorbents (MEPS) [7] have been successfully applied to extract and preconcentrate emerging organic contaminants from wastewater samples.

Among these techniques, MEPS as described by Abdel-Rehim [30,31], presents some interesting features. MEPS follow the same principles as SPE but on a miniaturized scale. A small amount of sorbent (1-4 mg) is packed between the body and the metallic needle of a chromatographic syringe (MEPS BIN). The lower amount of sorbent used in the MEPS BIN allows the reduction of sample volume needed from hundreds of mL to just 1-4 mL, while the amount of elution solvent needed to elute the target analytes decrease significantly to µL.

The technique also allows the direct injection of the complete elution volume into the chromatograph, making it a promising fully automated sample preparation approach for successful application for the determination of organic compounds in a broad group of samples (biological samples, environmental samples, etc.) [7,30,32-38]. To date, most of the studies combine MEPS with LC-MS or LC-MS/MS analysis [35,36,38], although new articles have recently been published with MEPS applications followed by GC-MS [7,32-34,37]. Following this trend, the aim of this work is the development of a fully automated MEPS-GC-IT-MS method for the determination of eight macrocyclic musk fragrances in wastewater samples.

2. Experimental

2.1. Chemical standards

Ethylenetridecanedioate (musk NN) as 100 mg L^{-1} solution in cyclohexane, oxacyclohexadecan-2-one (exaltolide), oxacyclo-hexadecen-2-one (habanolide), ethylenedodecanedioate (musk MC4) and 3-methylcyclopentadecanone (muscone) as 10 mg L^{-1} solution in cyclohexane, cyclopentadecanone (exaltone), oxacycloheptadec-8-en-2-one (ambrettolide) and d15-musk xylene (surrogate standard) as 100 mg L^{-1} solution in acetone were supplied by Symta (Madrid, Spain). The standard 9-cycloheptadecen-1-one (civetone) was purchase from Sigma-Aldrich (Steinheim, Germany).

Individual standard solutions of macrocyclic musks were prepared in cyclohexane at concentrations of 1,000 mg L^{-1}. A standard mixture solution of 10 mg L^{-1} was prepared in ethyl acetate. Musk NN standard was supplied already at a concentration of 10 mg L^{-1} which meant that no intermediate standard solution needed to be prepared. Cyclohexane and ethyl acetate were GC grade with purities of 99.8% (Merck, Darmstadt, Germany) and 99.0% (VWR International, Darmstadt, Germany), respectively. The main characteristics (molecular formula, CAS number, boiling point and molecular structure) of the target compounds are shown in Table 1.

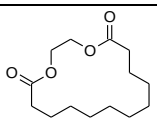
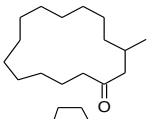
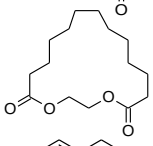
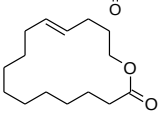
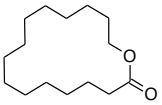
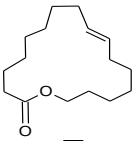
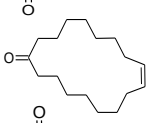
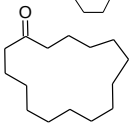
Ultrapure water was obtained using a purelab ultra-purification system (Veolia Water, Barcelona, Spain).

Helium gas with a purity of 99.999% (Carburos Metalicos, Tarragona, Spain) was used for the chromatographic analysis.

2.2. Samples

Influent and effluent wastewater samples were collected between October 2011 and January of 2012 at two wastewater treatment plants (WWTPs) located in Catalonia (NE Spain). The WWTPs are located in two cities with populations of around 120,000 inhabitants. For each sample, 200 mL was put in an amber glass bottle, filtered through a 0.22 µm nylon filter (Scharlab, Barcelona, Spain) and stored at 4 °C until analysis.

Table 1. Main characteristics of the target compounds.

Compound	CAS number	Molar mass (g mol^{-1})	Boiling point (°C) 760mmHg	$LogK_{ow}$	Molecular structure
Ethylenedodecanedioate (Musk MC4)	54982-83-1	255.33	464.5	2.33	
3-Methylcyclopentadecan-one(Muscone)	541-91-3	238.41	329.5	6.33	
Ethylenetridecanedioate (Musk NN)	105-95-3	270.36	330.5	2.90	
Oxacyclohexadecen-2-one (Habanolide)	34902-57-3	238.37	388.4	5.53	
Oxacyclohexadecan-2-one (Exaltolide)	106-02-5	240.39	344.8	6.10	
Oxacycloheptadec-8-en-2-one (Ambrettolide)	123-69-3	252.39	378.7	5.52	
9-Cycloheptadecen-1-one (Civetone)	542-46-1	250.41	371.4	6.31	
Cyclopentadecanone (Exaltone)	502-72-7	224.39	338.3	5.84	

2.3. MEPS and GC-MS equipment

The microextraction was performed with a MEPS device, supplied by SGE Analytical Science (Melbourne, Australia), consisting of a 100 µL gastight syringe body with a modified front fitting that permitted a small barrel to be incorporated into the conical shaped needle. This assembly, known as, barrel insert and needle (BIN), is filled with 4 mg of sorbent commonly used for reversed-phase chromatography or SPE. Two commercially available silica gel based sorbents (particle size 45 µm, pore size 60Å) provided by SGE Analytical Science (Melbourne, Australia) were taken into account in this study one modified with C8 and the other with C18. The MEPS syringe was used in connection with a Varian 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a 1079 injector and connected to a Varian 4000 ion trap mass detector. The extraction was performed using 10 mL screw top headspace vials from Varian that allow the extraction of different sample amounts and was processed by a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland). The instrument control and data processing have been performed by a Varian MS Workstation with v.6.9 software.

2.4. MEPs and GC-MS procedure

Under optimized conditions, the sorbent was sequentially conditioned with 25 µL of ethyl acetate and 25 µL of ultrapure water. The sample volume was then percolated by drawing and discarding 40 cycles of 100 µL at a withdrawal flow rate of 10 µL s^{-1}. In order to remove possible inorganic salts, the sorbent was washed twice with 25 µL of ultrapure water and dried by pumping 4×100 µL of air at 100 µL s^{-1}. Elution was carried out with 50 µL of ethyl acetate, pumped up through the sorbent at 1 µL s^{-1} and down directly into the 1079 injector of the gas chromatograph at 50 µL s^{-1}. A schematic of the extraction/injection system has been provided in Fig. 1.

After each extraction process, the sorbent was washed firstly with 4 cycles of 50 µL of ethyl acetate and then with another 4 cycles of 50 µL of ultrapure water, thereby avoiding the carry-over problems usually associated with MEPS extraction [19,23].

The 1079 injector operated in large volume injection (LVI) mode and a 2 mm i.d. insert liner packed with glass wool (Varian) was used. During injection in split mode at a rate of 50 mL min^{-1} the 1079 injector temperature was set at 70 °C. The ethyl acetate was purged out with a vent flow of 100 mL min^{-1} within 0.5 min (vent time). The splitless mode was then programmed for 2.5 min while the temperature increased at 100 °C min^{-1} to 300 °C for 5 min. A ZB-50 analytical column (30 m × 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex (Torrance, CA, USA) was used for the chromatographic separation. The oven temperature programme was as follows: initial temperature 100 °C held for 2.5 min, increased at 50 °C min^{-1} to 220 °C, 5 °C min^{-1} to 260 °C then 20 °C min^{-1} to 280 °C and held for 5 min. All of the compounds were separated within 10 min. Helium was used as the carrier and collision gas at a flow rate of

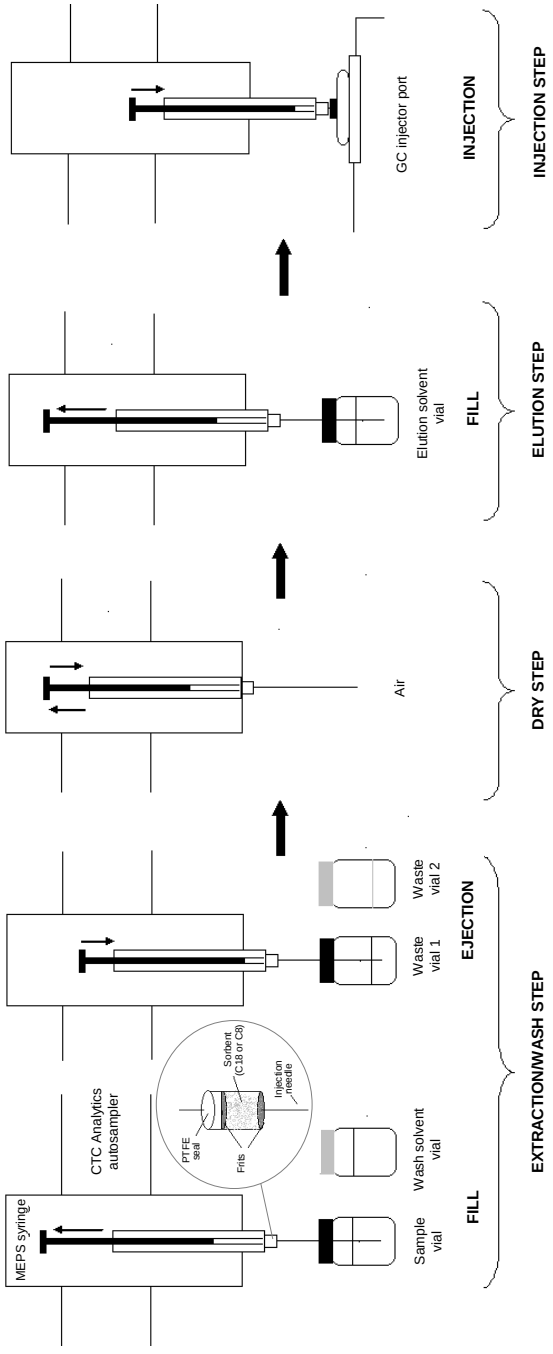


Fig. 1. Schematic of the automated extraction/injection system.

1 mL min^{-1}. The transfer line, manifold and trap temperatures were set at 280 °C, 50 °C, and 200 °C, respectively. A filament-multiplier delay of 3 min was set in order to prevent instrument damage. The mass spectrometer analysed the substances after electron impact ionization in selected ion storage (SIS) mode. The retention time and target ions of the analytes are listed in Table 2.

3. Results and discussion

3.1. Large volume injection GC-MS optimization

A mixed solution of 10 mg L^{-1} of eight macrocyclic musk fragrances and 1 mg L^{-1} of d15-musk xylene as a surrogate standard was prepared in ethyl acetate and 50 µL of this solution was directly injected into the GC-MS, using electron ionization fragmentation in full scan mode. All of the compounds were identified by their molecular ion and, afterwards, the chromatographic separation was optimized by testing several oven temperature programmes. All compounds were separated in just 10 min using the chromatographic conditions described in Section 2.4. Initially, in order to achieve maximum sensitivity/selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and then optimizing the ion trap MS/MS parameters (amplitude excitation voltage, CID storage level). However, due to the excessive fragmentation of the target compounds, which caused a significant decrease in the analytical signal, selective ion separation (SIS) mode in mass spectrometry was selected for the work. Table 2 also summarizes the ions selected for identifying and quantifying the target analytes.

3.2. Optimization of MEPS

As in SPE, the individual steps, namely extraction, clean-up and elution, have to be optimized for maximum analytes peak areas.
In addition, in an automated MEPS procedure, additional steps for post-cleaning, re-conditioning and the extraction regime have to be included in order to enable multiple use of the MEPS-BIN. The variables optimized in MEPS extraction were as follows: extraction regime, carry-over, fill and ejection speed, injection speed, elution solvent, and MEPS-BIN sorbent. Ultrapure water that contained 2.5 µg L^{-1} (n=3) of all the analytes and 1 µg L^{-1} of d15-MX as a surrogate standard was used for the optimization of the microextraction variables.

3.2.1. Extraction regime and carry-over

During the first optimization studies, 10 mL screw top headspace vials and 2 mL of sample volume were needed. The other experimental conditions were as follows: 5 µL s^{-1} as fill/ejection speed, 20 µL s^{-1} as injection speed, ethyl acetate (50 µL) as elution solvent and C8 and C18 as extraction sorbents. Two extraction regimes were applied in order to ascertain with which extraction regime the best analytical signal was achieved. Firstly, the sample may be pumped up only once and then discarded into waste (carrying out this

Table 2. Retention time. MS parameters, linear range, determination coefficients, MDLs, MQLs, intra-day and inter-day repeatability (% RSD, *n*=3, 1,000 ng L^{-1}) for effluent WWTP A.

Compound	Retention time (min)	Ions (SIS) [a] (m/z)	Linear range [b] (ng L^{-1})	Determination coefficient (r^2)	MDLs (ng L^{-1})	Intra-day Repeatability (RSD %)	Inter-day Repeatability (RSD %)
Exaltone	7.84	**225**, 135, 125	10-5,000	0.9991	5	3	7
Exaltolide	7.87	**241**, 223, 123	25-5,000	0.9992	10	2	8
Muscone	7.91	**238**, 209, 125	15-5,000	0.9992	7.5	1	8
Habanolide	7.91	239, 221, **95**	15-5,000	0.9991	7.5	3	9
Ambrettolide	8.58	235, 135, **95**	10-5,000	0.9990	5	5	8
Musk MC4	8.82	213, 149, **87**	10-5,000	0.9994	5	1	7
Civetone	9.12	**250**, 251, 121	10-5,000	0.9991	5	3	8
Musk NN	9.98	227, 211, **98**	10-5,000	0.9991	5	1	8
d15-Musk xylene [c]	8.30	**294**, 136, 122					

[a] Quantification ions (m/z) are shown in bold type.
[b] MQLs (ng L^{-1}), were fixed as the lowest calibration level.
[c] d15-Musk xylene is the surrogate standard.

operation with one or several aliquots of fresh sample), in a similar way to conventional SPE. Alternatively it may be pumped up and down several times from the same vial in the multiple draw-eject cycles procedure [19], as in in-tube SPME. With the SPE mode twenty 100 µL volume aliquots of fresh sample were drawn, passed through the MEPS-BIN and discarded into the waste, while with the multiple draw-eject cycles procedure, twenty 100 µL aliquots were discarded of the sample vial. The analytical signals obtained were slightly better in the SPE mode, independently of the sorbent used.

In addition, in order to improve the analytical signal without increasing the sample volume, multiple draw-eject cycles procedure was used, increasing the number of cycles from 20 to 100. In the analysis of the results, a slightly increase in the analytical response were observed when the number of cycles increase reaching the highest analytical signal working with 100 cycles.

However comparing the peak areas obtained working with SPE mode (20 cycles) with those obtained working with multiple draw-eject cycle mode (100 cycles), not significance differences were observed. Due to the less number of strokes needed in the SPE mode the extraction time is lower than working with multiple draw-eject mode and the mechanical stress to the MEPs syringe plunger is reduced which extends the lifetime of the MEPs syringe [20]. Therefore, this extraction regime was selected for further experiments. The influence of the sample volume on the target analytes peak areas was later studied in detail (Section 3.2.4).

The conventional SPE process with disposable cartridges or membranes, in comparison to reusing the MEPS sorbent, requires a detailed evaluation of potential carry-over phenomena [19,23]. This makes the inclusion of a thorough clean-up step necessary after each extraction process. In order to overcome possible carry-over problems and taking into account the SGE analytical science guidelines for the use of MEPS system, four wash-discard cycles were added after the elution each with 50 µL ethyl acetate followed by four clean-up-discard cycles each with 50 µL of ultrapure water. This cleaning procedure removed all the residual analytes and matrix components and left the MEPS-BIN ready for the next extraction.

3.2.2. MEPS syringe plunger speed

The syringe plunger speed is one of the most important variables for the optimization of a method that uses MEPS as the microextraction technique. In our study, fill/ejection speed (extraction step) and fill/ injection speed (elution step) were optimized.

The MEPS syringe plunger speed was evaluated within the range of 1 µL s^{-1} and 20 µL s^{-1} for the extraction and elution steps with an ultrapure water sample spiked with all the target analytes (2.5 µg L^{-1}) and d15-MX (1 µg L^{-1}) as a surrogate standard, C8 and C18 as extraction sorbents and ethyl acetate as the elution solvent. As can be seen in Fig. 2, the best peak

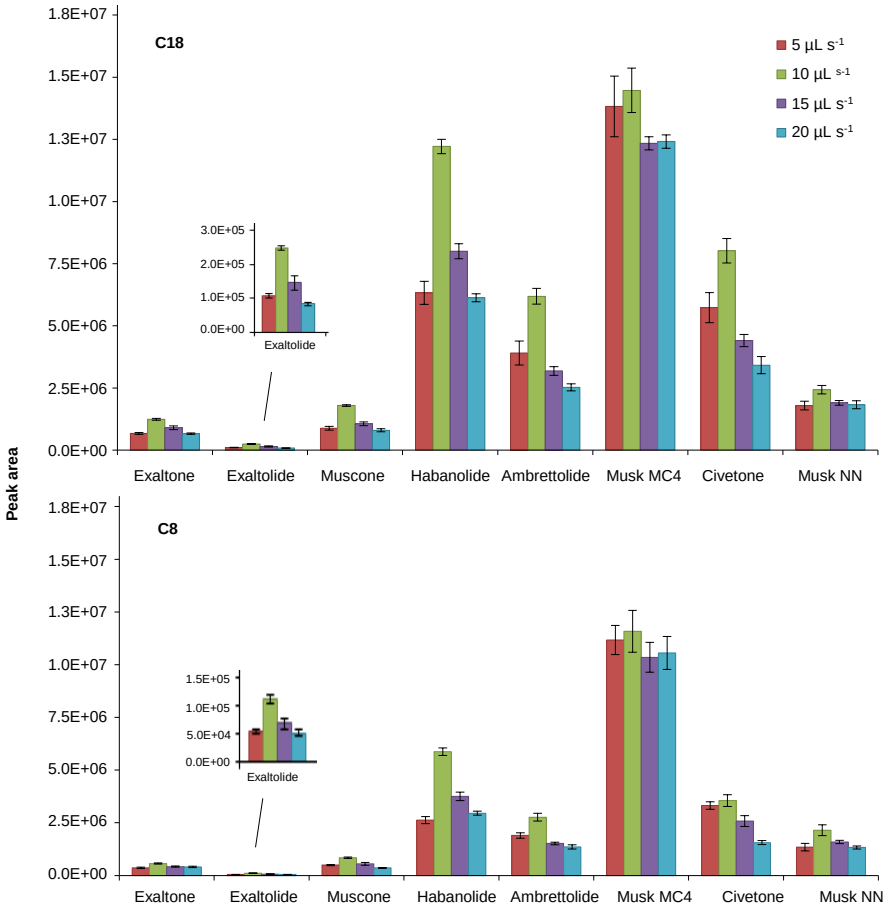


Fig. 2. Optimization of the fill/ejection speed during the MEPS extraction step with C8 and C18 BIN, 2 mL sample volume and ethyl acetate as elution solvent (n=3, 2.5 µg L^{-1} macrocyclic musk and 1 µg L^{-1} of surrogate standard).

areas were achieved for both sorbents when the extraction step was made at 10 µL s^{-1} fill/ejection speed, while at higher fill/ejection speeds such as 15 µL s^{-1} or 20 µL s^{-1} problems started to occur with cavitations (air bubbles) and the extraction volume could not be controlled. In the elution step, on the other hand, the best results were achieved working at 1 µL s^{-1} fill speed and 50 µL s^{-1} as the injection speed. When the elution solvent was filled at 1 µL s^{-1} the analyte/sorbent contact time increased, enhancing the extraction of the analytes retained in the sorbent. When the solvent was then injected into the GC injector port at 50 µL s^{-1} the analyte contact time was minimized and the analytes were forced to be eluted.

3.2.3. Elution solvent

Due to the small amount of sorbent used in MEPS, desorption may be performed with a relatively small volume of solvent that can be completely transferred into the LVI-GC instrument. A GC compatible, non-polar and volatile solvent is required. Ethyl acetate and cyclohexane were the two solvents tested.

The solvent evaluation was performed with two silica gel modified C8 and C18 sorbents, working at the optimized extraction and elution speeds with spiked ultrapure water (2.5 µg L^{-1} all macrocyclic musk plus 1 µg L^{-1} d15-MX, *n*=3). As can be seen in Fig. 3, with the C18 sorbent, all the target analytes have higher peak areas when ethyl acetate was used as elution solvent.

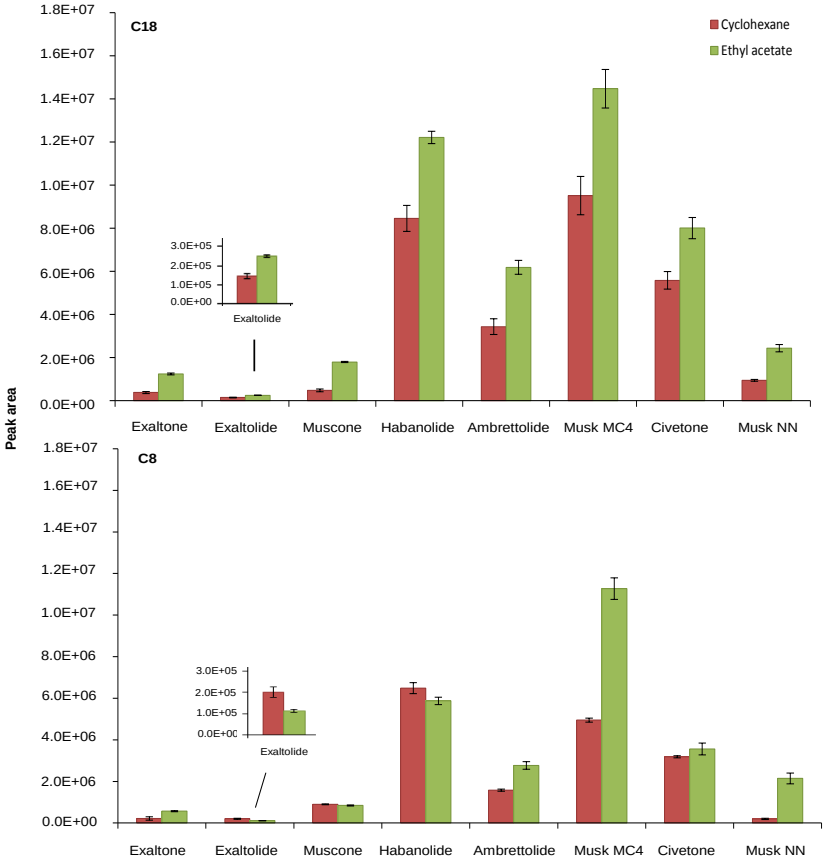


Fig. 3. Optimization of the elution solvent with C8 and C18 extraction sorbents and 2 mL sample volume. The fill/ejection speed during the extraction step was set at 10 µL s^{-1}, while the fill/ejection speed during the elution step were set at 1 µL s^{-1} and 50 µL s^{-1}, respectively (*n*=3, 2.5 µg L^{-1} macrocyclic musk and 1 µg L^{-1} of surrogate standard).

However, when the extraction was performed with the C8 sorbent, some analytes, such as muscone, habanolide and exaltolide achieved the highest peak areas when cyclohexane was used as elution solvent. While for the remaining target analytes ethyl acetate showed best results.

To summarize ethyl acetate was chosen as the optimal elution solvent because it was the solvent with which the highest peak areas were achieved for most of the analytes regardless of the MEPS sorbent used.

3.2.4. Comparison between C8 and C18

As mentioned above, two different sorbents commonly used for the solid-phase extraction of organic compounds were tested in this work: C8 and C18 modified silica gel. They were compared in terms of the maximum volume of sample that could be concentrated without losses, the overall extraction efficiency (absolute recoveries) and the selectivity when applied to a wastewater sample.

For sample volume comparison, volumes between 2 mL and 5 mL of ultrapure water were processed by C8 and C18 MEPS. Processing increasing volumes of spiked ultrapure water (2.5 µg L^{-1} all macrocyclic musks plus 1 µg L^{-1} surrogate standard, *n*=3), provided a proportional increase of the response of all analytes up to a 4 mL sample amount. Taking into account that the use of large sample quantities may improve the method detection limits (MDLs), 4 mL (40 × 100 µL) was selected as the optimal sample volume for both sorbents. Absolute recoveries were determined by comparison of the responses (peak areas) obtained by MEPS of 4 mL of ultrapure water that were spiked with the target analytes (2.5 µg L^{-1}) and the surrogate standard (1 µg L^{-1}) in comparison to those obtained working with a standard in ethyl acetate with an equivalent concentration of all the compounds mentioned above that was injected directly (50 µL) into the GC-MS system. Low recovery values, ranging from 7% to 28% with C8 and 13% to 39% for C18 were obtained for both sorbents, with slightly better results with the C18. In addition, corrected efficiency values were calculated in order to ascertain whether the surrogate standard could improve the extraction efficiencies. Higher recovery values were obtained, ranging between 49% and 98% and 56% and 97% for C8 and C18, respectively (Table 3). Finally, the analytical signals obtained in the chromatogram were studied in order to select the sorbent that provides the best selectivity. To perform this study, two different water matrices, namely influent wastewater and effluent wastewater, were spiked with 2.5 µg L^{-1} each of macrocyclic musk and 1 µg L^{-1} of the surrogate standard (*n*=3). Independently of the matrix analysed the macrocyclic musk recoveries (Table 3) and selectivity were better when C18 was used as MEPS extraction sorbent with corrected recovery values ranging between 52% and 92% for influent water and between 54% and 95% for effluent water. Therefore C18 was chosen as the optimal extraction sorbent to extract macrocyclic musk fragrances present in wastewater samples.

Table 3. Recovery values obtained with C8 and C18 sorbents with ultrapure water, influent and effluent wastewater.

Compound	Ultrapure water				Influent water				Effluent water			
	C8		C18		C8		C18		C8		C18	
	Recoveries[a] (%)	Corrected[b] recoveries (%)	Recoveries[a] (%)	Corrected[b] recoveries (%)	Recoveries[a] (%)	Corrected[b] recoveries (%)	Recoveries[a] (%)	Corrected[b] recoveries (%)	Recoveries[a] (%)	Corrected[b] recoveries (%)	Recoveries[a] (%)	Corrected[b] recoveries (%)
Exaltone	16	64	28	77	11	59	21	71	13	60	24	73
Exaltolide	7	49	13	56	5	46	10	52	6	48	11	54
Muscone	19	77	37	97	17	73	32	92	18	75	35	95
Habanolide	20	68	35	91	15	60	30	86	17	64	32	88
Ambrettolide	16	55	30	76	10	49	22	72	12	52	26	73
MC4	27	98	37	97	20	90	30	91	23	94	32	95
Civetone	16	52	37	97	13	47	29	90	14	49	34	94
Musk NN	28	98	36	93	22	91	31	87	24	93	33	90
d15-Musk xylene[c]	28		39		23		31		26		34	

[a] Recoveries in spiked ultrapure water, influent and effluent wastewater (2.5 μg L^{-1}), referred to the direct injection of a standard in ethyl acetate (200 μg L^{-1}).
[b] Recoveries in spiked ultrapure water, influent and effluent wastewater (2.5 μg L^{-1}), referred to the direct injection of a standard in ethyl acetate (200 μg L^{-1}) and corrected by the use of surrogate standard.
[c] d15-Musk xylene is the surrogate standard.

3.3. Method validation

The method was validated working with a C18 sorbent, 4 mL sample volume (40 cycles of 100 µL sample), 50 µL of ethyl acetate as the elution solvent, a fill/ejection speed of 10 µL s^{-1} in the extraction step and 1 µL s^{-1} fill and 50 µL s^{-1} injection speed during the elution step.

Before validating the method, the matrix effect was studied statistically by comparing the slopes of the calibration curves for influent and effluent WWTPs samples with those obtained using ultrapure water. The matrix effect was observed and could be corrected by the presence of a surrogate standard. The method was then analytically validated with an effluent WWTP B sample by establishing the linear ranges, method detection limits (MDLs), method quantification limits (MQLs), intra-day repeatabilities and inter-day repeatabilities. An effluent WWTP B was analysed for triplicate and peaks of exaltone, ambrettolide, MC4 and musk NN appeared in the chromatogram. The average peak area of each detected compound was subtracted from the peak area of each spiked sample.

The linear range of the method was obtained by analysing the WWTP B effluent sample spiked with macrocyclic musks at concentrations of between 7.5 ng L^{-1} and 10,000 ng L^{-1} while the surrogate standard concentration remained constant at 1,000 ng L^{-1}. The compounds showed a good linear range between 10 ng L-1 and 10,000 ng L^{-1} for most of the target analytes, except for muscone and habanolide, with a linear range of 15 ng L^{-1} to 10,000 ng L^{-1} and exaltolide with a linear range of 25 ng L^{-1} to 10,000 ng L^{-1}. The determination coefficients (r^2) were higher than 0.9990 for all the target compounds (Table 2).

The MDLs for each compound were calculated depending on their presence in the blank. For target compounds without blank signals, the MDLs were calculated as concentrations that give a signal of the quantifier ion three times higher than the noise signal. Whereas for target compounds with a blank signal (exaltone, ambrettolide, musk MC4 and musk NN), the MDLs were estimated as the concentration that gave a signal average of three times more than the standard deviation of the blank signals. In all cases, the MQLs were set at the lowest calibration level. As can be seen in Table 2, the MDLs and MQLs ranged from 5 ng L^{-1} to 10 ng L^{-1} and 25 ng L^{-1} to 10 ng L^{-1}, respectively.

The intra-day and inter-day repeatabilities were determined by spiking three replicates of the effluent WWTP B at 1,000 ng L^{-1}. The results obtained (Table 2), expressed as % RSD ranged between 5% and 1% for intra-day repeatability and between 9% and 7% inter-day repeatability.

3.4. Method application

The method developed was used to determine macrocyclic musk fragrances in different kinds of water samples: influent and effluent samples collected from urban WWTPs (A and B) over a period of four months (Section 2.2).

The analytes were quantified by using the calibration curves obtained with effluent WWTP B. Table 4

summarizes the results of the average concentrations (*n*=8) of the macrocyclic musk fragrances found in each type of sample. As expected, the influent musk concentrations were higher than the effluent ones. An analysis of the results shows that ambrettolide was the most abundant compound and appeared in all influent water matrices, ranging from 2,872 ng L^{-1} in WWTP A to 9,289 ng L^{-1} in the WWTP B. In the same way as ambrettolide, exaltone and civetone were present in all of the influent samples with concentrations ranging between 1,402 ng L^{-1} and 2,891 ng L^{-1} and 87 ng L^{-1} and 2,053 ng L^{-1}, respectively. With the exception of exaltolide and muscone, the remaining macrocyclic musks were detected in all influent samples with average concentrations higher than the quantification limits. Fig. 4 shows a chromatogram of a non-spiked influent WWTP B water sample.

Table 4: Concentrations of the target macrocyclic musks found in two WWTP (*n*=8), in ng L^{-1}.

Compound	WWTP A		WWTP B	
	Influent	Effluent	Influent	Effluent
Exaltone	1,402-1,997	970-1,677	1,810-2,891	450-2,263
Exaltolide	14-400	<MQL-161	n.d.-1,488	n.d.-477
Muscone	19-524	7-41	n.d.-2,462	n.d.-63
Habanolide	<MQL-497	<MQL-164	653-1,569	n.d.-534
Ambrettolide	2,872-4,360	424-1,428	3,133-9,289	<MQL-2,461
Musk MC4	39-64	7-25	8-97	23-41
Civetone	87-2,053	50-1,795	55-1,310	<MQL-51
Musk NN	8-131	<MQL-9	6-509	<MQL-18

n.d.; not detected.
<MQL; values under the method quantification limit.

In contrast, exaltone was the most abundant compound in effluent waters with values between 450 ng L^{-1} and 2,263 ng L^{-1}, followed by ambrettolide with concentrations ranging between <MQL and 2,461 ng L^{-1}.
The remaining compounds were detected in effluent waters at values significantly lower than the influent values and some of them, such as exaltolide, muscone, habanolide or musk NN, were detected at concentrations below the MQLs or were not detected at all.

4. Conclusions

An automated microextraction by packed sorbents procedure has been developed for the first time to determine eight macrocyclic musk fragrances from wastewater samples followed by a GC-MS (SIS) at lower ng L^{-1} concentrations. The combination of MEPS with GC-MS provides a promising alternative to laborious standard SPE enrichment. With good linearity and acceptable MDLs and MQLs ranging between 5 ng L^{-1} and

10 ng L^{-1} and 10 ng L^{-1} and 25 ng L^{-1} respectively. In addition, intra-day and inter-day repeatability values lower than 10% of RSD were achieved for all the target analytes.

Furthermore, the decrease of the amount of sorbent from 60 mg to 500 mg of SPE and from 1 mg to 4 mg of MEPS allowed us to work with low sample volumes (1-4 mL) and low elution solvent volumes (50 µL) and enabled the automation of all the MEPS steps (sample concentration, analytes enrichment and introduction of the extract into the GC-MS system). The applicability of the developed method has been demonstrated by the analysis of wastewater samples from two different wastewater treatment plants. The results show that all of the target analytes were present in influent wastewater samples while, in effluent samples, a decrease in the macrocyclic musk concentrations was observed. However, taking into account the presence of most of the target analytes in effluent wastewater samples, WWTPs treatments need to be evolved by incorporating tertiary treatments in order to increase the efficiency of the removal of the macrocyclic musk compounds.

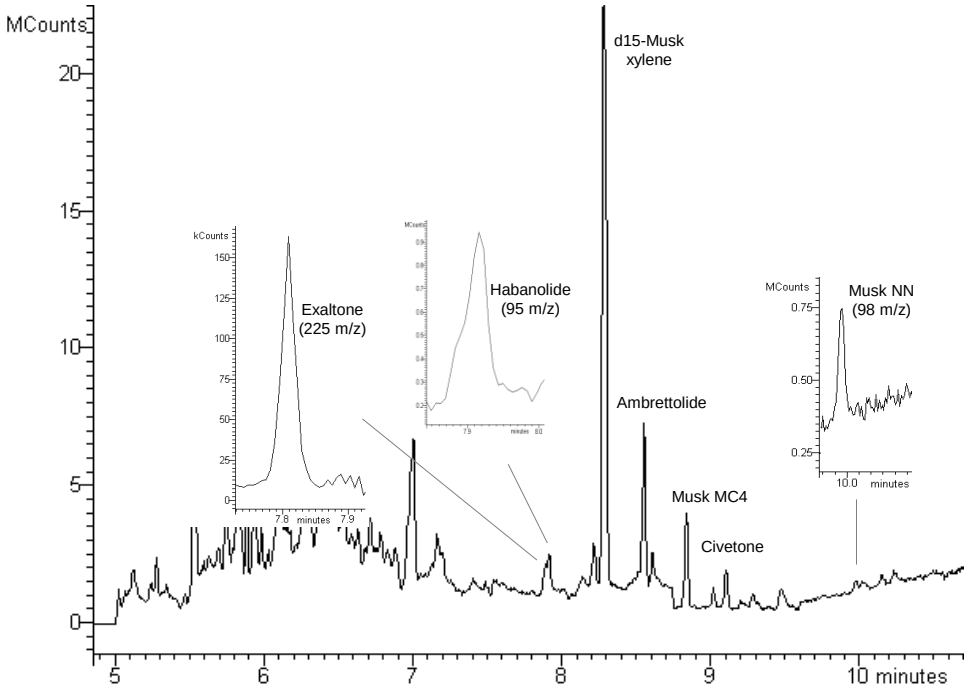


Fig. 4. Chromatogram of a non-spiked influent water sample from WWTP B and analytical signal enlargements.

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3.2.4. Sorbent-packed needle microextraction trap for synthetic musks determination in wastewater samples

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
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SORBENT-PACKED NEEDLE MICROEXTRACTION TRAP FOR SYNTHETIC MUSKS DETERMINATION IN WASTEWATER SAMPLES

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Abstract

A needle trap (NT) device filled with HF Bondesil-C18 as a sorbent material was evaluated for the dynamic headspace analysis of a family of nine synthetic musk fragrances that include two nitro musks, six polycyclic musks (with galaxolide and tonalide as the most widespread used polycyclic musks) and the degradation product of galaxolide (galaxolidone) in wastewater samples. Different parameters affecting the adsorption capacity of the sorbent were studied (e.g. extraction mode, extraction temperature, salt concentration, preincubation time fill and ejection speed or fill volume). Furthermore, injection parameters used with the NT device (e.g. desorption mode, desorption temperature and time) were evaluated to optimize the desorption and transfer of the target compounds into the GC column. Method detection limits obtained with gas chromatography-tandem mass spectrometry (GC-MS/MS) detection were found in the low ng L^{-1} range, between 2.5 and 12 ng L^{-1}, depending on the target compounds. Moreover, under optimized conditions, the method gave good levels of intra-day and inter-day repeatabilities in wastewater samples with relative standard deviations (n=5, 100 ng L^{-1}) less than 11 and 17%, respectively. The developed method was satisfactorily applied to the analysis of aqueous samples obtained from three wastewater treatment plants. All the polycyclic musk fragrances studied were detected in influent samples with cashmeran, galaxolide and tonalide as the most representative compounds. The analysis of effluent wastewater showed a decrease in the concentrations of all of the polycyclic musk fragrances detected in influent samples and an increase in the concentration of galaxolidone until a maximum value of 820 ng L^{-1}.

Keywords: *gas chromatography-tandem mass spectrometry; needle trap; synthetic musk fragrances; thermal desorption; wastewater samples.*

1. Introduction

Sample preparation is the cornerstone of chemical analysis. Preconcentration is a crucial step when synthetic musk fragrances often occurring in concentrations as low as mg L^{-1} or ngL^{-1} are to be determined in environmental water samples. Some preconcentration techniques such as liquid-liquid extraction (LLE) [1-3], solid-phase extraction (SPE) [4-7], dispersive liquid-liquid microextraction (DLLME) [8-10], solid phase-microextraction (SPME) [11,12], single-drop microextraction (SDME) [13,14], microextraction by packed sorbents (MEPS) [15,16] or dispersive microsolid-phase extraction (D-μ-SPE) [17] have been reported. Of all the extraction techniques mentioned, SPE is the most widely used in the environmental analytical field because it consumes minimal amount of organic solvents and a great diversity of sorbents is commercially available. Nevertheless the development of economical and ecological small scale sample preparation techniques that are able to meet requirements such as enhanced sensitivity and selectivity, robustness and simple handling are desirable [18,19]. In this way, solvent-free extraction methods based on the partitioning of analytes between gaseous or liquid phase and stationary phase have become important and have been widely applied in research over the last decade, with SPME as one of the most successfully approaches [20,21]. Although Raschdorf [22] developed the first device based on a needle filled with Tenax sorbent in the 1970s, needle trap (NT) extraction devices have only recently become popular due to their combination of advantages of SPME (e.g. solvent-free, fast, sensitive and one-step sample preparation and injection method) and SPE (e.g. sensitivity of the method can be increased by increasing the sample volume) with robustness, easier handling during sampling and desorption, and the fact that they permit a high degree of automation and on-line coupling to GC instruments [23-26]. The literature found up to now can be divided in two categories depending on the NT device used: (a) internally coated needles [27-29] and (b) needles packed with commercially available sorbents [26,30-36] or chemically synthesized polymers [37,38]. Regardless of the kind of NT used, the extraction by NT has the advantages of being solvent-free and of having sampling and analysis times that are significantly shorter than most existing methods. In this study, two different needles packed with 20 mm or 30 mm of HF Bondesil-C18 sorbent were evaluated in order to determine the optimal configuration to extract the synthetic musk fragrances present in wastewater samples prior to analysis by GC-MS/MS. The different parameters affecting the adsorption capacity of the NT as well as the desorption and transferring of the target compounds into the GC were also studied. Once the most appropriate experimental conditions were found, the NT methodology was compared in terms of method validation parameters with other microextraction techniques and was successfully applied for the analysis of synthetic musk fragrances in wastewater samples.

2. Experimental part

2.1. Chemical Standards

The nitro musk fragrances 2,4,6-trinitro-1,3- dimethyl-5-*tert*-butylbenzene (MX, musk xylene) and 1,1,3,3,5-pentamethyl-4,6-dinitroindane (MM, musk moskene) were purchased as 100 µg mL^{-1} solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The six polycyclic musk fragrances studied were supplied by Promochem Iberia (Barcelona, Spain) and were the following: 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)- indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-*tert*-butylindane (ADBI, celestolide), 6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI, phantolide), 5-acetyl- 1,1,2,6-tetramethyl-3-isopropylindane (ATII, traseolide), 1,3,4,6,7,8-hexahydro- 4,6,6,7,8,8- hexamethylcyclopenta-*(g)*-2-benzo-pyran (HHCB, galaxolide), 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4- tetrahydronaphthalene (AHTN, tonalide). International Flavors & Fragances Inc. (Barcelona, Spain) provided 1,3,4,6,7,8-hexahydro-4,6,6,7, 8,8-hexamethylcyclopenta-*[g]*-2-benzo-pyran-1-one (HHCB-lactone, galaxolidone) and the deuterated analogue d15-Musk xylene (d15-MX, surrogate standard) was supplied as a 100 mg L^{-1} solution in acetone by Symta (Madrid, Spain). Table 1 shows the boiling point and the octanol/water partition coefficient of each target compound.

Individual standard solutions of the synthetic musk fragrances were prepared in acetone at concentrations of 4,000 mg L^{-1} for polycyclic musks and 1,000 mg L^{-1} for HHCB-lactone. A standard mixture solution of 100 mg L^{-1} was prepared in ethyl acetate. MX, d15-MX and MM standards were supplied directly at a concentration of 100 mg L^{-1} and used as received. Acetone and ethyl acetate were GC grade with purity >99.9% from Prolabo (VWR, Llinars del Vallès, Barcelona, Spain).

Ultrapure water was obtained using an ultrapure water purification system from Veolia waters (Sant Cugat del Vallès, Barcelona, Spain). Helium gas with a purity of 99.999 % was used for the chromatographic analysis (Carburos Metálicos, Tarragona, Spain).

2.2. Sampling

Influent and effluent wastewater samples were collected from three urban wastewater treatment plants (WWTPs) located in Tarragona (WWTP A), Reus (WWTP B) and Vila-seca/Salou (WWTP C) between October and December 2013. The WWTPs receive urban sewage and industrial discharges from a population of about 130,000 inhabitants. The three WWTPs use activated sludge for biological treatment and the WWTP C also employs a tertiary treatment based on reverse osmosis (RO). All samples were collected by using pre-cleaned amber glass bottles and were filtered using a 1.2 µm glass fibre filter (Fisherbrand, Loughborough, UK) and a 0.22 µm nylon filter (Scharlab, Barcelona, Spain). Samples were analysed within three days of their collection (stored at 4 °C in the fridge).

Table 1. Boiling point, log K_{ow}, retention times (t_R) and parent and products ions of the target compounds.

No.	Compound	Boiling point (°C)	Log K_{ow} [a)]	t_R (min)	Parent ion (m/z)	Product ions (m/z) [c)]
1	Cashmeran (DPMI)	286.1	5.9	8.33	191	107, **135**, 173
2	Celestolide (ADBI)	309	5.4	10.24	229	131, **173**, 187
3	Phantolide (AHMI)	336.6	5.9	10.98	229	131, 145, **187**
4	Traseolide (ATII)	350	6.3	12.02	215	131, 171, **173**
5	Galaxolide (HHCB)	326	5.9	12.31	243	171, **213**
6	Tonalide (AHTN)	356.8	6.3	12.36	243	**145**, 159, 187
7	Musk xylene (MX)	392.3	3.8	13.24	282	**265**, 266, 281
8	Musk moskene (MM)	351.1	5.2	13.35	263	187, 201, **211**
9	Galaxolidone (HHCB-Lactone)	*	*	16.93	257	183, 201, **239**
10	d15-Musk xylene (d15-MX) [b)]	*	*	13.10	294	170, **276**, 295

* Information not found at the bibliography.

[a)] Log K_{OW} values predicted from SRC-K_{OW}Win software.

[b)] Surrogate standard (SS).

[c)] Quantification ions (m/z) are shown in bold type.

2.3. Preparation of the Needle Trap

Hamilton (Bonaduz, Switzerland) 22 gauge stainless steel (metal hub) needles (O.D.=0.718 mm, I.D.=0.413 mm and 51 mm length) with point style 5 (Fig.1) were filled with HF Bondesil-C18 sorbent (120 µm) from Agilent Technologies (Palo Alto, USA). Stainless steel wire (AISI 316L, GoodFellow, Huntingdon, UK) of 100 µm diameter was used to prepare spiral plugs to hold sorbent particles inside the needles. First, a small piece of spiral plug (five turns, ~1.5 mm) was fixed in the tip of the needle to prevent sorbent particles from being fixed in the side hole of the needle. Then, 20 mm or 30 mm of C18 sorbent particles were positioned inside the needle. Finally, another spiral plug was carefully introduced in the upper position of the needle until it reached the end of the sorbent layer to fix the sorbent particles. Using this needle configuration, needle traps were conditioned in the GC injector at 230 °C for 30 min to eliminate any contaminations from the manufacturing process or shipping. Each needle was stored inside a closed vial until analysis.

2.4. Needle Trap Extraction

A CombiPAL autosampler (CTC Analytics, Zwigen, Switzerland) equipped with a single Magnet Mixer, a 100 µL Hamilton syringe and controlled by the Cyclo Composer Macro Editor 1.4 Software was used for the fully automated needle trap micro-extraction (NTME). The general microextraction procedure was as follows: 10 mL of sample or standard solution was introduced into a 20 mL HS glass vial and immediately sealed

with a Teflon septum. When the temperature of the heat/stir accessory reached 60 °C, the vial was automatically transported there and the headspace was allow to equilibrate with the sample at the extraction temperature for 15 min. The needle with 30 mm of HF Bondesil-C18 sorbent was inserted in the vial to perform the HS dynamic extraction. 500 µL of headspace vapours were then percolated by pumping up through the sorbent 5 cycles of 100 µL at 10 µL s^{-1} fill and 30 µL s^{-1} ejection speeds. Afterward, the desorption was conducted at 230 °C for 3 min and the compounds were subsequently analysed by GC-MS/MS.

2.5. Gas chromatography-tandem mass spectrometry

The GC-MS/MS analyses were performed on a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) connected to a Varian 4000 ion trap detector.

The GC was equipped with a 1079 programmable temperature injector and a 0.8 mm i.d. insert liner (Varian) and a CombiPal autosampler (CTC Analytics, Zwigen, Switzerland). A fused silica capillary column (3 m x 0.25 mm i.d.) from Micron Phenomenex (Torrance, CA, USA) was used as a guard column. The chromatographic separation was done in a midpolarity phase capillary analytical column with 50% diphenyl/50% dimethylpolysiloxane (30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex. The oven temperature was programmed as follows: 70 °C hold for 3 min, raised at 40 °C min^{-1} to 180 °C, then 5 °C min^{-1} to 220 °C and finally 20 °C min^{-1} to 280 °C (hold 3.25 min). The carrier gas employed was helium with purity of 99.999%, at a constant flow of 1 mL min^{-1}. During the thermal desorption the injector was operated in splitless mode at 230 °C. Temperatures of transfer line, manifold and trap were 280 °C, 50 °C and 200 °C, respectively. A filament-multiplier delay of 3 min was established in order to increase filament's service life. The mass spectrometer analysed the substances after electron impact ionization in tandem mass spectrometry mode (MS/MS).

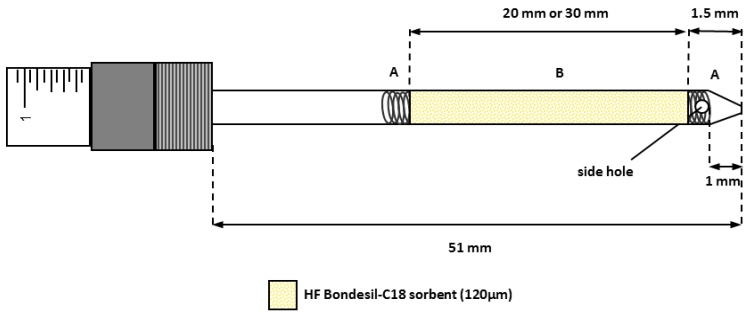

Fig. 1. Sorbent-packed needle trap device: (A) spiral plugs and (B) sorbent material.

3. Results and discussion

3.1. GC-MS/MS Optimization

A mixed solution of 10 mg L^{-1} of the target musk fragrances and 1 mg L^{-1} of d15-MX as surrogate standard (SS) was prepared in ethyl acetate and 1 µL of this solution was directly injected into the GC-MS, using electron ionization fragmentation in full scan mode. All compounds were separated in just 17 min using the chromatographic conditions described in Section 2.4. Under optimum chromatographic conditions, the chromatogram (Fig. 2a) also shows the separation of two galaxolide stereoisomers (4S,7S; 4S,7R). The galaxolide was quantified by integrating the 4S and 7R/S peaks together due to the fact that both stereoisomers are responsible for the musky odour [39]. In order to achieve maximum sensitivity/selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and the MS/MS parameters optimized in a previous paper [40]. The three product ions selected for a correct identification of the target analytes as well as the retention time and the parent ion of each analyte are summarized in Table 1. Each compound was acquired separately in one segment, except, HHCB and AHTN and the target nitro musks (d15-MX, MX and MM).

3.2. Needle Trap Optimization

To optimize needle trap microextraction (NTME) conditions, two different NT were tested, the first one contained 20 mm of HF Bondesil-C18 sorbent while the second one was filled with 30 mm of the same sorbent. HFBondesil-C18 sorbent was selected as extraction sorbent due to its ability to extract a wide range of non-polar compounds, including synthetic musk fragrances, by establishing non-polar interactions [41-43]. More selective SPE sorbents previously used to extract synthetic musk fragrances from environmental samples, as Oasis HLB [44] or Bond Elut Nexus [45], were discharged because are not commercially available at higher particle size (100-250 µm). The particle size would affect the packing density and consequently affect the capacity, the extraction efficiency and desorption efficiency of the NT device. As has been demonstrated by Zhan and Pawliszyn [46], NTs packed with small particles possess higher extraction capacity and efficiency but much higher resistances to flow as well. In addition, the use of low particle size sorbents could make pneumatic restrictions and generation of bubbles and important factor to take into account.

The main parameters that affect the sorption and desorption process in NTME were optimized for each needle in order to maximize the chromatographic peak area of the compounds by analysing a standard mixed solution of 1 µg L^{-1} of all the target musks and the SS d15-MX in 10 mL of ultrapure water. To obtain the best conditions for each needle, such variables as extraction mode, extraction temperature, salt concentration, preincubation time, fill and ejection speed, fill volume and desorption parameters (temperature and time) were optimized

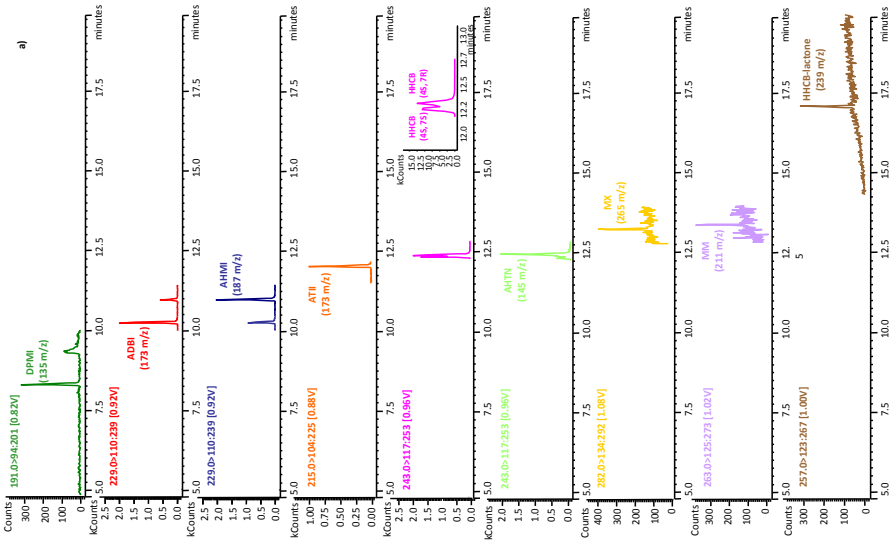

Fig. 2. MRM chromatograms of a influent wastewater sample from WWTP B. (a) Sample spiked with 1 µg L^{-1} of musk fragrances and (b) non-spiked sample.

because these factors were expected to be the most influential in the extraction process. Taking into account our previous experience in the determination of synthetic musk fragrances in wastewater samples by SDME and previous literature on synthetic musks [40,47-49], the initial experimental conditions selected were as follows: 300 g L^{-1} NaCl, 80 °C extraction temperature, 5 min preincubation time, 200 µL fill volume (2 cycles x 100 µL), 30 µL s^{-1} fill and ejection speed, 3 min of desorption time at 230 °C. In the same way, sample volume and stirring rate were fixed at 10 mL (20 mL HS vials) and 750 rpm, respectively.

Firstly the extraction mode was optimized. As musk fragrances are semi-volatile compounds two extraction modes were tested, immersion [50] and headspace [51,52]. The results showed that immersion mode provided higher peak areas than those obtained by headspace mode for all of the target compounds using both 20 mm and 30 mm NTs (data not shown). However, due to the trap's pneumatic restrictions and the bubble formation it was impossible to ensure a constant flow rate of the sample inside the needle and the exact sample volume that pass through the sorbent, obtaining non reproducible results.

So, headspace mode was selected to optimize the NTME. In the same way NT desorption with organic solvent was discarded and replaced by thermal desorption.

Then the extraction temperature was studied by comparison of the peak areas obtained at 30 °C, 45 °C, 60 °C, 80 °C and 100 °C. The other extraction conditions were the same as described previously. Independently of the NT used, a progressive increase in the peak areas was observed for all of the polycylic musks up to an optimum extraction temperature of 60 °C after which there was a decrease when the extraction temperature was extended up to 100 °C (Fig.3). While nitro musks compounds reached the highest peak areas at 80 °C with slightly better peak areas than those obtained at 60 °C. However, as a compromise 60 °C was chosen as the optimum extraction temperature.

To study the influence of adding salt on the efficiency of NTME, the ionic strength of the ultrapure water solutions was modified by adding NaCl in the the range of 0-360 g L^{-1}. 300 g L^{-1} was selected as the optimal salt concentration because maximal peak areas were obtained for most of the target compounds (data not shown). DPMI and HHCB-lactone showed maximal peak areas at 200 g L^{-1} NaCl but only a slight decrease in those analytical signals was observed at 300 g L^{-1} NaCl. It is clear that the addition of NaCl increased the ionic strength and thus promotes the transport of the analytes to the headspace and hence to the NT [53]. Therefore, it is to be expected that this will drive additional analytes into the headspace or gaseous phase and NT. Regarding the preincubation time, 5, 15 and 30 min were also studied in order to enhance the efficiency of the NT efficiency. The best results were obtained working with 30 min (data not shown). However due to the fact that no statistical differences were observed between 15 and 30 min (differences

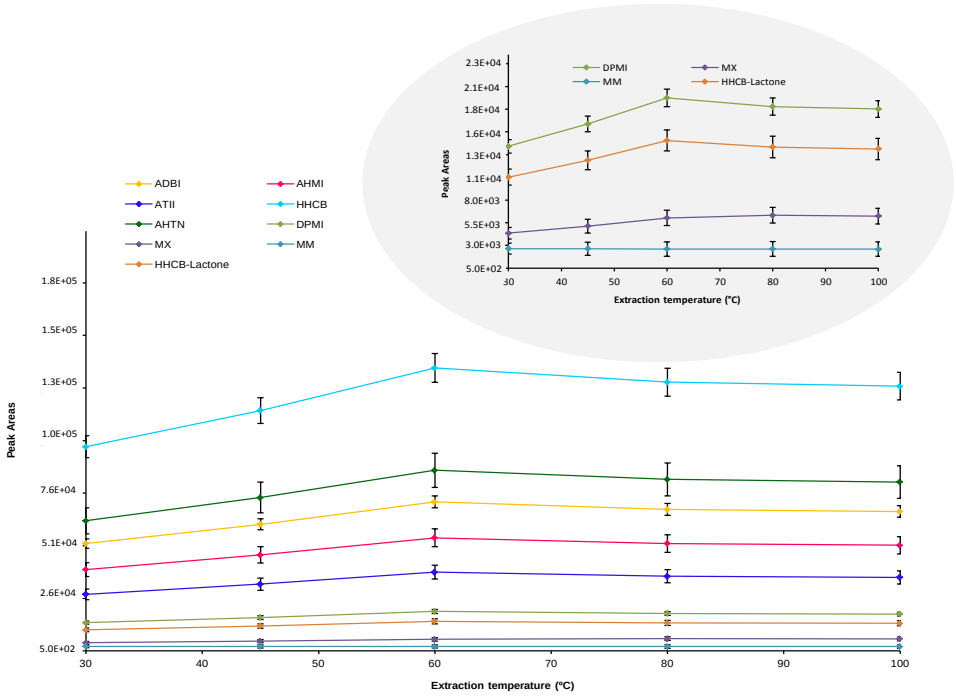

Fig. 3. Effect of the extraction temperature on the chromatographic peak areas obtained with NTME (1 µg L^{-1}, *n*=3). Experimental conditions: 30 mm HF Bondesil-C18 sorbent NT, 10 mL sample poured in a 20 mL HS vial stirred at 750 rpm, 30% NaCl addition, preincubation time of 5 min, 200 µL fill volume, 30 µL s^{-1} fill and ejection speed and 3 min desorption time (230 °C).

<10% of peak areas), 15 min of preincubation time was selected as optimum in order to reduce the analyses time.

Fill and ejection speed were studied in the range of 1 µL s^{-1} to 100 µL s^{-1} with a 100 µL Hamilton syringe. Higher fill/ejection speeds were discarded for pneumatic restrictions of the NT that can lead in repeatability problems [54]. Theoretically, lower fill speeds increase the sampling time to help musks to diffuse through the sorbent, so the extraction efficiency of the NT should be better working at low fill speeds than working at high fill speeds [54]. Experimental results (Fig. 4) showed that the highest peak areas and therefore the best extraction efficiency was achieved working at 1 µL s^{-1} fill and 30 µL s^{-1} ejection speeds for all of the target analytes independently of the NT used. However, since no statistical differences were observed between working at 1 µL s^{-1} or 10 µL s^{-1} fill speeds and not to lengthen the extraction time, 10 µL s^{-1} fill speed was chosen as optimal.

Then, fill volume was optimized under optimal conditions for both 20 mm and 30 mm sorbent NTs. The range of volumes studied was between 100 µL to 1,000 µL and were percolated by drawing and discarding between 1 and

10 cycles of 100 µL. In concordance with the results obtained by Eom *et. al.* [55] and Alonso *et. al.* [56] for the determination by NTME of BTEX (benzene, toluene, ethylbenzene and *p*-xylene) and volatile organic compounds in water and blood, respectively, increasing sampling volume, the extracted amount of analyte was increased: this occurred because sampling more headspace induced the transfer of more target analytes from the solution to the headspace, resulting in higher trapping efficiency. The results show that working with a 20 mm sorbent NT a proportional increase of the response of all of the target compounds up to 300 µL was observed (data not shown). At higher fill volumes the analytical signal remains constant. Whereas the 30 mm sorbent NT reached the highest peak areas at 500 µL or 750 µL depending on the target compounds. Due to the compounds that reached the highest peak areas at 500 µL are the less sensitive ones (nitro musk), 500 µL was chosen to work with a 30 mm sorbent NT.

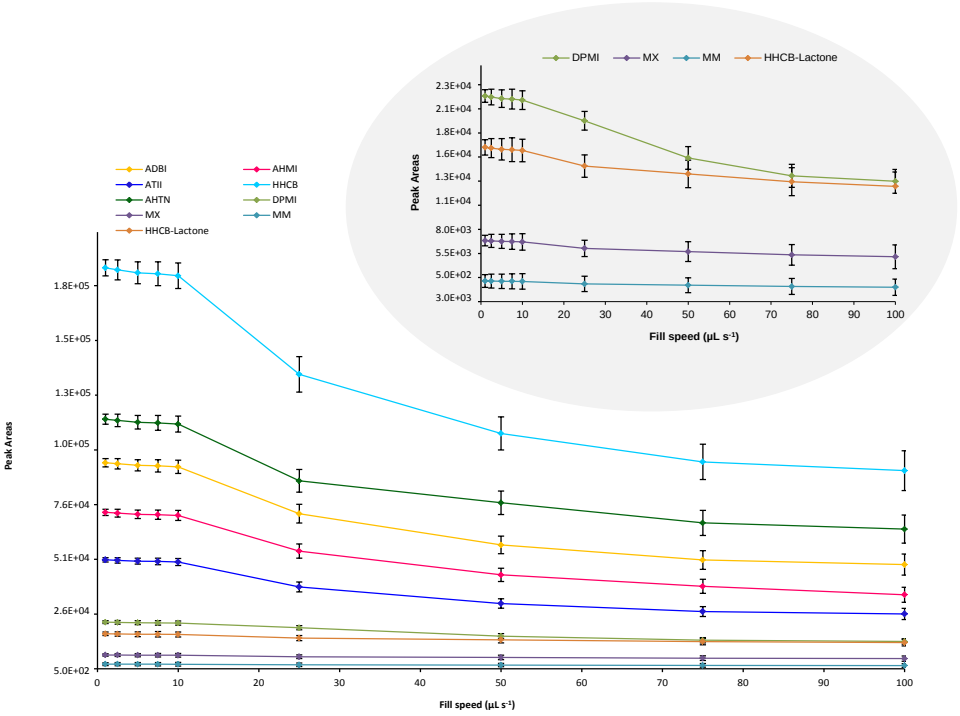

Fig. 4. Effect of the fill speed on the chromatographic peak areas obtained with NTME (1 µg L^{-1}, *n*=3). Experimental conditions: 30 mm HF Bondesil-C18 sorbent NT, 10 mL sample poured in a 20 mL HS vial stirred at 750 rpm, 30% NaCl addition, an extraction temperature of 60 °C, preincubation time of 15 min, 200 µL fill volume, 30 µL s^{-1} fill and ejection speed and 3 min desorption time (230 °C).

Finally desorption parameters as desorption time and temperature were tested. Working with a 20 mm sorbent NT the highest peak areas were obtained at holding for 1 min the NT at 230 °C while not statistical differences were observed increasing the desorption time to 3 or 5 min, and carry-over problems were not detected. Moreover, with a 30 mm NT the best peak areas were reached at 3 min desorption time and 230 °C desorption temperature. Not statistical differences were observed for the vast majority of the target compounds between 1 or 3 min of desorption time but carry-over problems were detected for HHCB and AHTN working at 1 min desorption time. Under the optimized conditions described at Table 2, as can be seen in Fig. 5, the 30 mm sorbent NT presented higher peak areas for all the target analytes, so this was the NT selected to validate the method.

3.3. Method Validation

As NTME may be strongly influenced by the sample matrix, before validating the method, the matrix effect was studied by statistically comparing the slopes of the calibration curves for influent and effluent WWTPs samples with that obtained with ultrapure water. As expected, the matrix effect was observed in both kinds of water, especially in influent samples. The feasibility of the microextraction procedure must be demonstrated with real samples spiked at different concentrations.

The method was then analytically validated with an influent and an effluent sample from WWTP A by establishing the linear ranges, method detection limits (MDL), method quantification limits (MQL), intra-day and inter-day repeatabilities (expressed as relative standard

Table 2. NTME optimum conditions for each HF Bondesil-C18 needle studied.

Process	Parameters	20 mm sorbent NT	30 mm sorbent NT
Extraction	Mode	Headspace	Headspace
	Temperature (°C)	60	60
	NaCl (g L^{-1})	30	30
	Preincubation time (min)	15	15
	Fill/ ejection speed (µL s^{-1})	10/ 30	10 /30
	Fill volume (µL)	200 (2 cycles x 100 µL)	500 (5 cycles x 100 µL)
Desorption	Mode	Temperature	Temperature
	Temperature (°C)	230	230
	Time (min)	1	3

deviation (RSD)).Procedural blanks of the conditioned NT were performed before NTME in order to prevent carry-over effects and ensure the repeatability of the analytical method.

The WWTP A samples used to validate the method were analysed (*n*=5), and the peaks of HHCB and AHTN appeared in the chromatogram of the influent sample, while in the effluent sample peaks of HHCB, AHTN and also HHCB-lactone were found. The averaged peak area of each detected compound was subtracted from the corresponding peak area of each spiked sample.

The linear range of the method was obtained by analysing the WWTP A samples spiked with all of the target analytes at concentrations between 2.5 ng L^{-1} and 5,000 ng L^{-1} while the SS concentration remained constant at 1,000 ng L^{-1}. The method was linear in all ranges between MQL and 5,000 ng L^{-1} for all of the target analytes and matrices. In addition, the presence of the SS enabled us to improve the determination coefficients (r^2) of the calibration curves to values higher than 0.997 for all the target compounds. MQLs were the lowest point of the calibration curve and MDLs were evaluated by spiking WWTP A influent or effluent samples in order to obtain a signal-to-noise ratio higher than three, for the compounds that did not appear in these samples. MDLs for HHCB, AHTN and HHCB-lactone were estimated as the concentration that gave a signal average of plus three times the standard deviation of the influent or effluent sample signal (*n*=10). These limits are shown in Table 3. Thus, MQLs ranged from 5 ng L^{-1} to 25 ng L^{-1} and MDLs ranged from 2.5 ng L^{-1} to 12 ng L^{-1} depending of the compound and matrix studied. These limits are better than those reported in

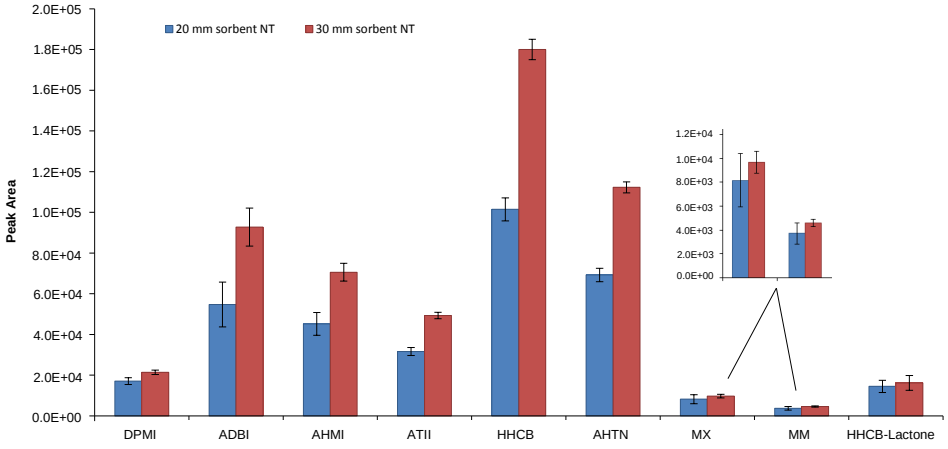

Fig. 5. Comparison of the chromatographic peak areas obtained with the 2 sorbent-packed needle traps in the optimal NTME conditions for extracting the target musk fragrances from 10 mL water samples spiked at 1 µg L^{-1} (*n*=3).

the literature, which reports MDLs between 5 ng L^{-1} and 63 ng L^{-1} for influent and effluent waters working with microextraction by packed sorbents [41], dispersive liquid-liquid microextraction [29,57] or ultrasound-assisted emulsification-microextraction [58] as extraction technique. However, slightly better MDLs (from 0.25 ng L^{-1} to 9 ng L^{-1}) were obtained working with solid-phase microextraction as preconcentration technique [48,59].

Intra-day and inter-day repeatability were obtained with five replicates of influent and effluent sample spiked at 100 ng L^{-1} (see Table 3). Intra-day repeatability (% RSD, *n*=5) was always less than 11% for both influent and effluent samples. Inter-day repeatability was always less than 17% or 15% (% RSD, *n*=5) for influent and effluent samples, respectively.

3.4. Needle trap microextraction versus ionic liquid-based headspace single-drop microextraction

The NT methodology was compared in terms of method validation parameters with an ionic liquid-based headspace single-drop microextraction (IL-HS-SDME) methodology, which was successfully applied by our research group for the determination of musk fragrances in wastewater samples [40].

Both microextraction techniques are considered environmentally friendly because of the use of 1 µL of an ionic liquid as extraction solvent instead of an organic solvent in the case of the IL-HS-SDME or because it is a solventless technique as NT. In addition both extraction procedures were fully automated by a CombiPal autosampler to ensure the repeatability of the methodology when dispensing a drop of ionic liquid (IL-HS-SPME) or to control the fill/ejection speed (NT).

Method validation parameters were obtained by the analysis of 10 mL influent and effluent samples spiked at different concentrations by using GC-MS/MS as separation and detection technique. The results showed that better MDLs were obtained with NTME with values between 2.5 ng L^{-1} and 12 ng L^{-1}, while IL-HS-SDME MDLs ranging between 10 ng L^{-1} 30 ng L^{-1}. The main reason of the increase of the MDLs when working with IL-HS-SDME is that because of the low volatility of the ionic liquids, residues could be accumulated in the glass wool placed inside the GC liner. In the same way, higher MQLs were obtained with IL-HS-SDME (50 ng L^{-1}-100 ng L^{-1}). Both methods were linear between the MQLs and 5,000 ng mL^{-1} with NTME and up to 10,000 ng mL^{-1} with IL-HS-SDME. On the other hand, slightly better repeatability results were obtained with IL-HS-SDME, with intra-day repeatability values between 2-6% and inter-day repeatabilities ranging between 5-11%.

Summarizing, both methodologies were successfully applied for the determination of musk fragrances in wastewater samples but the validation results as well as daily work have led me to conclude that the NTME is preferable to IL-HS-SDME.

Furthermore, NTME validation values can also be compared with those obtained by Moeder *et. al.* [15] working with MEPS as extraction technique. MEPS is a microextraction

Table 3. Method linear ranges, MDLs, MQLs, intra-day repeatabilities and inter-day repeatabilities.

Compound	Influent				Effluent		
	Linear range [a] (ng L^{-1})	MDLs (ng L^{-1})	RSD [b] (%)	RSD [c] (%)	Linear range [a] (ng L^{-1})	MDLs (ng L^{-1})	RSD [b] (%)
DPMI	10-5,000	3.5	7	11	7.5-5,000	2.5	6
ADBI	7.5-5,000	3	8	15	5-5,000	2.5	6
AHMI	7.5-5,000	2.5	6	16	5-5,000	2.5	5
ATII	20-5,000	5	11	11	5-5,000	2.5	8
HHCB	7.5-5,000	3*	10	13	7.5-5,000	2.5*	3
AHTN	7.5-5,000	3.5*	8	17	7.5-5,000	2.5*	5
MX	20-5,000	10	7	12	20-5,000	10	5
MM	25-5,000	12	6	13	20-5,000	10	9
HHCB-lactone	20-5,000	10	10	13	10-5,000	5*	8

[a] MQL (ng L^{-1}): were fixed as the lowest calibration level.
[b] Intra-day repeatability (% RSD): n=5, 100 ng L^{-1}.
[c] Inter-day repeatability (% RSD): n=5, 100 ng L^{-1}.
* Estimated.

technique that can be considered as a miniaturized solid-phase extraction in which the sorbent (≈1 mg) is inserted between the needle and the barrel as a cartridge but not in the needle. Moeder *et. al*. [15] obtained MDLs of 42 ng L^{-1} and 37 ng L^{-1} for HHCB and AHTN, respectively, while working with NT as extraction technique slightly better MDLs, between 2.5 and 3.5 ng L^{-1} for HHCB and AHTN, were obtained. These values can be explained by the differences in sorbent position. NT design allows working in headspace mode and thermal desorption, and that makes this technique ideal for the extraction of volatile organic compounds from air or wastewater samples. On the other hand, MEPS bin is ideal for work in immersion mode and desorption with organic solvents and is normally used to extract polar compounds from environmental or biological samples [48].

3.5. Method application

To demonstrate the applicability of the NTME-GC-MS/MS, influent and effluent wastewater samples from three WWTPs (A, B, C) on different dates were analysed (*n*=8). All the polycyclic musk compounds were detected in some of the influent samples from WWTP A and WWTP B, with HHCB (20 ng L^{-1}-1,160 ng L^{-1}) and AHTN (<MQL-430 ng L^{-1}) as the only compounds present in all the samples analysed (Table 4). For instance, Fig. 2b shows MRM chromatograms of the influent from WWTP B for the sample spiked with 1,000 ng L^{-1} of musk fragrances a), and the un-spiked sample b). As expected, the concentrations of polycyclic musk found in effluent WWTP A and B samples were lower than those detected in influent samples and with DPMI (n.d.-180 ng L^{-1}), HHCB (10 ng L^{-1}-550 ng L^{-1}) and AHTN (<MQL-240 ng L^{-1}) as the compounds that showed the highest concentrations. None of the samples contained detectable traces of nitro musk compounds. HHCB-lactone was only detected in effluent WWTP A and B samples as a result of the degradation of HHCB to HHCB-lactone during the WWTP treatment [60,61].

In the same way, HHCB (70 ng L^{-1}-240 ng L^{-1}) and AHTN (<MQL-50 ng L^{-1}) are the only compounds present in all the influent samples of RO taken from WWTP C. The remaining polycyclic musks as well as HHCB-lactone were detected in some of the samples in minor concentrations. In effluent RO samples all targets musks were detected at values below the MQL or were not detected.

Previous works [42,45,62,63] that have focused on the determination of synthetic musk fragrances in waste-water samples confirms the finding of the present study, i.e. that the most abundant fragrances found in waste-water samples are the polycyclic musks HHCB and AHTN, although other polycyclic musks as DPMI, ADBI or AHMI can also be present in minor concentrations.

Table 4. Concentrations of the target musks found in wastewater samples (n=8) in ng L^{-1}.

Compounds	WWTP A		WWTP B		WWTP C	
	Influent	Effluent	Influent	Effluent	Influent RO	Effluent RO
DPMI	260-630	<MQL-10	<MQL-880	n.d.-180	n.d.-<MQL	n.d.-<MQL
ADBI	n.d.-30	n.d.-<MQL	<MQL-40	n.d.-<MQL	n.d.-30	n.d.-<MQL
AHMI	n.d.-20	n.d.-<MQL	<MQL-110	n.d.-<MQL	n.d.- 40	n.d.-<MQL
ATII	n.d.-70	n.d.-<MQL	<MQL-180	n.d.-30	n.d.-90	n.d.-<MQL
HHCB	560-600	160-210	20-1,160	10-550	70-240	<MQL
AHTN	130-260	40-70	<MQL-430	<MQL-240	<MQL-50	<MQL
MX	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HHCB-Lactone	n.d.	<MQL-820	n.d.	<MQL-290	n.d.-270	n.d.

n.d.; not detected.
<MQL; values under the method quantification limit.

4. Conclusions

In this study, a NTME followed by GC-MS/MS procedure was developed for determining nine musk fragrances in water samples. The NTME-GC-MS/MS method was shown to be completely automated, simple and environmentally friendly. It also provided low ng L^{-1} MDLs and satisfactory precision (RSD between 3 and 11%) for wastewater samples.

The following main parameters involved in the NTME were evaluated and optimized: sorbent-packed needle trap, extraction mode, extraction temperature, salt concentration, preincubation time, fill and ejection speed, fill volume and desorption parameters (temperature and time). Two sorbent-packed needle traps were tested (20 mm HF Bondesil-C18 sorbent and 30 mm HF Bondesil-C18 sorbent), and the best conditions were found to be the following: 30 mm HF Bondesil-C18 sorbent needle trap, 10 mL sample volume placed in a 20 mL HS vial and stirred at 750 rpm, 30% NaCl addition, an extraction temperature of 60 °C, preincubation 15 min, 10 µL s^{-1}/ 30 µL s^{-1} fill and ejection speed, 500 µL fill volume and 3 min desorption time (230 °C).

The applicability of the method was also tested with water samples from influent and effluent WWTPs. The most abundant compounds were found to be DPMI, HHCB and AHTN while the remaining polycyclic musk fragrances were present at lower concentrations. None of the samples analysed contained detectable traces of nitro musk compounds and HHCB-lactone was only detected in effluent samples as a degradation product of HHCB.

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3.2.5. Discussion of results

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Automated IL-HS-SDME followed by GC-IT-MS/MS was successfully applied for the determination of PCM and NM fragrances in wastewater samples and sewage sludge that had previously undergone PLE. Under optimized conditions the methods also provided acceptable MDLs between 10 ng L^{-1} and 30 ng L^{-1} for aqueous samples and ranging between 1 ng g^{-1} (d.w.) and 3 ng g^{-1} (d.w.) for sewage sludge samples. The automation of the microextraction procedure also provided repeatability values below 10% for most of the target fragrances.

The performance of HS-SDME is influenced by several variables, such as extraction solvent, drop volume, stirring rate, extraction temperature, salt concentration, sample volume and extraction time [1,2]. After the simultaneous study of the first four variables with four extractants, two organic solvents (toluene and *n*-heptane) and two ILs ([OMIM][PF_6] and [HMIM][PF_6]), the results showed that [OMIM][PF_6] (1 µL) and toluene (3µL) gave the highest peak areas. However, [OMIM][PF_6] was selected as the extraction solvent because its low volatility enables the extraction temperature to be increased up to 60 °C with the consequent increase in the extraction efficiency. Moreover, the RSD values obtained with [OMIM][PF_6] (4-9%) were significantly lower than those obtained with toluene (7-24%). The rest of optimal conditions were: 10 mL of water (20 mL vial) containing 300 g L^{-1} of NaCl and stirred at 750 rpm for 45 min at 60 °C.

With respect to sewage sludge, the influence of the main factors on the efficiency of PLE was studied [3]. The highest recovery values were achieved using 1 g (d.w.) of sewage sludge, water:methanol (1:1, v/v) as extraction solvent at 80 °C for 5 min, 2 cycles, 100% flush volume, 120 s of purge time and 1 g of florisil as the in-cell clean-up sorbent. The use and optimization of an in-cell clean-up sorbent was shown to be a critical step because of the presence of fatty interferents in the PLE extracts that strongly influence the IL-HS-SDME. Although the best recovery values were obtained with diatomaceous earth (72-98%), florisil was selected as in-cell clean-up sorbent because of the recovery values obtained (63-100%) and the efficiency in the removal of fatty interferents present in PLE extract. Moreover, the use of in-cell clean-up sorbents results in an attractive alternative to laborious standard SPE clean-up protocols reducing sample manipulation and time.

With respect to GC, some preventive steps needed to be taken to work with ILs as the extraction solvent. A liner was used with a large internal diameter (3.4 mm) to improve the IL evaporation, into which a piece of glass wool was introduced to prevent ILs entering the GC column, as well as a guard column of 3 m to ensure analytical column protection. The non-modification of the GC injector permitted the development

of a completely automated, simple and environmentally friendly method. However the piece of glass wool which was placed inside the liner needed to be removed after every 10 analyses due to the presence of ILs residues that may compromise the sensitivity of the method. For this reason, the detection was performed in MS/MS mode with an IT as the analyzer. The MS/MS fragmentations were optimized for each compound by selecting an amplitude excitation voltage that gave a product ion with an abundance of 100% and a relative abundance of the parent ion between 10% and 20%. Each compound was acquired separately in one segment, except traseolide, galaxolide and tonalide (segment 4), and musk xylene, musk moskene and the internal standard d15-musk xylene (segment 5), because, even under optimal chromatographic conditions, these compounds appeared separated by less than 0.5 min.

Even working in MS/MS mode, the MDLs obtained with IL-HS-SDME and 10 mL of sample volume (10-30 ng L^{-1}) were slightly higher than those obtained in Section 3.1.5 working with on-line SPE followed by GC-Q-MS in SIM mode (1-7 ng L^{-1}, 10 mL sample volume). There are two main reasons that may explain the obtained results: the high recovery values obtained with on-line SPE (>80% for most of the target fragrances) and the loss of sensitivity caused by the presence of IL residues in the liner with SDME. However, when working with PLE followed by IL-HS-SDME and GC-IT-MS/MS the MDLs obtained were slightly lower (1-3 ng g^{-1} (d.w.), 1 g (d.w.) of sample amount) than those obtained with SBSE followed by TD-GC-Q-MS (5-25 ng g^{-1} (d.w.), 0.1 g (d.w.) of sample amount). With the differences in samples amount and the applicability of a high split during the thermal desorption of the stir bar as the most probable causes of the differences obtained.

With respect to the third study, MEPS was successfully applied for the first time to determine MCM fragrances in wastewater samples followed by GC-IT-MS. This combination was shown to be a promising alternative to time consuming standard SPE protocols which also provided acceptable MDLs between 5 ng L^{-1} and 10 ng L^{-1} and repeatability values lower than 10% in all cases. Moreover, the use of only 1-4 mg of extraction sorbent allowed us to decrease sample volumes from hundreds of millilitres to 1-4 mL and enabled the automation of all the MEPS steps by reducing the elution solvent volume to 50 µL. However, in an automated MEPS procedure, apart from the individual steps of the SPE, additional steps for post-cleaning, re-conditioning and the extraction regime have to be studied in depth to prevent carry-over problems and to enable multiple use of the MEPS-BIN [4,5]. Optimal conditions, with corrected recoveries between 52% and 92% for influent samples and between 54% and 95% for effluent samples, were achieved with C18 as extraction sorbent, 4 mL of sample volume

(40 cycles of 100 µL sample), 50 µL of ethyl acetate as elution solvent, a fill/ejection speed of 10 µL s^{-1} in the extraction step and 1 µL s^{-1} fill and 50 µL s^{-1} ejection speed. Fill/ejection speed was one of the most important variables to be optimized when working with MEPS, because at higher fill/ejection speeds (20 µL s^{-1}), problems started to occur with air bubbles and the extraction volume could not be controlled. Additionally, four wash-discard cycles with ethyl acetate (50 µL) followed by four clean-up discard cycles with ultrapure water (50 µL) were required to remove residual analytes and matrix components and leave the MEPS-BIN ready for the next extraction.

With respect to the injection of 50 µL in the GC-IT-MS system, the injector port operated in LVI mode and a liner packed with glass wool was required. The temperature programme needed to be optimized accurately, as well as split rates, to obtain well defined peaks and prevent losses of the target fragrances. After that, the target fragrances were injected individually into the GC-MS system because of the lack of information about their identification/quantification ions, and the chromatographic separation was optimized. As mentioned in Section 3.1.5, even under optimal conditions, exaltone and exaltolide co-eluted, as well as muscone and habanolide. They could be quantified separately because they had different ions and fragments. Acquisition was performed in SIM mode in order to improve the selectivity/sensitivity of the method. MS/MS could not be applied due to the excessive fragmentation of MCM fragrances (see Section 3.1.5).

The MDLs obtained with MEPS followed by GC-IT-MS (5-10 ng L^{-1}, 4 mL sample volume) for the determination of MCMs in water samples are comparable with those obtained in Section 3.1.5 working with HS-SPME followed by GC-IT-MS (1-5 ng L^{-1}, 10 mL) and both methodologies are also fully automated. MDLs obtained with MEPS are also comparable with those obtained with on-line SPE followed by GC-Q-MS (10-30 ng L^{-1}, 10 mL sample volume). However MEPS or HS-SPME are preferable to on-line SPE because they are easier to perform and enables the automation of the whole analytical method.

Lastly, a dynamic HS-NTME followed by GC-IT-MS/MS method was developed for the determination of a group of synthetic musk fragrances, including PCMs and NMs, in wastewater samples. In line with the methods previously developed, this method is completely automated, simple and environmentally friendly. It also provided MDLs between 2.5 and 12 ng L^{-1} and repeatability values between 3% and 11%.

For the optimization of NTME, two NTs were tested. The first contained 20 mm of HF Bondesil-C18 sorbent, while the second was filled with 30 mm of HF Bondesil-C18

sorbent. Other more selective sorbents, such as Oasis HLB [6] or bond Elut Nexus [7], were ruled out because they are not commercially available in larger particle sizes (100-250 µm). The selection of the particle size of the sorbents in this case is crucial, as demonstrated by Zhan and Pawliszyn [8]. Needles packed with small particles possess higher extraction capacity and efficiency but much higher resistance to flow as well, and may sometimes cause pneumatic restrictions and the generation of bubbles, an important factor to take into account. The following main parameters involved in NTME were evaluated and optimized for both NTs and the best results were found with a 30 mm HF Bondesil-C18 sorbent NT, exposed in the HS of 10 mL sample volume (20 mL vial) stirred at 750 rpm, 30% NaCl addition, an extraction temperature of 60 °C, preincubation time of 15 min, 10 µL s^{-1} fill/30 µL s^{-1} ejection speed, 500 µL fill volume and 3 min desorption time (230 °C). Although the results showed that immersion mode provided significantly higher peak areas than those obtained by HS for all of the target fragrances regardless of the NT used, this option was ruled out due to the formation of bubbles that made it impossible to ensure a constant flow rate inside the NT and the exact sample volume that pass through the sorbent, obtaining non-reproducible results.

The TD of the NT was directly performed in the injector port of the GC system in splitless mode. It is important to take into account the internal diameter of the liner because the desorption of the NT take place inside the liner. In our case, a liner with a 0.8 mm i.d. was used [9]. With respect to chromatographic separation, as happened when working with IL-HS-SDME, a guard column was installed just before the analytical column to prevent column damage. The chromatographic separation was performed in a mid-polarity analytical column (50% diphenyl/50% dimethylpolysiloxane) in just 17 min. To improve the sensitivity/selectivity of the method the detection was performed in MS/MS mode using the fragmentation voltages optimized previously during the IL-HS-SDME study. Although NTME and IL-HS-SDME were followed by the same chromatographic separation and, in both cases, the MS detector acquired in MS/MS mode, using the same sample amount (10 mL) better MDLs ranging between 2.5 and 12 ng L^{-1} were achieved with NTME. As shown in Section 3.1.5, the MDLs obtained with IL-HS-SDME were between 10 and 30 ng L^{-1}. Apart from that, NTME presents some advantages compared with IL-HS-SDME, such as being a solvent-less technique and avoiding the problems derived from the injection of ILs in the GC system. Moreover, the MDLs obtained with NTME are comparable with those obtained with on-line SPE followed by GC-Q-MS, 1 and 7 ng L^{-1} (see section 3.1.5). The use of a more specific sorbent, such as Oasis HLB (60 µm), for the on-line SPE instead of the C18 (120 µm) used for the NTME favours good recovery values and this counteracts the sensitivity enhancement obtained working with MS/MS instead of MS in our case.

The applicability of the aforementioned methodologies was assured by analysing influent and effluent samples from WWTP A (Tarragona) and WWTP B (Reus), from the tertiary treatment of WWTP C (Vila-seca) and from DWTP D (L'Ampolla). Sewage sludge from WWTP A and B was also analysed. It is important to emphasize that it was the first time that MCM fragrances were determined at these WWTPs.

In line with the literature [10,11], the most abundant fragrances found were galaxolide and tonalide with maximum concentrations of 2,060 ng L^{-1} and 430 ng L^{-1}, respectively in influent samples. However, other PCMs studied were present in some of the influent samples analysed at lower concentrations, such as cashmeran and phantolide, among others. A significant decrease in musk concentrations was detected in effluent samples with galaxolide and tonalide as the most abundant compounds. In the same way, galaxolide (70-240 ng L^{-1}) and tonalide (<MQL-50 ng L^{-1}) were the only compounds present in all influent samples taken from WWTP C. The remaining PCMs were detected in some of the samples at minor concentrations. After the tertiary treatment based on reverse osmosis (RO), all of the target PCMs were detected at values below MQL or were not detected. With reference to DWTP D, only galaxolide was found at concentrations higher than the MQL, 290 ng L^{-1} in influent samples and down to 90 ng L^{-1} in effluent samples. None of the samples analysed contained detectable traces of NMs. HHCB-lactone was only detected in effluent samples as degradation product of galaxolide. PCMs were also found in sewage sludge of WWTP A and B, with concentrations between <MQL and 6.1 ng g^{-1} (d.w.) and between <MQL and 90.8 ng g^{-1} (d.w.), with galaxolide being the only PCM found in all the samples analysed. Meanwhile, NMs, were only detected at values below MQL and musk xylene was not detected in any of the samples analysed. HHCB-lactone was also found in all of the samples analysed at concentrations between <MQL and 530.5 ng g^{-1} (d.w.).

In contrast, MCM fragrances were found in influent samples with ambrettolide (2,872-9,289 ng L^{-1}), exaltone (1,402-2,891 ng L^{-1}) and civetone (55-2,053 ng L^{-1}) as the most common compounds. In effluent samples, exaltone was the most abundant compound with values in the range of 450 ng L^{-1} to 2,263 ng L^{-1}, followed by ambrettolide with values between <MQL and 2,461 ng L^{-1}. The remaining MCMs studied were found in effluent samples at concentrations significantly lower than those detected in influent samples or were not detected.

During the course of this Doctoral Thesis, conventional microextraction techniques as well as novel microextraction techniques (see Figure 3.2.1) were applied for the determination of synthetic musk fragrances in environmental samples (i.e. wastewater and sewage sludge). All of the microextraction techniques used share certain advantages, based on which they are all considered environmentally friendly techniques. These advantages include the use of small volumes of sample (4-10 mL) or sample amount (0.1-1 g (d.w.)), leading to a decrease in the amount of extraction sorbent used and lower volumes of organic solvents are required to elute the target analytes, between 1 µL for SDME and 50 µL for MEPS and on-line SPE. Moreover, some microextraction techniques such as SPME and NTME, are solvent-free and no use of organic solvent is required during the extraction or desorption steps. In contrast, there are disadvantages that have limited the applicability of the aforementioned microextraction techniques for the determination of synthetic musk fragrances in environmental samples.

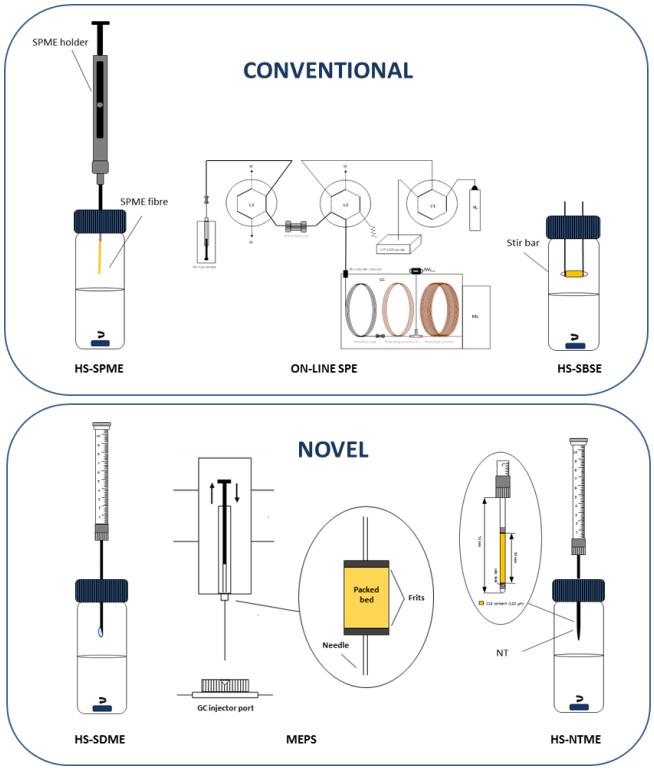

Figure 3.2.1. Conventional and novel microextraction techniques studied in this Doctoral Thesis.

In this respect, the use of ILs as the extraction solvent minimizes the problems of drop instability and precision characteristics of SDME when working with organic solvent. However, the presence of IL residues in the liner requires specific maintenance every ten analyses and also limits the sensitivity of the method. Although the use of MS/MS detection partly counteracts this loss of sensitivity, IL-HS-SDME cannot compete with the other microextraction techniques from an everyday work point of view for the routine analysis of the presence of synthetic musk fragrances in wastewater samples.

With respect to MEPs and on-line SPE, both microextraction techniques provided good recovery values for the determination of the synthetic musk fragrances studied in wastewater samples and MEPS also resolved the problems of instrumental requirements in the case of on-line SPE and allows the automation of the whole SPE procedure. In our case, blockage problems associated with MEPS were not observed and carry-over problems were resolved by adding a washing step of the syringe with the elution solvent and ultrapure water just before the next extraction. In any case, MEPS and on-line SPE methods provided MDLs slightly better than those obtained with IL-HS-SDME, without the need to use MS/MS detection. However, from our point of view, on-line SPE is less robust than MEPS because, as shown in Table 3.3.1 (page 373) its set-up results in a higher number of potential points of leaks and requires a trained analyst to build it up. Thus, MEPS is more suitable for routine analysis.

In the case of the solvent-less microextraction techniques evaluated in the present Thesis for the determination of synthetic musk fragrances in wastewater samples namely SPME and NTME, both techniques are shown to be completely automated and provided the best MDLs. Due to the wide variety of commercially available SPME fibres, the extraction efficiency of SPME is higher than that obtained with NTME. For this reason, an MS detection acquiring in SIM mode is sufficient for achieving MDLs at low ng L^{-1} levels. Meanwhile, in the case of NTME, to avoid pneumatic restrictions, the use of sorbents with a large particle size (100-250 µm) is recommended, which limits the availability of commercially available sorbents and, therefore, the extraction efficiency. In the case of NTME, MS/MS is needed to achieve MDLs low enough for the determination of synthetic musk fragrances in wastewater samples at trace levels. Although both microextraction techniques could be used for the determination of synthetic musk fragrances in wastewater samples for routine analysis, SPME has been shown to be a more reliable technique in terms of precision than NTME.

Of the microextraction techniques evaluated in this Thesis for the determination of synthetic musk fragrances in wastewater samples, SPME and MEPS are the techniques

which provided the best MDLs, and both methodologies are fully automated. In our opinion, to date, SPME offers a higher throughout than MEPS due to the wide variety of commercially available sorbents. In addition, it also enables working with an autosampler or performing the microextraction manually, which is not possible with MEPS due to the need to control the fill/ejection speed of the syringe during the SPE extraction procedure.

In contrast, of the microextraction techniques used for the extraction of synthetic musk fragrances present in sewage sludge samples, SPME, SBSE and the combination of PLE and IL-HS-SDME, the best MDLs were obtained by large with SPME and it is also the only methodology that is fully automated. With SBSE, the extraction efficiency was limited by the split applied during the thermal desorption of the stir bar. It should be highlight that, in both methodologies, the microextraction was performed directly on the sewage sludge without any pretreatment step. With respect to IL-HS-SDME, a pretreatment of the sample was applied, consisting of PLE extraction with water:methanol (1:1, v/v) as the extraction solvent and an in-cell clean-up with florisil. This resulted in longer analysis time, more sample manipulation and ruled out the possibility of automation of the whole analytical procedure. Although the MDLs obtained with the combination of PLE with IL-HS-SDME followed by GC-MS/MS are between those obtained with SPME and SBSE followed by GC-MS, this methodology can not compete with SPME and SBSE in terms of analysis time and sample manipulation.

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UNIVERSITAT ROVIRA I VIRGILI
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Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

3.3. Determination of musk fragrances in fish and mussel samples

UNIVERSITAT ROVIRA I VIRGILI
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As mentioned in the Introduction, synthetic musk fragrances are widely used as fragrances in a great variety of consumer products such as cosmetics and detergents. Due to their widespread use, they enter the environment both directly (disposal and wastage from external application) and indirectly (excretion, washing and swimming) [1]. As synthetic musk fragrances are generally not subjected to structural alterations, resulting in the release of large quantities of unaltered compounds into the environment [2]. The incidence of synthetic musk fragrances in the environment may have a negative impact on human and wildlife individuals either by their accumulation or long-term chronic exposure of aquatic organisms to these compounds [2,3]. For instance, *in vivo* and *in vitro* anti-oestrogenic effects of PCMs in zebrafish have been documented by Schreurs *et al.* [4].

Due to their high lipophilicity, synthetic musk fragrances have been detected in surface waters, wastewater, soil and sediments as summarized in recent reviews [2,5] as well as in the experimental part of this Doctoral Thesis. In contrast, relatively few studies have documented the occurrence of synthetic musk fragrances in marine organisms [6,7].

The study included here focuses on the development of a rapid, sensitive and accurate method based on GC-IT-MS/MS for the determination of PCM and NM fragrances in marine organisms such as fish and mussels. Two extraction techniques were tested, the first involving a conventional PLE, which was successfully applied for determining EOCs [8,9] in solid samples in general and for the determination of PCM and NM fragrances from sludge samples, as reported in particular in Section 3.2.5 of this Thesis. Meanwhile, the second extraction technique tested consisted of QuEChERS extraction, a consolidated extraction methodology in the field of food analysis [10] of growing interest in the field of environmental analysis in recent years [11,12] due to its speed, ease of implementation (instrumentation is not required), minimal solvent requirement and low cost when compared with instrumental extraction techniques, which can be very useful for monitoring studies. Other extraction techniques previously used for the determination of synthetic musk fragrances such as SPME and SBSE in sluge samples were discharge due to the limited extraction efficiency compared with PLE.

To achieve the efficient extraction of the target compounds from fish or mussel samples using a PLE system, several operational parameters were optimized, including the extraction solvent, temperature, time and number of cycles. In addition, three in-cell clean-up sorbents (florisil, alumina and silica) were tested to reduce the matrix effect (ME) as much as possible. With respect to QuEChERS, certain variables such as the salts

were adopted from the European Standard Method EN15662 [13]. Other significant parameters that affect extraction and clean-up performance were optimized, such as, the ratio between the sample mass and the volume of solvent, type of extraction solvent and dSPE.

The PLE and QuEChERS extraction procedure were compared in terms of validation parameters, analysis time and ME when applied to fish and mussel samples. The preferred method was applied to determine musk fragrances in gilt head bream (*Spaurus aurata*), turbot (*Psetta maxima*), red mullet (*Mullus surmuletus*) and mussels (*Mytilus galloprovincialis*), all purchased locally in the Tarragona market (NE Spain) and mostly caught in the Mediterranean Sea. Perch (*Preca fluviatilis*), sheat fish (*Silurus glanis*) and carp (*Cyprius carpio*) samples, which had been caught in the wild and collected from the River Ebro, were also analysed.

The results of this study have been published in Talanta *134 (2015) 690-698.*

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UNIVERSITAT ROVIRA I VIRGILI
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3.3.1. Influence of pretreatment process on matrix effect for the determination of musk fragrances in fish and mussel

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INFLUENCE OF PRETREATMENT PROCESS ON MATRIX EFFECT FOR THE DETERMINATION OF MUSK FRAGRANCES IN FISH AND MUSSEL

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Abstract

Musk compounds are widely used as fragrances in personal care products. On account of their widespread use and their low biodegradation, they can be found in environmental samples. In our study two extraction methodologies were compared and different clean-up strategies were also studied in order to develop a reliable analytical method, with minimum matrix effect and good detection limits, to determine synthetic musk fragrances -six polycyclic musks, three nitro musks and the degradation product of one polycyclic musk- in fish and mussel samples. The first extraction technique involves a QuEChERS extraction, a consolidate extraction methodology in the field of food analysis of growing interest over recent years, followed by a dispersive solid-phase extraction (dSPE) as clean-up strategy. The second extraction technique consists of a conventional pressurized liquid extraction (PLE) with dichloromethane and an in-cell clean-up to decrease the matrix effect and remove the undesired components present in PLE extracts. Large volume injection (LVI) followed by gas chromatography-ion trap-tandem mass spectrometry (GC-IT-MS/MS) was chosen as the separation and detection technique. Validation parameters, such as method detection limits and method quantification limits were found at ng g^{-1} dry weight (d.w.) levels for both fish and mussel matrices. Good levels of intra-day and inter-day repeatabilities were obtained analysing fish and mussel samples spiked at 50 ng g^{-1} (d.w.) (n=5, RSDs <17%). The developed PLE/GC-IT-MS/MS method was successfully applied to determine the target musk fragrances present in fish and mussel samples from the local market in Tarragona and fish samples from the Ebro River. The results showed the presence of galaxolide (2.97-18.04 ng g^{-1} (d.w.)) and tonalide (1.17-8.42 ng g^{-1} (d.w.)) in all the samples analysed, while the remaining polycyclic musks such as cashmeran, celestolide and phantolide, were only detected in some of the fish samples analysed. None of the samples analysed contained detectable traces of the nitro musks studied.

Keywords: *Fish samples; LVI-GC-IT-MS/MS; matrix effect; musk fragrances; pressurized liquid extraction; QuEChERS.*

1. Introduction

Musk compounds are a family of cyclic personal care products (PCPs), that include polycyclic musks, nitro musks and macrocyclic musks, widely used as fragrances in consumer products such as cosmetics, toiletries, detergents, soaps, body oils, toothpaste and also as flavours in foods and drinks: in short, they are used in a broad range of everyday products. They belong to the so-called emerging organic compounds (EOCs), which have been of increasing interest, to scientists in recent years [1-8] .

Discussions on the toxicology of nitro musks soon arose because of the presence of a nitro aromatic compound in their structure. In this respect, the European Directive 98/62/EEC [9] relating to cosmetic products prohibits the use of musk ambrette, musk moskene and tibetene in cosmetics and limits musk xylene and musk ketone content. Recently, the European Comission under the new chemical regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), considered MX a very persistent and very bioaccumulative substance and therefore decided to ban it as well [10]. Furthermore, nitro musks can be transformed in wastewater treatment plants (WWTPs) -as well as in biota- into amino metabolites [11], and these transformation products can be even more problematic than the parent compounds [12,13]. This has led to a significant decrease in their use, while polycyclic musk production has increased significantly. Polycyclic musks are the musk fragrances that dominate the global market today, and two of them, galaxolide and tonalide, have been included on the EPA's high production list [14]. The use of tonalide in the cosmetic industry has in fact been regulated through European directive 2008/42/EC [15]. Macrocyclic musks, which smell more intensive than polycyclic musks and so less mass is needed to achieve the same performance in perfumery, are not as widely used as polycyclic musks because of the cost of their synthesis. Nevertheless, they are becoming more generally available because of advances made in synthesis methods over the last few years [2,16,17]. It is expected that over the next few years the decrease in the price of synthesizing macrocyclic musks and their environmentally friendly properties will mean that they will replace polycyclic musks in the market.

On account of their widespread use, musk compounds can be considered ubiquitous throughout the world, and due to their lipophilic characteristics and slow biodegradation, they can be found in surface water [18-20], sewage [21,22], sediments [23] and fish species living in contaminated rivers and estuaries [24-26].

A wide range of analytical methods have been developed to determine musk fragrances in fish tissue. These methods have used a varied assortment of extraction techniques (Soxhlet, microwave assisted extraction (MAE), focused ultrasound-solid liquid extraction (FUSLE), and pressurized liquid extraction (PLE) usually followed by a clean-up step (silica gel, florisil

and/or gel permeation chromatography (GPC)) prior to analysis with GC-MS or GC-MS/MS [24,27-30]. In this paper a new extraction methodology of growing interest in the field of food analysis over recent years [31-33] - QuEChERS (quick, easy, cheap, effective, rugged and safe)- was tested and compared in terms of validation parameters with PLE. Special effort was on the reduction of matrix effect.

The QuEChERS methodology was first developed by Anastassiades *et al.* [34] for the extraction of pesticides from food matrices and involves two basic steps. At first QuEChERS methods use a single step buffered acetonitrile extraction and simultaneously salt out water from the aqueous sample using anhydrous magnesium sulfate to induce liquid-liquid partitioning. Subsequently, a clean-up step using a dispersive solid-phase extraction (dSPE) is often conducted to clean up the mixture, removing any undesired sample components. The main advantages of this extraction methodology are its speed, ease of implementation (instrumentation is not required), minimal solvent requirement and low cost when compared with instrumental extraction techniques.

The aim of this investigation was therefore to develop a rapid, sensitive and accurate analytical method based on GC-IT-MS/MS for determining ten synthetic musk fragrances in fish and mussels. PLE or QuEChERS as extraction procedures were compared and different clean-up strategies as in-cell clean-up sorbent for PLE or dSPE for QuEChERS were assayed to minimize the matrix effect. To the best of our knowledge, this is the first time that QuEChERS has been used to extract musk fragrances present in fish samples.

2. Experimental part

2.1. Reagents and standards

The six polycyclic musks studied were supplied by Promochem Iberia (Barcelona, Spain) and were the following: 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-*tert*-butyllindane (ADBI, celestolide), 6- acetyl-1,1,2,3,3,5- hexamethylindane (AHMI, phantolide), 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (ATII, traseolide), 1,3,4,6,7,8- hexahydro- 4,6,6,7,8,8- hexamethylcyclopenta-(*g*)-2-benzopyran (HHCB, galaxolide) and 7- acetyl-1,1,3,4,4,6- hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN, tonalide). The nitro musk fragrances 2,4,6- trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX, musk xylene) and 1,1,3,3,5-pentamethyl-4,6-dinitroindane (MM, musk moskene) were purchased as 100 mg L^{-1} individual solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The standard 4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (MK, musk ketone) was provided by Fluka (Buchs, Switzerland). International Flavors & Fragances Inc. (Barcelona, Spain) supplied 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran-1-one (HHCB-lactone, galaxolidone) while the internal standard d15-musk xylene (d15-MX) came as a 100 mg L^{-1} solution in acetone from Symta (Madrid, Spain). Suppl. Table 1

shows the main characteristics (formula name, molecular structure, CAS number, molar mass and boiling point) of the target compounds [1,35,36].

Individual standard solutions of the synthetic musk fragrances were prepared in acetone at concentrations of 4,000 mg L^{-1} for polycyclic musks and 1,000 mg L^{-1} for musk ketone and HHCB-lactone. A working mixture solution of 100 mg L^{-1} was prepared in ethyl acetate except for MX, MM and d15-MX which were supplied directly at a concentration of 100 mg L^{-1} in acetonitrile and used as received. Acetone and ethyl acetate were GC grade with purity >99.9 % from Prolabo (VWR, Llinars del Vallès, Barcelona, Spain).

The extraction solvents dichloro-methane, methanol, and hexane were GC grade (of>99.9% purity) from Prolabo, while acetonitrile was HPLC grade from Prolabo. Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallès, Barcelona, Spain). Helium gas with a purity of 99.999% was used for the chroma-tographic analysis (Carburos Metálicos, Tarragona, Spain).

2.2. Sampling and sample pre-treatment

Red mulltet (*Mullus surmuletus*) and mussels (*Mytilus galloprovincialis)* were selected for method development, optimization and validation. The method was successfully applied to determine musk fragrances in gilt head bream (*Sparus aurata*), turbot (*Psetta maxima*), red mullet (*Mullus surmuletus*) and mussels (*Mytilus galloprovincialis*), which had been purchased locally (Tarragona market) and mostly caught or collected in the Mediterranean Sea between May and December 2013. Perch (*Perca fluviatilis*), sheatfish (*Silurus glanis*) and carp (*Cyprinus carpio*) samples, which had been caught in the wild (between May and November 2013) and collected from the River Ebro, were also analysed.

After collection, the samples were immediately preserved in a refrigerated box. Lateral fillets were then dissected from the fish, homogenized and stored in a freezer until analysis. Frozen samples were lyophilized using the freeze-drying system (Labconco, Kansas City, MO, USA), crushed using a mortar and pestle and sieved through a 125 µm screen to homogenize the diameter of the particles.

2.3. Sample extraction

2.3.1. Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)

A total of 0.5 g (d.w.) of freeze-dried sample was weighed into 50 mL centrifuge tubes from Scharlab (Barcelona, Spain), 10 mL of ultrapure water was added to the tube, and the tube was shaken vigorously for 1 min. Then, 10 mL of acetonitrile was added, followed by an extraction salt packet (Scharlab) for the European Committee for Standardization (CEN) extraction method [37], which contains 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate. The mixture was then vortexed (3 min) and centrifuged for

5 min at 7,000 rpm (Hettich Universal 32R, Tuttlingen, Germany). The supernatant (acetonitrile layer) was removed and transferred to a 15 mL centrifuge tube containing 1 g of florisil (Sigma-Aldrich) for the dSPE clean-up. The tube was vortexed for 3 min and centrifuged again at 7,000 rpm for 5 min and the supernatant was evaporated under a gentle stream of nitrogen to a final volume of ≈1 mL. The internal standard (IS, 50 ng g^{-1} (d.w.)) was added to the extract before it was reconstituted to 2 mL with ethyl acetate. Extracts were filtered with a 0.22 µm PTFE syringe filter and analysed by GC-IT-MS/MS.

2.3.2. Pressurized liquid extraction (PLE)

Extraction of fish and mussel samples was carried out using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA; USA). Stainless steel extraction cells and glass collecting vials of 11 mL and 20 mL volume respectively were used. A cellulose filter was placed at the bottom of the 11 mL stainless steel extraction cell. It was then filled with 1 g of florisil (in-cell clean-up sorbent) previously conditioned at 400 °C overnight, 0.5 g (d.w.) of freeze-dried sample mixed with 1 g of diatomaceous earth (conditioned at 400 °C for 8 hours), and 1 g of diatomaceous earth. This was finally compacted and closed before extraction. The extraction was carried out with one cycle of dichloromethane at 60 °C and 1,500 psi for 5 min. The preheating time was 5 min, flush volume was 100% of cell volume and purge time was 90 s. The sample extract was evaporated with a rotary evaporator (R-114, Büchi, Switzerland) set at 30 °C, the IS (50 ng g^{-1} (d.w.)) was added to the residue (≈1 mL) before it was reconstituted to 2 mL with ethyl acetate and filtered with a 0.22 µm PTFE syringe filter, and finally analysed by GC-IT-MS/MS system.

2.4. Gas chromatography-ion trap-tandem mass spectrometry

The GC-IT-MS/MS analyses were performed using a Varian ion trap GC-MS system (Varian, Walnut Creek, CA, USA), equipped with a 3800 gas chromatograph, a 4000 ion trap mass detector, a 1079 programmable vaporizing temperature injector and a CombiPal autosampler (CTC Analytics, Zwigen, Switzerland). The mass spectrometer was operated in the electron ionization (EI) mode (70 eV) and the system was controlled by Varian MS Workstation v.6.9 software. A fused silica capillary column (3 m x 0.25 mm i.d.) from Micron Phenomenex (Torrance, California, USA) was used as a guard column. The chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl/50% dimethyl-polysiloxane, 30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Micron Phenomenex. The oven temperature was programmed as follows: 70 °C hold for 3.5 min, raised at 50 °C min^{-1} to 200 °C, then 5 °C min^{-1} to 240 °C and finally 20 °C min^{-1} to 290 °C (hold 3.4 min). The carrier gas employed was helium with a purity of 99.999% at a constant column flow of 1 mL min^{-1}. During the injection of the 10 µL, the

1079 injector operated in large volume injection (LVI) mode and a 2 mm i.d. insert liner packed with glass wool (Varian) was used. During injection in split mode at a rate of 50 mL min^{-1} the 1079 injector temperature was set at 70 °C. The ethyl acetate was purged out with a vent flow of 100 mL min^{-1} within 0.5 min (vent time). The splitless mode was then programmed for 2.5 min while the temperature was increased at 100 °C min^{-1} to 300 °C for 5 min. Transfer line, manifold and trap temperatures were 280 °C, 50 °C and 200 °C respectively. For quantitative analysis of the target compounds, the tandem mass spectrometry (MS/MS) mode was applied. Retention times as well as optimal MS parameters of the target compounds are summarized at Suppl. Table 2.

3. Results and discussion

3.1. Large volume injection GC-IT-MS/MS optimization

A mixed solution of 10 mg L^{-1} of the target musk fragrances and 1 mg L^{-1} of d15-MX as IS was prepared in ethyl acetate and 10 µL of this solution was directly injected into the GC-IT-MS, using electron impact ionization in full scan mode. All the compounds were identified by their molecular ion and afterwards the chromatographic separation was optimized by testing several oven temperature programmes. All compounds were separated in just 16 min using the chromatographic conditions described in Section 2.4. In order to achieve maximum sensitivity/ selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/ product ions and IT-MS/MS parameters based on a previous paper [38]. In Suppl. Table 2 are also summarized, the parent ion, optimum amplitude excitation voltage, CID storage level, product ions (quantifiers and qualifiers), the m/z range of ions analysed by EI-MS/MS and scan time of each target compound. Each compound was acquired separately in one segment, except HHCB and AHTN and d15-MX, MX and MM; because of this, the scan time of these compounds was shorter than the others.

3.2. QuEChERS optimization

QuEChERS extraction involves two extraction steps, the first of which, a salting-out liquid-liquid extraction to extract the analytes of interest from the matrix while the second, a dSPE for the clean-up of the sample. To achieve efficient extraction of the target compounds from a fish or mussel sample using the QuEChERS system, certain variables such as the salts were adopted from the European Standard Method EN15662 [37]. Other significant parameters that affect extraction and clean-up performance, i.e. the ratio between the sample mass and the volume of solvent, type of extraction solvent and dSPE sorbents, were optimized.

Lyophilized fish (red mullet) and mussel samples were spiked at a concentration of 1 µg g^{-1} (d.w.) for each compound to ensure that peak areas of the analytes present in the samples (<10% of peak areas from spiked samples) do not affect the accurate quantification of analytes during the optimization of the

QuEChERS variables. To calculate the QuEChERS recoveries, internal standard calibration curves were constructed by using fish and mussel samples spiked after the extraction. Then, samples spiked previously to the extraction were analysed and calculated concentrations by those calibration curves and theoretical concentrations were compared. Thus, recoveries do not take into account the differences caused by the matrix effect, only the extraction yield [39].

The influence of the sample mass/solvent volume ratio was studied by mixing different sample amounts (0.25, 0.50, 0.75 and 1.00 g (d.w.)) with 10 mL of ultrapure water followed by the addition of 10 mL of acetonitrile and the QuEChERS extraction salts. The other parameters are described in Section 2.3.1. The best QuEChERS recoveries (between 54-97% for fish samples and between 47-85% for mussel samples) were obtained working with 0.50 g (d.w.) of sample amount. Some agglomerates were formed for higher sample amounts, indicating that the amount of $MgSO_4$ used was not enough to remove all the water. This situation negatively affected the extraction method, obtaining QuEChERS recoveries 10% and 20% lower for fish and mussel samples, respectively. Furthermore, the use of large sample quantities can lead to a higher co-extraction of matrix interferents.

For the best extraction of musk fragrances and to guarantee a minimal co-extraction of matrix interferences, the selection of an appropriate solvent is a crucial step in this phase of the optimization process. Four different solvents were tested: dichloromethane, acetonitrile, ethyl acetate and hexane. Acetonitrile is the common solvent used in QuEChERS methodology and the other solvents were chosen based on literature [26,40,41].

The acetonitrile showed the best extraction performance with QuEChERS recoveries between 54% and 97% for fish and 47% and 85% for mussels. No significant differences were obtained working with dichloromethane, ethyl acetate or hexane, with QuEChERS recoveries in any case lower than those obtained with acetonitrile. Therefore acetonitrile was chosen as the extraction solvent because it was the only one capable of fully dispersing the matrix and increasing the surface contact area between the sample and the extraction solvent, resulting in higher QuEChERS recoveries.

One of the major drawbacks in the analysis of biological samples is the high matrix effect observed, especially when MS is used, which involves ion suppression or enhancement of the signal. Consequently to achieve better quantification limits of the target analytes, a dSPE was tested to clean-up the sample. In this clean-up step, a commercially available dSPE tube containing primary and secondary amine exchange sorbent (PSA) and octadecyl-silica (C18) was used. The PSA sorbent is used to remove sugars, fatty acids, organic acids, lipids and certain pigments, while the C18 sorbent is used to remove long chain fatty acid compounds and other non-polar interferences [42]. Home-made dSPE tubes containing florisil (1g), silica (1g) and alumina (1g) as clean-up sorbents were also tested due to their

ability in the removal of lipids, oils and waste from PLE extracts [43,44].

The matrix effect (ME, %) was calculated with Eq. 1:

$$ME\ (\%) = \frac{(C_{sample} - C_{standard})}{C_{standard}} \cdot 100 \quad \text{Eq. 1}$$

Where C_{sample} is the concentration determined by spiking a fish or mussel extract after QuEChERS and using an internal standard calibration curve obtained by direct injection of the standards. $C_{standard}$ is the theoretical concentration. Moreover, apparent recoveries (R_{app}), which include QuEChERS recovery and matrix effect, were calculated by analysing a spiked fish or mussel sample and using the same calibration curve as before. Working without a clean-up step a high ME was observed for all of the target analytes with R_{app} between 6% and 90% independently of the kind of sample analysed. It is worth noting that ME values were between -95% and -85% for MM, MK and HHCB-lactone. However, when a dSPE was applied different behaviour of the target analytes was observed. For polycyclic musks, florisil was the best dSPE sorbent with ME between -28% and 16% and between -52% and 31% for fish and mussel samples, respectively and R_{app} ranging between 59-110% (see Fig. 1). Nitro musks and HHCB-lactone showed the highest R_{app} (36-66%) and lowest ME, between -58% and -15% for fish samples and -62% and -28% for mussel samples, working with a mixture of PSA and C_{18} sorbents as dSPE sorbent. Therefore florisil was chosen as the dSPE sorbent as a compromise.

To summarize, working with QuEChERS as extraction technique, optimum results for both fish and mussel samples were achieved when 10 mL of ultrapure water was mixed with 0.5 g (d.w.) of sample. Then 10 mL of acetonitrile followed by 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate were added. A dSPE with 1 g of florisil as a clean-up step was performed. Table 1 summarizes the R_{app} and ME found under optimal conditions working with fish and mussel matrices.

3.3. PLE optimization

To achieve efficient extraction of the target compounds from a fish or mussel sample using a PLE system, several operational parameters as extraction solvent, temperature, time and number of cycles must be optimized. In addition, an in-cell clean-up sorbent was tested in order to reduce ME. Other parameters such as pressure, flush volume and purge time can also be optimized, but it is well known that these parameters have no significant effect on extraction efficiency.

Lyophilized fish and mussel samples (0.5 g (d.w.)) spiked at 1 μg g^{-1} (d.w.) were mixed with 1 g (d.w.) of diatomaceous earth and they were placed into a stainless-steel cell. The initial experimental conditions were set according to previous literature [22,24]: 80 °C, 2 cycles, 5 min static time, 120 s of purge time, 1,500 psi and 100% flush volume.

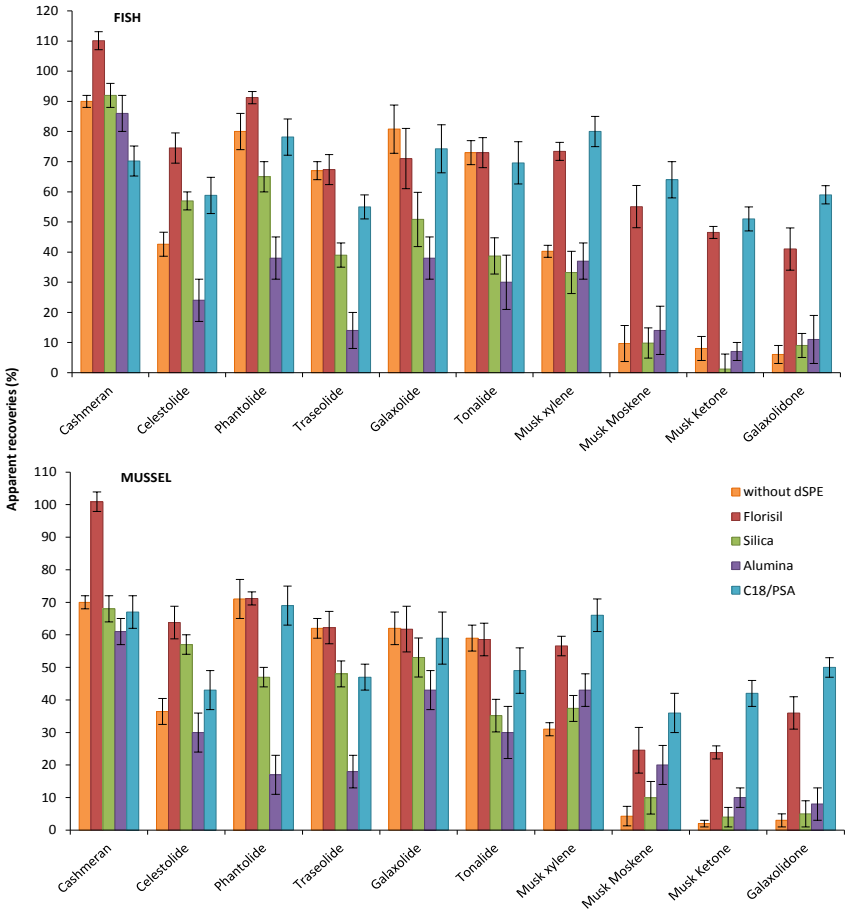

Fig. 1. Optimization of the dSPE clean-up sorbent under QuEChERS optimal conditions: 0.5 g (d.w.) sample amount, 10 mL acetonitrile as extraction solvent and QuEChERS containing 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate (n=3, 1 μg g^{-1} (d.w.)).

First the extraction solvent was optimized. Hot water, which was successfully applied by our group for the determination of nitrosamines and aliphatic amines in sewage sludge [45,46], was chosen as the initial extraction solvent instead of organic solvents because makes the extraction more environmentally friendly. However, the high lipid percentage of the mussels caused a solid mass to form inside the extraction cell that made it impossible to extract the target compounds present in mussels by PLE using water as the extraction solvent. When working with fish samples, PLE recoveries were below 30% for all the target compounds. Therefore it was

decided to work with organic solvents previously used as extraction solvents to extract musk fragrances or other personal care products from sludge samples [22,47] and biota samples [24,26]. Of the organic solvents studied -methanol (polarity index=5.1), ethyl acetate (polarity index=4.4, dichloromethane (polarity index=3.1) and hexane (polarity index=0.0), as can be seen in Fig. 2, dichloromethane was the most efficient solvent for extracting the target analytes from fish and mussels, with PLE recoveries between 57%-86% and 51%-91%, respectively. A non-polar solvent (polarity<dichloromethane) as hexane did not provided good PLE recovery values, below 50% for fish samples and up to 38% for mussel samples. Among relatively polar solvents (polarity>dichloromethane, methanol was also capable of extracting musk fragrances from fish or mussels samples with PLE recoveries between 49%-82% for fish and 44%-90% for mussels, respectively. However due to the lipophilic properties of musk fragrances, they can be retained in the fatty precipitates that appear in the PLE extracts. While, as can be seen in Fig. 2 the target compounds were not effectively extracted with ethyl acetate as extraction solvent with PLE recovery values lower than 48% and 31% for fish and mussels, respectively. As a compromise between analyte recoveries and co-extracted matrix components, dichloromethane was therefore been selected as extraction solvent.

The extraction temperature was then studied by comparing PLE recoveries obtained at 60 °C, 80 °C and 100 °C. The other extraction conditions were the same as described above. A temperature of 60 °C provided the best PLE recoveries for the entire target analytes (between 67-95% for fish and 64-101% for mussel) independently of the matrices analysed. PLE recoveries decreased when the temperature was increased to 80 °C and were below 40% for most of the compounds when the temperature was 100 °C. This is probably due to the presence of high amounts of fatty precipitates in the PLE extract increasing with the temperature, which makes a filtration step with a PTFE filters (0.45 µm) previous to filtration with PTFE filters of 0.20 µm before GC-MS mandatory.

Static time and number of cycles were also studied in order to enhance the efficiency of PLE extraction. Static times of 5, 10 and 15 min were studied. The best results were obtained working with 5 min. Static times of 10 and 15 min did not result in a significant increase in extraction efficiency. Regarding the number of cycles, 1, 2 and 3 cycles with a static extraction time of 5 min were tested. Two extraction cycles did not improve PLE recoveries significantly for the vast majority of the target compounds, and so any increase in the number of cycles was discarded. Therefore one cycle and a static time of 5 min were chosen as the optimal parameters for PLE extraction.

Due to the optimization of the PLE parameters, the presence of fatty precipitates in the PLE extract was considerably reduced until turbidity. However, in order to minimize the ME and avoid a clean-up step previous to GC-IT-MS/MS, an in-cell clean-up sorbent was tested. So 1 g of in-cell

clean-up sorbent was placed on the cellulose filter at the bottom of the extraction cell to retain the interfering substances when the PLE was carried out under optimum conditions. Three sorbents were tested to work as in-cell clean-up sorbent -florisil, alumina and silica- all conditioned at 400 °C overnight. R_{app} as well as ME, calculated as has been described in section 3.2, were taken into account to select the optimal in-cell clean-up sorbent. Results showed that florisil was the only sorbent that provided lower ME, ranging between -49% to 16% for fish samples and between -58% to 19% for mussel samples and an enhancement of R_{app} for all of the target analytes until the values placed in Table 1. Silica seemed not to affect the response of analytes and working with alumina a decrease of between 10-20% of R_{app} of nitro musks and also HHCB-lactone was observed, probably because the analytes were adsorbed by alumina.

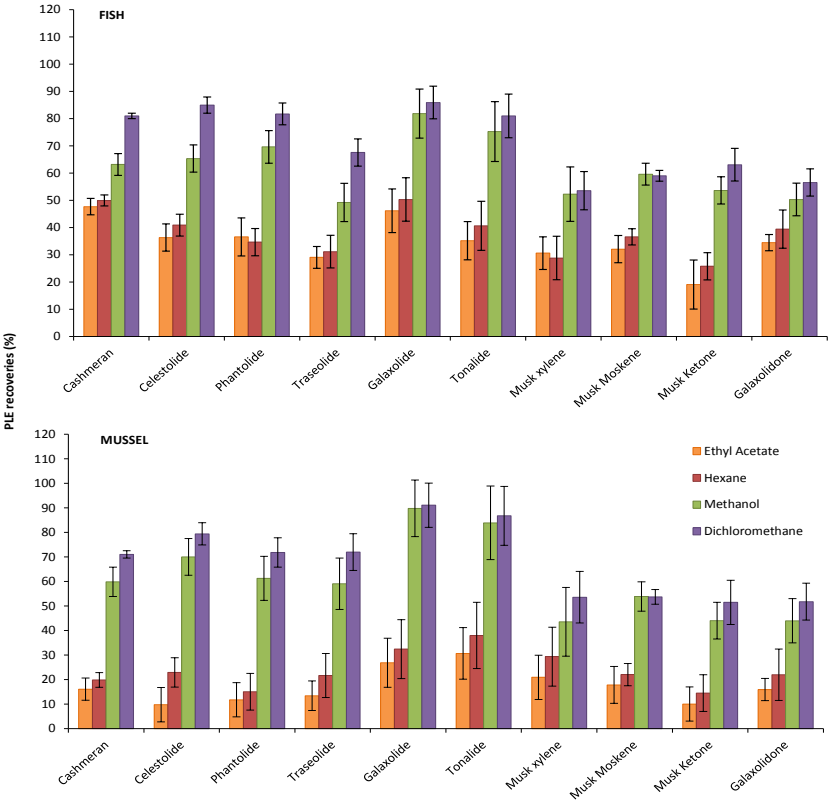

Fig. 2. Optimization of the PLE extraction solvent under the following initial experimental conditions: 80 °C, 2 cycles, 5 min static time, 120 s purge time, 1,500 psi, 0.5 g (d.w.) sample amount and 100 % flush volume (n=3, 1 µg g^{-1} (d.w.)).

Table 1. Apparent recoveries (R_{app}, %) and matrix effect (ME, %) obtained under PLE and QuEChERS optimal conditions for both fish and mussel samples (n=3, 1 µg g^{-1} (d.w.)).

Compounds	FISH				MUSSEL			
	PLE		QuEChERS		PLE		QuEChERS	
	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)
Cashmeran	109	8	110	16	79	19	110	31
Celestolide	94	-19	75	-28	88	-20	64	-32
Phantolide	91	10	91	-15	80	-13	71	-21
Traseolide	95	9	67	-10	87	-11	62	-23
Galaxolide	95	16	71	-17	91	-53	62	-52
Tonalide	96	5	73	-21	86	-41	59	-46
Musk xylene	67	-29	73	-20	67	-39	57	-34
Musk moskene	64	-33	55	-54	57	-41	25	-65
Musk ketone	69	-47	46	-56	54	-58	24	-67
Galaxolidone	61	-49	41	-62	45	-58	36	-70

The best $R_{app,}$ which were summarized in Table 1, were achieved under the following conditions: 60 °C, 1 cycle 5 min static time, 120 s purge time, 1,500 psi, 0.5 g (d.w.) sample, 100% flush volume and 1g florisil as in-cell clean-up sorbent.

3.4. Method validation

As both extraction methodologies are suitable for the extraction of the target analytes present in fish and mussel samples, both methods were validated. Linear range, method detection limits (MDLs), method quantification limits (MQLs), intra-day and inter-day repeatability (expressed as % Relative Standard Deviation) were the validation parameters evaluated. Although ME had been significantly reduced by the optimization of the clean-up step, it was decided to use a matrix-matched calibration curve for the quantification of analytes in order to obtain more accurate results. In addition, as large volume injection mode was used (10 µL), the IS d15-MX was used to improve the repeatability.

The fish (*Mullus surmuletus*) and mussel (*Mytilus galloprovincialis*) samples used to validate the method were analysed (*n*=5) to determine if any target analyte was present, and the results revealed peaks of HHCB and AHTN in the chromatogram. The average peak area of each compound detected was subtracted from the corresponding peak areas of each spiked sample.

Linear range, MQLs and MDLs were obtained experimentally by spiking fish and mussel samples at different levels (IS=50 ng g^{-1} (d.w.)) prior to the extraction procedure by PLE or QuEChERS (Tables 2 and 3). The linear range started at the MQL (defined as the lowest calibration point) and went up to 100 ng g^{-1} (d.w.) or 250 ng g^{-1} (d.w.) depending on the target analyte, with good linearity for all of the target compounds (r^2>0.994) provided by the presence of the IS. The MDLs were calculated by the S/N of 3 for the compounds that did not appear in the fish and mussel samples. MDLs for HHCB and AHTN were estimated as the concentration that gave a signal average of plus three times the standard deviation of the signal obtained for blank samples. Thus MQLs and MDLs were between 1 ng g^{-1} (d.w.) and 20 ng g^{-1} (d.w.) and 0.5 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.), respectively, independently of the matrix analysed. As can be seen in Tables 2 and 3, slightly better MQLs and MDLs were obtained working with PLE as extraction technique for both fish and mussel samples. In addition, the developed methods provided better MDLs than those reported in the literature by Subedi *et. al.* [26] and Mottaleb *et. al.* [27]. Considering that MDLs representing the lowest concentration of each analyte that may be reported in a defined matrix with 99% confidence that the concentration is non zero [48]. Subedi *et al.* [26] reported MDLs between 1.6-38 ng g^{-1} (d.w.) working with PLE followed by GPC and GC-IT-MS/MS. While Mottaleb *et al.* [27] obtained MDLs between 12-397 ng g^{-1} (d.w.) working with LLE followed by GPC and GC-IT-MS/MS as separation and detection technique. Intra-day and inter-day repeatability were obtained with five replicates of a

Table 2. Method validation parameters obtained working with fish (*Mullus surmuletus*) samples and PLE or QuEChERS as extraction technique.

Compounds	MDLs (ng g^{-1} (d.w.))		Linear range [a] (ng g^{-1} (d.w.))		Intra-day Repeatability [b] (%)		Inter-day Repeatability [b] (%)	
	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS
Cashmeran	0.5	0.5	2.5-250	2.5-100	4	6	11	9
Celestolide	0.25	0.5	1-100	2.5-250	5	4	7	8
Phantolide	0.25	0.25	2.5-250	2.5-250	3	3	6	6
Traseolide	0.5	1	2.5-250	5-250	5	2	9	5
Galaxolide	0.25	0.25	1-100	1-100	4	5	8	7
Tonalide	0.25	0.25	1-100	1-100	3	5	6	7
Musk xylene	5	10	10-250	20-250	7	6	8	16
Musk moskene	5	10	10-250	20-250	9	7	11	13
Musk ketone	5	10	10-250	20-250	8	7	8	11
Galaxolidone	0.25	0.5	2.5-100	5-250	10	4	12	7

[a] MQLs (ng g^{-1} (d.w.)) were fixed as the lowest calibration point.
[b] RSD (%), n=5, 50 ng g^{-1} (d.w.).

Table 3. Method validation parameters obtained working with mussel (*Mytilus galloprovincialis*) samples and PLE or QuEChERS as extraction technique.

Compounds	MDLs (ng g^{-1} (d.w.))		Linear range [a] (ng g^{-1} (d.w.))		Intra-day Repeatability [b] (%)		Inter-day Repeatability [b] (%)	
	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS
Cashmeran	0.5	1	2.5-250	5-250	6	6	9	9
Celestolide	1	2.5	5-250	5-250	4	7	9	8
Phantolide	2.5	2.5	5-250	5-250	2	3	4	16
Traseolide	1	2.5	5-250	5-250	7	5	8	18
Galaxolide	0.5	0.5	2.5-100	2.5-250	7	7	9	9
Tonalide	0.5	0.5	2.5-100	2.5-250	7	6	9	13
Musk xylene	5	7.5	10-250	20-250	9	4	12	9
Musk moskene	5	7.5	10-250	20-250	12	14	12	19
Musk ketone	5	7.5	10-250	20-250	10	5	14	10
Galaxolidone	2.5	5	7.5-250	10-250	2	14	16	16

[a] MQLs (ng g^{-1} (d.w.)) were fixed as the lowest calibration point.
[b] RSD (%), n=5, 50 ng g^{-1} (d.w.).

fish sample and a mussel sample spiked at 50 ng g^{-1} (d.w.). The presence of the IS improved the method repeatabilities obtaining intra-day repeatability values always less than 10% for fish samples and 14% for mussel samples, and no significant differences were observed between work with PLE or QuEChERS as the extraction technique. Inter-day repeatability was always less than 16% or 19% (%RSD, *n*=5) for fish and mussel samples respectively.

PLE and QuEChERS extraction procedures were compared in terms of validation parameters, analysis time and ME when applied to fish and mussel. Although both extraction techniques are suitable for the extraction of musk fragrances from fish and mussel tissues, the results showed that PLE was the extraction procedure that provided the lowest ME. As can be seen in Fig. 3a and 3b the chromatograms obtained by PLE showed lower base lines, well-defined peaks for all of the target musk fragrances and an absence of interfering peaks. As a result slightly better validation parameters were obtained for both fish and mussel samples when the PLE based method was used. That together with the absence of significant differences in terms of extraction time meant that PLE was chosen to determine the target musks present in the different kinds of fish samples. However, QuEChERS could be used to determine musk fragrances in fish and mussel samples if PLE is not available.

3.5. Method application

The PLE GC-IT-MS/MS method was applied to determine musk fragrances in fish samples of red mullet (*Mullus surmuletus*), gilt head bream (*Sparus aurata*), turbot (*Psetta maxima*) and mussels (*Mytilus galloprovincialis*) from Tarragona market, and also in perch (Perca fluviatilis), sheatfish (Silurus glanis) and carp (*Cyprius carpio*) samples from the River Ebro (section 2.2). As it has been already described, two matrix-matched calibration curves were used, one for fish samples and the other for mussel samples, for the quantification of analytes in order to obtain more accurate results.

Table 4 presents the results of the average concentrations of musk fragrances found in each sample (*n*=8) analysed. HHCB and AHTN, which were usually determined in wastewater [18-20] or river waters [24-26] at concentrations ranging from µg L^{-1} to mg L^{-1}, were present in all the samples analysed at concentrations ranging between 2.97 ng g^{-1} (d.w.)-18.04 ng g^{-1} (d.w.) and 1.17 ng g^{-1} (d.w.)-8.42 ng g^{-1} (d.w.) for HHCB and AHTN, respectively. Perch and sheatfish were the fish samples with the highest concentrations of HHCB (18.04 ng g^{-1} (d.w.)) and AHTN (8.42 ng g^{-1} (d.w.)), respectively. DPMI was found in all the samples analysed (12.83 ng g^{-1} (d.w.)-33.53 ng g^{-1} (d.w.)), except red mullet and mussels. ADBI was determined in red mullet, turbot and carp samples at concentrations between 1.56 ng g^{-1}

Table 4. Musks concentrations (ng g^{-1} (d.w.)) determined in samples analysed.

Compounds	Tarragona Market				Ebro River		
	Red Mullet *(Mullus surmuletus)*	**Gilt head bream** *(Sparus aurata)*	**Turbot** *(Psetta maxima)*	**Mussel** *(Mytilus galloprovincialis)*	**Perch** *(Perca fluviatilis)*	**Sheatfish** *(Silurus glanis)*	**Carp** *(Cyprinus carpio)*
Cashmeran	n.d.	12.83	15.69	n.d.	13.36	33.53	14.06
Celestolide	6.25	n.d.	8.26	n.d.	n.d.	n.d.	1.56
Phantolide	n.d.	n.d.	2.61	n.d.	n.d.	n.d.	12.51
Traseolide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Galaxolide	2.97	6.12	9.67	8.94	18.04	16.23	12.68
Tonalide	1.17	3.61	5.19	5.65	7.53	8.42	1.38
Musk xylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Musk moskene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Musk ketone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Galaxolidone	n.d.	n.d.	n.d.	n.d.	15.99	17.94	n.d.

n.d.: not detected.

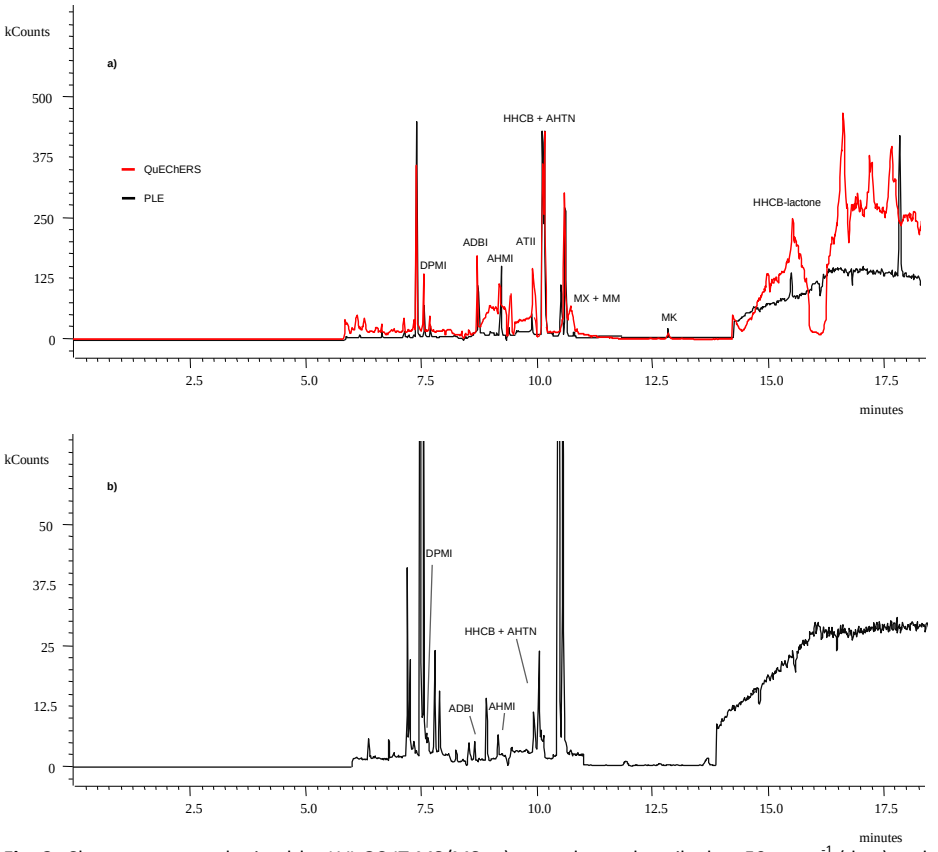

Fig. 3. Chromatograms obtained by LVI-GC-IT-MS/MS: a) mussel sample spiked at 50 ng g^{-1} (d.w.) and extracted by QuEChERS (red line) and PLE (black line), respectively. b) non-spiked turbot sample extracted by PLE and analysed by LVI-GC-IT-MS/M. DPMI (Cashmeran), ADBI (Celestolide), AHMI (Phantolide), ATII (Traseolide), HHCB (Galaxolide), AHTN (Tonalide), MX (Musk xylene), MM (Musk moskene), MK (Musk ketone), HHCB-lactone (Galaxolidone).

(d.w.) and 8.26 ng g^{-1} (d.w.). The presence of AHMI was demonstrated only in the turbot and carp samples, with an average concentration of 2.61 ng g^{-1} (d.w.) and 12.51 ng g^{-1} (d.w.), respectively. None of the samples contained detectable traces of MX, MM or MK. HHCB-lactone was found only in the perch (15.99 ng g^{-1} (d.w.)) and sheatfish (17.94 ng g^{-1} (d.w.)) samples from the River Ebro. Fig. 3b shows the PLE GC-IT-MS/MS chromatogram of a turbot sample in which the presence of DPMI, ADBI, AHMI, HHCB and AHTN is shown.

Previous research [24-27,29,49] that has focused on determining musk fragrances in fish samples (lateral fillets) from river or sea waters confirm the findings of this study, i.e. that the most abundant polycyclic musks are HHCB and AHTN, although other

polycyclic musks such as DPMI, ADBI, AHMI and ATII can also be present in fish samples in minor concentrations. However, the nitro musks (MX, MM and MK) show significant differences in concentration depending on the location of the fish samples, and if the study was carried out before nitro musk fragrances became subject to regulation [26, 29, 49].

4. Conclusions

Two extraction methodologies, QuEChERS and PLE, were compared for determining ten musk fragrances in fish and mussel samples. A dSPE clean-up with florisil or an in-cell clean-up with florisil was applied to de-fat the sample extract and reduce the matrix effect in QuEChERS and PLE, respectively. Despite the reduction of ME observed by applying those strategies, matrix-matched calibration curves were applied to ensure more accurate results.

The methods were validated for both extraction procedures, with slightly better results being obtained working with PLE plus in-cell clean-up as the extraction technique, with MDLs ranging between 0.5 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.) depending on the target analyte, intra-day repeatabilities lower than 14% for all the compounds analysed, and inter-day repeatabilities between 4% and 16%.

The applicability of the PLE/GC-IT-MS/MS method has been demonstrated by analysing different kinds of fish such as red mullet, gilt head bream, turbot and mussels, from the local market in Tarragona and perch, sheatfish and carp taken from the Ebro River. The results showed that HHCB and AHTN were present in all the samples analysed, with perch and sheatfish being the samples that present the highest concentrations of HHCB (18.04 ng g^{-1} (d.w.)) and AHTN (8.42 ng g^{-1} (d.w.)) respectively.

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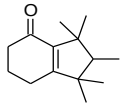
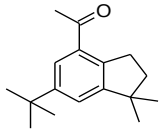
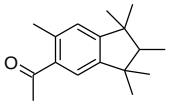
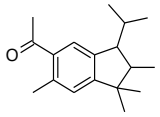
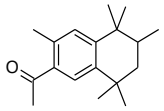
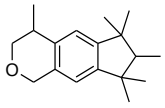
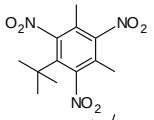
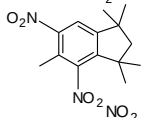
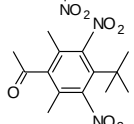
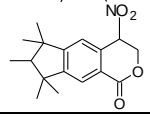
Supplementary material

Suppl. Table 1. Main characteristics of the target compounds.

nº	Formula name	Molecular structure	CAS number	Molar mass (g mol^{-1})	Boiling point (°C) 760 mmHg
1	6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)-indanone (Cashmeran, DPMI)		33704-61-9	206.32	286.10
2	4-acetyl-1,1dimethyl-6-*tert*-butylindane (Celestolide, ADBI)		13171-00-1	244.38	309.00
3	6-acetyl-1,1,2,3,3,5-hexamethylindane (Phantolide, AHMI)		15323-35-0	244.37	393.00
4	5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (Traseolide, ATII)		68140-48-7	258.40	350.00
5	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran (Galaxolide, HHCB)		1222-05-5	258.40	326.00
6	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN)		1506-02-1	258.40	393.00
7	2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (Musk xylene, MX)		81-15-2	297.26	392.30
8	1,1,3,3,5-pentamethyl-4,6-dinitro indane (Musk moskene, MM)		116-66-5	280.32	351.10
9	4-aceto-3,5-dimethyl-2,6-dinitro-*tert*-butylbenzene (Musk ketone, MK)		81-14-1	294.30	369.00
10	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran-1-one (Galaxolidone, HHCB-Lactone)		*	272.40	*

* Information not found at the bibliography.

Suppl. Table 2. Retention times and MS conditions.

Compounds	Retention time (min)	Parent ion (m/z)	Products ions [c] (m/z)	CID Amplitude (V)	CID Storage level (m/z)	m/z range	Scan time (s/scans)
Cashmeran	7.77	191	107, **135**, 173	0.82	84.1	94-201	1.08
Celestolide	8.98	229	131, **173**, 187	0.92	100	110-239	1.01
Phantolide	9.46	229	131, 145, **187**	0.92	100	110-239	1.01
Traseolide	10.17	215	131,171, **173**	0.88	94.7	104-225	1.01
Galaxolide [a]	10.42	243	171, **213**	0.96	122	132-253	0.53
Tonalide [a]	10.44	243	**145**, 159, 187	0.96	103	113-253	0.53
Musk aylene [b]	11.07	282	**265,** 266, 281	1.08	124.2	134-292	0.59
Musk moskene [b]	11.15	263	187, 201, **211**	1.02	115.9	125-273	0.59
Musk ketone	12.97	279	**191**, 247, 280	1.07	122.9	132-289	1.05
Galaxolidone	15.50	257	183, 201, **239**	1.00	113.2	123-267	1.03
^{2}H15- Musk xylene (IS)[b]	10.89	294	170, **276**, 295	1.11	129.5	139-304	1.04

[a], [b] Compounds were separated using the Multiple Reaction Monitoring.
[c] Quantification ions (m/z) are shown in bold type.

3.3.2. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

In this study two extraction methodologies, QuEChERS and PLE followed by GC-IT-MS/MS, were developed for determining PCMs and NMs in marine organisms, namely fish and mussels. Different clean-up techniques were evaluated, including dSPE and in-cell clean-up both with florisil as clean-up sorbent, to de-fat sample extracts and reduce the ME as much as possible. The developed methods provided MDLs between 0.5 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.) with intra-day repeatabilities lower than 14% and inter-day repeatabilities between 4% and 16%.

With respect to the GC-MS system, the injector operated in LVI mode and a 2 mm i.d. insert liner packed with glass wool was used. The use of this kind of injector enabled us the injection of volumes between 10 and 50 µL to improve the sensitivity of the method. However, due to the high ME generated by the fish and mussel samples analysed, only 10 µL of samples extract were injected. At higher injection volumes the analytical signals of NMs and HHCB-lactone decrease significantly and with mussel samples problems with peak distortion started to appear. A fused silica capillary column was used as a guard column. The chromatographic separation was performed with the mid-polarity analytical column (50% phenyl/dimethylpolysiloxane) previously used for the determination of synthetic musk fragrances in wastewater and sewage sludge samples (see Sections 3.1.5 and 3.2.5) which allows the separation of galaxolide enantiomers, even with fish and mussel samples. To achieve maximum sensitivity/selectivity of the compounds, the MS detector acquired in MS/MS mode and was carried out by selecting appropriate precursor/product ions and IT-MS/MS parameters based on previous studies discussed in section 3.2.5 of this Thesis.

To achieve efficient extraction of the target fragrances from marine organisms using QuEChERS, some variables, such as the salts, were taken from the European Standard Method EN 15662 [1], while other significant variables that affect extraction and clean-up were optimized, such as the ratio between the sample mass and the volume of solvent, extraction solvent and dSPE sorbent [2,3]. With respect to the sample amount, the best recoveries between 54 and 97% for fish and between 47 and 85% for mussels, were achieved working with 0.5 g (d.w.) of sample. At higher sample amounts, some agglomerates started to appear, indicating that the amount of magnesium sulphate used was not enough to remove all of the water content. Although four solvents extensively used for extracting musk fragrances (dichloromethane, acetonitrile, ethyl acetate and hexane [4,5]) were evaluated for the best extraction of musk fragrances and to guarantee a minimal co-extraction of matrix interferents, the best recovery values were provided with acetonitrile (54-97% for fish and 47-85% for mussels). For good reason, acetonitrile is the most commonly used extraction solvent in the QuEChERS

methodology [6], because it is capable of fully dispersing the matrix and increasing the surface contact area between the samples and the extraction solvent, resulting in higher QuEChERS recoveries. Moreover, dSPE was performed to clean-up the sample and decrease the ME. In this respect, a commercial dSPE tube containing PSA and C18 was used and dSPE tubes built in house containing florisil, silica and alumina were also tested. The results showed that florisil provided the best R_{app} between 59% and 110% and ME values between -28% and 16% and between -52% and 31% for fish and mussel samples respectively for PCMs. Meanwhile, NMs and HHCB-lactone showed the highest R_{app} (36-66%) and lowest ME, between -58% and -15% for fish samples and -62% and -28% for mussel samples, working with the commercial mixture of PSA and C18. Therefore, florisil was chosen as the dSPE sorbent as a compromise.

With respect to PLE, the variables expected to be the most influential were optimized, such as extraction solvent and temperature, among others. Moreover, an in-cell clean-sorbent was required for reducing ME. Initially, water was chosen as the extraction solvent rather than organic solvents, to make the extraction more environmentally friendly. However, working with fish samples, the recoveries obtained were below 30% for all of the target compounds and the high lipid percentage of mussels caused a solid mass to form inside the extraction cell that made it impossible to extract the target compounds present in mussels by PLE using water as the extraction solvent. Therefore, it was decided to test organic solvents previously used to extract musk fragrances present in biota samples (methanol, ethyl acetate, dichloromethane and hexane) [7,8]. Dichloromethane was the most efficient solvent for extracting the target musk fragrances from fish and mussels, with PLE recoveries between 57% and 86% and 51% and 91%, respectively. In the case of the extraction temperature, contrary to what was expected, the best PLE recoveries (67%-95% for fish and 64%-101% for mussels) were found at 60 °C. At higher temperatures, probably due to the presence of high amounts of fatty precipitates in the PLE extracts, recovery values decrease gradually to reach below 40% obtained at 100 °C. Although the presence of fatty precipitates in the PLE extract was considerably reduced down to turbidity, an in-cell clean-up sorbent was tested to minimize ME. Three sorbents were tested, florisil, alumina and silica. The obtained results showed that florisil was the only sorbent capable of decreasing the ME to values between -49% and 16% for fish and between -58% and 19% for mussel samples and increase the R_{app} to values between 45% and 109%, regardless of the matrix analysed. Silica seemed not to affect the response of the target analytes, while, working with alumina, the R_{app} of NMs and HHCB-lactone decreased between 10% and 20%.

Both PLE and QuEChERS are characterized by their speed, low volume of organic solvent used, high throughout and good recovery values obtained. In both cases, a clean-up step was required to minimize ME and obtain good recovery values, an in-cell clean-up with florisil for PLE and dSPE for QuEhERS. Although both extraction techniques are suitable for the extraction of musk fragrances from fish and mussel samples, PLE is preferable because of its low MEs. Moreover, the chromatograms obtained with PLE showed lower base lines, well-defined peaks for all the target musk fragrances and the absence of interfering peaks resulting in slightly better validation parameters. However, QuEChERS could be used for the determination of synthetic musk fragrances in fish and mussel samples if PLE is not available.

The PLE GC-IT-MS/MS method was applied to the determination of musk fragrances in fish samples from the Tarragona market such as red mullet, gilt head bream, turbot and mussels, and also in perch, sheatfish and carp from the River Ebro. The results showed that galaxolide and tonalide were found in all the samples analysed at concentrations between 2.97 ng g^{-1} (d.w.) and 18.04 ng g^{-1} (d.w.) and 1.17 ng g^{-1} (d.w.) and 8.42 ng g^{-1} (d.w.), respectively. As can be seen in Figure 3.3.1, the highest concentrations of these compounds were found in perch (18.04 ng g^{-1} (d.w.) of galaxolide) and sheatfish (8.42 ng g^{-1} (d.w.) of tonalide). Cashmeran was also detected in all the samples analysed, except red mullet and mussels, at concentrations ranging between 12.83 ng g^{-1} (d.w.) and 33.53 ng g^{-1} (d.w.). Meanwhile, other compounds, such as phantolide, celestolide and HHCB-lactone were only found in some of the samples analysed at concentrations between 2.61 ng g^{-1} (d.w.) and 17.94 ng g^{-1} (d.w.). Specifically, HHCB-lactone was only found in perch and sheatfish from the River Ebro. None of the samples analysed contained detectable traces of the NM fragrances studied.

Previous works [4,8-10] focused on the determination of musk fragrances in fish samples from river or sea waters confirm the findings of this study, i.e. that the most abundant polycyclic musks are galaxolide and tonalide, although other polycyclic musks, such as cashmeran, celestolide and phantolide, can also be present in fish samples at lower concentrations. However, the NM concentrations vary significantly depending on the sampling site or whether the study was performed before or after the regulation of these compounds [4,10,11].

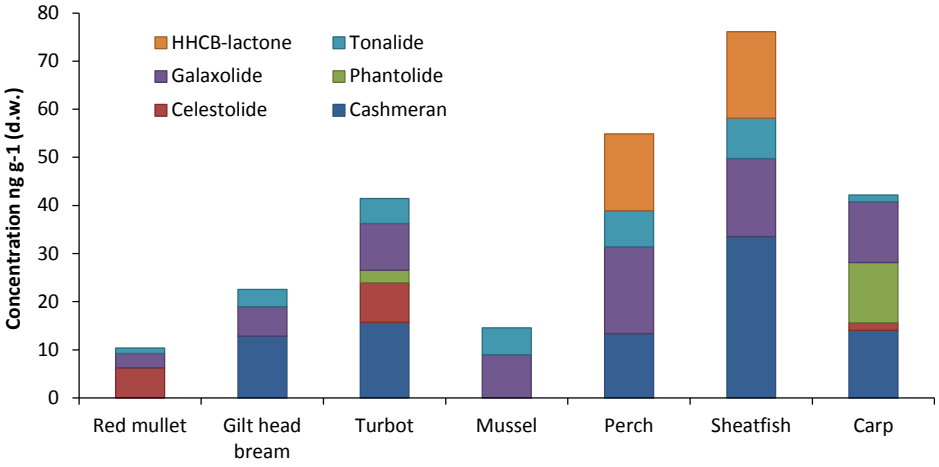


Figure 3.3.1. Concentrations of musk fragrances found in fish samples and mussels from the Tarragona market (red mullet, gilt head bream, turbot and mussels) and from the River Ebro (perch, sheatfish and carp), expressed as ng g^{-1} (d.w).

In this Doctoral Thesis, conventional microextraction techniques such as SPME, on-line SPE and SBSE, as well as novel microextraction techniques like SDME, MEPS and NTME, were applied for the determination of synthetic musk fragrances in wastewater and sewage sludge samples. Moreover, two extraction techniques namely PLE and QuEChERS were applied for the determination of synthetic musk fragrances in marine organisms namely fish and mussel samples. In Table 3.3.1 are summarized the most important parameters to take into account of each developed methodologie such as sample volume/amount, the microextraction procedure, the kind of injector, the GC analytical column and the detector used as well. Other parameters as the MDLs and the advantages and disadvantages of the corresponding microextraction techniques are also included to make easier the comparison between methodologies. In this respect, in Section 3.2.5 (pages 328-330) there is a detailed comparison between the conventional and novel microextraction techniques used in function of the kind of sample analysed. While the applicability of PLE versus QEChERS for the determination of syntethic musk fragrances was discussed previously in this section.

Table 3.3.1. List of methodologies derived from this doctoral Thesis for the determination of synthetic musk fragrances in environmental samples.

Compounds	Sample	Sample volume/amount	Microextraction technique	Advantages	Disadvantages	GC injector	GC analytical column	Detector	MDLs
MCMs	Influent/effluent wastewater	10 mL	HS-SPME PDMS/DVB TD	Solvent-free and on-line	Losing and breakable fibre, desorption, low sensitivity	Splitless	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (IT) EI	1-5 ng L^{-1}
MCMs	Sewage sludge	0.25 g (d.w.)	HS-SPME PDMS/DVB TD			Splitless	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (IT) EI	0.005-0.025 ng g^{-1} (d.w.)
PCMs NMs MCMs	Influent/effluent wastewater	10 mL	on-line SPE Oasis HLB Ethyl acetate	Small volume of solvent, on-line, good extraction recovery, high selectivity, sensitivity	Instrumental requirements, injection, time consuming	On-column	Retaining gap (5 m x 0.53 mm id) Retaining precolumn (2 m x 0.25 mm id, 0.25µm film thickness) ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (Q) EI	1-30 ng L^{-1}
PCMs NMs MCMs	Sewage sludge	0.1 g (d.w.)	HS-SBSE PDMS TD	High sensitivity, high extraction recovery, packed and coated sorbent	Coating loss, drying and desorption, on-line disability, long equilibrium time	Splitless	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (Q) EI	5-30 ng g^{-1} (d.w.)
PCMs NMs	Influent/effluent wastewater	10 mL	IL-HS-SDME [OMIM][PF_6]	Small volume of solvent, on-line, high enrichment factor	Instability of the solvent drop, limited precision and sensitivity	Splitless	Retaining gap (3 m x 0.53 mm id) ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS/MS (IT) EI	10-30 ng L^{-1}
PCMs NMs	Sewage sludge	1 g (d.w.)	PLE ultrapure Water:MeOH (1:1, v/v) In-cell clean-up: Florisil IL-HS-SDME [OMIM][PF_6]	*	*	Splitless	Retaining gap (3 m x 0.53 mm id) ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS/MS (IT) EI	1-3 ng g^{-1} (d.w.)
MCMs	Influent/effluent wastewater	4 mL	MEPS C18 Ethl acetate	High throughout, sensitivity, fast and easy, good extraction recovery	Blockage and carry-over desorption	LVI	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS (IT) EI	5-10 ng L^{-1}
PCMs NMs	Influent/effluent wastewater	10 mL	NTME HF-Bondesil C18 TD	Solvent-free and on-line	Desorption, limited precision, pneumatic restrictions	Splitless	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS/MS (IT) EI	2.5-12 ng L^{-1}
PCMs NMs	Fish Mussels	0.5 g (d.w.)	PLE DCM In-cell clean-up: Florisil QuEChERS Acetonitrile dSPE: Florisil	PLE: quick extraction, small volume of solvent, selectivity, high throughout and good recovery QuEChERS: speed, ease of implementation, low cost, high throughout and good recovery	PLE: insturmental requirements QuEChERS: low selectivity and high matrix effect	LVI	ZB-50 (30 m x 0.25 mm id, 0.25 µm film thickness)	MS/MS (IT) EI	PLE: 0.25-5ng g^{-1} (d.w.) QuEChERS: 0.25-10 ng g^{-1} (d.w.)

ZB-50=50% phenyl/50% dimethylpolysiloxane.

* Both PLE and SDME advantages and disadvantages.

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UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

3.4. Biodegradation of synthetic fragrances by lignin

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The resistance of synthetic fragrances to conventional wastewater treatment, as well as their potential adverse impacts on humans and ecosystems, has been reported in many studies [1-3]. This has prompted research into synthetic fragrance degradation via oxidative treatments with ozone (O_3) [4,5], photochemical processes under UV irradiation [4,5] and visible light irradiation [5], and biodegradation assays performed with fungi derived from freshwater environments or commercial enzymes [6]. Lignin degradation promoted by fungi is relatively inexpensive and represents one of the most important low environmental impact processes [7,8]. Moreover, it is considered an attractive alternative in the development of an effective technology for the treatment of wastewater.

In this section, a Laccase-mediator system consisting of the commercial laccase from *Trametes Versicolor* and the redox mediator 2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used for the biotransformation of three major representative of synthetic fragrances; 2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethylnaphthalen-2yl)ethan-1-one (Iso-E-Super, OTNE), 1,3,4,6,7,8,-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-[g]-2-benzopyran (Galaxolide, HHCB), 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydro-naphthalene (Tonalide, AHTN) and the transformation product of HHCB, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-[g]-2-benzo-pyran-1-one (Galaxolidone, HHCB-lactone) in tap water. A particular focus was assessing the effects of the enzyme laccase on the enantioselective degradation of the target compounds by means of enantioselective GC separation followed by MS detection and the subsequent evaluation of the enantiomeric fractions. Moreover, compost samples collected between one and six weeks of composting time were analysed for the target fragrances using the method previously described by Chen and Bester [9] and Sadef *et al.* [10] to check whether the target fragrances are degraded enantioselectively during composting as they are in wastewater, sewage sludge or biota samples [11-13]. To the best of our knowledge, there have been no previous evaluation of either the degradation of OTNE and HHCB-lactone by the enzyme laccase or the enantiomeric fractions over time during degradation assays by the enzyme laccase and composting procedure.

The study presented in this section was developed during my research stay in the Department of Environmental Science at the University of Aarhus (Roskilde, Denmark) under the supervision of Prof. Dr. Kai Bester. The results of this study were submitted to Analytical and Bioanalytical Chemistry on 29th July 2015.

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3.4.1. Degradation of synthetic fragrances by laccase-mediated system

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DEGRADATION OF SYNTHETIC FRAGRANCES BY LACCASE-MEDIATED SYSTEM

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Abstract

Laccase mediator systems are important biodegradation agents as the rate of reaction could be enhanced in the presence of redox mediators. In the present study the commercial enzyme laccase from *Trametes versicolor* and the redox mediator 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used for the biotransformation of the synthetic fragrances 1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethylnaphthalen-2yl)ethan-1-one (Iso-E-Super, OTNE), 1,3,4,6,7, 8,-hexahydro-4, 6,6,7,8,8-hexamethylcyclopenta-(*g*)-2-benzopyran (Galaxolide, HHCB), 7-acetyl-1,1,3, 4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN) and the transformation product of HHCB, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(*g*)-2-benzo-pyran-1-one (Galaxolidone, HHCB-lactone) in water. A particular focus was to assess the effects of the enzyme laccase from *Trametes versicolor* in the enantioselective degradation of the target compounds, for this reason gas chromatography with an enantioselective column was used as separation technique followed by mass spectrometry detection. In addition, as enantioselective degradation of musk fragrances was observed in wastewater, sewage sludge and fish samples, enantiomeric fractions of selected compounds were studied during composting of sludge. In a period of 144 h, the target fragrances could be effectively removed by the enzyme laccase with removal percentages greater than 70%, except AHTN with a removal percentage of 42%. On the other hand, enantioselectivity was neither found during the degradation with laccase nor during the composting process.

Keywords: *biotransformation, enantioselective chromatography, laccase, synthetic fragrances.*

1. Introduction

Personal care products (PCPs) include a broad range of compounds widely used as additives in cosmetics, flavourings body oils, soaps: in short, in a broad range of daily products. They are included in the so-called emerging organic contaminants, which have been of increasing interest to scientists in recent years [1-4]. These compounds are partially removed in wastewater treatment by sorbing them into sludge [5,6]. However, their presence in wastewater effluent water around the world shows that conventional treatment technologies are ineffective in removing those compounds from wastewater [7-10]. PCPs were also reported in sludge from wastewater treatment plant which can be used as fertiliser on land either after pre-treatment such as composting or directly [5,11-13]. The resistance of certain PCPs to conventional wastewater treatment as well as their potential adverse impacts in humans and in ecosystems have been reported in many studies [14-16]. This has prompted research on PCPs biodegradation by lignin [17, 18].

Lignin degradation promoted by fungi represents one of the most important low environmental impact processes [17,19-21]. Most basidiomycetes that cause the white rotting of wood produce laccases as ligninolytic enzymes. Laccases have received much attention from researchers in last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds [18,22,23]. These enzymes which are regarded to be environmentally friendly and of relative low-cost, are considered an attractive alternative in the development of an effective technology for the biotreatment of wastewater. These enzymes are multi-copper oxidases that catalyze the direct oxidation of electron rich aromatic substrates, like phenols and anilines, via a four-electron reduction of oxygen to water [24]; however, the redox potential of laccase alone is not high enough to break carbon-hydrogen aliphatic bonds. The redox potentials reported for laccases are lower than those of non-phenolic compounds, so these enzymes cannot oxidize such substances. However, the range of compounds that can be oxidized by these enzymes can be expanded by the presence of appropriate low molecular-weight compounds capable to act as electron transfer mediators, thus, allowed the oxidation of non-phenolic benzylic substrates, characterized by relatively high redox potentials [17,25,26]. 1-hydroxybenzotriazole (HBT) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), are the most extensively used laccase mediators [23,27].

Redox-mediated laccase catalysis has been used for the degradation of a wide range of organic compounds like polycyclic aromatic hydro-carbons [28,30], musk fragrances [23], polychlorinated biphenyls [31] phenolic compounds [18,32,33] pesticides or insecticides [34], pharmaceuticals [22,35-37], UV-filters [35,37], polybrominated flame retardants [37]. This study considers the biodegradability of three major representatives of synthetic fragrances as well as the degradation product of

one of them from tap water by means of the commercial enzyme laccase from *Trametes Versicolor*. The synthetic fragrances included in this study are 1,2,3,4,5,6,7,8- octahydro-2,3,8,8-tetramethylnaphthalen- 2yl) ethan- 1- one (Iso-E-Super, OTNE), 1,3,4,6,7,8,-hexahydro- 4,6,6,7,8,8 -hexamethylcyclopenta- (*g*)-2- benzopyran (Galaxolide, HHCB), 7- acetyl- 1,1,3,4,4,6- hexamethyl- 1,2,3,4- tetrahydronaphthalene (Tonalide, AHTN) and the transformation product of HHCB, 1,3,4,6, 7,8- hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- (*g*)-2- benzopyran- 1- one (Galaxolidone, HHCB-lactone). The effects of the enzyme laccase from *Trametes versicolor* in the enantiodegradation of the target compounds were also evaluated by means of an enantioselective separation and the subsequent evaluation of the enantiomeric fractions. Moreover, compost samples collected between one and six weeks of composting time were studied to check if the target analytes are degraded enantioselectively during composting as they are in wastewater, sewage sludge or biota samples [38-40]. To the best of our knowledge, neither the degradation of OTNE and HHCB-lactone by the enzyme laccase nor the enantiomeric fractions over time during degradation assays by the enzyme laccase and composting procedure has been evaluated before.

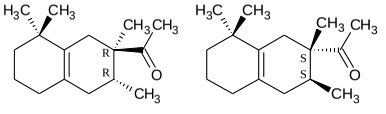
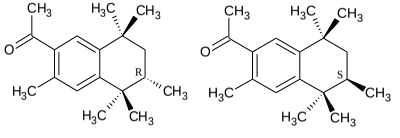
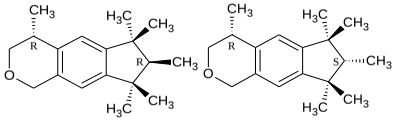
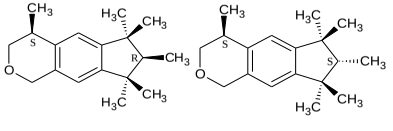
2. Material and methods

2.1. Reagents and standards

The bicyclic hydrocarbon fragrance compound 1,2,3,4,5,6,7,8-octahydro-2,3,8,8- tetramethylnaphthalen- 2yl) ethan-1-one (Iso-E-Super, OTNE) was provided from the market stock of International Flavour&Fragrances (Hilversum, Netherlands). The polycyclic musk fragrances 7-acetyl-1,1, 3,4,4,6- hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta - (*g*)- 2 - benzopyran (Galaxolide, HHCB) were supplied by Dr. Ehrenstorfer (Augsburg, Germany) and Sigma Aldrich (Steinheim, Germany), respectively. The degradation product of HHCB, 1,3,4,6,7,8-hexahydro- 4,6,6,7,8,8 -hexamethylcyclopenta- (*g*)- 2 -benzopyran -1 -one (Galaxolidone, HHCB-lactone) was provided by International Flavour &fragrances (Hilversum, Netherlands). The internal standard (IS) d15-musk xylene (d15-MX) was from Dr. Ehrenstorfer (Augsburg, Germany). The synthetic fragrances selected for the study of the enantiodegradation by the laccase mediator system and kinetic studies are listed in Table 1.

Commercial laccase from *Trametes versicolor* as well as the redox mediator 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were both purchased from Sigma-Aldrich (Steinheim, Germany). Citric acid and sodium citrate used to prepare the sodium citrate buffer and Tween 80 were also delivered by Sigma Aldrich. The derivatization reagents evaluated in this sutdy: trimethyl-sulfonium hydroxide and Sylon BTZ containing trimethylchlorosilane, N,O-bis(trimethylsilyl)-acetamide, and N-trimethylsilylimidazole were provided by Fluka (Buchs, Switzerland) and Supelco (Bellefonte, PA), respectively.

Table 1. Synthetic fragrances used for the study of the enantiodegradation by the laccase mediator system and chromatographic parameters of the enantioselective GC of the target compounds.

Name	CAS number	Enantiomers/ Diastereoisomers	Retention time (t_R, min)	Separation [a] factor (α)	Target ions [b] (m/z)	Structure
OTNE	54464-57-2	OTNE 1 OTNE 2	30.33/31.31	1.018	135, **191**, 219	
AHTN	1506-02-1	AHTN (3S) AHTN (3R)	59.40/60.13	1.012	159, **243**, 258	
HHCB	1222-05-5	HHCB (4S,7S) HHCB (4R,7R)	61.67/80.49	α_1=1.305	213, **243**, 258	
		HHCB (4S,7R) HHCB (4R,7S)	63.28/79.04	α_2=1.249		

[a] Separation factor: $\alpha=k_2/k_1$, k_1=retention factor of the first peak and k_2=retention factor of the second peak. Retention factor: $k=t_R-t_M/t_M$, t_R=retention time of the compound and t_M=retention time of an unretained compound.

[b] Quantifier ions are shown in bold type.

Table 1. (Cont.). Synthetic fragrances used for the study of the enantiodegradation by the laccase mediator system and chromatographic parameters of the enantioselective GC of the target compounds.

Name	CAS number	Enantiomers/ Diastereoisomers	Retention time (t_R, min)	Separation [a] factor (α)	Target ions [b] (m/z)	Structure
HHCB-lactone	1222-05-5	HHCB-lactone (4S,7S)	123.01/126.99	α_1=1.032	239, **257**, 272	
		HHCB-lactone (4R,7R)				
		HHCB-lactone (4S,7R)	124.39/125.48	α_2=1.009		
		HHCB-lactone (4R,7S)				

[a] Separation factor: $\alpha=k_2/k_1$, k_1=retention factor of the first peak and k_2=retention factor of the second peak. Retention factor: $k=t_R-t_M/t_M$, t_R=retention time of the compound and t_M=retention time of an unretained compound.
[b] Quantifier ions are shown in bold type.

Solvents were purchased from Merck (Darmstadt, Germany). Toluene was of GC (Suprasolv®) grade; acetone and ethyl acetate were of analytical (p.a.) grade. All glassware were cleaned three times with acetone and then with ethyl acetate; selected instruments and syringes additionally with the solvents later inserted.

2.2. Sample collection and preparation

Sampling was carried out in a full-scale compost pile at an indoor commercial composting facility (Komtek Composting Company, Southern Denmark), with 15 °C as indoor air temperature during composting time. Taking into account the normal composition of compost processed at Komtek, the pile consisted of; 19.2% yard waste, 9.3% trolltex (cement and and wood fibre-based insulation material), 1.9% straw, 7% paper, 20.6% screening residue (from screening of finished compost) and 42.1% sewage sludge by mass. The pile was turned once immediately following construction, after which composting was initiated. Samples were collected between one and six weeks after pile formation, because based on Poulsen and Bester [41] the pile was assumed to have become adequately mixed after seven days of composting. Further details about pile construction and composting process as well as sampling procedure can be found in Sadef *et al.* [42].

Compost samples were analysed for the target fragrances (Table 1) using the following procedure, which was described in more detail by Chen and Bester [43] and Sadef *et al.* [11]. In summary: compost samples were lyophilized overnight at 2 mbar and 50 °C. Then, lyophilized samples were sub-divided in 5 g portions, blended with 5 g of diatomaceous earth and homogenized in a Philips kitchen blender for 30 seconds. The homogenates were transferred to a 33 mL stainless steel extraction cells that contained 1 cm of general purpose Otawa sand (20-30 mesh) at the bottom and extraction was carried out using an accelerated solvent extraction system (ASE 200, Thermo Fisher, Denmark A/S, Hvidovre, Denmark) with ethyl acetate at 90 °C and 150 bar [11, 43]. After addition of 100 µL of an internal standard solution containing 1,000 ng of d15-Musk xylene (d15-MX), the extracts were concentrated to 1 mL on a Büchi Syncore multiport concentrator (Büchi, Switzerland) at 180 mb and 50 °C. Concentrates were centrifuged at 1,000 rpm for 2 minutes and the supernatant cleaned up with a size exclusion chromatography system consisting of a Gilson 322 pump, a Gilson 155 UV-Vis-detector and a Gilson-233 fractionation/autosampler unit using a phenol gel column of 30 mm length, 21.2 mm ID, 100 Å of pore size and 10 µm as particle size with ethyl/acetate/cyclohexane (1:1, v/v) at a flow rate of 5 mL min^{-1} as mobile phase as previously described by Sadef *et al.* [11] and Bester and Hühnerfuss [44]. Extracts were condensed to 1 mL on a Büchi Syncore multiport concentrator and transferred into 10 mL toluene and again concentrated on the Büchi concentrator to 1 mL at 50 °C and 40 mbar. Concentrates were then subsequently cleaned up with silica solid-phase extraction cartridges

by packing 1 g of silica 60 (dried over night at 105°C) into a glass column (60 mm long, 12 mm inner ø) from IST, Biotage, Sweden and two polyethylene frits (Supelco, Bellefonte, PA, USA) on the top and bottom of the silica. A detailed point by point explanation of the clean-up with silica solid phase cartridge can be found in Sadef *et al.* [11]. Solid phase extracts were concentrated on the Büchi concentrator and were transferred into 10 mL toluene and condensed to 1 mL. These fractions were finally analysed by gas chromatography-mass spectrometry (GC-MS) as described in section 2.4.

2.3. Degradation of musk fragrances by Laccase mediator system

All the experiments were performed in 10 mL reaction tubes containing 5 mL of 100 mM citric acid/sodium citrate buffer (pH 4.0) with the enzyme laccase at a concentration of 0.2 U mL^{-1}. The target fragrances were added from between 9.18 mM-10.66 mM (2,500 µg mL^{-1}) individual stock solutions in acetone, to give a final concentration of between 91.8 µM-106.6 µM (25 µg mL^{-1}). To improve the solubility of the fragrances, 0.1% (w/v) Tween 80 was added. The redox mediator ABTS was also included at 1 mM. Reaction tubes were tightly closed with Teflon-lined screw caps and the enzymatic incubations were carried out under agitation at 120 rpm with an Unimax 2010 shaker (Heidoplh, Schwabach, Germany), at room temperature in the dark for 0-144 h. Control samples were prepared with heat-inactivated enzyme Laccase (120 °C, 30 min).

Incubation solutions were acidified to pH 2.0 with concentrated acetic acid (Sigma Aldrich, Steinheim, Germany) to stop the oxidation process [23,33,45], and extracted twice with 5 mL ethyl acetate. Extracts were combined and dried over anhydrous sodium sulphate (Sigma Aldrich, Steinheim, Germany). For metabolites identification, 100 µL of the derivatization reagent Sylon BTZ was applied to the dry extracts and derivatization was carried out at 60 °C for 1 h. Excess of derivatization agent was removed with 200 µL of ultrapure water at pH 3.0. Derivatization products were dried over anhydrous sodium sulphate and after addition of 100 µL of an internal standard solution containing 1,000 ng of d15-MX; the extracts were concentrated to 1 mL on a Büchi Syncore multiport concentrator (Büchi, Switzerland) at 50 °C and 100 mbar. Recoveries of the extraction procedure were calculated with the control samples and range between 68% and 106% with standard deviation values less than 10% (means from *n*=21) over time (0-144h) (see Table 2). This indicates a negligible contribution of processes not attributable to enzyme laccasse system, such as adsorption onto surfaces of the glassware.

2.4. Analysis by enantioselective GC-MS

Analysis was performed by gas chromatographic separation on a Trace GC ultra-system obtained from Thermo Scientific (Copenhagen, Denmark) with quadrupole mass

spectrometric detection system using an ISQ. The GC-MS was equipped with a split injector in a splitless mode and Combi-Pal autosampler. A 1 µL injection was performed into a splitless injector at the injection temperature of 230 °C with a splitless time of 0.5 minutes. Enantioselective GC separation was performed with a 25 m 0.25mm ID column, with a film of heptakis-(2, 3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin in OV1701 obtained as FS-Hydrodex β-6TBDM (Macherey-Nagel, Düren, Germany). Film thickness was between 0.2-0.3 µm according to the manufacturer. Helium was used as carrier gas. The transfer line and ion source temperatures were set at 180 °C and 200 °C, respectively. A filament-multiplier delay of 3 min was set in order to prevent instrument damage. Qualitative analysis was performed in full scan mode (50-400 m/z). Quantitative analysis was performed in selected ion (SIM) mode.

Identification of the target compounds as well as optimization of the enantioselective separation were performed by direct injection of 1 µL of 10,000 ng mL^{-1} standard solution of all the target fragrances in full scan mode (40-50 amu). All the target compounds were identified by their molecular ion working with the following oven temperature programme: initial temperature 110 °C [3 min], increased at 1 °C min^{-1} to 200 °C [20 min], and a helium flow rate of 1.2 mL min^{-1}. The target ions chosen for the identification as well as the quantification of the target analytes are listed in Table 1. Afterwards, taking into account that column's temperature limit is given as 230 °C by the manufactures, the chromatographic separation was optimized by testing several oven temperature programmes and helium flow rates between 0.5-1. 2 mL min^{-1}. The best separation factors (α); α=1.018 for OTNE, α=1.012 for AHTN, α_1=1.305/ α_2=1.249 for HHCB and α_1=1.032/ α_2=1.009 for HHCB-lactone, were obtained using a multi-plateau oven temperature programme as follows: initial temperature 90 °C [3 min], increased at 5 °C min^{-1} to 132 °C [25 min], 5 °C min^{-1} to 145 °C [40 min], 5 °C min^{-1} to 166 °C [50 min] then 10 °C min^{-1} to 200 °C [20 min].

As can be seen in Fig. 1, on the first plateau (132 °C) the two enantiomers of OTNE were observed. On the second plateau (145 °C) the enantiomers of AHTN (3S and 3R) as well as the enantiomers and diastereoisomers of HHCB were separated. According to Gatermann *et al.* [40] the elution order of the HHCB enantiomers and diastereoisomers are: (4S,7S), (4S,7R), (4R,7S) and (4R,7R). Finally, on the third plateau (166 °C), the separation of the enantiomers and diastereoisomers of HHCB-lactone was performed. As the elution order determined by Meyer [46] is used and due to the two chiral centres of HHCB-lactone, as shown in Fig. 1. HHCB-lactone has two enantiomeric pairs: (4S,7S) vs. (4R,7R) as well as (4S,7R) vs. (4R,7S). Table 1 shows the chromatographic parameters as well as the structures of each analysed compound.

In addition an HP-5MS (30 m length, 0.25 mm inner diameter and 0.25 µm film thickness) analytical column from Agilent Technologies (Agilent Technologies, PA, USA) was used for the

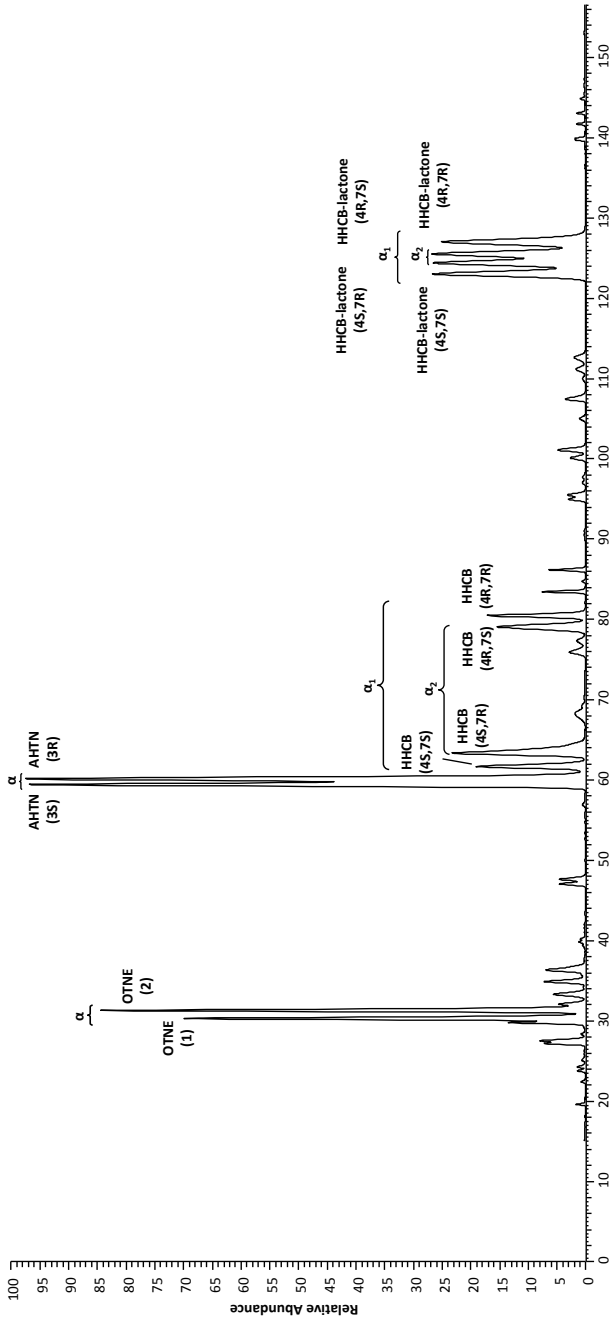


Fig. 1. Total ion chromatogram (TIC) of a standard mixture of OTNE, AHTN, HHCB and HHCB-lactone (10,000 ng mL^{-1}) separated on the FS-Hydrodex-β-6TBDM column.

detection of biotransformation products during degradation assays with laccase. The oven temperature programme was as follows: initial temperature 75 °C [1 min], increased at 10 °C min^{-1} to 280 °C [10 min] with helium as carrier gas at a flow rate of 1.2 mL min^{-1}. The chromatographic separation obtained with an HP-5MS column is shown at Suppl. Fig. 1.

Calibrations were performed as a multistep internal standard calibration. Individual stock solutions of the target fragrances were prepared in acetone at a concentration of 2,500 µg mL^{-1} and stored at 4 °C in the dark. The weight of these flasks was controlled before and after each operation. Calibration standards (100, 300, 1,000, 3,000, 10,000, 25,000, 50,000, 100,000 and 200,000 ng mL^{-1} in ethyl acetate) were made by serial dilution of the stock solution. Calibration standards contained the internal standard d15-MX at a concentration of 100 ng mL^{-1}.

3. Results and discussion

3.1. Reaction of synthetic fragrances with laccase mediator system

Under the conditions described in Section 2.3, the concentrations of the OTNE enantiomers decreased from 418±31 µM to 121±4 µM and from 410±29 µM to 123±4 µM (mean ± standard deviation for triplicate enzyme assays), respectively. As shown in Fig. 2 most of the OTNE has been degraded between the first 72 hours (removal percentage 56±1% and 55±2% for OTNE 1 and OTNE 2, respectively), after that the degradation rate decrease until the concentration remain constant at 144 h with a final removal percentage of 71±3% ONTE 1 and 70±2% OTNE 2, respectively. AHTN concentrations decreased from 527±27 µM to 308±2 µM for AHTN (3S) and from 540±14 µM to 311±1 µM for AHTN (3R). As happened with OTNE, most of the AHTN has been removed before 72 h, 35±3% AHTN (3S) and 36±2% AHTN (3R), while at higher incubation times as 144 h only a little increase in the removal percentage has been observed (42±2% for both AHTN enantiomers). In the same way, the concentrations of the HHCB enantiomers decreased from 590±14 µM to 104±5 µM for HHCB (4S,7S), 509±20 µM to 94±5 µM for HHCB (4S,7R), 500±21 µM to 92±4 µM for HHCB (4R, 7S) and 515±11 µM to 97±6 µM for HHCB (4S,7S), by the end of the assays at 144 h. This means that, independently of the HHCB enantiomer studied, an 80±1% of the initial HHCB concentration has been removed by the enzyme laccase and that more than 60±3% of HHCB has been removed in just 48 h. The study of the degradation of the main degradation product of HHCB, HHCB-lactone, by the enzyme laccase revealed a decrease of the concentrations of all the HHCB-lactone enantiomers/ disatereoisomers (see Fig. 2.); from 443±22 µM to 36±5 µM for HHCB-lactone (4S,7S), 474±23 µM to 42±5 µM for HHCB-lactone (4S,7R), 462±24 µM to 45±3 µM for HHCB-lactone (4R,7S) and 455±24 µM to 42±5 µM for HHCB-lactone (4R,7R), until reached a maximal removal percentage of 92±1%, 91±1%, 90±1% and 91±1% for HHCB-lactone (4S,7S), HHCB-lactone (4R,7S), HHCB-lactone (4S,7R) and HHCB-lactone (4R,7R),

Table 2. Recoveries of the extraction procedure, target fragrances concentrations [a] in laccase assays at the beginning (time = 0 h) and after six days (time = 144 h) of incubation and removal percentages after 144 h.

Compound	Recoveries [b] (%)	Concentration $_{initial}$ (μM)	Concentration $_{final}$ (μM)	Removal [c] percentage (%)
OTNE 1	103±6	418±31	121±4	71±3
OTNE 2	106±4	410±29	123±4	70±2
AHTN (3S)	70±3	527±27	308±2	42±3
AHTN (3R)	68±3	540±23	311±1	42±2
HHCB (4S,7S)	82±2	590±14	104±5	82±1
HHCB (4S,7R)	84±3	509±20	94±5	82±1
HHCB (4R,7S)	86±4	500±21	92±4	82±1
HHCB (4R,7R)	86±4	515±11	97±6	81±1
HHCB-lactone (4S,7S)	75±3	443±22	36±5	92±1
HHCB-lactone (4S,7R)	75±2	474±23	42±5	91±1
HHCB-lactone (4R,7S)	76±4	462±24	45±3	90±1
HHCB-lactone (4R,7R)	75±3	455±24	42±5	91±1

[a] Concentrations=means±standard deviation for triplicate assays.
[b] Recoveries; control samples spiked at 25 μg mL^{-1} with all the target musks (*n*=21).
[c] Removal percentage=100- $Concentration_{final}$*100/$Concentration_{initial}$.

HHCB-lactone (4R,7S), HHCB-lactone (4S,7R) and HHCB-lactone (4R,7R), respectively. Table 2 shows the initial and final concentrations found for each target musk fragrance as well as the removal percentage after six days (144 h) of incubation time. AHTN and HHCB degradation plots are placed in Suppl. Fig.2.

3.2 Kinetic studies

Rate constants for the degradation of the target fragrances by the enzyme laccase in tap water were calculated. The reaction was considered pseudo-first order as the enzyme laccase concentration kept constant at 0.2 U mL^{-1} for 144 h. According to bibliography found up to now [23,47-49], the following equation was considered (1):

$$C=C_0 \exp^{-Kt}=C_0 \exp^{-K\,t} \quad (1)$$

where C (μM) is the fragrance concentration at a given time, C_0 (μM) is the fragrance concentration at t=0h, K (h^{-1}) is the first order degradation rate constant accounting for biological fragrance elimination. Accordingly, K was calculated as (2):

$$K=k_{assay}-k_{control} \quad (2)$$

where k_{assay} was determined upon linear regression of the plots ln C/C_0 versus incubation time in enzyme laccase assays (see Fig. 3 and Suppl. Fig. 3) and $k_{control}$ (h^{-1}), which refers to

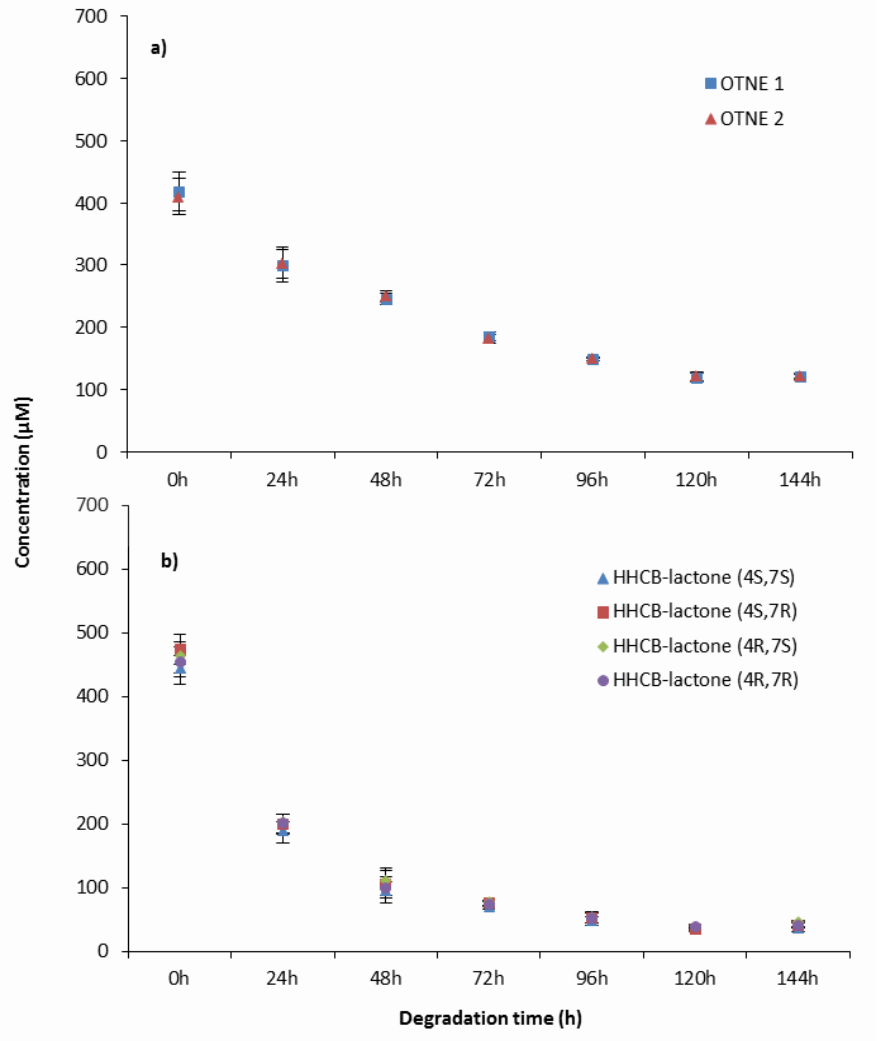


Fig. 2. Time-courses of a) OTNE and b) HHCB-lactone in enzyme Laccase mediator system. Symbols and error bars represent means and standard deviations for triplicate assays.

the abiotic fragrance removal, was obtained under linear regression of the plots ln C/C_0 versus incubation time in control samples. Half-life's times were also calculated as $t_{1/2}=\ln(2)/K$. First order reaction constants and the corresponding half-life times of the target fragrances are listed in Table 3. First order reaction constants found range between 0.0063±0.0007 h^{-1} for the AHTN (3S) enantiomer to 0.0251±0.0017 h^{-1} for HHCB-lactone (4S,7S), while the removal of the target fragrances in control samples were

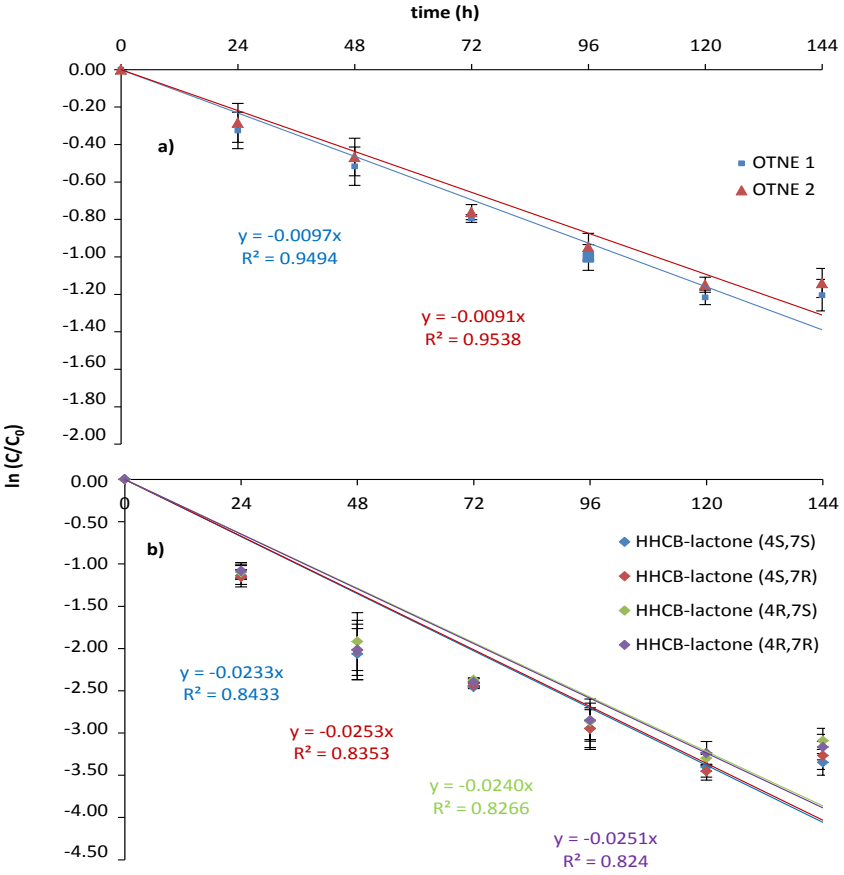


Fig. 3. Pseudo-first order reaction plots for the removal of a) OTNE and b) HHCB-lactone enantiomers/diastereoisomers in tap water taking enzyme Laccase to be permanently constant at 0.2 U mL^{-1}. The slope of the linear regression yielding the first order constant K_{assay} (h^{-1}).

negligible. The corresponding half-life times were found to be between 110±11 h (AHTN enantiomers) and 28±2 h (HHCB-lactone (4S,7S). In addition, not significantly differences were observed between the first order reaction constants of the enantiomers/ diastereoisomers of each target compound. The removal of HHCB ($t_{1/2}$=38±1-43±1 h) and AHTN ($t_{1/2}$=109±7-110±11 h) working with enzyme laccase system is comparable with that observed by Balk and Ford [50] in pure cultures of the terrestrial white rot fungus *Phanerochaete chrysosporium*, where HHCB and AHTN disappeared within 72 h and 144 h, respectively. In contrast, a slower HHCB and AHTN degradation were observed in active *Myrioconium sp.*cultures [23] with first order reaction constants of 0.0031 h^{-1} and 0.0042 h^{-1} for HHCB and

AHTN, respectively. The corresponding half-life time values were about 220.8 h for HHCB and 165.6 h for AHTN. Thus, HHCB is more resistant to degradation by *Myrioconium sp.* cultures than AHTN.

3.3. Detection of biotransformation products during laccase reactions by GC-MS

For the study of the transformation products generated during degradation assays with the enzyme laccase two derivatization agents, sylon BTZ and trimethylsulfonium hydroxide, as well as two analytical columns, FS-Hydrodex β-6TBDM (Macherey Nagel, Düren, Germany) and HP-5MS (Agilent Technologies, PA, USA), were evaluated. The GC methods as well as analytical columns characteristics were described in section 2.4.

Independently of the derivatization agent or the analytical used, no metabolites were detected upon laccase treatment of OTNE and AHTN enantiomers. In addition, essentially no differences in the HHCB-lactone concentrations between assays containing enzyme laccase and ABTS and those containing the heated-inactivated enzyme were monitored, whereas HHCB was clearly removed by the enzyme. This suggests that products other than HHCB-lactone might also be formed, for instance in further reactions between ABTS radicals and primary HHCB oxidation products. Laccase oxidation of PAHs in the presence of redox mediators as ABTS resulted in the formation of quinones as well as oxidative coupling products, and laccase-redox mediator systems were shown to further oxidize quinones resulting from PAHs oxidation

Table 3. Pseudo-first order constants concerning the degradation of the target fragrances in tap water and the corresponding half-life's ($t_{1/2}$) having laccase concentration of 0.2U mL^{-1}.

Compound	k_{assay} (h^{-1})	$k_{control}$ (h^{-1})	K (h^{-1})[a)]	$t_{1/2}$ (h)
OTNE 1	0.0097±0.0006	-0.0003±0.0004	0.0110±0.0006	63±3
OTNE 2	0.0091±0.0006	-0.0001±0.0002	0.0091±0.0007	76±5
AHTN (3S)	0.0066±0.0007	0.0002±0.0003	0.0064±0.0006	109±7
AHTN (3R)	0.0068±0.0007	0.0005±0.0004	0.0063±0.0007	110±11
HHCB (4S,7S)	0.0180±0.0004	0.0002±0.0004	0.0178±0.0006	38±1
HHCB (4S,7R)	0.0171±0.0008	0.0001±0.0004	0.0170±0.0007	41±2
HHCB (4R,7S)	0.0167±0.0009	0.00001±0.0003	0.0165±0.0007	42±2
HHCB (4R,7R)	0.0163±0.0003	0.00003±0.0002	0.0163±0.0003	43±1
HHCB-lactone (4S,7S)	0.0233±0.0013	-0.0005±0.0002	0.0238±0.0011	29±1
HHCB-lactone (4S,7R)	0.0253±0.0016	0.0002±0.0005	0.0251±0.0017	28±1
HHCB-lactone (4R,7S)	0.0240±0.0014	0.0002±0.0002	0.0238±0.0013	29±1
HHCB-lactone (4R,7R)	0.0251±0.0024	0.0004±0.0003	0.0247±0.0021	28±2

[a)] $K=k_{assay}-k_{control.}$
[b)] $t_{1/2}=\ln(2)/K$.

[28,29]. In the same way, despite the decrease in the concentration of HHCB-lactone, no metabolites were detected upon laccase treatment of HHCB-lactone. Oxidative coupling products potentially formed upon target compounds oxidation by the laccase might have been inaccessible with the applied GC-MS method, covering a mass range of 50 to 400 amu.

3.4. Enantiomeric fraction studies

The target fragrances, as illustrated in Table 1, are chiral compounds. It has been shown that biotransformation reactions of such compounds in fish, surface water or sewage sludge are often enantioselective processes [38-40]. As experiments work well under the assumption that enzymes are the responsible for enantioselective degradation [51], enantiomeric fractions (EFs) of the degradation results obtained working with the enzyme laccase were calculated to check if the enzyme laccase can degrade enantioselectively the target fragrances. In addition EFs obtained working with compost samples collected between one and six weeks of composting time were evaluated to verify if enantioselective processes are present during composting. EFs were calculated as described by Harner *et. al* [48]. As HHCB and HHCB-lactone have two chiral centres, four peaks are separated (see Fig. 1), EFs have been calculated taking into account two enantiomeric pairs: (4S,7S) *vs.* (4R,7R) as well as (4S,7R) *vs.* (4R,7S).

3.4.1. Degradation assays

The EFs measured for the target fragrances in the standard solutions ($EF_{STANDARD}$) (concentrations between 100 and 200,000 ng mL^{-1}, *n*=18) were compared with those obtained under degradation assays with the enzyme laccase ($EF_{LACCASE}$) (incubation time 0-144h, *n*=21). In both cases, EFs were calculated with two ions in order to confirm the results. As shown in Suppl. Fig. 4a, $EF_{LACCASE}$ found by OTNE remained constant over time at 0.390 ±0.005 (mean±standard deviation, *n*=21, ion=191) and not significantly differences were observed between the $EF_{LACCASE}$ and $EF_{STANDARD}$ (0.391±0.017, ion=191), the results were also confirmed by the EFs obtained with the ion 219 (Table 4). As found with OTNE, the $EF_{LACCASE}$ of AHTN, HHCB and HHCB-lactone remained constant over time (see Suppl. Fig. 4b, c, d)at values between: 0.471±0.005 (ion=243), 0.504 ±0.003 (ion=243), 0.480±0.004 (ion= 243), 0.492±0.032 (ion=257), 0.482± 0.008 (ion=257) for AHTN, HHCB (4S,7S) vs. HHCB (4R,7R), HHCB (4S,7R) vs. HHCB (4R,7S), HHCB-lactone (4S,7S) vs. HHCB-lactone (4R, 7R) and HHCB-lactone (4S, 7R) vs. HHCB-lactone (4R,7S), respectively. As shown in Table 4, the $EF_{STANDARD}$ of AHTN, HHCB and HHCB-lactone are similar to those determined under degradation assays. Therefore, considering that no changes in the EFs of the target fragrances have been detected under degradation assays with the enzyme laccase, it can be concluded that reaction

Table 4. Enantiomer fractions found for each target fragrance in a standard solution, over degradation assays with the enzyme laccase and compost samples.

Compound	Ions (m/z)	$EF_{STANDARD}$ [b]	$EF_{LACCASE}$ [d]	$EF_{COMPOST}$ [c]
OTNE 1 *vs.* OTNE 2 [a]	191	0.391±0.017	0.390±0.005	0.377±0.012
	219	0.381±0.020	0.384±0.006	0.385±0.012
AHTN (3S) *vs.* AHTN (3R)	243	0.465±0.013	0.471±0.005	0.484±0.022
	258	0.469±0.011	0.472±0.006	0.463±0.042
HHCB (4S,7S) *vs.* HHCB (4R,7R)	243	0.529±0.037	0.504±0.003	0.506±0.023
	213	0.513±0.024	0.502±0.004	0.518±0.015
HHCB (4S, 7R) *vs.* HHCB (4R,7S)	243	0.497±0.025	0.480±0.004	0.487±0.009
	213	0.499±0.019	0.485±0.003	0.462±0.026
HHCB-lactone (4S,7S) *vs.* HHCB-lactone (4R,7R)	257	0.492±0.032	0.498±0.011	*
	272	0.495±0.025	0.497±0.017	*
HHCB-lactone (4S,7R) *vs.* HHCB-lactone (4R,7S)	257	0.482 ± 0.008	0.480±0.005	*
	272	0.499 ± 0.015	0.477±0.008	*

[a] Enantiomer fractions (EF)=$Area_{OTNE\ 1}/(Area_{OTNE\ 1} + Area_{OTNE\ 2})$.
[b] $EF_{STANDARD;}$ standard solutions at concentrations between 100 and 200,000 ng mL^{-1} (*n*=18).
[c] $EF_{COMPOST;}$ compost samples collected between one and six weeks of composting time (*n*=36).
[d] $EF_{LACCASE;}$ Laccase assays between 0 and 144h of incubation time (*n*=21).
* Not calculated.

mechanism involved in the degradation of the target fragrances is not enantioselective.

3.4.2. Compost samples

The EFs found for the target fragrances, except HHCB-lactone, in compost samples ($EF_{COMPOST}$) (1 and 6 week composting time, *n*=26) were compared with those obtained working with standard solutions (see Table 4).
The EFs of HHCB-lactone, which elutes as a quartet signal at the end of the GC run (Fig. 1), could not be calculated because the stereoisomers of HHCB-lactone were not fully resolved in compost samples. The results showed that $EF_{COMPOST}$ of OTNE (Suppl. Fig. 5a) remained constant at 0.377±0.012 (ion=191) and were similar to $EF_{TANDARD}$. The EFs of AHTN (0.484±0.022, ion=243) and the respective stereoisomers of HHCB (0.506±0.023 and 0.487±0.009 for HHCB (4S,7S) and HHCB (4S,7R), ion=243) have also been determined. No significant changes in these have been detected (Suppl. Fig. 5b, c). These results are in agreement with those found by Bester [39] and Berset *et al.* [38] when the enantiomeric ratios (ERs) of AHTN and HHCB in influent/effluent wastewater samples as well as river samples were

evaluated. However, the ERs of AHTN and HHCB found by Berset *et al.* [38] showed enantiodegradation when sewage sludge samples were analysed. As ERs found by Bester [39] and Berset *et al.* [38] differ significantly depending on whether the wastewater treatment plant procedure favours the bio-degradation of fragrances or not.

Conclusions

The potential of laccase mediator system as important biodegradation agent with the presence of the redox mediator ABTS for the degradation of three of the most representative synthetic fragrances; OTNE, AHTN, HHCB and the main degradation product of HHCB, HHCB-lactone, was demonstrated. The results showed that the studied fragrances could be effectively removed by the enzyme laccase with removal percentages higher than 70% after 144 h of incubation time except AHTN (removal percentage 42%, 144 h). Especially HHCB-lactone enantiomers showed removal percentages higher than 90% after 144 h. Neither laccase nor the organisms performing the degradation in compost perform the respective degradation enantioselectively.

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Conflict of interest

The authors do not have any potential conflict of interest to declare.

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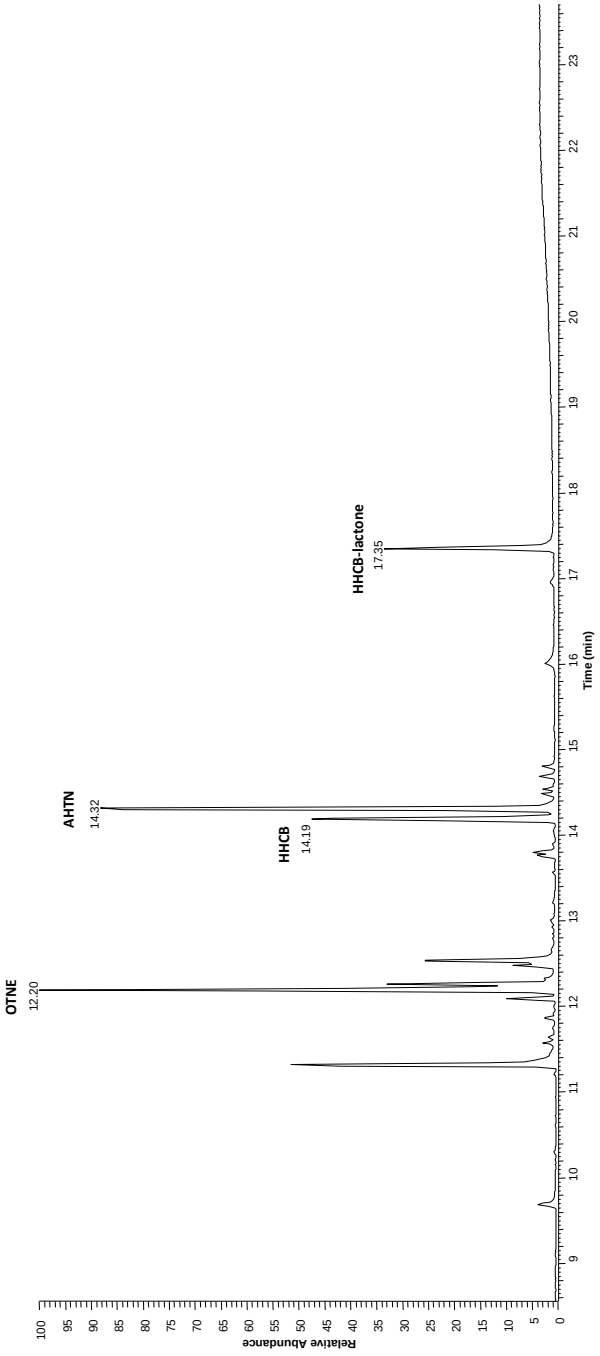
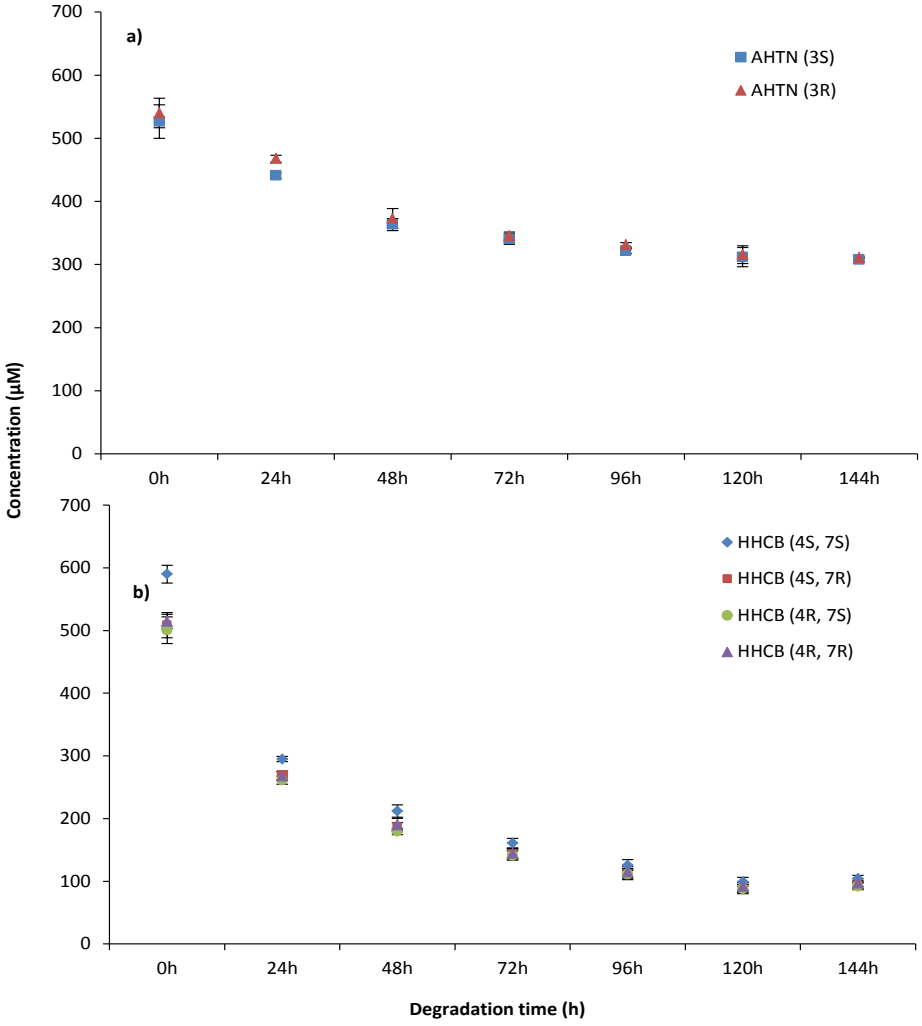
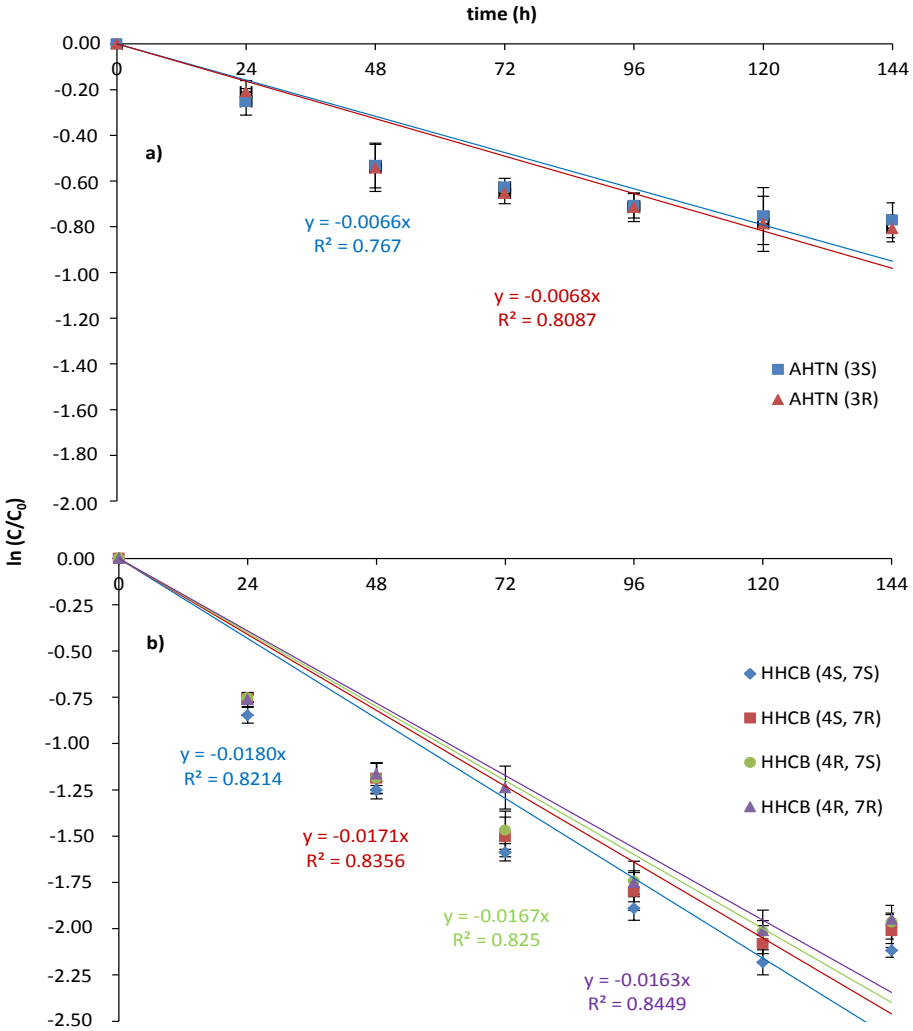
Supplementary material



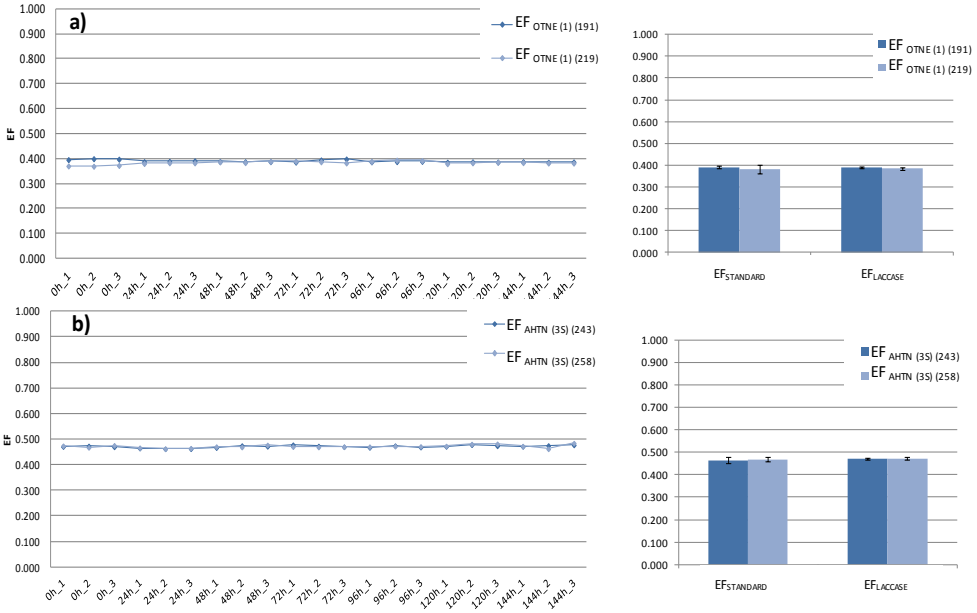
Suppl. Fig. 1. Total ion chromatogram (TIC) of a standard mixture of OTNE, AHTN, HHCB and HHCB-lactone (10,000 ng mL^{-1}) separated on the HP-5MS column.



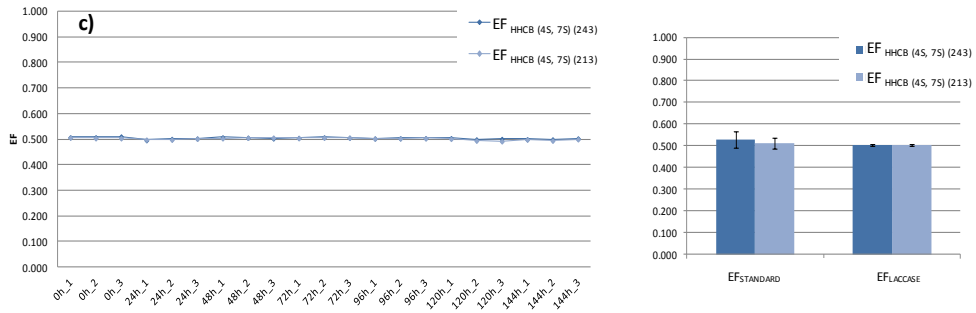
Suppl. Fig. 2. Time-courses of a) AHTN and b) HHCB in enzyme laccase mediator system. Symbols and error bars represent means and standard deviations for triplicate assays.



Suppl. Fig. 3. Pseudo-first order reaction plots for the removal of a) AHTN and b) HHCB enantiomers/diastereoisomers in tap water taking enzyme laccase to be permanently constant at 0.2 U mL^{-1}. The slope of the linear regression yielding the first order constant K_{assay} (h^{-1}).



PAIR 1



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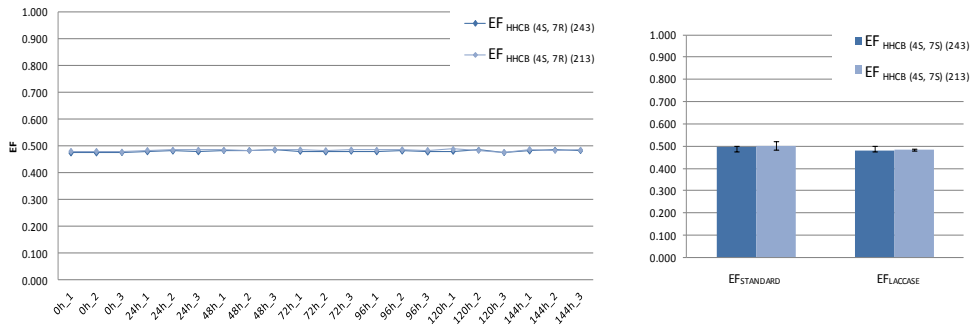
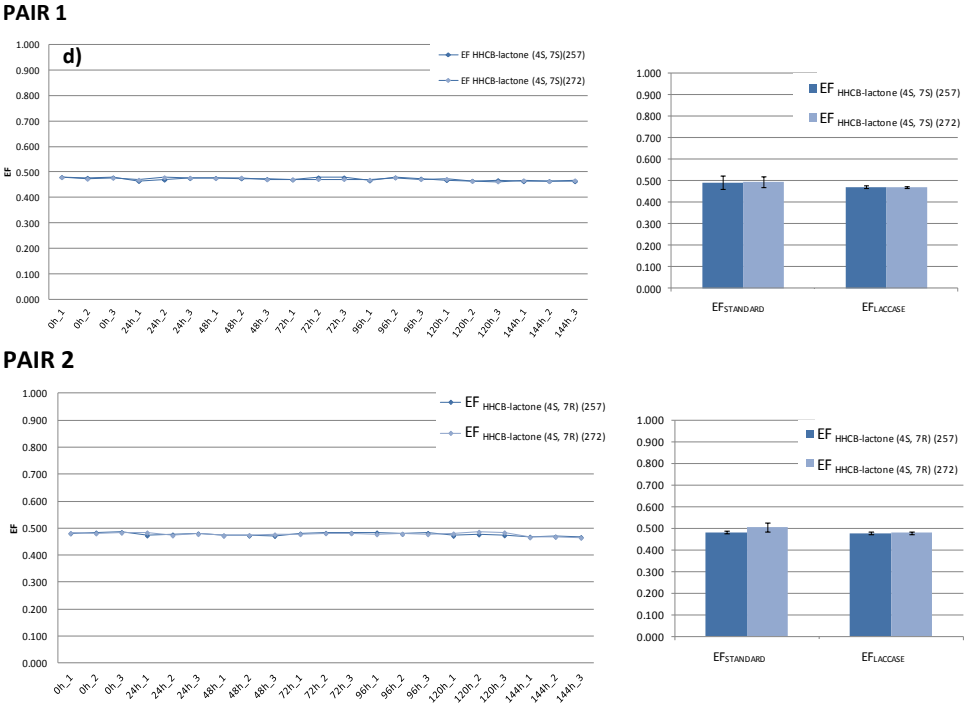
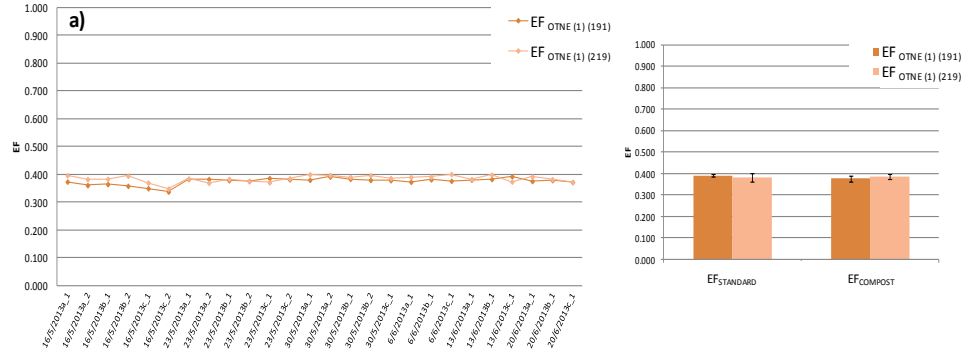


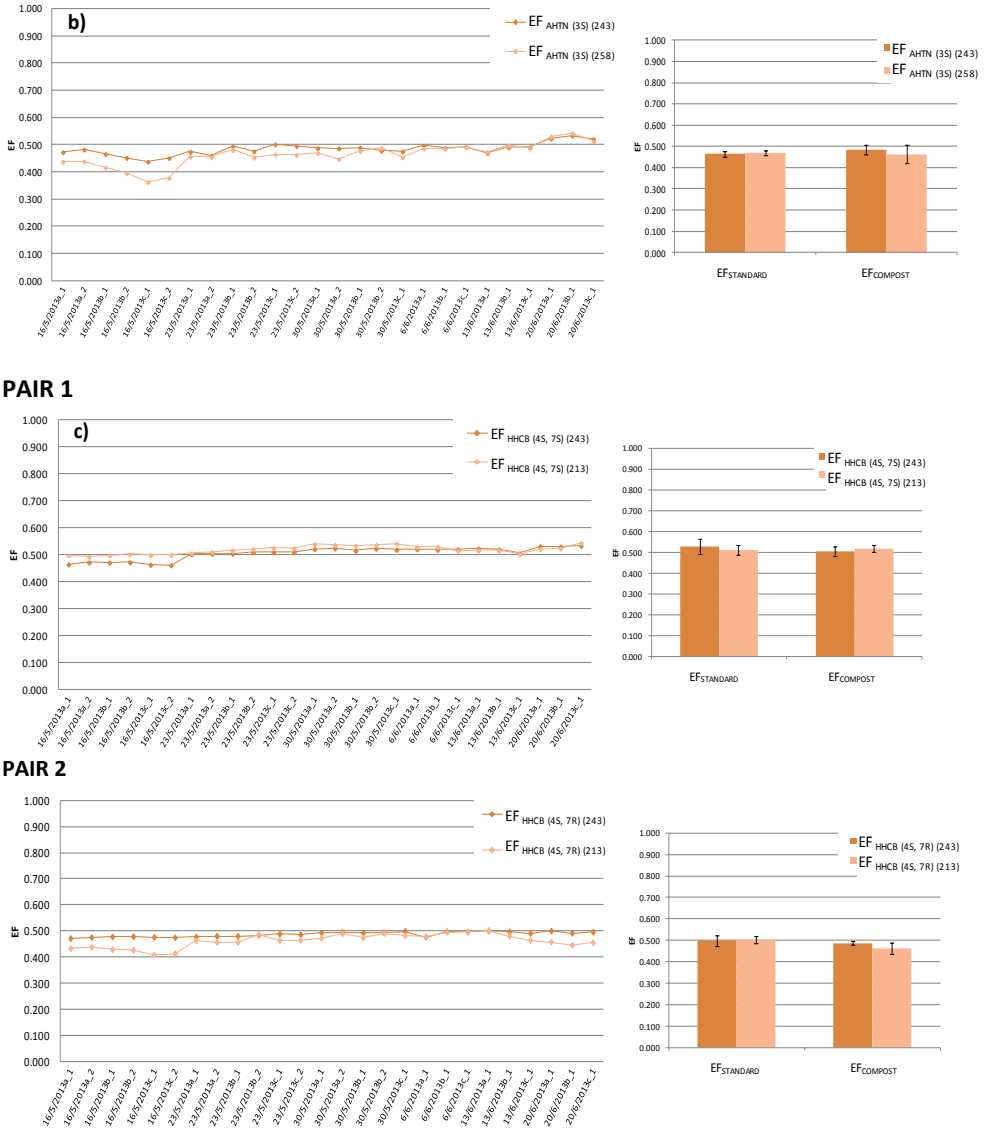
Suppl. Fig. 4. Enantiomeric fractions of a) OTNE, b) AHTN, c) HHCB and d) HHCB-lactone obtained under degradation assays with the enzyme laccase as well as comparison of $EF_{STANDARD}$ versus $EF_{LACCASE}$.



Suppl. Fig. 4. (Cont.). Enantiomeric fractions of a) OTNE, b) AHTN, c) HHCB and d) HHCB-lactone obtained under degradation assays with the enzyme laccase as well as comparison of $EF_{STANDARD}$ versus $EF_{LACCASE}$.



Suppl. Fig. 5. Enantiomeric fractions of a) OTNE, b) AHTN, and c) HHCB found in compost sample (1 to 6 weeks of composting time) as well as comparison of $EF_{COMPOST}$ versus $EF_{STANDARD}$.



Suppl. Fig. 5 (Cont.). Enantiomeric fractions of a) OTNE, b) AHTN, and c) HHCB found in compost sample (1 to 6 weeks of composting time) as well as comparison of $EF_{COMPOST}$ versus $EF_{STANDARD}$.

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Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

3.4.2. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
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In the last study included in this Thesis, the applicability of the laccase-mediator system was shown as an important biodegradation agent with the presence of the redox mediator ABTS for the degradation of three of the most widely used synthetic fragrances: OTNE, galaxolide, tonalide, and the main degradation product of galaxolide, known as HHCB-lactone. It was the first time that OTNE and HHCB-lactone were included in biodegradation studies by a laccase-mediator system as parent compounds. All of the studied fragrances were effectively removed by the enzyme laccase with removal percentages higher than 70% after 144 h of incubation time, except tonalide with a removal percentage of 42% after 144 h. The removal percentages obtained for HHCB-lactone were particularly notable at over 90% after 144h.

Rate constants for the degradation of the target fragrances by the enzyme laccase were also calculated by considering the reaction of pseudo-first order as the enzyme concentration kept constant [1] and ranged between 0.0063±0.0007 h^{-1} for tonalide (3S) enantiomer and 0.0251±0.0017 h^{-1} for HHCB-lactone (4S,7S). The corresponding half-life times were found to be between 110±11 h (tonalide enantiomers) and 28±2 h (HHCB-lactone (4S,7S)). The half-life times found for galaxolide (38±1 h and 43±1 h) and tonalide (109±7 h and 110±11 h) working with the enzyme laccase system were comparable with those observed by Balk and Ford [2] in pure cultures of the terrestrial white rot fungus *Phanerochaete chrysosporium*, where galaxolide and tonalide disappeared within 72 h and 144 h, respectively. Meanwhile, slower galaxolide and tonalide degradation was observed in active *Myrioconium sp. Cultures* [3] with first order reaction constants of 0.0031 h^{-1} (220.8 h) and 0.0042 h^{-1} (165.6), respectively.

With respect to the chromatographic separation, it was performed in an enantioselective GC column with a film of heptakis-(2, 3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin in OV1701. Several oven temperature programmes and helium flow rates between 0.5 and 1.2 mL min^{-1} were tested and the best separation factors (α), ranging between 1.009 and 1.249, were obtained using the following multi-plateau oven temperature programme: 90 °C (3 min), increased at 5 °C min^{-1} to 132 °C (25 min), increased at 5 °C min^{-1} to 145 °C (40 min), 5 °C min^{-1} to 166 °C (50 min) then 10 °C min^{-1} to 200 °C (20 min). As shown in Figure 3.3.1, OTNE enantiomers appeared on the first plateau. It should be highlighted that this was the first time that the stereoselective separation of OTNE was performed. On the second plateau, the enantiomers of tonalide and the enantiomers/ diastereoisomers of galaxolide were separated. Meanwhile, on the third plateau,

the enantiomers/diastereoisomers of HHCB-lactone were separated. As reported by Gatermann *et al.* [4] and Meyer *et al.* [5], the elution order of HHCB/HHCB-lactone enantiomers/diastereoisomers in this column was: (4S,7S), (4S.7R), (4R,7S) and (4R,7R).

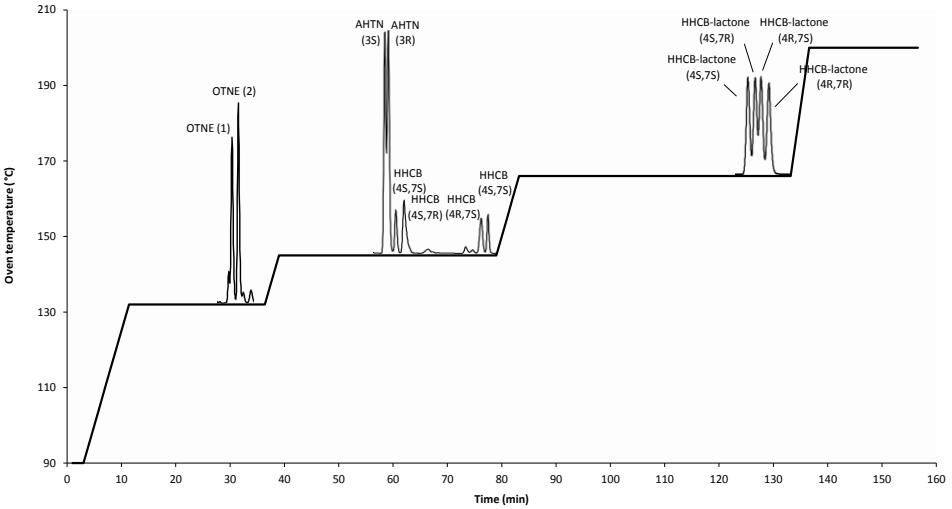

Figure 3.4.1. Multi-plateau oven temperature programme chart and chromatographic signals obtained for each synthetic fragrances studied.

As the experiments worked well under the assumption that enzymes are responsible for enantioselective degradation [6], EFs [7] for the target fragrances in the standard solutions were compared with those obtained under degradation assays with the enzyme laccase in order to evaluate the possible enantiodegradation of the target fragrances by the enzyme laccase. Therefore, considering that no changes in the EFs of the target fragrances have been detected under degradation assays with the enzyme laccase (for instance, EFs of OTNE remained constant over time at 0.390±0.005), and no significant differences were observed between the EF obtained under degradation assays and those obtained with the standard solution, it can be concluded that reaction mechanisms involved in the degradation of the target fragrances by laccase are not enantioselective.

Moreover, EFs obtained working with compost samples collected between one and six weeks of composting times were evaluated to verify whether enantioselective processes are present during composting. In contrast to what happens in

biotransformation reactions of the target fragrances in fish, surface water or sewage sludge [4,8,9], the results showed that EFs remained constant over time regardless of the target fragrance, and were comparable with the EFs obtained with standard solution, confirming that the degradation processes involved in the composting are not enantioselective.

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UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
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UNIVERSITAT ROVIRA I VIRGILI
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4. CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
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Dipòsit Legal: T 72-2016

The main conclusions drawn from the studies presented in this Doctoral Thesis can be summarized as follows:

1. The methods developed in this Doctoral Thesis to determine polycyclic musks (PCMs), nitro musks (NMs), macrocyclic musks (MCMs) and the degradation product of galaxolide have limits of quantification low enough for their determination in wastewater, sewage sludge and fish and mussel samples.

2. When both a low-polarity and a mid-polarity gas chromatography columns were tested for the separation of the target musk fragrances, short analysis times, good resolution and high efficiency were obtained working with a mid-polarity column under the optimized conditions. Moreover, the four enantiomers/diastereoisomers of galaxolide were separated in a mid-polarity column.

3. When tandem mass spectrometry (MS/MS) was compared to mass spectrometry (MS) for the determination of synthetic musk fragrances, better sensitivity and selectivity were obtained for PCMs and NMs. However, MS/MS is not suitable for MCMs due to the excessive fragmentation of these compounds, which leads to the loss of sensitivity.

4. The use of conventional and novel microextraction techniques can contribute to the development of environmentally friendly methodologies. Compared with conventional extraction techniques such as solid-phase (SPE) and liquid-liquid extraction (LLE), microextraction techniques are faster, require lower preconcentration volumes and reduce or avoid the use of organic solvents. Furthermore, the risk of contaminations is minimized as the whole extraction procedure can be automated.

5. Solventless techniques as solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE), both in headspace (HS) mode were found to be effective for the extraction of MCMs from wastewater and sewage sludge and for the extraction of PCMs, NMs and MCMs from sewage sludge, respectively. Five commercially available SPME fibres were tested (PDMS 7 µm, 30 µm and 100 µm, PDMS/DVB 65 µm and PA 85 µm), with PDMS/DVB 65 µm providing the best results. Meanwhile, SBSE was performed with a PDMS stir bar. In the case of sewage sludge samples, SPME and SBSE extractions were directly performed on the HS of sewage sludge samples avoiding pretreatment steps and facilitating the automation of the whole methodology.

6. Miniaturized SPE techniques, such as on-line SPE and microextraction by packed sorbents (MEPS), provided excellent recoveries for all of the compounds studied in this Thesis and MCMs, respectively. A balanced hydrophilic/hydrophobic polymeric sorbent, such as Oasis HLB, and a silica gel modified with C18 sorbent were evaluated for on-line SPE, with Oasis HLB providing the better recoveries for all of the compounds studied in this Thesis. Meanwhile, different silica gel sorbents modified with C8 and C18 were tested for MEPS, with C18 providing better recoveries for all of the studied MCMs.

7. Single-drop microextraction (SDME) with an ionic-liquid (IL), [OMIM][PF_6], as the extraction solvent was successfully applied for the extraction of PCMs and NMs from wastewater and sewage sludge samples. Due to the properties of ILs, extraction drop can be subjected to higher extraction temperatures and long extraction times obtaining higher extraction efficiencies.

8. Needle trap microextraction (NME) followed by GC-IT-MS/MS procedure was applied for the determination of PCMs and NMs from wastewater samples. The needle trap was filled with 30 mm of HF Bondesil C18 sorbent with a particle size of 120 µm to avoid problems of pneumatic restrictions. The developed method was found to be completely automated, simple and environmentally friendly.

9. Of the microextraction techniques evaluated in this Thesis for the determination of synthetic musk fragrances in wastewater samples, SPME and MEPS are the techniques that provided the best results, and both methodologies are fully automated. However, to date, SPME is a microextraction technique with a higher throughout than MEPS due to the wide variety of commercially available sorbents and the option of performing the microextraction with an autosampler and manually.

10. Pressurized liquid extraction (PLE) is a useful technique for extracting PCMs and NMs included in this Thesis from solid samples, such as sewage sludge and fish and mussel samples. In the case of sewage sludge, the extraction solvent that provided the best recoveries was a mixture of water:methanol (1:1, v/v), while, for fish and mussel samples, high recoveries were obtained with dichloromethane. It has been shown that the use of in-cell clean-up PLE strategies helps to reduce matrix interferences.

11. QuEChERS extraction was successfully applied for the determination of PCMs and NMs in fish and mussel samples for the first time, and high recoveries were obtained for most of the compounds. It was shown that the use of dispersive SPE (dSPE) as the clean-up technique helps to reduce the high matrix effect (ME) found in fish and mussel samples. In addition, new horizons have been opened with QuEChERS in terms of sample extraction procedures for fish and mussel samples.

12. Enantioseparation of the synthetic fragrances OTNE, galaxolide, tonalide and the degradation product of galaxolide, HHCB-lactone, was successfully performed in a 25 m 0.25 mm i.d. analytical column, with a film of heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin in OV1701. This allows the evaluation of the enantiodegradation of the target compounds during biodegradation by the laccase-mediator system and during composting by means of the enantiomeric fractions (EF).

13. Laccase-mediator system with the redox mediator ABTS was shown to be an important biodegradation agent in the case of three of the most extensively used synthetic fragrances: OTNE, galaxolide, tonalide and the main degradation product of galaxolide, HHCB-lactone.

14. Neither laccase nor the organisms performing the degradation in compost perform the respective degradation enantioselectively. EFs remained constant over time and are comparable with those obtained with standard solutions.

15. The studies presented in this Thesis have further demonstrated the presence of PCMs, NMs and MCMs in wastewater, sewage sludge and fish and mussel samples. Moreover, the presence of MCMs in wastewater and sewage sludge samples was reported for the first time in this Thesis.

16. The PCMs galaxolide and tonalide and the MCMs ambrettolide, exaltone and musk NN were the most abundant compounds of each group of synthetic musks included in this Thesis found in wastewater and sewage sludge samples. Meanwhile, most of the samples analysed did not contain detectable traces of NMs. The degradation product of galaxolide, HHCB-lactone, was found in influent/effluent samples with slightly higher concentrations found in effluent samples due to the degradation of galaxolide during WWTP treatment.

17. PCMs were found at trace level in fish and mussel samples from the Mediterranean Sea and fish samples from the River Ebro, with galaxolide and tonalide being the most abundant compounds.

Annex I. List of abbreviations

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

ABTS	2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ACM	Alicyclic musk
ACN	Acetonitrile
ADBI	Celestolide
AEE	Versalide
AHTN	Tonalide
AHMI	Phantolide
APPI	Atmospheric pressure photoionization
ASE	Accelerated solvent extraction
ATII	Traseolide
BIN	Barrel insert and needle
BOD_5	Biological oxygen demand
BPA	Bisphenol A
$[BMIM][PF_6]$	1-Butyll-3-methylimidazolium hexafluoro-phosphate
BSTFA	N,O-bis(trimethylsylil)trifluoroacetamide
C8	Octyl bonded silica sorbent
C18	Octadecyl bonded silica sorbent
CE	Capillary electrophoresis
CI	Chemical ionization
CID	Collision-induced dissociation
d15-MX	d15- musk xylene
DBPs	Disinfection by-products
DCM	Dichloromethane
DI	Direct immersion
DLLME	Dispersive liquid-liquid microextraction
DPMI	Cashmeran
dSPE	Dispersive solid-phase extraction
DVB	Divinylbenzene
d.w.	Dry weight
DWTP	Drinking water treatment plant
ECD	Electron capture detector
EDC	Endocrine disrupting compound
EF	Enantiomer fractions
EI	Electron impact
EN	European Normative
EOC	Emerging organic compound
ER	Enantiomer ratio
ESI	Electrospray ionization
EU	European Union

FD	Flourescence detection
FHTN	Vulcanolide
FID	Flame ionization detector
FMAE	Focused microwave assisted extraction
FUSALE	Focused ultrasound assisted extraction
GC	Gas chromatography
GPC	Gel permeation chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
HFLPME	Hollow-fibre liquid-phase microextraction
HHCB	Galaxolide
HHCB-lactone	Galaxolidone
[HMIM][PF_6]	1-Hexyl-3-methylimidazolium hexafluorophosphate
HPLC	High performance liquid chromatography
HPV	High production volume
HRMS	High resolution mass spectrometre
HS	Headspace
IFF	International Flavors&Fragrances
IFRA	International Fragrance Association
IL	Ionic liquids
IPCS	International Programme on Chemical Safety
IS	Internal standard
IT	Ion trap
LC	Liquid chromatography
LDS	Low-density solvent
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LPME	Liquid-phase microextraction
LIS	Labelled internal standard
LVI	Large volume injection
l.w.	Lipid weight
MA	Musk ambrette
MAE	Microwave assisted extraction
MALLE	Membrane-assissted liquid-liquid extraction
MCM	Macrocyclic musk
MDL	Method detection limits
MEPS	Microextraction by packed sorbents
MeOH	Methanol
ME	Matrix effect
MIP	Molecularly-imprinted polymer
MK	Musk ketone

MM	Musk moskene
MQL	Method quantification limits
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid-phase dispersion
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MX	Musk xylene
NM	Nitro musk
MT	Musk tibetene
MW	Molecular weight
N	Northern
NaCl	Sodium chloride
n.d.	Not detected
NE	Nord east
NP	Nitrogen/phosphorus
NPD	Nitrogen-phosphorus detector
NW	Nord west
NTME	Needle trap microextraction
OC	On-column
[OMIM][PF_6]	1-Octyl-3-methylimidazolium hexafluorophosphate
OSPAR	Oslo and Paris Commission
OTNE	Iso-E-Super
PA	Polyacrylate
PAHs	Polycyclic aromatic Hydrocarbons
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ester
PCB	Polychlorinated biphenyl
PCM	Polycyclic musk
PCP	Personal care product
PCSE	Partially concurrent solvent evaporation
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
PF	Preconcentration factor
PFA	Perfluorinated acid
PFO	Perfluoroctanesulfonamide
PFS	Perfluorosulfonate
PHWE	Pressurized hot water extraction
PLE	Pressurized liquid extraction
PLRP-S	Polystyrene-polyvinylbenzene copolymer

PMAE	Pressurized microwave assisted extraction
PPCP	Pharmaceutical and personal care products
PSA	Primary-secondary amine
PS-DVB	Polystyrene-divinylbenzene copolymer
PTV	Programmed temperature-vaporizer
PUF	Polyurethane foam
Q	Quadrupole
QqQ	Triple quadrupole
QTOF	Quadrupole-time-of-flight
QuEChERS	Quick, Easy, Cheap, Rugged and Safe
RAM	Restricted access material
RP	Reverse-phase
RDSE	Rotating disk sorptive extraction
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RO	Reverse osmosis
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SCX	Strong cation exchanger
SDME	Single-drop microextraction
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SIS	Selected ion storage
S	Southern
SPE	Solid-phase extraction
SPDE	Solid-phase dynamic extraction
SPME	Solid-phase microextraction
SS	Surrogate standard
SSL	Split/splitless
SVE	Solvent vapour exit
SWE	Subcritical water extraction
TFME	Thin-film microextraction
TD	Thermal desorption
TNO	Toegepast-natuurwetenschappelijk onderzoek
TNT	Trinitrotoluene
TOF	Time-of-flight
UHPLC	ultra-high performance liquid chromatography
USAE	Ultrasound assisted extraction
USAEME	Ultrasound-assisted emulsification-microextraction

USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VOC	Volatile organic compound
WHO	World Health Organization
w.w.	Wet weight
WWTP	Wastewater treatment plant

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

Annex II. Publications

UNIVERSITAT ROVIRA I VIRGILI
APPLICABILITY OF MICROEXTRACTION TECHNIQUES FOR THE ETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN ENVIRONMENTAL SAMPLES.
Laura Vallecillos Marsal
Dipòsit Legal: T 72-2016

List of publications derived from this Doctoral Thesis:

- L. Vallecillos, F. Borrull, E. Pocurull, Recent approaches for the determination of synthetic musk fragrances in environmental samples, *Trends Anal. Chem.* 72 (2015) 80-92 (Section 1.2.3.).

- L. Vallecillos, F. Borrull, E. Pocurull, An automated headspace solid-phase microextraction followed by gas chromatography-mass spectrometry method to determine macrocyclic musk fragrances in wastewater samples, *Anal. Bioanal. Chem.* 405 (2013) 9547-9554 (Section 3.1.1.).

- L. Vallecillos, E. Pocurull, F. Borrull, A simple an automated method to determine macrocyclic musk fragrances in sewage sludge samples by headspace solid-phase microextraction and gas chromatography-mass spectrometry, *J. Chromatogr. A* 1314 (2013) 38-43 (Section 3.1.2.).

- L. Vallecillos, F. Borrull, E. Pocurull, On-line coupling of solid-phase extraction to gas chromatography-mass spectrometry to determine musk fragrances in wastewater, J. Chromatogr. A 1364 (2014) 1-11 (Section 3.1.3.).

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Complementary environmental research:

- S.C. Cunha, J.O. Fernandes, L. Vallecillos, G. Cano-Sancho, J.L. Domingo, E. Pocurull, F. Borrull, A.L. Maulvault, F. Ferrari, M. Fernandez-Tejedor, F. Van den Heuvel, M. Kotterman, Co-occurrence of musk fragrances and UV-filters in seafood and macroalgae collected in European hotspots, Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2015.05.003.

- N. Ramírez, L. Vallecillos, A. C. Lewis, F. Borrull, R. M. Marcé, J. F. Hamilton, Comparative study of GCxGC-NCD and GC-IT-MS/MS for determining nicotine and carcinogen organic nitrogen compounds in thirdhand tobacco smoke, J. Chromatogr. A. (submitted).