SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.

Laura Trabalón Escoda

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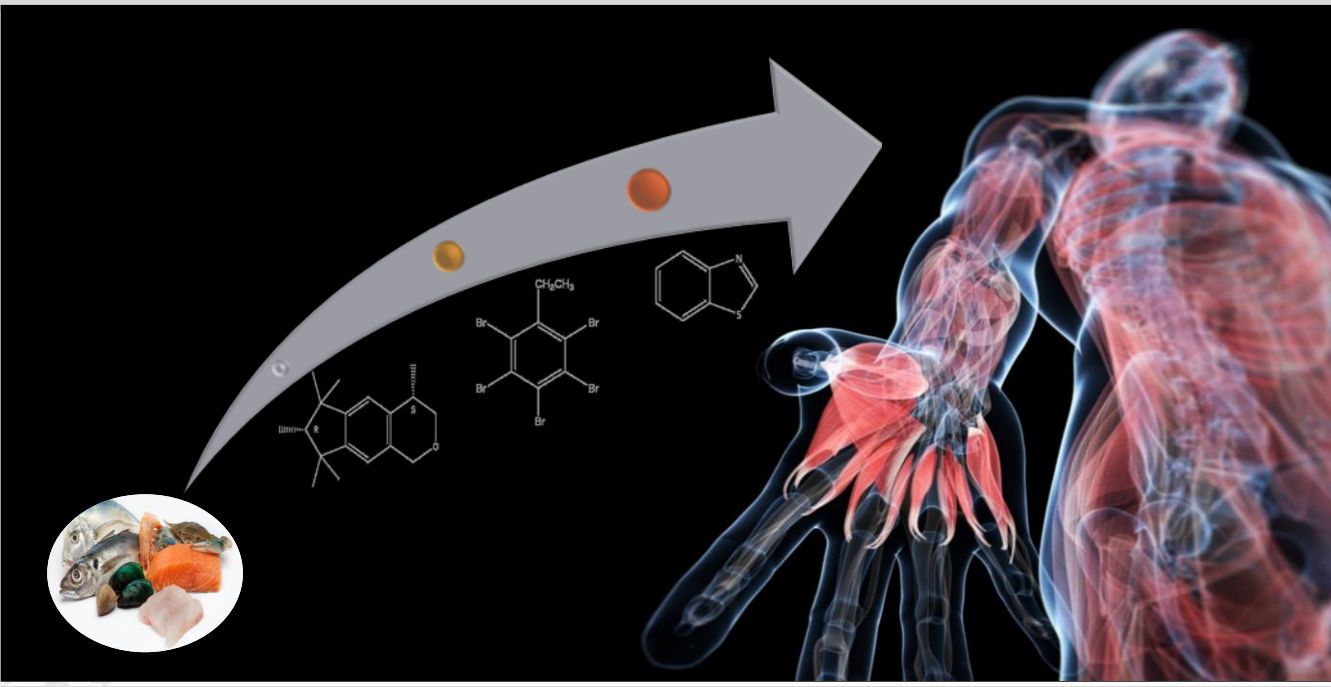
LAURA TRABALÓN ESCODA



DOCTORAL THESIS
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DOCTORAL THESIS

Supervised by

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Departament de Química Analítica I Química Orgànica

UNIVERSITAT ROVIRA i VIRGILI
Tarragona

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DEPARTAMENT DE QUÍMICA ANALÍTICA I QUÍMICA ORGÀNICA

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WE STATE that the present study, entitled "SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN", presented by LAURA TRABALÓN ESCODA for the award of the degree of Doctor, has been carried out under our supervision at the Department of Analytical Chemistry and Organic Chemistry of this university, and it fulfils all the requirements to be eligible for the distinction of International Doctor.

Tarragona, 1st September 2017.

Prof. Francesc Borrull Ballarín Dr. Eva Pocurull Aixalà Dr. Martí Nadal Lomas

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Qui m'ho havia de dir a mi que acabaria fent un doctorat. Sempre m'havien fet molt de respecte els passadissos de la facultat, on hi ha els laboratoris de recerca i els despatxos dels professors i moltes vegades pensava: Què hi deu fer tanta gent amb bata blanca per aquí? Doncs bé, aquí estem. Finalment he aconseguit un somni que no havia somiat mai però que m'ha pres moltes nits. Quan em vaig aventurar a fer el màster no hagués pensat mai que m'oferissin l'oportunitat d'optar a una beca predoctoral però, el Prof. Francesc Borrull així ho va fer.

Primerament, voldria agrair als meus directors de Tesi, el Prof. Francesc Borrull, la Dra. Eva Pocurull i el Dr. Martí Nadal, tota la seva dedicació per a que aquest treball es fes realitat. També totes les hores dedicades i tots els consells que m'han brindat. Quantes vegades hauré picat a la porta del despatx de l'Eva amb la mateixa cantarella de: Perdona Eva, tens un moment? I sempre m'hi he trobat un somriure com a resposta. Doncs bé, a tots vosaltres, moltes gràcies de tot cor. També voldria agrair tota l'ajuda rebuda per part de la resta de persones que formen part del grup, les Dres. Rosa Maria Marcé, Núria Fontanals, Carme Aguilar i Marta Calull, amb les quals he compartit des de congressos i reunions de qualitat fins a sobretaules.

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trobar, xerrant, xerrant ens vam tornar a passar l'entrada. La meva estança a Noruega va ser genial quan vau venir a visitar-me. Van ser tres dies de riures incontrolats i cansament màxim per voler-ho fer tot en poc temps. Us estimo molt.

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El plaer més noble és la satisfacció de comprendre.
- Leonardo da Vinci -

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ABSTRACT

Emerging organic contaminants (EOCs), a term which refers to chemical substances whose continuous emission into the environment may be hazardous for the environmental ecosystems and mankind, are generally lipophilic, bioaccumulative and semivolatile compounds. Some EOCs are produced on a mass scale in industrial processes, while others are released as subproducts of activities such as combustion. These compounds are found in several worldwide ecosystems as a result of anthropogenic activities and can affect human health due to bioaccumulation through the food chain and possible toxic effects.

The risks on human health derived from environmental exposure to EOCs are still the subject of considerable research, regulation and debate. It is well-known that human exposure to EOCs mainly occurs by inhalation of air, ingestion of dust, dermal absorption and the daily intake of foodstuffs. Certain dietary habits can compromise health by being a source of exposure to environmental toxic contaminants. Many EOCs are fat soluble and, therefore, any food which contains lipids often has high levels. Although human diet embraces different types of food, it has been demonstrated that seafood intake could be one of the main pathways for the absorption of chemical contaminants by the human body.

Thus, this doctoral thesis aims to provide greater insight into the presence of various families of EOCs (synthetic musk fragrances, brominated flame retardants and benzothiazoles) in seafood samples widely consumed in Tarragona County, and characterise the risk of dietary intake.

In the first section, three different analytical methods based on gas chromatography-ion trap-tandem mass spectrometry have been adapted or developed and successfully used to determine each family of compounds in seafood samples. It should be pointed out that it is the first time that benzothiazoles have been determined in seafood samples. Then, risk has been characterised by considering different subpopulation groups divided by age and gender, and various intake scenarios.

In order to perform a more accurate risk assessment, an *in vitro* digestion has been evaluated to determine bioaccessibility, in the second section. Galaxolide (HHCB), the most widely used synthetic musk fragrance, was selected as the compound to be studied because there is no information about its bioaccessibility. In addition, the most common cooking processes – namely, steaming and grilling – have been tested to determine whether they affect the concentration of ingested HHCB.

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CHAPTER 1. INTRODUCTION

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The natural environment has been subject to the effects of chemicals emitted by both anthropogenic and natural processes. Anthropogenic chemicals can originate from industrial production or be generated as by-products of chemical synthesis or combustion processes. Once they have been released into the environment, the effects they have will depend on their physicochemical properties, the properties of the environmental compartments they are mostly associated with, and meteorological and even sociological parameters. Anthropogenic chemicals can be released into the atmosphere, water, or soils but they rarely stay in the compartment they were originally released into. Some chemicals may be applied to soils and plants or used in daily products but, eventually they can pass into ambient air, wastewater, sludge and food, by evaporation or wash-out. Some chemicals are given to animals or humans but they eventually make it into wastewaters, sewage sludge and fields for agriculture. Emerging organic contaminants (EOCs) are a group of compounds that has been attracting the attention of scientific communities for decades because of their potential for both long-range transport and adverse effects. EOCs are chemical substances that are continuously emitted into the environment and may be hazardous for the ecosystem and human population. Some examples are pharmaceutical compounds, personal care products, industrial additives and plasticisers. Although some of them are not expected to be very dangerous, they are an inherent risk because of the large amounts used daily [1]. As many of these compounds have lipophilic properties and accumulate in biological tissues [2], they have been considered by several international conventions and treaties in an attempt to protect the environment and human health.

Water, especially the oceans, occupies 70% of the Earth's surface and is the largest environmental compartment. In conjunction with the atmosphere, it plays an important role in the fate and distribution of chemicals. It has an extremely high capacity to retain quantities of EOCs introduced by the discharge of contaminated water and atmospheric depositions. For example, some chemicals used as additives in paints to improve their adhesiveness (PCBs) or fire resistance (BFRs) are applied on a variety of indoor and outdoor surfaces. As they are semivolatile, they can evaporate into indoor or outdoor air. In air, they can partition between the gas and particulate phase depending on availability and quality of particulate matter and the properties of compounds. This partitioning further determines their life time in the atmosphere and their potential for long-range transport. The reactivity of chemicals is different in the gas and particulate phase but degradation products are often more stable and toxic than their parent compounds. Particle-bound chemicals can be deposited in different surface waters, soils or biota (depending on the size of the particles and the availability of surfaces). The interactions of gas-phase associated chemicals at the air-water or air-soil interfaces are determined by their physicochemical properties and the matrix itself. Once in the aquatic environment, chemicals can again partition between water and particulate matter. While dissolved chemicals are transported, particles can either float or sediment to the bottom. Truly dissolved chemicals play an important role in research into the contamination of aquatic food chains as they can accumulate in aquatic biota and are biomagnified in higher trophic levels [3]. Although the aquatic environment has been the most widely

studied environmental compartment in terms of the determination of EOCs, the aquatic organisms present in it have not been studied in so much depth. All these pathways are important for evaluating the routes of exposure of organisms and quantifying the bioaccumulation factors of chemicals.

All the main pathways for assessing the routes by which humans are exposed and quantifying the bioaccumulation factors of most chemicals are shown in Figure 1. As can be seen, there are numerous pathways by which humans can be exposed to EOCs.

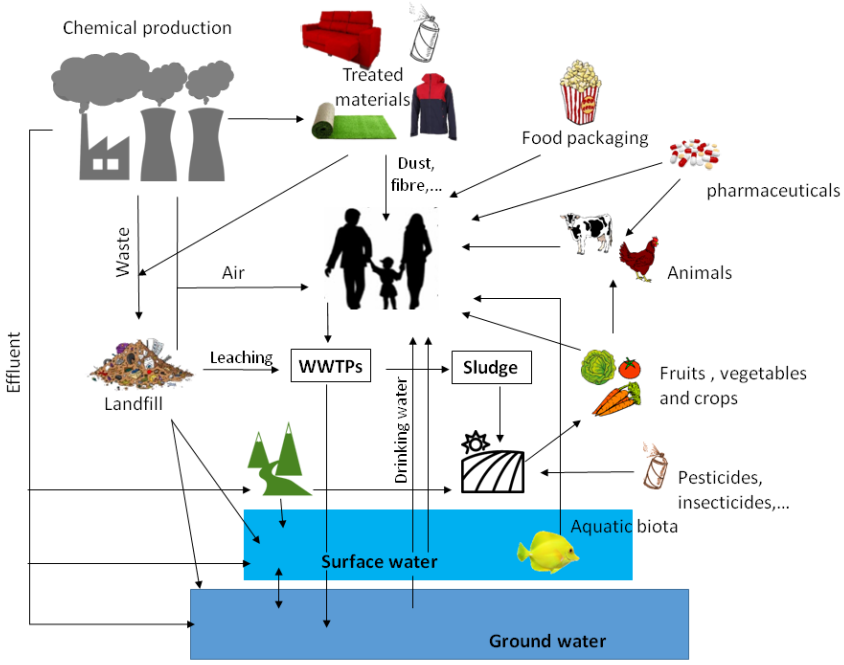

Figure 1. Fate of EOCs in the environment and their impact on human exposure. WWTPs: wastewater treatment plants.

EOCs enter our bodies, through the treated materials we use every day, the consumption of food or the air we inhale and remain there, until they are excreted or bioaccumulated. As an example of the number of chemicals currently used in the European Union, more than 100,000 chemicals are registered and their consumption is estimated at around 300 million tonnes per year [4]. It is therefore obvious that these large quantities of chemicals can result in an unhealthy impact on the environment and living species. This is demonstrated 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 [5].

In order to ensure that appropriate decisions are taken about risk management, technical advances in more sensitive and selective techniques have been used to help the scientific community to verify that numerous EOCs are present in environmental matrices, especially in situations in which new compounds have been identified. As risk assessment improves and the level of risk to human health becomes clearer, the legislative limits for contaminants in food are decreasing, which drives the need for a more sensitive methodology. Public health and safety risk assessments require that the data be reliable and that analytes be unequivocally identified so that the data cannot be questioned [6].

For all of the above, this doctoral thesis focuses on the determination of several families of EOCs - synthetic musk fragrances, brominated flame retardants and benzothiazoles - in seafood samples using different extraction techniques and gas chromatography (GC) and mass spectrometry (MS) as separation and detection techniques. Firstly, the main characteristics of these contaminants are reviewed and their occurrence and main environmental and health effects are described. This is followed by an overview of the most widely used analytical techniques for seafood samples and, finally, a vision of how to perform the dietary exposure assessment. 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. Emerging organic contaminants

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A variety of EOC families are discussed in this doctoral thesis: for example, synthetic musk fragrances, which are chemicals used in daily products; brominated flame retardants, which have fire resistant properties and are ubiquitous in the environment; and benzothiazoles, which are high volume production chemicals used in industrial and medical processes. The following sections give a detailed description of the compounds in each group, as well as their physicochemical properties, their occurrence in the environment through different emission sources and their ecotoxicological impact on both human health and the environment.

1.1.1. Synthetic musk fragrances

Synthetic musk fragrances, known as white musks in the perfume industry, are a class of synthetic man-made chemicals produced in large amounts and used extensively in a wide range of daily products such as household products, air fresheners, perfumes, cosmetics and personal care products. Although the first perfumes were all natural, synthetic ones were introduced at the end of the nineteenth century. Synthetic musk fragrances were created to enhance the scent of products such as perfume and to replace the use of natural musks obtained from animal sources (musk deer and musk ox). Nowadays, because they are easier and cheaper to produce than natural musks, they are used worldwide [7–10].

These kinds of compound have structures and chemical properties that are unlike those of natural musks. In fact, the chemical and physical properties of synthetic musks present more similarities with those of persistent organic contaminants. For instance, they have bioacummulative and lipophilic characteristics similar to those of PCBs and some pesticides. Synthetic musk fragrances have been detected in wastewater [11–16], river water [3, 17–19], lake water [17, 20], seawater [17], sediments [3, 19, 21, 22], soils [23] and sewage sludge [24, 25]. Moreover, due to their lipophilic nature and their slow biodegradation, synthetic musk fragrances have been shown to be present in biota such as fish and shellfish [26–30], mammals and terrestrial organisms [31], and in human samples, including urine [32], blood [32, 33], adipose tissue [34] and breast milk [35]. Furthermore, some synthetic musk fragrances can be transformed in wastewater treatment plants (WWTPs) and in biota, and such transformation products as nitroso and hydroxylamino derivatives [36, 37] can be even more problematical than the initial musks.

Synthetic musk fragrances also comprise a broad range of different compounds which, from a chemical point of view, can be classified as nitro, polycyclic, macrocyclic and alicyclic musks [38]. The group of nitro musks contains five synthetic alkylated nitro benzenes: musk xylene (MX), musk ketone (MK), musk moskene (MM), musk ambrette (MA) and musk tibetene (MT), which are shown in Figure 2. The first man-made nitro musk was obtained by Albert Bauer in 1888. It was discovered accidentally as a result of Bauer's attempt at producing a more effective form of trinitrotoluene (TNT) [10, 38, 39]. As a curiosity, in 1921, Ernest Beaux used a combination of nitro musks to make the world's most famous perfume, Chanel Nº 5 [10]. As far as environmental effects are

concerned, wastewater treatment plants only remove some of these compounds before their effluent is released back into the environment. Nitro musks have low octanol-water partition coefficients and, therefore, have lipophilic characteristics [34, 40, 41].

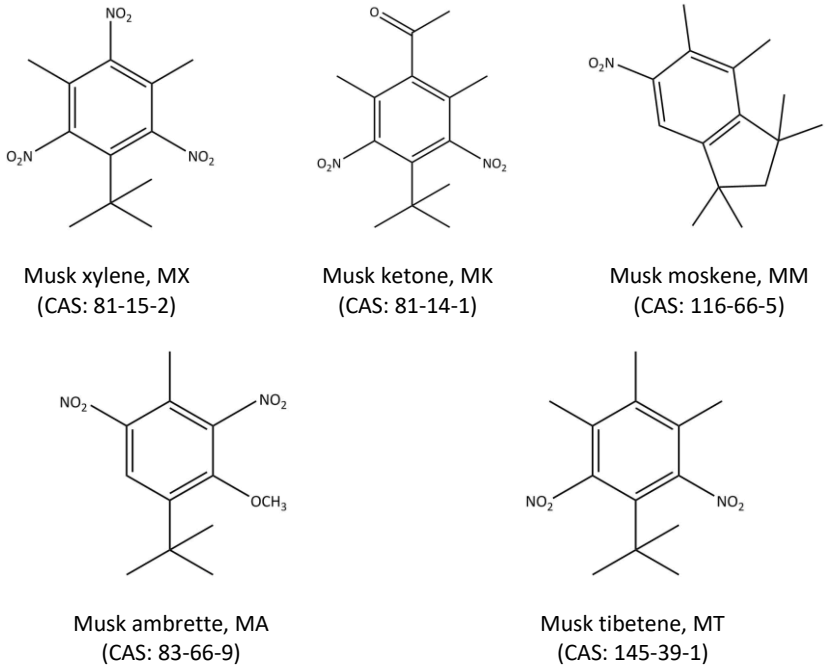


Figure 2. Chemical structures and CAS number of nitro musk fragrances. MX, MK and MM are the ones studied in this doctoral thesis.

The first study that identified nitro musks in aquatic ecosystems was performed in 1981 by Yamagishi *et al*. [36, 42]. Their results indicated the bioaccumulative and lipophilic nature of nitro musks in marine organisms. MX and MK were the most extensively used and for this reason they were the ones that were most commonly detected in water sample [38]. Although dermal exposure of nitro musks from personal care products can be an important contamination route, so too is exposure by inhalation or ingestion of fish and drinking water [26, 43, 44].

The toxicity of some nitro musks can lead to different types of dermatitis, have carcinogenic effects and cause endocrine dysfunctions [39, 45]. MA was prohibited [46–48] in 1994 since it had proved to be mutagenic and neurotoxic [43, 49, 50]. There are

two nitro musks, MM and MT, which showed no hazardous effects [43] but they were also banned in Europe [46, 48] because their structures were similar to those of estrogen receptors and they competitively bound with them [43]. MX and MK showed carcinogenic effects during long-term exposure and reproductive dysfunctions, respectively [37, 42, 43, 49]. In response, the use of MX and MK was restricted by European Directive 2002/34/EC [46]. Moreover, a new chemical regulation has classified MX as a persistent and extremely bioaccumulative substance and it has, therefore, been banned [51]. However, since most European companies stopped production in the 1990s, China is now the principal producer of nitro musk fragrances and the main source of European imports [19, 21, 24, 52]. Some articles on the determination of nitro musk in environmental samples can be found in the literature [41, 53, 54]. Most research has focused on samples of water and sludge although fish and shellfish have also been studied to a lesser extent [38]. There is also an interesting article that suggests that melting alpine glaciers could be a source of nitro musks [43].

Nitro musks, then, are being used less and less but another group of synthetic musk fragrances (polycyclic musks) has appeared. This class of musks was created largely because of the need to eliminate the nitro functional group from nitro musks because of its photochemical reactivity and its instability in the alkaline medium, which causes discolouration problems in functional products. The group includes acetylated and methylated pyran, tetralin and indane compounds and the main representatives are: cashmeran (DPMI), celestolide (ADBI), phantolide (AHMI), traseolide (ATII), galaxolide (HHCB) and tonalide (AHTN) (Figure 3). Because of their structure (see Figure 3), almost all polycyclic musks are chiral compounds and this provides some interesting insight into their odour profile [38, 55]. For example, in the case of HHCB stereoisomers the strong odour of musk comes almost entirely from C-4 because of this configuration is more important than the configuration at C-7 [56, 57]. Common characteristics of these compounds are their hydrophobic behaviour, excellent chemical stability, non-biodegradability and poor water solubility. Therefore, polycyclic musks are expected to be absorbed into organic matter and lipids. Nowadays, polycyclic musks are the most important and commercially successful synthetic musk fragrances used in the fragrance industry. The most commonly used compounds are galaxolide and tonalide [9]. The volume of European production of these two substances is assumed to be between 1000 and 5000 tonnes per year for HHCB and, in recent years, for AHTN it has been reported to be 358 tonnes [58, 59]. Thus, as a consequence of the massive production of these synthetic musk fragrances and their chemical characteristics, they have been found in such matrices as wastewater, sediments and biota [38]. Since HHCB is one of the most abundant compounds, as mentioned above, the oxidation process during wastewater treatment means that one of its degradation products, HHCB-Lactone or galaxolidone (Figure 3), is now commonly found in wastewater [15, 18, 38, 60]. HHCB-Lactone has also been found in sediments [61], household dust [62], aquatic biota [28, 63] and even human fluids [64].

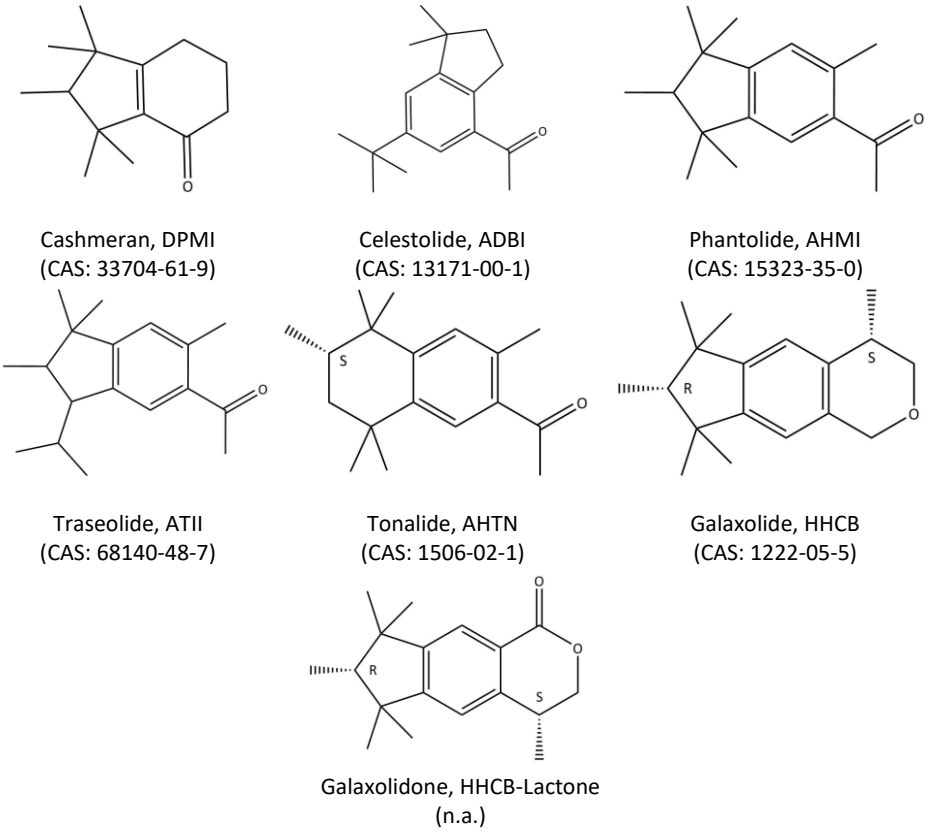


Figure 3. Chemical structures and CAS number of polycyclic musk fragrances, all of which have been studied in this doctoral thesis. (n.a.: not available).

Table 1 collects the most representative recent studies of the occurrence of HHCB and AHTN in a variety of environmental matrices in different countries. Most of the studies focus on water samples from European countries and China but recently, aquatic biota and seafood samples have been considered as target samples. The highest concentrations of HHCB and AHTN, in aquatic organisms, have been found in China at concentrations of up to 38.3 ng g^{-1} (w.w. (wet weight)) and 12.8 ng g^{-1} (w.w.), respectively. These values are high because the sampling site was a river into which the effluents from WWTPs were discharged. Moreover, the highest concentrations found in Europe are 96.4 ng g^{-1} (d.w.) for HHCB and 12.99 ng g^{-1} (d.w. (dry weight)) for AHTN. The most bioaccumulative samples are fish with a high fat content and mussels (Table 1).

Therefore, the major environmental source of these musks is the discharge of effluent wastewater because conventional treatment plants are not efficient enough to remove these compounds totally. HHCB and AHTN, then, are found in sediments, aquatic biota, etc., as can be seen in Table 1. Toxicological studies have been made to assess how these synthetic musk fragrances can affect the environment and human health. There is evidence to suggest that exposure to polycyclic musks can have hormone-disrupting effects and cause anti-estrogenic activity, among others effects [55, 57]. In particular, HHCB and AHTN can bind to human oestrogen receptors and stimulate them, and they have both been shown to affect androgen receptors and significantly decrease progesterone and cortisol synthesis in a human carcinoma cell line. AHTN has also been reported to increase the proliferation of oestrogen-responsive human breast cancer cells [34, 54, 57].

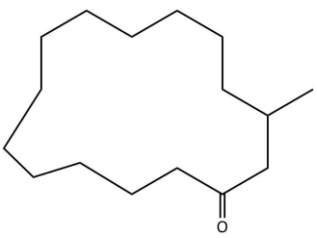
Moreover, AHTN has been identified as a photosensitizer, a chemical that becomes more toxic when exposed to sunlight on the skin, and has also been linked to liver toxicity [57]. Thereby, HHCB and AHTN are included on the Priority List of high volume substances by the EU Authorities responsible for evaluating and controlling the risk of existing substances [70]. The EU published the final reports on HHCB [71] and AHTN [59] in 2008 and estipulate that risk measures need to be reduced but that the use of AHTN should be restricted because of its photosensitizing effects. Thus, the use of AHTN in the cosmetic industry was regulated [72]. Moreover, HHCB and AHTN represent a high percentage of the market in both Europe and the US, and for this reason both substances are included on the REACH list for the EU [73] and on the EPA's high production volume chemical list [74]. The use of polycyclic musks in Europe and the US has been decreasing since the second half of the nineties. This could be due to the fact that consumer products which contain polycyclic musks are increasingly being exported outside Europe, while polycyclic musk-free products have replaced them. Also, because of their bioacummulative potential, polycyclic musks are being substituted by macrocyclic musks [58].

Macrocyclic musks are cyclic personal care products consisting of a single ring of more than 6 carbons (often 10-15). They can be extracted from plants or synthesised into large ringed lactones. The first macrocyclic musk was discovered by Leopold Ruzicka in 1926. He found the structure of muscone (Figure 4) to be a 15-membered ring ketone with one methyl substituent in the 3-position (3-methylcyclopentadecane-1-one) [50].

Table 1. Occurrence of HHCB and AHTN in a variety of environmental matrices in recent years.

Matrix	Location	HHCB Conc.	AHTN Conc.	References
Water samples		mg L^{-1}	mg L^{-1}	
Tap	Portugal China	<LOD - <LOQ	<LOD	[11], [65]
Sea	Portugal	336	<LOD	[11]
River	Portugal China	109 - 828	6.41 - 462	[11], [65]
Inffluent sewage	Portugal China Spain	7.9 - 11123	<LOQ - 1236	[11], [65], [12], [15]
Effluent sewage	Portugal China Spain	<LOQ - 4816	<LOQ - 524	[11], [65], [12], [15]
Sediment samples		ng g^{-1} (d.w.)	ng g^{-1} (d.w.)	
River	China Tunisia	<0.5 - 17.5	<LOQ - 3.63	[21], [66], [19]
Air samples		μg m^{-3}	μg m^{-3}	
Outdoor	Canada Spain	<LOQ - 1.5	0.0054 - 0.3	[20], [67]
Indoor	Spain	1 - 10	-	[67]
Aquatic biota		ng g^{-1}	ng g^{-1}	
Seafood products	Europe	<LOQ - 96.4 (d.w.)	<LOQ - 12.99 (d.w.)	[26], [69]
Fish	China Spain	<LOQ - 38.3 (w.w.)	<LOQ - 12.8 (w.w.)	[68], [28]
Mussels	Spain	8.94 (d.w.)	5.65 (d.w.)	[28]
Human samples		μg L^{-1}	μg L^{-1}	
Blood	China	0.98	<LOD	[32]
Urine	China	<LOD	<LOD	[32]
Breast milk	Korea	<5-1346 ng g^{-1} (l.w.)	<5-350 ng g^{-1} (l.w.)	[35]

(d.w.: dry weight), (w.w.: wet weight), (l.w.: lipid weight).
<LOD: concentration below limit of detection.
<LOQ: concentration below limit of quantification.



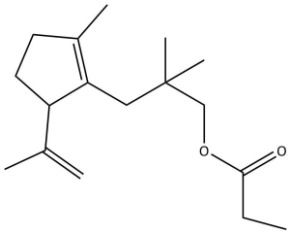
Muscone
(CAS: 541-91-3)

Figure 4. Chemical structure and CAS number of Muscone, the first macrocyclic musk fragrance discovered.

Despite this discovery and the discovery of other pathways for the synthesis of macrocyclic musks, musks of this class were not commercially produced and commonly used until the late 1990s due to difficulties in their synthesis and, consequently, their higher price [58]. From some points of view, they do have advantages over the polycyclic ones, though they are currently more expensive than nitro musks and polycyclic musks. They are two-to-four times more expensive to produce than polycyclic musks [58] and their use in perfume industry is only about 3-4%. Therefore, macrocyclic musks are not as extensively used as polycyclic musks [38]. Notwithstanding, new advances in synthetic methods and their advantageous properties, the use of these musks has increased in recent years from 25% in 1998 to 60-65% in 2008 [58]. Comparing them with the two families of musks mentioned above, this group of musks seems to be more degradable, to smell more intense and less mass is needed to achieve the same perfumery results. Hence, they are interesting substitutes [75, 76]. Some studies have shown that they are increasingly being used in perfumes and personal care products [54]. As a consequence, some macrocyclic musks have been found at low concentrations ($\mu g\ L^{-1}$) in wastewater [75, 77], sewage sludge [25] and indoor dust [54, 62]. Because they degrade easily, they are not expected to have any toxicological effects on the environment or human health, but the transformation products formed in various treatment processes or metabolomic pathways could be a source of risk. Nevertheless, the few studies of exposure assessment that have been done all show low acute toxicity from macrocyclic musks and no genotoxic and mutagenic activity through dermal and oral exposure [78–80].

Continuing with the search for new artificial fragrances which are easily biodegradable and simple to manufacture, the fourth generation of synthetic musk fragrances appeared with the name of alicyclic musks, also referred to as linear musks or cycloalkyl esters [38, 58]. Alicyclic musks were made possible by the discovery of cyclomusk (Figure 5), a modified alkyl ester, in 1975 by Hoffmann and Fraunberg at BASF [81]. They have a very different structure from previous musks. Some of the best known musks in this class are cyclomusk, helvetolide and romanolide [50]. Although they show good properties, their

use in personal care products is very limited and no further information is currently available on these types of musk [38].



Cyclomusk
(CAS: 84012-64-6)

Figure 5. Chemical structure and CAS number of Cyclomusk, the first alycyclic musk fragrance discovered.

1.1.2. Brominated flame retardants

Flame retardants (FRs) are a group of compounds that are mainly used in plastics, textiles and electronic equipment, among others things, to prevent material from igniting. These compounds allow the material containing them to resist fire through low energy sources of ignition such as cigarettes, candles, or heaters [82, 83]. The history of flame retardants goes back to at least 450 B.C. when, as noted by Herodotus, the Egyptians soaked wood in alum to prevent it from combusting. The first patent on a flame retardant substance was granted in 1735 to a man named Obadiah Wyld who used a mixture of alum, borax and ferrous sulphate to make paper and textiles flame resistant. However, it was not until the nineteenth century that Gay-Lussac began the first scientific research on this type of compound, in response to King Louis XVIII's concern to prevent the curtains in Parisian theatres from igniting. The results of these studies led to various compositions and chemical substances, many of which are the basis of the commercial mixtures used today. But it was not until World War II that flame retardants came into widespread use after the development of the synthetic polymers used in textiles. These chemicals are now added to our couches, TVs, and computers in response to the flammability standards developed in the 1970s [83, 84].

On the other hand, although FRs have good properties and applications, they also represent a risk for human health. As mentioned above, World War II accelerated the modern development of FRs and the Michigan case in 1973 was the first sign that they had environmental implications. A commercial mixture of polybromobiphenyl (PBB) flame retardants and a feed additive for cattle were produced by Michigan Chemical Co. (St Louis, U.S.). By mistake, the FR mixture was sent to the Michigan Agricultural Services Office instead of the food additive. When the error was discovered in April 1974, PBBs had entered the food chain through milk and other dairy products such as beef, pork,

sheep, chickens and eggs. More than nine million people were exposed and 25 years later PBB levels could still be detected [83, 85]. Since then, the dispersion of flame retardant compounds and their effects on humans and the environment has been investigated by scientific community.

Nowadays, there are many different types of flame retardant and they are classified by the relationship they have with materials. There are reactive FRs and additive FRs. The former are incorporated into the material through covalent bonds between FRs and the material, while the latter are dissolved, not chemically bound, into the material and, will therefore be more prone to be discharged [83]. The principal objective of FRs is to increase resistance to material ignition and reduce fire propagation without losing any material properties. For this reason, inhibition or breakdown mechanisms are the typical routes by which the chemical/physical equilibrium in the polymer combustion cycle is reduced (Figure 6). All of these mechanisms attempt to reduce the heat flow towards the polymer and, therefore, reduce its pyrolysis. Depending on its nature, the FR will act chemically and/or physically in the solid and/or gas phase of the combustion cycle [83].

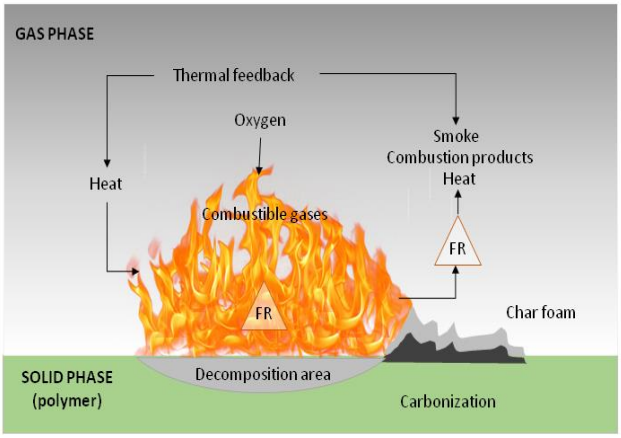


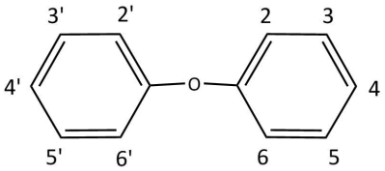
Figure 6. Polymer combustion cycle. Flame retardants (FRs) act to disrupt the formation of combustible gases and to minimise the generation of smoke. If these two pathways (solid and/or gas phase) are impeded, FRs will be effective.

FRs can also be classified according to the substance of which they are made. According to this classification, there are four classes of FRs: phosphorated, nitrogenated, inorganic and halogenated [86]. This doctoral thesis focuses on halogenated FRs and particularly on brominated FRs (BFRs). The most common halogenated FRs used are brominated and chlorinated substances, the former being the most commonly used because the bonds between the material and the FR are weaker than in other FRs so they

are more resistant to fire. As a consequence, these BFRs interrupt the combustion process easily and show good balance between benefit and cost [86, 87]. The annual global consumption of FRs is around two million tons, of which 35% are brominated and organophosphorus compounds [86, 88]. They are mainly used in the electric and electronic sector and the construction sector. Textiles, rubber, furniture and other materials also contain them. These BFRs include polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDEs) [82, 87].

Polybrominated diphenyl ethers are additive FRs which have two phenyl rings linked by an ether bond. They are produced by the bromination of diphenyl ether in the presence of a Friedel-Craft catalyst in a solvent such as dibromomethane. Diphenyl ether rings contain 10 hydrogen atoms that can be substituted for bromine atoms (Figure 7), giving rise to 209 possible PBDE congeners: 3 monoBDE (brominated diphenyl ether), 12 diBDE, 24 triBDE, 42 tetraBDE, 46 pentaBDE, 42 hexaBDE, 24 heptaBDE, 12 octaBDE, 3 nonaBDE and 1 decaBDE congeners [89–91]. Moreover, PBDEs can also present twist or skew conformations depending on the number of bromine atoms (more bromine substituents, more twist conformation) [91]. Their chemical stability depends on their structure, the PBDE congeners with nine and ten bromine substituents being the most vulnerable, whereas the PBDE congeners with four to eight bromine substituents have high stability. Also, PBDEs are photosensitive and can be affected by reductive debromination [91]. The main physicochemical characteristics of PBDEs are low vapour pressure, very low water solubility and high octanol-water partition coefficients. For these reasons, they can be expected to be released into the environment and to bind to the organic fraction [92]. PBDEs were introduced into the market to replace PBBs as brominated flame retardants following the Michigan accident in 1973. As mentioned above, they are added to the material during the manufacturing process, so these PBDEs could be released by leaching through use or the recycling of the material containing them. PBDEs have been used extensively as three different commercial formulations: Deca-BDE, Octa-BDE and Penta-BDE [83, 89]. Deca-BDE was first produced in U.S. in 1976 and was the most used PBDE technical mixture (82% of global production) [91]. This mixture is a white powder that contains 83% bromine and its formulation consists, basically, of BDE209 (97-98%) and nonaBDEs (0.3-3%). Deca-BDE is used in a broad range of polymers such as polyester resins, polyolefins, acrylonitrile-butadiene-styrene (ABS), polyvinyl chloride (PVC) and its high thermal stability means that it is used in materials that need high temperatures in their production process (for instance, TV sets, circuit boards, other electric and/or electronic sets, among others). It is also used in textiles and furnishing fabric [83, 89, 91]. The technical mixture of Octa-BDE is a white powder consisting mainly of hepta and octaBDE congeners (43-44%, 31-35%, respectively) and the rest is around 10-12% of hexaBDE [83, 93]. This mixture has been extensively used in ABS and also in nylon, polycarbonate, phenol-formaldehyde resins and unsaturated polyesters in coatings and adhesive products [91]. Penta-BDE formulation is a viscous liquid and it is defined, chiefly, as penta and tetraBDEs. For pentaBDE (44-45%), the main congener is BDE99 and to a lesser extent BDE100. For tetraBDE (41-42%) the major congener is BDE47. The rest

consists of hexaBDE (6-7%) and the two major congeners are BDE153 and BDE154 [83]. It is mainly used in polyurethane foams as an additive and also, to a lesser extent, in textiles, rubber, and other materials [91]. Due to their widespread use in recent decades, PBDEs have been found in the environment, wildlife and humans [94–96], because they are released through industrial and waste disposal processes, wastewater treatment plants, incinerators, among others, into a variety of environmental compartments. This doctoral thesis focuses on the PBDEs listed in Figure 7.



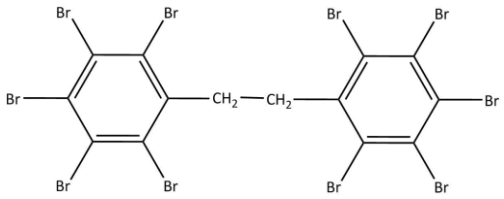
Abbreviation	Formula name	CAS
BDE28	2,4,4'-Tribromodiphenyl ether	41318-75-6
BDE47	2,2',4,4'-Tetrabromodiphenyl ether	5436-43-1
BDE100	2,2',4,4',6-Pentabromodiphenyl ether	189084-64-8
BDE99	2,2',4,4',5-Pentabromodiphenyl ether	60348-60-9
BDE154	2,2',4,4',5,6'-Hexabromodiphenyl ether	207122-15-4
BDE153	2,2',4,4',5,5'-Hexabromodiphenyl ether	68631-49-2
BDE183	2,2',3,4,4',5',6-Heptabromodiphenyl ether	207122-16-5
BDE209	Decabromodiphenyl ether	1163-19-5

Figure 7. PBDEs studied in this doctoral thesis.

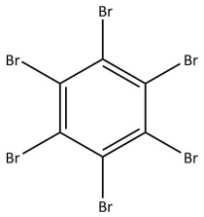
Recently, strict bans have been imposed on the use of Penta-, Octa- and Deca-BDE mixtures around the world and some congeners have been added to the list of Persistent Organic Pollutants (POPs) by the Stockholm Convention [86]. The European Union decided to ban the use of PBBs and, in particular, PBDEs in electric and electronic devices. As defined by the European Commission Decision 2005/717/EC, Deca-BDE cannot be used in electric and/or electronic equipment [97]. Moreover, articles may not be sold if they contain PBDE concentrations (Deca-, Octa- and/or Penta-BDE) higher than 0.1% by mass [98]. PBDEs are ubiquitous in the environment and according to the Institute of Health and the consumer protection assessment report, exposure to them may pose health risks. The evidence from that report indicated that PBDEs may be toxic to the liver, thyroid and neurodevelopment [99]. Also in U.S., EPA proposed new uses and restrictions of Deca-, Octa- and Penta-BDE [100] and in recent years, several states have taken measure to phase out the production and importation of Deca-BDE [101]. In Canada, there are strict

policies about environmental contaminants. Hence, regulations prevent the manufacture of decaBDE products and restrict the use of tetraBDE products [93, 102]. Nevertheless, the regulation of PBDEs in Asia is still limited and the use of Deca-BDE is not restricted [93]. Safety requirements regulate the use of PBDEs in Regulation Nº 178/2002 of the European Parliament [103], which says that "The Authority shall contribute to a high level of protection of human life and health". Thus, the EFSA's scientific opinion report shows that eight PBDEs (BDE28, 47, 99, 100, 153, 154, 183 and 209) are ubiquitously present in biota and also food and feed [91] and they are listed in the Panel on Contaminants in the Food Chain [104]. Owing to all these restrictions, new FRs have now replaced older ones and they have been classified as emerging flame retardants (EFRs) [86].

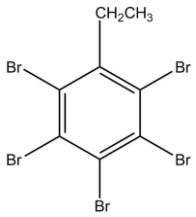
Decabromodiphenyl ethane (DBDPE) was proposed as a replacement for the BDE209 congener because it had similar applications. DBDPE has been marketed since the mid-1980s and can be found in the environment because it is released during manufacturing and use [89]. The first study to show the presence of DBDPE was published in 2004 [105] and, since then, other studies have demonstrated their dispersion and bioaccumulation [95, 106]. Although the use or production of this substance is not regulated [89], DBDPE is registered in Registration, Evaluation and Authorisation of Chemicals (REACH) [107]. Another emerging retardant described in this doctoral thesis is hexabromobenzene (HBB), an aromatic perbrominated compound. HBB has been used in Japan as an additive to plastics, paper, and electronic devices, among other things. It is not reported to have been produced in Europe [89] but HBB has been found in such environmental matrices as fish and sewage sludge [94, 95]. Finally, the last EFR discussed in this doctoral thesis is pentabromoethylbenzene (PBEB). PBEB is a bioaccumulative compound [94] and it is mainly used as an additive in textiles, foams and unsaturated polyesters [107]. In general, there is little experimental data on EFRs and the EFSA's scientific report shows that there is little information on their occurrence in food and in human samples. Therefore, there is insufficient information for a meaningful risk assessment of any of the EFRs and information about their production volumes and uses is needed [107]. Figure 8 shows the chemical structures of these three EFRs.



DBDPE (CAS: 84852-53-9)



HBB (CAS: 87-82-1)



PBEB (CAS: 85-22-3)

Figure 8. Chemical structures and CAS number of emerging flame retardants (EFRs) studied in this doctoral thesis.

As mentioned above, of all the various classes of BFRs, PBDEs have received the highest attention because of their widespread use, persistent environmental distribution and bioaccumulative characteristics. However, the focus of attention has recently changed to structural analogues of PBDEs such as hydroxylated (OH-) and methoxylated (MeO-) PBDEs. Unlike PBDEs, neither OH-PBDEs nor MeO-PBDEs are produced commercially [108] and some of these compounds are lipophilic, which can cause bioaccumulation and biomagnification in environmental matrices. Many of these natural products are used in chemical defence or as hormones [109]. Evidence from marine sponges and algae strongly suggest that these compounds are common in the marine environment [110] and their concentrations, in some instances, exceed those of PBDEs [94]. The occurrence of OH-PBDEs is of particular interest since they are more toxic than PBDEs [111]. Moreover, it has been suggested that ortho- substituted OH- and MeO-PBDEs are formed from naturally occurring compounds in marine ecosystem, whereas meta-/para- substituted compounds could originate from the biotransformation of synthetic PBDEs [112]. However, the natural origin of MeO-PBDE has been confirmed by the analysis of stable isotopes of ^{14}C in North Atlantic whales [111]. Two abundant congeners of MeO-PBDEs (6-MBDE47 and 2-MBDE68) have been reported to be natural products of marine organisms and it has been suggested that all MeO-PBDEs are formed via the methylation of OH-PBDEs or by biotransformation (Figure 9) [113].

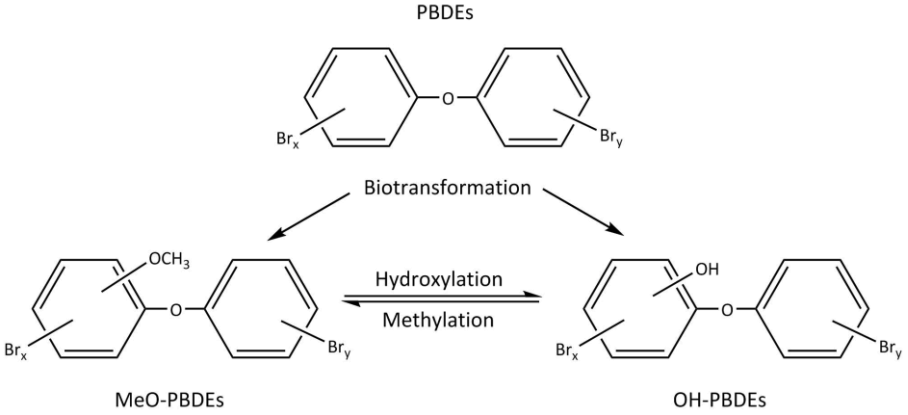
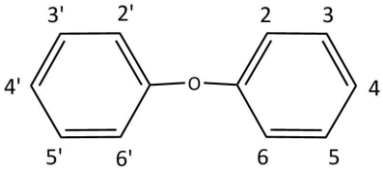


Figure 9. Pathways based on knowledge of biotransformation of PBDEs in MeO- and OH-PBDEs.

MeO-PBDEs are present in fish [94, 114], seals [82] and in other environmental matrices [115]. In addition, to their presence in wildlife, OH-PBDEs and MeO-PBDEs have been identified in health products, including commercial fish oil [116]. Due to the dietary intake of fish products, it is not surprising that both OH- and MeO-PBDEs are found in humans [111]. Although some studies show concentrations of these compounds in human samples, information on their toxicity is still limited, but since their chemical structure is similar to that of PBDEs, adverse effects on wildlife and the environment cannot be ruled out [111]. Figure 10 shows the chemical structures of the MeO-PBDEs studied in this doctoral thesis.



Abbreviation	Formula name	CAS
2-MBDE68	2',3,4',5-Tetrabromo-2-methoxydiphenyl ether	n.a.
6-MBDE47	2,2',4,4'-Tetrabromo-6-methoxydiphenyl ether	n.a.
5-MBDE47	2,2',4,4'-Tetrabromo-5-methoxydiphenyl ether	n.a.
4-MBDE49	2,2',4',5-Tetrabromo-4-methoxydiphenyl ether	n.a.
5-MBDE100	2,2',4,4',6'-Pentabromo-5-methoxydiphenyl ether	n.a.
4-MBDE103	2,2',4',5,6'-Pentabromo-4-methoxydiphenyl ether	n.a.
5-MBDE99	2,2',4,4',5-Pentabromo-5'-methoxydiphenyl ether	n.a.
4-MBDE101	2,2',4,5,5'-Pentabromo-4'-methoxydiphenyl ether	n.a.

Figure 10. MeO-PBDEs studied in this doctoral thesis (n.a.: not available).

1.1.3. Benzothiazoles

Benzothiazoles (BTs) belong to the family of heterocyclic compounds that has a benzene nucleus fused with a five-membered ring consisting of nitrogen and sulphur atoms [117]. Heterocyclic compounds are very widely distributed in nature and are particularly important because of the wide variety of physiological activities associated with this class of substances. Some of these natural compounds that contain benzothiazoles are vitamin B, genetic material, antibiotics, plant pigments, aminoacids, dyes, enzymes and other complex structures [118]. Moreover, BTs are present in a range of marine and terrestrial natural compounds that have useful biological activities [119] and, interestingly, luciferin (a natural benzothiazole derivative) has been found to supply fireflies with their own bioluminescence [117]. Due to their biochemical characteristics, they are also present in clinical drugs and since 1950, several compounds of benzothiazoles used as drugs [118] have been studied, revealing that heterocyclic compounds could modulate drug properties such as lipophilicity, polarity and, solubility. Benzothiazoles have a broad spectrum of antimicrobial, antiviral, antidiabetic [120], antimalarial, analgesic, and anticancer therapeutic functions [121]. One example of a marketed drug comprising benzothiazoles is riluzole (Figure 11), which is used to treat amyotrophic lateral sclerosis and it is also known to possess antidepressant activity [119]. As mentioned above, benzothiazoles have a wide range of drug applications and there are some methods available to synthesise derivative products such as 2-substituted benzothiazoles which also have therapeutic applications and they are used extensively in industrial processes [122]. In 1882, 1-mercapto-benzothiazole was synthesised first by A. W. Hofmann in an effort to prepare the disulfhydryl derivative of thiocarbanilide by the action of carbon disulfide on o-aminophenol. A few years later, Jacobson and Frankenbacher synthesised the same product as Hofmann while studying the formation of benzothiazole. However, the most common classical method for synthesising benzothiazole is the Jacobson synthesis based on the cyclization of thiobenzamides which involves the use of potassium ferricyanide with sodium hydroxide and the cyclization of substituted aniline in the presence of potassium thiocyanate through oxidation by bromine [117, 123–125].

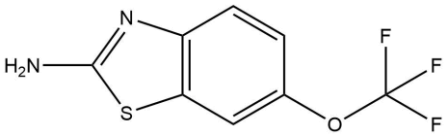


Figure 11. Riluzole, a marketed benzothiazole drug.

From the environmental point of view, BTs are classified as high production volume (HPV) chemicals because they are manufactured in amounts that exceed 1000

tonnes/years to produce chemical and household products [126]. Their uses range from rubber, as vulcanization accelerators, biocides in paper and leather, fungicides and herbicides [127], antifreezes [128], ultraviolet stabilizers in plastics and textiles [129] and to the aforementioned medical applications.

In this doctoral thesis, five benzothiazoles have been studied: benzothiazole (BT), 2-aminobenzothiazole (NH_2BT), 2-hydroxybenzothiazole (OHBT), 2-(methylthio)-benzothiazole (MeSBT) and 2-chlorobenzothiazole (ClBT) (Figure 12). As mentioned above, HPV chemicals are compounds manufactured or imported in high amounts into the European Union as defined by the Organisation for Economic Co-operation and Development (OECD) [4]. For instance, the annual production of benzothiazole derivatives used in the rubber industry, between 2000-2010, was around 38000 tonnes/year [130]. Since large quantities of several chemical products are used today, high production is an indicator of high exposure and could be associated with ecotoxicological and human health risks. In addition, BTs are easily released into the environment because it seems that they are not entirely eliminated during wastewater treatment processes [126]. Hence, it is not surprising that these compounds have been detected in a wide variety of matrices such as different types of water (wastewater [131–135], river water [128, 136], tap water [137]), sewage sludge [134, 135, 138, 139], human urine [140, 141], house dust and indoor air [142], exhaled breath [143], adipose tissue [144], clothing textiles [129], synthetic turfs [145] and, in urban particulate matter [146].

However, although they are found in a wide range of environmental matrices, these compounds are only rarely regulated. The European Chemical Agency (ECHA) gives some data about BT, such as its exposure to the aquatic environment, but it has no public registered indicating whether or which chemical products the BT might be used [147, 148]. In addition, BT is used as a flavouring substance in food applications and has been recognized as a safe compound by the European Food Safety Authority (EFSA), which has set a limit in food of 0.05 mg kg^{-1} [149]. Moreover, only one of benzothiazole derivatives is mentioned by ECHA, the 2-aminobenzothiazole (NH_2BT). It is suspected that this compound produces acute oral toxicity and it may be carcinogenic and mutagenic and that it is harmful to the aquatic system and persistent in the environment. Nevertheless, various benzothiazoles have shown toxic effects in several *in vivo* tests with fish cell cultures when concentrations are higher than those reported in environmental matrices [150]. BT, especially, might have a toxic effect on the kidney and liver at sufficient exposure [145] and also cause dermatitis and respiratory problems [140, 145, 151].

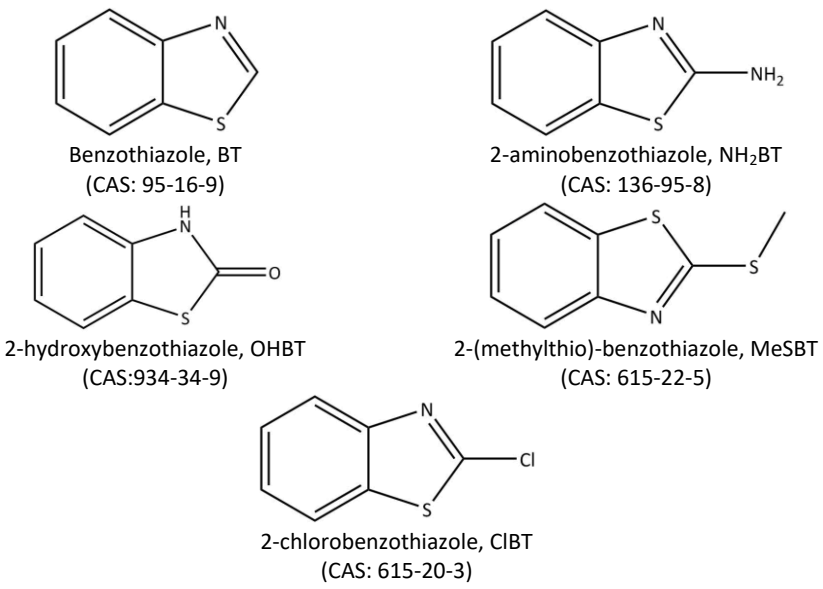


Figure 12. Chemical structures and CAS number of benzothiazole derivatives studied in this doctoral thesis.

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

1.2. Determination of emerging organic contaminants in seafood

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Food matrices are known to be highly heterogeneous, since they are composed of water, carbohydrates, fats, proteins, and minerals, and also have a potential diversity of micronutrients, additives and contaminants. This complexity may be further increased after food thermal processing. Consequently, food samples are inherently difficult to analyse accurately. From an analytical point of view, seafood is amongst the most complex food products, not only because of its chemical composition but also because of what is considered as being edible. Additionally, the seafood tissues/organs to be analysed represent key points in the overall analytical process, especially when attempting to estimate the dietary intake of a certain nutrient or contaminant [152].

As mentioned above, EOCs are frequently present at low concentrations. For this reason, accurate methods of analysis are therefore required to identify and quantify them in different matrices and to help identify the origins of the contamination and provide data for reliable consumer safety risk assessments [153]. However, for many EOCs no toxicological studies have been carried out so good risk assessment cannot be guaranteed.

Therefore, considering that the compounds studied in this doctoral thesis are usually found at concentrations ranging from less than one part per trillion to more than one part per billion in seafood samples, they cannot be directly determined and extraction and preconcentration steps are applied in order to achieve the appropriate LODs. The extraction techniques most commonly used for seafood samples are Soxhlet, pressurised liquid extraction (PLE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction but these techniques alone are not selective enough to meet the needs of food safety and food regulatory requirements and generally require a further clean-up and/or preconcentration step such as solid-phase extraction (SPE) and gel permeation chromatography (GPC) [26, 28, 94, 154]. Miniaturised techniques have also been used more recently, such as solid-phase microextraction (SPME) [27, 155]. The development of more selective sample preparation methods that can be applied to complex matrices such as food will enable analysis at the low levels now required by legislation for many residues and contaminants but, more importantly, results from more robust methods will produce more reliable data to support food safety risk assessment [156].

Generally, in seafood samples some sample pretreatments are required before the extraction process such as drying, lyophilisation, grinding and sieving. These pretreatments help to improve the mass transfer process, which in turn improves the extraction processes. Moreover, in order to characterise seafood samples and report their concentrations in dry, wet or lipid weight, moisture and lipid content are generally evaluated prior to extraction. Dry and wet weight should be determined gravimetrically, while the lipid content is often determined gravimetrically but with a previous extraction with organic solvent [26, 68, 157] or the same sample that is used for analysis [82, 94]. However, several procedures could be used to determine the lipid content instead of gravimetric measures [158, 159].

Therefore, the typical steps involved in sample preparation can be represented by a flow chart (Figure 13).

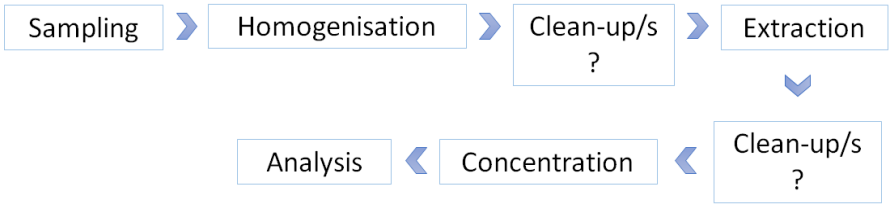

Figure 13. Scheme of seafood sample preparation.

As well as using various extraction methods for this sort of EOCs, to ensure that the analytical process is free from contamination, blank samples are analysed together with seafood samples. It should not be forgotten that BFRs are widespread so it is very likely that they are present in the materials and equipment used for sample treatment. Hence, no plastic material except polyethylene should be used for sampling [160, 161], and analysts should be careful not to wear personal care products during sampling and sample pretreatment for the determination of synthetic musk fragrances [23, 38]. Likewise, to prevent contamination, all material needs to be cleaned carefully with organic solvents and deionised water. Scented detergents should not be used in the case of synthetic musk fragrances. For these, the glass material is cleaned thoroughly by soaking in a chromic mixture overnight [16, 33] or being subject to high temperature for several hours (250-400 ºC) [29].

After the extraction and preconcentration steps, chromatographic techniques are normally required because of the similar physicochemical properties of the compounds belonging to the same family groups. In addition, efficient chromatographic separations reduce the effect of the matrix due to the separation between the compounds of interest and the undesired components of the co-eluent matrix. However, there is no universal chromatographic technique because of the wide range of chemical compounds considered as emerging organic contaminants. Therefore, the most commonly used techniques are gas chromatography (GC) and liquid chromatography (LC). Choosing one or the other will depend on the physicochemical properties of the analytes, such as their volatility, thermal stability and polarity. In the case of synthetic musk fragrances and brominated flame retardants, as they are semi-volatile and thermostable compounds, they are mainly separated by GC [38, 158]. On the other hand, benzothiazoles are mainly determined by LC [117]. In addition to the separation technique used, highly sensitive and selective detection techniques are also necessary. In this regard, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are the detection techniques that are most widely used for determining these EOCs and, today, they are an essential part of any analytical method applied in the environmental analysis.

The next section gives a brief review of the techniques that are most commonly used for extracting and determining the three group of EOCs this doctoral thesis is focused on, with examples relating to their applications in seafood analysis.

1.2.1. Extraction techniques

The extraction of EOCs from seafood is a process by which solutes are desorbed from the sample matrix and then dissolved into the solvent. Extraction efficiency is influenced by several factors, the most important of which are solubility, which depends on the type of solvent selected, mass transfer and matrix. Mass transfer refers to analytes being transported from the inside of the matrix to the solvent. It involves solvent penetrating the matrix and solutes being removed from the adsorbent sites. Mass transfer is dependent on the diffusion coefficient, the particle size and the structure of the matrix. The effects of the matrix are difficult to avoid: a highly soluble compound might be non-extractable because it is blocked in the matrix pore or tightly bound to its surface [162].

In most cases, classical techniques such as shaking and Soxhlet extraction are used with seafood samples [157, 163, 164]. Alternatively, classical techniques have been replaced with new extraction techniques which are more efficient, use less solvent and take less time although they are still used in some analytical methods. Nonetheless, very recent papers use solvent extraction to determine brominated flame retardants in seafood samples [82, 154, 165]. For example, Sun *et al*. [154] and Subedi *et al* [157] used Soxhlet extraction for the determination of PBDEs in seafood samples, obtaining recoveries above 92% and 80%, respectively. Although the Soxhlet technique is exhaustive it is not selective and further clean-up steps are necessary. This may be one of the reasons why Sun *et al* [154] obtained higher recoveries because they combined gel permeation chromatography (GPC) with SPE as clean-up steps, while Subedi *et al* [157] only used SPE as a clean-up step. However, their results cannot be compared because the seafood matrices and the analytes were not the same, although in both studies PBDEs were determined. Moreover, Soxhlet extraction has also been used for the determination of synthetic musk fragrances in seafood samples [18, 157, 166] and Subedi *et al* [157] report good recoveries (between 76-101%) for HHCB and AHTN in mussel samples.

Nowadays, the most widely used extraction techniques are PLE and QuEChERS. However, depending on the characteristics of the compounds to be analysed other extraction techniques can be applied such as matrix solid-phase dispersion (MSPD), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and microextraction techniques such as SPME. Furthermore, almost all analytical methods used to determine EOCs in seafood samples apply some preconcentration/clean-up steps to improve the selectivity of the extraction method. The most common clean-up technique for seafood samples is SPE and dispersive-SPE (dSPE) but, for samples with high lipid content, more robust clean-up procedures have to be employed such as acid treatments followed by GPC or dSPE so that fats and oils can be removed from seafood

samples. Moreover, SPME techniques have also been applied after some extraction techniques as a preconcentration /clean-up step.

UAE and MAE extraction techniques have been used to analyse seafood samples and determine the compounds presented in this doctoral thesis but they have not been used as extensively as the other extraction techniques mentioned below. UAE uses the energy of ultrasound waves to increase the contact between the solid and liquid phases, thereby accelerating the extraction process. Moreover, UAE is a simple extraction technique as it requires no sophisticated equipment. Xu *et al* [167] applied a combination of UAE and SPE called ultrasonication and vacuum assisted extraction (UVAE) to determine PBDEs, EFRs and other FRs. The extraction was carried out by 5 mL of a mixture of acetonitrile and toluene (9:1, v/v) for 1 hour and then an SPE was applied with anhydrous magnesium sulphate ($MgSO_4$) as sorbent. The authors performed a clean-up step using florisil SPE cartridges and used various solvents to extract the analytes. PBDEs and EFRs such as DBDPE were eluted from the florisil cartridge with 8 mL of hexane, while organophosphate flame retardants (PFRs), and others, were eluted with 5 mL of acetonitrile. Then, for each fraction a clean-up process was applied. For example, the fraction which contained PBDEs and EFRs was preconcentrated to 1 mL and loaded onto a cartridge with 2 g of 10% acidified silica for further clean-up and 12 mL of a mixture of hexane:dichloromethane (1:1, v/v) was used to elute these compounds. After that, the authors mixed this fraction with the fraction obtained using 5 mL of acetonitrile to make a new one, which underwent another clean-up process using APC cartridges. To elute PBDEs and EFRs 10 mL of hexane was used and 12 mL of a mixture of hexane:dichloromethane (1:1, v/v) was used to elute the other compounds studied. The recoveries obtained for PBDEs were good (78-137%) although in comparison with the previous extraction techniques, the extraction took longer.

MAE uses the energy of microwaves to heat the extraction solvent, which increases the kinetics of the extraction procedure. Thus, good extraction efficiencies can be achieved using less volume of solvent and shorter extraction times than UAE. For example, Dias *et al* [168] developed a method to determine PBDES, among other FRs, in seafood samples from Brazil. In this study, 40 mL of a mixture of acetonitrile and hexane (1:1, v/v) was used as an extraction solvent at 115 ºC for 15 min. As usual, a clean-up step was done using a packed column with a salt mixture followed by purification with HPLC and GPC to remove excess lipids. One disadvantage of these two extraction techniques is that because both selectivity and sample preparation enrichment capabilities are limited, further clean-up and/or preconcentration steps are required. Moreover, because they are not suitable for volatile compounds, literature on the determination of synthetic musk fragrances from seafood samples is scarce.

PLE involves extractions with liquid solvents but at elevated temperature and pressure. Several other names have been used for this technique including accelerated solvent extraction (ASE), pressurised fluid extraction (PFE), high pressure solvent extraction (HPSE), pressurised hot solvent extraction (PHSE), subcritical solvent extraction

(SSE) and, when water is used as solvent, pressurised hot water extraction (PHWE) or subcritical water extraction (SBWE). The main parameters of PLE are the extraction solvent, extraction temperature, extraction time and the number of extraction cycles. Under these conditions, solvents have enhanced solvation power and increased extraction rates. Extraction is quicker than classical extraction techniques such as Soxhlet extraction. PLE uses a dispersant agent such as diatomaceous earth or hydromatrix, which is added directly to the extraction cell although a sorbent material can also be used to provide *in situ* clean-up [28]. PLE also offers the option of performing a preliminary extraction with non-polar solvents to eliminate lipid compounds prior to the extraction of the analytes of interest [169]. Nowadays, PLE is one of the most extensively used extraction techniques for analysing seafood samples because it is compatible with most of the solvents currently used for solid-liquid extraction and it provides more exhaustive extractions than other less sophisticated techniques. Nonetheless, PLE extracts normally need one or more preconcentration/clean-up steps. For example, Cunha *et al* [69] used PLE to successfully extract nitromusk, polycyclicmusk and HHCB-Lactone in seafood such as mussels, mullet, flounder, clams and seaweeds. The authors used dichloromethane at 60 ºC for 5 min and one extraction cycle. Although the authors did not report the recoveries obtained, Vallecillos *et al* [28] applied the same technique to determine the same analytes in fish and mussel species obtaining recoveries between 61-109% and 45-91%, respectively. PLE have also been used to extract brominated flame retardants from seafood samples [94, 170, 171]. This technique uses various solvents or mixtures of solvents such as dichloromethane, hexane, hexane:acetone, dichloromethane:hexane and ethanol:toluene, as reported by Cruz *et al* [158]. Aznar-Alemany *et al* [94] used PLE to determine PBDEs, MeO-PBDEs, HBB, DBDPE and PBEB, among other flame retardants in seafood such as cod, mackerel, monkfish, mussels, Nile perch, plaice, salmon, seabream, shrimps and tuna. The extraction conditions were 100 ºC, 1500 psi, 10 min and one cycle, using a mixture of hexane:dichloromethane (1:1, v/v). The authors applied an exhaustive clean-up process based on an acid treatment to remove all the lipid content from the samples, followed by SPE with Al-N cartridges to obtain recoveries ranging between 51 and 109%. Additionally, Martellini *et al* [170] developed a method using PLE to determine PBDEs in foodstuffs commonly consumed in Italy, including fish and mussels. The extraction was performed with one cycle of 15 min. Unlike the clean-up used by Aznar-Alemany *et al* [94], this study applied an automated SPE as clean-up step which reduced the total analysis time.

As mentioned before, SPME techniques have become preconcentration or clean-up techniques, which are applied after extraction, rather than as a self-extracting technique, in which seafood samples are analysed. SPME is a solid-phase extraction technique that uses a fibre coated with an extraction phase, which may be a liquid or solid, to extract different types of volatile or non-volatile analyte, from different types of matrix. The amount of analyte extracted by the fibre is proportional to its concentration in the sample whenever equilibrium is reached or the equilibrium time can be shortened with the aid of convection or agitation. The extraction selectivity and efficiency of SPME mainly depend on the coating's properties and size, and its interaction with the analytes. For the

analysis of volatile compounds, the use of solvent can be avoided by analysing the head-space (HS) of the sample. HS-SPME sampling is based on the equilibrium between three phases: sample matrix, vapour and fibre. Optimization of the temperature is important since, unlike other kinds of analysis, in which an increase in temperature means an increase in analyte response, HS-SPME at high temperature may result in less deposition onto the fibre due to the volatility of the analytes and, as a result, the analytes will go into the vapour phase. Synthetic musk fragrances are commonly determined with both SPME and HS-SPME modes due to their volatility although they are not the most frequently used techniques with seafood samples. However, a recent study developed an *in vivo* SPME method to determine MX, MK, HHCB and AHTN in Tilapia fish [27]. Chen *et al* [27] used a lab-made PDMS (polydimethylsiloxane) fibre (1 cm x 44 μm thickness) attached to stainless steel wire as a syringe. Then, after 10 min of extraction, the fibre was remove from the fish muscle, rinsed with deionised water, dried and finally, directly introduced into the separation system to be analysed. Wu *et al* [155] performed a method combining MAE and HS-SPME for the determination of MK, MX, HHCB and AHTN in fish samples. PDMS/DVB (polydimethylsiloxane/divinylbenzene) (65 μm) fibre was used as usual to determine synthetic musk fragrances. The whole process was done in 5 min because the MAE extraction conditions were 5 min at 80 W (watts). The extraction solvent was a mixture of 4 mL of methanol and 15 mL of deionised water with 4 g of NaCl and the pH was adjusted to 2. Salt is commonly added and/or pH values adjusted in HS-SPME to increase the partitioning between the HS and the sample. The PDMS/DVB fibre is the most commonly used to determine synthetic musk fragrances in aqueous and sediment samples [76, 77]. In addition, Naccarato *et al* [141] also used SPME to analyse benzothiazoles in aqueous matrix and human urine but PA (polyacrylamide) fibre was selected to analyse the samples. SPME techniques are not usually applied to the determination of BFRs since there is no related literature.

Another extraction technique, which has been used for extracting seafood samples but to a lesser extent, is matrix solid-phase dispersion (MSPD). The simplicity of MSPD makes it one of the most advantageous in the field of miniaturization. MSPD blends solid samples with a solid support. Then, the mixture is transferred to an empty cartridge to form a packing column, and finally, the analytes are eluted with an appropriate solvent. Moreover, extraction and clean-up are performed in the same step, saving analysis time and organic solvent. The major factors affecting MSPD are the solid support material, the solvent and the matrix. In fact, MSPD methods using acidic silica and florisil, primary-secondary amine (PSA) and C_{18} have been employed to isolate various EOCs in food and biological matrices [29, 172]. MSPD can be miniaturised by reducing the amount of sample, which correspondingly reduce the solvent volume, sorbent and time. For example, Ziarrusta *et al* [29] developed a method to determine the main polycyclic musk fragrances, HHCB and AHTN, in molluscs from Colombia and Nicaragua. In this study, florisil was selected as the solid support material and was used as the extraction and clean-up sorbent. Dichloromethane (25 mL) was used for solvent extraction and the column was packed with active and deactivated silica. Thus, the extraction technique gave recoveries between 45 and 123%. Villaverde de Sáa *et al* [172] used PSA as a sorbent

instead of florisil to extract PBDEs and DBDPE, among other BFRs from mollusc samples. The clean-up step was carried out by a packed column with florisil as clean-up sorbent with acidified silica. Although the two studies determined different compounds both Ziarrusta *et al* [29] and Villaverde de Sáa *et al* [172] concluded that MSPD was a good alternative to other extraction techniques because it was simple to implement and economic.

QuEChERS extraction has been widely used for the determination of pesticides in food matrices but numerous research studies have extended the scope of the analytical applicability of QuEChERS to other compounds such as synthetic musk fragrances and BFRs [173]. QuEChERS is a sample preparation technique entailing the solvent extraction of high moisture samples with acetonitrile and partitioning with $MgSO_4$ alone or in combination with other salts followed by clean-up using dSPE. It is very flexible technique and since it was first used it has undergone several method modifications depending on the analyte and matrices studied. Today, there are two commonly used methods: the European Committee for Standardization (CEN) standard method EN 15662, which uses 4 g of $MgSO_4$, 1 g of NaCl, 1 g of trisodium citrate dehydrate (TSCD) and 0.5 g of disodium hydrogencitratesesquihydrate (DHS), and the AOAC Official method 2007.01 which uses 6 g of $MgSO_4$ and 1.5 g of sodium acetate (NaOAc) [173]. As mentioned above, dSPE is applied after the compounds have been extracted. The supernatant layer resulting from the extraction step is treated with a sorbent to clean it. Various sorbents have been used: PSA or C_{18} to remove fatty acids and sugars, graphitised carbon black (GCB) to remove pigments and sterols; and sorbents such as florisil in recent studies [26, 28]. Thus, the main advantages of this technique are that equipment is not required, little solvent is consumed and little time is taken. Overall, QuEChERS provides good recoveries. For example, Vallecillos *et al* [28] compared PLE and QuEChERS extractions for the determination of nitromusks, polycyclic musks and HHCB-Lactone in fish and mussel samples, in terms of validation parameters, analysis time and matrix effect. They confirmed that both techniques were suitable for the extraction of these compounds from fish and mussel samples. In this study, QuEChERS recoveries ranged between 41 and 110% and 24 and 110%, and PLE recoveries ranged between 61 and 109% and 45 and 91% (for fish and mussel samples, respectively). However, the authors reported that QuEChERS extraction showed the highest matrix effect. As far as the length of analysis is concerned, the QuEChERS technique took less than 10 min to analyse a sample, whereas PLE took at least 10 min. Saraiva *et al* [26] and Yao *et al* [68] determined nitro and polycyclic musk fragrances in seafood using different QuEChERS methods. The first study used the EN 15662 method with 1 mL of acetonitrile while the second study used the AOAC method with 10 mL of acetonitrile. The dSPE clean-up was carried out with a mixture of $MgSO_4$, C_{18} and PSA as clean-up sorbents for both studies and, although the methods used were different, recoveries were good (46-100% and 74-96%, respectively). Furthermore, for the determination of PBDEs, Romanelli *et al* [174] used a modified QuEChERs method to determine these compounds in mussel samples and, they compared it with PLE extraction. A mixture of 0.6 g of $MgSO_4$ and 0.3 g of NaCl was used as extraction salt and 2 mL of ethyl acetate was used instead of acetonitrile, which is the most

commonly used solvent. Moreover, after the extraction step an SPE was performed using Extrelut-NT3 cartridges instead of dSPE. Then, PBDEs were eluted using 8 mL of hexane:dichloromethane (3:1, v/v). Although both techniques reported comparable results, the QuEChERs method was less time consuming and more environmentally friendly.

1.2.2. Separation techniques coupled to mass spectrometry

As mentioned at the beginning of the section, GC and LC are commonly used for separating compounds in extracts from seafood samples. GC is the preferred analytical technique for determining volatile and semi-volatile organic contaminants whereas more polar and less volatile organic contaminants are determined by LC, because chromatographic signals are good and sensitivity is high. MS is the most commonly used detection technique because of its high sensitivity and selectivity. This section discusses the main features of the separation techniques used and MS detection for the determination of the compounds covered in this doctoral thesis in seafood samples.

LC is the most widely used chromatographic technique reported for the determination of benzothiazoles in environmental samples due to its low volatility. Herrero *et al* [117] reviewed scientific literature about benzothiazoles, among others, and pointed out that they are commonly used in LC studies, which have analysed them in wastewater, sewage sludge, and other matrices. Recent studies have also analysed them in exhaled breath [143] and clothing textiles [175] using UHLC and LC coupled to Orbitrap MS, respectively. Furthermore, Speltini *et al* [176] determined them in agricultural soil using LC coupled to ultraviolet (UV) detection.

GC has been used to determine these compounds but to a lesser extent. Nowadays, few studies determine benzothiazoles by GC. The most usual matrices analysed are aqueous samples [132, 136, 141], human urine [141] and tire extracts [127]. Capillary columns with a stationary phase of 5% polydimethylsiloxane and 30 m x 0.25 mm x 0.25 μm in size are the most commonly used columns for benzothiazoles [127, 136, 141]. As well as these columns, Fries *et al* [132] used a capillary column with polyethyleneglycol as the stationary phase to separate BT and MeSBT for wastewater samples. This column has a high polar capacity so BT and MeSBT showed higher retention times than in other studies. All these studies used the same injection and ionization modes. The semi-volatile compounds were injected into the GC system in the splitless injection mode at temperatures between 250-290 ºC. Once separated, they are usually detected by MS or MS/MS using electron impact (EI) and simple quadrupole (Q), triple-quadrupole (QqQ), ion trap (IT) or quadrupole-time-of-flight (Q-TOF) [136, 141]. Naccarato *et al* [141] used two deuterated internal standards (I.S) at the beginning of the study, d_4-BTR and p-TSA-d_4, to determine benzothiazole, benzotriazole and benzenesulfonamide families. Of these, they discarded the first because the results showed poor chromatographic behaviour with pronounced tailing peaks. Therefore, p-TSA-d_4 was the I.S used to correct losses of analytes during sample preparation and/or sample inlet. The I.S selected

depends on the analytes and it needs to be similar to the compounds analysed so the best choice is to select the same deuterated analyte. In contrast, Jover *et al* [136] determined the same compounds by GCxGC-TOF-MS using triphenylamine as I.S to correct both the MS response and the chromatographic retention time in both dimensions. Different columns were used for each family of compounds and separation with GCxGC was compared with the separation obtained with the same first-dimension column in a single GC. As expected, some coelutions cannot be prevented by the single GC but these issues were resolved by GCxGC. In addition, it has been reported that derivatization may be useful for improving the shape of the semi-volatile analyte peaks and increasing their resolution. In this study, trimethylsulfonium hydroxide (TMSH)] and a [bis (trimethylsilyl) trifluoroacetamide (BSTFA) were tested but the results did not improve. Normally, the derivatization step is used when the volatility of the analytes is poor or the analytes respond poorly to a specific detector. Derivatization can improve resolution between coeluting compounds and overlapping peaks but the process can sometimes be complex and laborious without significantly improving the results.

Unlike benzothiazoles, the main technique for determining synthetic musk fragrances and brominated flame retardants in seafood samples is GC due to their volatility.

GC-MS and GC-MS/MS have proved to be versatile and widely used techniques for determining synthetic musk fragrances in seafood matrices because of their inherent properties (high thermal stability, volatility, etc.). Therefore, many studies have used these techniques [18, 26–30, 68, 69, 155, 157, 166, 177]. LC has been used by some authors but few studies have been published and seafood is not involved in any of them [178–180]. Samples are usually injected into GC systems by split/splitless injector or programmed temperature vaporising (PTV) injector in order to provide greater analytical flexibility and enhanced sensitivity by means of large-volume-injection (LVI). This option has been reported by many authors for the analysis of seafood samples [26, 28, 29, 181]. For example, Ziarrusta *et al* [29] compared PTV (10 μL) and splitless (2 μL) injection mode in the determination of HHCB and AHTN in molluscs. PTV was applied in order to increase sensitivity but the system became dirty so it was discarded. To prevent issues of this sort and to prolong the lifetime of the GC columns, guard columns (3 m x 0.25 mm i.d) are normally used [28, 69]. In contrast, Saraiva *et al* [26] injected 50 μL by PTV inlet and neither used the guard column or reported any problem regarding the dirtiness of the GC column. Chromatographic separation is usually performed by means of low polarity capillary columns with stationary phases of 5% phenyl-95% dimethylpolysiloxane (DB-5MS, ZB-5MS, among others) or 50% phenyl-dimethylpolisiloxane (DB-50MS among others). In some studies, the use of mid-polarity columns is also reported. For example, Lange *et al* [18] used a mid-polar GC column (VF-XMS from Agilent Technologies) of arylene/methyl modified polysiloxane to separate HHCB, AHTN and HHCB-Lactone from fish samples. This increase in polarity requires a long analysis time (33 min). The most common dimensions of GC columns to separate synthetic musk fragrances are 30 m x 0.25 mm i.d x 0.25 μm film thickness and, as a result, the analysis times are between 10-17 min [28, 29, 177]. Yao *et al* [68] used a DB-5MS column with a higher amount of

stationary phase (250 mm i.d x 0.25 mm film thickness) but no significant differences were observed in retention times.

Mass spectrometric determination has been carried out primarily as single MS. However, some authors prefer to use MS in tandem because it improves selectivity and sensitivity by reducing background noise [182]. Otherwise, when the analyses are carried out by LC, MS/MS is the chosen technique [178–180]. Of all the ionisation sources used in GC-MS and GC-MS/MS, EI is the most common because polycyclic musk fragrances have no functional groups and the sensitivity for quantitative targeted analysis is good. Because the EI is a strong ionization, mass spectra show several characteristic m/z fragments which are well suited as indicative mass ions for the identification of these compounds and can be applied to routine analysis. Therefore, GC-EI-MS in SIM mode can screen synthetic musk fragrances at a high level of specificity. However, to determine nitro musk fragrances, the presence of nitro groups make chemical ionisation (CI) more sensitive than EI [183]. Quadrupole (Q) MS has been the most widely used analyser for determining synthetic musk fragrances in seafood samples [18, 26, 27, 68, 155, 157, 166] while ion-trap (IT) and triple quadrupole (QqQ) in MS/MS mode are the second most common analyser [28, 29, 69]. For the trace analysis of these compounds tandem MS has been successfully applied and, as has been mentioned above, has considerably increased sensitivity and selectivity. The method developed by Ziarrusta *et al* [29] to determine organic pollutants, including HHCB and AHTN, in molluscs is an example of the suitability of the MS/MS mode. Results were better in terms of LODs, which were 14-29 ng g^{-1} when GC-MS was used and 4-6 ng g^{-1} with GC-(QqQ) MS/MS. In some recent studies, IT has been used. LODs were between 0.25-10 ng g^{-1} [15, 69]. In all these methods, selected-reaction monitoring (SRM) was used to determine the compounds analysed and multiple-reaction monitoring (MRM) was selected for those compounds that coelute but can be identified by their product ions, as happens with the deuterated compounds used as I.S. Of all the studies on MS determination and cited in this section, it should be pointed out that Saraiva *et al* [26] reported better LODs (0.001-1.94 ng g^{-1}) than the studies which worked with MS/MS.

The last group of compounds discussed in this section are brominated flame retardants (BFRs) which involve polybrominated diphenyl ethers (PBDEs), methoxylated polybrominated diphenyl ethers (MeO-PBDEs) and some emerging flame retardants (EFRs). Once again, gas chromatography is the preferred technique for BFRs separation. They are often detected after GC separation by MS or MS/MS using either IT, Q or QqQ analysers. There is an interesting and very recent review by Cruz *et al* [158] that focuses on analytical aspects for the determination of PBDEs and their metabolites in seafood matrices. This review reports both extraction techniques and GC-MS and GC-MS/MS methods, and summarises the most important information from sample preparation to instrumental analysis up to 2006, as well as spectrometry ionisation and fragmentation for these compounds.

One of the major pitfalls in the separation of BFRs is that most of these compounds have a very similar structure and, therefore, it is difficult to chromatographically separate them completely. In the chromatographic separation of BFRs, non-polar columns such as ZB-5MS, DB-5MS and HP-5MS are normally used. Highly brominated PBDEs can also undergo degradation in retention gaps, so column length and film thickness are two important characteristics that affect the retention time of the analyte and, as a result, their degradation. Short GC columns (15 m) with less film thickness (0.1-0.25 μm) should be used to reduce residence time and avoid possible thermal degradation. This is the case of BDE 209 which easily degrades. Thus, it is separated using a shorter column while other PBDEs are usually separated using 30 m columns. It should be noted that the more bromine atoms there are, the faster the degradation is [89]. For example, Geng *et al* [82], Aznar-Alemany *et al* [94] and Fuchao *et al* [167] used GC columns of 15 m to separate PBDEs and MeO-PBDEs, including BDE 209. Geng *et al* [82] also used an RTX GC column which is optimized for the analysis of PBDEs by the 1614 EPA method. Martellini *et al* [170] used two different columns to determine PBDEs (30 m) and BDE 209 (15 m). Likewise, Peng *et al* [164] used a 30 m column to separate two EFRs (HBB and PBEB) and a 15 m column to separate BDE 209 and other EFRs (DBDPE). These compounds are usually injected into the GC system, using splitless, PTV and on-column injectors [158]. However, one of disadvantages of on-column injection is that it requires exhaustive clean up steps since the sample extracts are injected directly into the column. Therefore, this injection mode is not usually used with seafood matrices. The split and split/splitless injection modes require high injection temperatures so thermal degradation of higher brominated congeners can be observed and a careful optimization is needed. On the other hand, PTV injection makes it possible to programme the injection temperature, avoid any possible degradation of compounds and inject high volume of extracts, usually up to 10 μL with seafood samples, in order to increase the LODs. However, almost all studies on the determination of BFRs in seafood matrices use the splitless injection mode [94, 157, 164] but PTV is becoming more popular. Fuchao *et al* [167] used PTV to determine PBDEs and other organophosphate compounds in fish samples. Although they injected only 1 μL, they used a guard column to protect the GC column. Ziarrusta *et al* [29] compared both splitless and PTV injection to determine PBDEs in mollusc samples but since 10 μL was injected into the GC column without a guard column, it became dirty and was discarded for further analysis.

As mentioned above, BFRs are usually detected by tandem mass spectrometry operating with Q, IT or QqQ. Due to the properties of these compounds, EI and electron capture negative ionisation (ECNI) were the most commonly used ionisation sources because of the abundance of molecular and other characteristic ions. GC-EI-MS methods can efficiently quantify PBDE congeners between 1 and 7 bromine atoms but not higher ones because they are predisposed to interfere with other analytes with similar structures. Higher sensitivity and selectivity can also be achieved using GC-EI-MS/MS either with IT or QqQ because the matrix has less effect and there are fewer interferences but EI in MS/MS mode considerably fragments compounds and makes it difficult to select specific and abundant precursor ions. Ziarrusta *et al* [29] applied EI with both (Q) MS and

(QqQ) MS/MS and the PBDEs studied were between tri- and hexa-bromo congeners because these compounds can be quantified by EI-MS without problems of interference. GC-EI-MS/MS reported better LOD values (0.24-8.1 ng g^{-1}) than GC-EI-MS (4.6-25 ng g^{-1}). Moreover, the authors used deuterated chrysene and perylene as surrogate standards for the PBDEs whereas many other studies used ^{13}C-labelled standards [94, 165, 170]. GC-ECNI-MS shows higher sensitivity than EI for those compounds with a high number of bromine atoms (8-10) but the recorded spectra are dominated by the two bromine ions (79 and 81 m/z) which make it difficult to distinguish PBDEs from different homologue congeners or any bromo-compounds. However, Peng *et al* [164] reported very low instrumental LODs for BFRs, using ECNI, with tetra-, penta- and hexa-BDEs (0.04-0.05 pg g^{-1} (w.w.)) although they have fewer than 8 bromine atoms in their structures. They also found the same for BDE 209 and DBDPE (5 pg g^{-1} (w.w.) and 10 pg g^{-1} (w.w.), respectively). Likewise, Sun *et al* [154] used ECNI to determine tetra- and hexa- PBDE congeners, among others, and Fuchao *et al* [167] used this mode to determine PBDEs from tri- to deca-bromo compounds. In the recent years, atmospheric pressure chemical ionisation (APCI) has increasingly being applied to determine PBDEs and their metabolites by GC-MS/MS. APCI works with soft ionisation in the gas phase and, as a result, fragmentation is less. Selectivity and sensitivity increase because more molecular ions ($[M]^+$) are formed. This ionisation mode makes it possible to operate in the positive ion mode for BFR compounds but negative ion mode was also used to determine of halogenated analytes. Two studies [82, 184] report their advantages over GC-EI-MS/MS and GC-ECNI-MS (for example, they select sensitive and specific precursor ions, they operate in SRM mode, and the lower fragmentation gives good LODs and LOQs).

The fragmentation pattern of PBDEs [82, 185, 186] and MeO-PBDEs [185, 187] is well elucidated. Cruz *et al* [158] summarise the fragmentation patterns of PBDEs and MeO-PBDEs in both EI and ECNI from the study by Hites *et al* [185]. For PBDEs, molecular ions ($[M]^+$) and ions which lose 2 bromine atoms with both single or double charges ($[M\text{-}Br_2]^+$ and $[M\text{-}Br_2]^{++}$, respectively) are commonly used for identification and quantification of target analytes. Although the $[M]^+$ ion has been observed as the most abundant ion, $[M\text{-}Br_2]^+$ would be optimum in SIM mode for most PBDEs because compounds with high bromine substituents are dominated by the cluster corresponding to $[M\text{-}Br_2]^+$. Hites *et al* [185] presented a graphical bar which showed the abundances of three different ion clusters from PBDEs in EI mode. For tri- and tetra-BDE congeners the predominant ions were $[M]^+$ and fragmentation was low while for penta- and deca-BDE congeners the most abundant ions were $[M\text{-}Br_2]^+$. They showed that BDE-77 was an exception because its spectra did not show the presence of $[M\text{-}Br_2]^+$. As BDE-77 does not have ortho-bromine substituents, it cannot form a stable dibromobenzofuran ion (corresponding to the loss of 2 Br). Overall, EI has some limitations when identifying the substitution pattern of the bromine atoms on the aromatic rings as reported by La Guardia *et al* [186]. Nevertheless, ECNI can be used to distinguish the position of bromine atoms in some cases. The most abundant ions generated by the ECNI spectra of PBDEs are 79 m/z and 81 m/z, followed by HBr_2^- ions in the PBDEs congeners which have 7 or fewer bromine atoms and then brominated phenoxide ions in the case of compounds with 8 to 10 bromine atoms [185,

186]. These brominated phenoxide ions are generated due to cleavage of the C-O bond and the negative charge is located in the oxygen atom $[C_6Br_yH_zO]^-$. Hites *et al* [188] reported an exception related to BDE-206 (nona-BDE), the spectra of which showed abundant intensities of M-Br_3^- and M-Br_4^- ions instead of its corresponding brominated phenoxide ion. They also showed a dependency between the ion source temperature (150 ºC-250 ºC) and the increase in Br^- ions (expressed as the sum of 79 m/z and 81 m/z). For all the PBDEs studied, with the exception of BDE-209, an increase in the Br^- fragmentation pattern was observed. As mentioned above, ECNI mode can differentiate between PBDEs congeners through their bromine substituents. For example, La Guardia *et al* [186] demonstrated the differences in the spectra for two octa-BDE (BDE-204 and BDE-197). BDE-204 has 3 bromine atoms in one aromatic ring and 5 in the other one while BDE-197 has 4 bromine atoms in each aromatic ring. In the case of BDE-197 the only abundant cluster corresponded to $[C_6Br_4H_5O^-]$ (408 m/z) whereas for the spectra of BDE-204 two major clusters appeared $[C_6Br_5H_5O^-]$ and $[C_6Br_3H_5O^-]$ (486 m/z and 328 m/z, respectively).

In the determination of MeO-PBDEs by ECNI, the substitution position of methoxylated groups cannot be distinguished as reported by Hites *et al* [185]. Their mass spectra also show abundant intensities of 79 m/z and 81 m/ such as PBDEs in the same ionization mode but meta- and para-substituted congeners show more HBr_2^- ions than ortho-substituted congeners. Unlike ECNI for MeO-PBDEs, when EI is used to determine these PBDE metabolites the ortho-, meta- and para- positions for the aromatic ether bond and methoxy group can be differentiated. MeO-PBDE spectra are dominated by the $[M]^+$ ion but ortho- MeO-PBDEs also show the $[M\text{-}BrCH_3]^+$ ion while para- MeO-PBDEs show $[M\text{-}CH_3]^+$ ion. Hites *et al* [185] and Yu *et al* [187] are in agreement but Yu *et al* [187] reported that only the $[M]^+$ ion was used to identify meta-MeO-PBDEs and Hites *et al* [185] reported that meta- MeO-PBDEs showed $[M\text{-}Br_2]^+$ as the predominant fragment. The possible reaction mechanisms that explain these different fragment patterns have also been reported [185].

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

1.3. Dietary intake

UNIVERSITAT ROVIRA I VIRGILI
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Exposure to chemical substances may pose significant risks to the general population because of potential health effects. Risks caused by the toxic materials in our environment are a matter of serious concern for modern societies. As mentioned above, environmental organic pollutants, even toxic chemicals, are now found all over the world: traces of EOCs can be found in high mountains, deep oceans and polar regions. They are spread by the wind, carried by water and bioaccumulated in the food chain. They ultimately reach humans. Therefore, it is virtually impossible for people to live in a toxicant-free environment. In this context, it is extremely important to evaluate whether any traces of these EOCs can mean a risk for human health. The most typical procedure to conduct this evaluation is through health risk assessment of human exposure to EOCs. In the following sections, there is an overview of the risk assessment process and detailed information about dietary intake, specially focused on seafood consumption, as well as a review of the most recent studies about human exposure/dietary intake via fish and shellfish consumption.

Toxicology is the area of medicine which investigates the adverse effects of chemicals on living organisms [189]. Toxicology is one of the oldest fields in medicine, which came into being when our prehistoric ancestors tried to introduce substances into their diets that they had not previously found in their environments. By observing which substances could satisfy hunger without causing illness or death, humans started to develop eating habits that improved the survival and proliferation of the species in their traditional environment, and allowed them to adapt to new environments. Despite the number of intoxications in Ancient Greece and the Roman Empire, toxicology did not emerge as an objective science until the end of the 15th century, with Paracelsus, who is considered to be its founder. He was the first person to attribute the adverse effects of certain substances to the substance itself, and not to its association with evils or god(s). Moreover, he is known by the famous expression: "*All substances are poison; there is none that is not a poison. The right dose differentiates the poison from a remedy*" [190].

Humans are exposed to a wide range of chemicals in indoor and outdoor environments, through a number of pathways (inhalation, ingestion or dermal contact). The first step in the poisoning of an organism is to expose it to a toxic substance. Exposure is defined as contact between an agent and a target, where contact takes place on an exposure surface (air, foods, beverages, etc.) over an exposure period. After that, the chemical is absorbed by the organism. The term absorption usually includes not only entry into the barrier tissue but also further transport into circulating blood. Metabolism can take place at the point of uptake and, as a general rule, metabolism can convert lipid-soluble chemicals to water soluble metabolites that can be effectively excreted. The distribution of a substance within the organism is a dynamic process which depends on uptake and elimination rates, blood flow to the different tissues and their affinities for the substance. ADME, the acronym of “adsorption, distribution, metabolism and excretion” is the term that is most often used to explain the processes of a toxicant within the human body. Water-soluble, small, uncharged molecules, univalent cations, and most anions diffuse easily and will eventually reach a relatively even distribution in the body

[191]. As stated above, humans are exposed to chemicals in daily environments, and may be contaminated. Some of these toxic chemicals, or even non-toxic chemicals, are present in the environment. Although they can come from natural sources, they mainly come from industrial processes and consumer products. Ultimately, chemicals reach different environmental compartments, such as water, soils and foods. A conceptual model of the main exposure routes and materials is depicted in Figure 14.

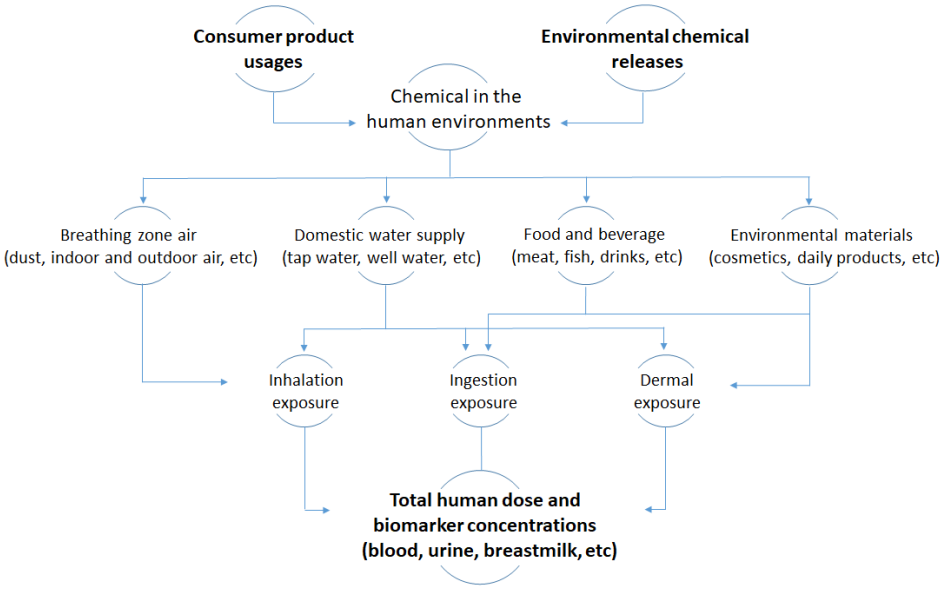

Figure 14. Conceptual model of human exposure to environmental pollutants.

Threshold levels for individual chemicals are usually generated as a combination of scientific and political consensus [6]. Also, exposure assessment can provide information about the amount of pollutant consumed by the population. However, exposure assessment relies on previous procedures in the general process of risk assessment. Overall, risk assessment may be considered as a systematic process for arriving at estimates of all the significant risk factors associated with an entire range of exposure scenarios in connection with some hazard situations. It is indeed a scientific process that can be used to identify and characterise human health problems related to chemical exposure [192, 193]. Risk assessment has traditionally included four different steps: 1) *hazard identification*, which determines whether exposure to a chemical can increase the incidence of a particular adverse health effect and determines the likelihood of occurrence in humans; 2) *dose-response assessment* or hazard characterization, which determines the relationship between dose and incidence of effects in humans (data are derived from animal studies or, less frequently, from studies in exposed human populations); 3) *exposure assessment*, which is a key tool in the risk assessment process

since without exposure, even the most toxic chemical would not present a threat; and 4) *risk characterization*, which involves predicting the frequency and severity of effects in exposed populations (Figure 15) [6, 192, 193]. However, in recent years, two subsequent stages, risk management and risk communication, have been identified as key steps in the whole process of risk analysis (Figure 16).

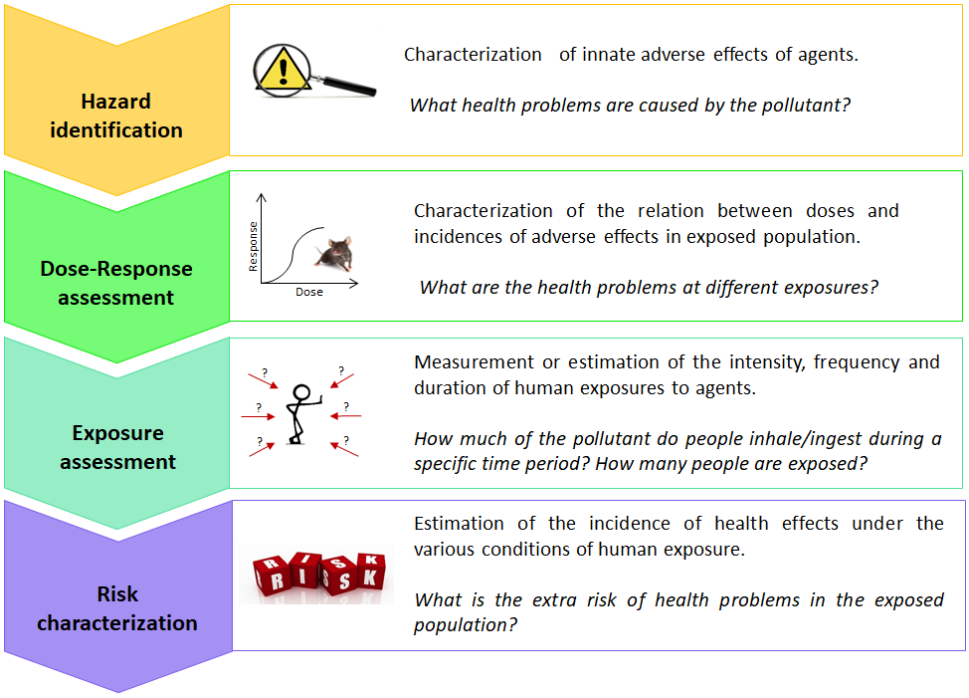

Figure 15. Elements of the risk assessment process.

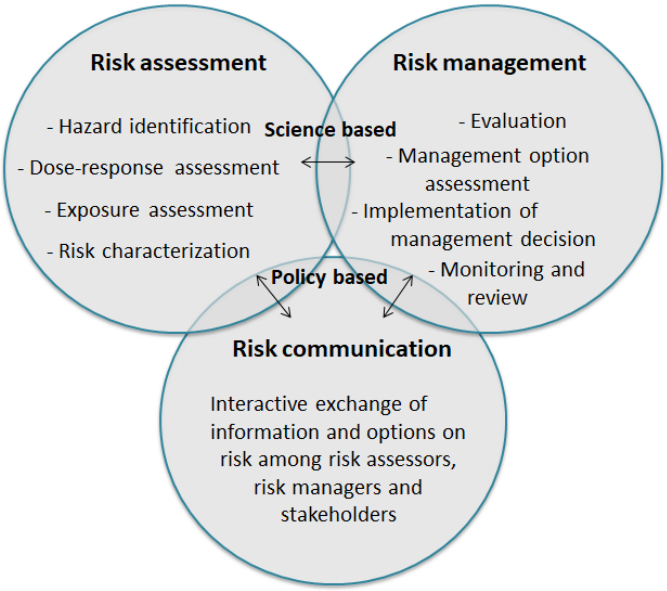

Figure 16. Relationship between the three components of Risk Analysis.

Risk management is the decision-making process used to establish policies to address hazards identified during risk assessments. The decision process includes the development of regulatory options or policies, and the evaluation of public health consequences. Risk managers consider the risk assessment data along with the social, economic, statutory and political factors. Although risk assessments may contain risk management considerations, the process of risk management should be considered a separate activity. Risk management should be considered as the control or mitigation step in risk assessment. The decision to control a pollutant will involve costs. The cost-benefit trade-offs and social impact of the policy are all key components in managing risk. One definition of risk communication is that it is the process by which understanding of the hazard or potential hazard is raised. While effective risk communication may not ensure acceptance by all stakeholders, poor risk communication will almost certainly lead to disagreement and outrage [6, 193, 194].

The present doctoral thesis focuses on the exposure assessment and risk characterization of the dietary intake of emerging pollutants through the consumption of fish and shellfish by the general population. Contaminated fish and shellfish species are potential sources of human exposure to toxic chemicals. EOCs are not only transported in surface waters; they can also be stored and accumulated in sediments, as a result of complex physicochemical processes. Most of these environmental pollutants are stored in fatty tissues, whereas others are found in non-lipid compartments. Fish and shellfish

species are important contributors to the dietary intake of contaminants because of their bioconcentration capacity through the food web [152, 195–197]. Exposure assessment combines concentration data of a chemical in food with the amounts consumed (intake). There are various methods for combining these two parameters. The resulting dietary intake (or dietary exposure) may then be compared with databases of health guideline values for the food chemical of concern, as a part of risk characterization [192, 193]. Intake data is usually based on data collected from surveillance studies, food consumption surveys and/or food consumption databases [153, 198, 199]. Food consumption surveys include food diaries, food frequency questionnaires (FFQs), or dietary recalls, among others. Moreover, the information gathered involves factors that could influence dietary intake, such as demographic characteristics (age, sex, etc.), body weight, healthy habits, etc [153, 192].

The most usual approach to determine the concentration of a chemical in food (e.g., fish and shellfish) is targeted sampling by analytical methods [153]. The concentration data obtained from these methods should be carefully used when evaluating dietary intake, since values may not be representative of all food available for sale. The greatest limitation of monitoring data is that normally not all contaminants entering the food chain are monitored. The accuracy, selectivity and sensitivity of the analytical methods are important if the detection and quantification limits are to be suitable. In addition, non-detected (ND) and non-quantified (NQ) concentrations need to be managed but there are different scientific approaches and no international guidelines on how to report values [200–202]. It should be assumed that samples without detectable or quantifiable concentrations could contain the chemical below their limit of detection (LOD) or limit of quantification (LOQ). To assess dietary intake in these situations, one option is to assume a value of one-half of their LOD or LOQ [203]. A second option is to use different level-bound scenarios (lower, middle and upper) [153]. The upper-bound scenario (UB) is obtained by assuming the respective concentrations of LOD and LOQ and in the lower-bound scenario (LB), a concentration of zero is assumed for non-detected chemicals and a concentration equal to the LOD is assumed for chemicals with their levels below the LOQ. The middle-bound scenario (MB) is obtained by assuming one-half of the LOD or LOQ of the concentrations.

The general equation of dietary intake is (Eq. 1):

$$E_t = \frac{\sum_{f=1}^{p} C_f X_{t,f}}{bw} \qquad \text{Eq. 1}$$

where E_t is the dietary intake (exposure) of chemical *t* in the general population (ng kg^{-1} bw day^{-1}), C_f is the mean consumption of the food *f* (g kg^{-1} bw day^{-1}), and $X_{t,f}$ represents the concentration of chemical *t* in the food *f* (ng g^{-1}). The mean consumption is normalised

by dividing the dietary intake with the mean body weight (bw), which varies for different subpopulation groups [6].

Another factor to consider in terms of concentration is how to express it. All solid analytical results are usually reported on dry weight (d.w.) basis [204] because food samples are generally freeze-dried prior analysis. However, if instead of using freeze-dried samples wet samples are used, the results could also contain the moisture present in the samples and also some of their interferences, not just the solid portion. On the other hand, when the concentration is expressed in terms of dietary intake, it is usually reported in wet weight (w.w.) [205, 206]. In some cases, as mentioned above, contaminants are stored in fatty parts of the body, hence, the concentration can also be expressed as lipid weight (l.w.) [111, 205]. Conversions between dry and wet weight and dry and lipid weight are suitable using the percentage of moisture content (Eq. 2) and the percentage of lipid content (Eq. 3), respectively:

$$[\text{w.w.}] = \frac{[\text{d.w.}]\ \%\ \text{moisture content}}{100} \qquad \text{Eq. 2}$$

$$[\text{l.w.}] = \frac{[\text{d.w.}]\ 100}{\%\ \text{lipid content}} \qquad \text{Eq. 3}$$

where [w.w.], [l.w.] and [d.w.] are the contaminant concentrations in wet, lipid and dry weight, respectively. The reported concentrations on dry weight are higher than the same wet weight results, because in wet weight the concentrations are diluted at a specific moisture percentage. For lipophilic contaminants, the concentrations reported on a lipid weight are higher than dry weight because these chemicals have a high affinity for the lipid content. To obtain the moisture of samples, the most usual approach is to weight the amount of fish before and after the freeze-drying process. The differences between these two values, multiplied by 100, is the % of moisture content. Likewise, various lipid extraction methods can be used to obtain the lipid content of samples [159]. If these values are divided by the amount of fish and multiplied by 100, the % lipid content is obtained.

In addition, cooking methods can contribute to changes in contaminant concentrations in some foods. However, there is not general consensus on these issues. While some studies suggest that there is a decrease in the presence of contaminants in cooked fish [205, 207], others do not [137, 208, 209].The increase or decrease in the presence of EOCs after cooking seafood not only depends on the cooking process itself but also on the properties of the compounds studied. What is more, once the raw or cooked fish has been ingested, only the amount of contaminant absorbed by the

intestine, the bioaccessible part, would be considered for risk assessment studies [207, 210–212]. Unfortunately, the sort of study that target environmental contaminants and their relation with seafood consumption is scarce.

Overall, exposure assessment is important because it provides information about the nature of the source, the exposure route and the individuals who are exposed. Risk cannot be reliably estimated if exposure and its uncertainty are not properly characterised and sufficiently quantified. Uncertainties in risk assessment include considerations about different groups of the population, exposure time, food consumption data, sampling methods, body weight, concentration data and the, handling of non-detected and non-quantifiable limits [213]. Uncertainties should be characterised as transparently as possible to ensure adequate consideration in decision-making concerning the need for appropriate risk characterization.

Ultimately, the risk of human exposure to a particular chemical is determined to be a function of the intake and potency of the substance (Eq. 4):

$$\text{Risk} = [\text{intake of chemical}] \times [\text{chemical potency}] \qquad \text{Eq. 4}$$

Risk characterization is the process of estimating the likely incidence of adverse impact on potential receptors under a set of exposure conditions. The process normally includes specifying the uncertainties associated with risk estimates. Risk characterization involves integrating data and analysis of the first three components of the risk assessment process to determine the likelihood that the human being will experience any of the various forms of toxicity associated with a substance [193, 214]. The toxic characteristics of a substance are generally classified according to the main target organ/system (that is, that which is mostly affected) [189].

Chemicals may also be classified as carcinogenic and non-carcinogenic. Non-carcinogenic chemicals work with threshold mechanisms; in other words, the manifestation of the systematic effect requires a threshold level of exposure or dose that will be exceeded over a period of continuous exposure. Therefore, non-cancer toxicity is generally treated as if there is an identifiable exposure threshold level below which there are no observable adverse effects, and which means that continuous exposure to levels below the threshold will produce no adverse effects on health. Consequently, there is a range of exposure from zero to some finite value (threshold level) that may be tolerated by the exposed organism with no probability of adverse effects. This characteristic distinguishes the assessment criteria of non-carcinogenic and carcinogenic groups, since carcinogens are often treated as non-threshold processes, since there is no threshold for this group [200]. In general terms, the relationship between the degree of exposure to a chemical and the magnitude of the chemical-induced effects is typically described by a

dose-response curve. The dose-response curve is classified according to whether the chemical of concern is considered to be a threshold chemical, where no response is observed until a minimum dose is reached, or if it is considered to be a non-threshold chemical, which means that a response is expected for any dose [190]. The most important part of the dose-response curve for a threshold chemical is the dose at which significant effects first begin to show. The highest dose that does not produce an observable adverse effect is the "no-observed adverse effect level" (NOAEL) and the lowest dose that produces an observable adverse effect is the "lowest-observable adverse effect level" (LOAEL) [BFR34] (Figure 17).

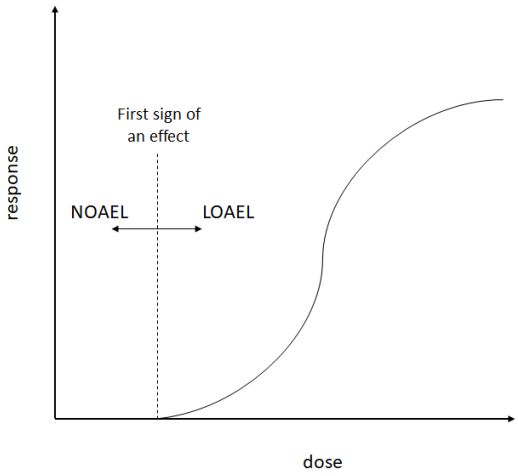

Figure 17. Dose-response curve for a threshold chemical.

Data are generally provided by animal studies. However, in the absence of complete monitoring information, mathematical exposure assessment models may be used. Traditionally, risk decisions on toxicity are made using the concept of "acceptable daily intake" (ADI) or by using the reference dose (RfD). ADI is the amount of a chemical (ng g^{-1} bw day^{-1}) to which a receptor can be exposed daily for an extended period of time without suffering a pernicious effect. To derive an ADI, for a cancer-free effect, it is common practice to apply uncertainty factors (UF) to NOAEL, LOAEL, or a RfD [214]. UFs are used to account for the uncertainties of extrapolation such as inter-individual variations, differences between species, duration of exposure, etc [213]. ADIs are usually calculated by dividing a NOAEL or LOAEL, derived from animal toxicity studies, by one or more uncertainty factors (Eq. 5):

$$ADI = \frac{NOAEL}{UF(s)} \qquad \text{Eq. 5}$$

Generally, the UF multiplies by 10 each factor that represents a specific area of uncertainty in the extrapolation of the available data [6, 213]. As mentioned above, the ADI for humans is often derived from animal experiments, and it is calculated on the basis of NOAEL. However, using NOAEL to determine ADI does have some drawbacks, as it is limited to one of the doses in the study and it depends on the study design. Alternatively, other methodologies are becoming more popular. One of these is the "benchmark dose" (BMD) approach. An important objective of the BMD approach is to define a starting point for the calculation of a reference value or a slope factor, which is more independent of the study design. The BMD method requires mathematical models to be fitted to dose-response data and the results are then used to select a BMD that is associated with a predetermined benchmark response (such as a 10% increase in the incidence of a particular lesion or a 10% decrease in body weight changes). The BMD has been defined as a lower confidence limit on the effective dose associated with a defined level of effect (5% or 10% increase in response). A dose-response relationship is adjusted to the bioassay data points and a confidence limit is determined for that ratio. BMD is the dose that gives the desired response rate (5% or 10%) based on the curve representing the confidence limit. Unlike NOAEL, BMD uses all the bioassay data and is not restricted to the doses administered in the experiment. On the other hand, there is no scientific basis for selecting a specific response rate for BMD and it is not easy to target BMD for continuous responses, as the dose at which a particular fraction of animal is affected by the toxicant needs to be identified [192].

Risk characterization serves as a bridge between risk assessment and risk management, which is a decision-making process that involves weighing policy alternatives and then selecting the most appropriate regulatory actions [156, 193].

In this doctoral thesis, two main approaches have been used for the chemical threshold (see below). The potential non-cancerous health effect resulting from a problem of exposure to chemicals is usually the risk factor (R_t). The R_t is defined by the relationship between the estimated chemical exposure level and the specific reference dose, represented as follows (Eq. 6):

$$R_t = \frac{E_t}{ADI_t} \times 100 \qquad \text{Eq. 6}$$

where E_t is the dietary intake of the chemical *t* (ng g^{-1} bw day^{-1}) and ADI_t is the acceptable daily intake of the chemical *t* (ng g^{-1} bw day^{-1}), obtained from a NOAEL value and selected

UFs. As R_t is expressed as a percentage value, the R_t values close to 100% represent a higher risk of the chemical studied, while values with a percentage far below 100% indicate a lower risk.

Another way of calculating or estimating risk is through the margin of exposure (MOE) approach, which is based on BMD values. MOE is the numerical value obtained by dividing the BMD value of the defined effect level (5% or 10%) by dietary intake (Eq. 7). Various expert committees, such as EFSA panels, have proposed the numerical band system for the MOE when it is based on the dose of BMD obtained from 10% of animal studies, indicating that when MOE is higher than 10000 concern is low [192, 200].

$$MOE_t = \frac{BMD_t}{E_t} \qquad \text{Eq. 7}$$

where, MOE_t is the margin of exposure for the dietary intake exposure to chemical *t*, E_t is the dietary intake of chemical *t* (ng g^{-1} bw day^{-1}), and BMDt is the benchmark dose corresponding to 10% incidence by the effect of chemical *t* (ng g^{-1} bw day^{-1}).

Nowadays, analytical studies tend to include a dietary intake assessment. However, risk characterization is sometimes lacking, as there is no information available in the scientific literature, especially regarding organic contaminants. Indeed, a summary of recent studies on the dietary intake of various contaminants through seafood consumption is provided in Table 2, as an overview of the importance that this field has acquired.

Table 2. Summary of recent studies on dietary intake through seafood consumption of various contaminants.

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Italy	Fish and mussels	PBDEs	∑PBDEs (fish): 524 ∑PBDEs (mussels): 494	∑PBDEs (fish and mussels): 0.29 ng kg^{-1} bw day^{-1}	• Mean concentration values (pg g^{-1} (w.w.)) • Dietary intake expressed as mean consumption • Risk was attainable for BDE47, BDE99, BDE153 and BDE209	[170]
Spain	Fish oil	Dechlorane plus (DP), dechlorane 602, 603, 604, anti-DP and syn-D	<LOQ-384.2	DP: 39-105 pg day^{-1}	• Range of all concentrations (pg g^{-1}) • Dietary intake expressed as mean consumption • Risk was calculated	[215]
Austria	Fish and shellfish	Tryptamine	2130	Children: 0.003-0.010 mg kg^{-1} bw day^{-1} Women: 0.007-0.023 mg kg^{-1} bw day^{-1} Men: 0.006-0.021 mg kg^{-1} bw day^{-1}	• Mean concentration values (mg kg^{-1}) • Dietary intake expressed as mean consumption	[216]

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Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Spain	Seafood	DDTs, PCBs, Benzo[a]pyrene, HCHs, HCB, As, Cd and Hg	∑DDTs: 0.1-2.2 ∑HCHs: 0.01-0.4 ∑HCB: 0.02-1 ∑PCBs: 0.4-3.6 Benzo[a]pyrene: 0.04-0.2 As: 177.7-538.7 Cd: 58.1-14.3 Hg: 27.3-45.2	Adult / Children ∑DDTs: 85 /43.7 ng day^{-1} ∑HCHs: 15.4 / 7.9 ng day^{-1} HCB: 36.7 / 18.4 ng day^{-1} ∑PCBs: 144.3 / 74.5 ng day^{-1} Benzo[a]pyrene: 9.3 / 5.3 ng day^{-1} As: 31849.8 / 22755.9 ng day^{-1} Cd: 2191.8 / 1458.4 ng day^{-1} Hg: 3569.3 / 2571.5 ng day^{-1}	• Range of concentrations (ng g^{-1} (d.w.)) • Dietary intake expressed as mean consumption • Risk was calculated	[217]
Korea	Fish and shellfish	Total As	Shrimps: 2.431 Mackerel: 1.296 Mussels: 1.407	105.5 μg day^{-1}	• Mean concentration values (mg kg^{-1} (w.w.)) • Dietary intake expressed as mean consumption in men and women	[218]

Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
China	Fish and seafood	BDE209, DBDPE, HBB, PBT, PBEB, PBBz and TBX	1900	8200 pg kg^{-1} bw day^{-1}	• Mean of all concentration values (pg g^{-1} (w.w.)) • Dietary intake expressed as mean consumption of all contaminants	[164]
Norway	Fish	BDE47, BDE99, BDE153, BDE209	-	BDE47: 0.29-1.91 ng kg^{-1} bw day^{-1} BDE99: 0.11-0.65 ng kg^{-1} bw day^{-1} BDE153: 0.03-0.42 ng kg^{-1} bw day^{-1} BDE209: 0.35-2.82 ng kg^{-1} bw day^{-1}	• Dietary intake expressed as range values	[219]
Italy	Seafood	Hg and Cd	Hg: 0.04-0.80 Cd: 0.02-0.26	Hg: 0.04-1.44 µg kg^{-1} bw $week^{-1}$ Cd: 0.04-0.32 µg kg^{-1} bw $week^{-1}$	• Range of concentrations (µg g^{-1} (w.w.)) • Dietary intake expressed as range values • Risk was estimated	[220]

Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Spain	Fish and seafood	As, Cd, Hg, Me-Hg and Pb	As: 3.2	As: 161 μg day^{-1}	• Mean concentration values (μg g^{-1} (w.w.))	[221]
			Cd: 0.050	Cd: 1.8 μg day^{-1}	• Dietary intake expressed as mean consumption value for adult men and comparison between ENCAT and ENIDE values were done	
			Hg: 0.22	Hg: 8 μg day^{-1}		
			Me-Hg: 0.17	Me-Hg: 5.7 μg day^{-1}		
			Pb: 0.028	Pb: 0.63 μg day^{-1}		
Spain	Fish and seafood	PBDEs	∑PBDEs: 3790.2	Adult: 0.178 ng kg^{-1} bw day^{-1}	• Mean concentration values (pg g^{-1} (w.w.))	[222]
				Children: 0.196 ng kg^{-1} bw day^{-1}	• Dietary intake expressed as mean consumption value for adult men (UB)	
					• Risk was calculated	

Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Spain	Fish	PCDDs, PCDFs	∑PCDDs: 0.007-0.179 ∑PCDFs: <0.003-0.277	11.6 pg WHO-TEQ	• Range of concentrations (ng kg^{-1} (w.w.)) • Dietary intake expressed as mean consumption of all contaminants using WHO-TEQ values	[223]
Spain	Fish and shellfish	PFASs	8.7-2700	Children: 0.06-5.83 ng kg^{-1} bw day^{-1} Boys: 0.02-5.59 ng kg^{-1} bw day^{-1} Girls: 0.02-5.77 ng kg^{-1} bw day^{-1} Male adult: 0.02-5.05 ng kg^{-1} bw day^{-1} Female adult: 0.02-6.37 ng kg^{-1} bw day^{-1} Male senior: 0.02-5.50 ng kg^{-1} bw day^{-1} Female senior: 0.02-4.55 ng kg^{-1} bw day^{-1}	• Range of concentrations (pg g^{-1} (w.w.)) • Dietary intake expressed as range values and assumed ND = 1/2 LOD	[224]

Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Hong Kong	Freshwater fish and marine fish	PBDEs, MeO-PBDEs, OH-PBDEs and BRPs	Freshwater fish/marine fish ∑PBDEs: 4.3 / 6.3 ∑MeO-PBDEs: $5.1 \cdot 10^{-1}$ / 1.4 ∑OH-PBDEs: 45.1 / 78.1 ∑BRPs: 32 / 35.4	Freshwater fish/marine fish ∑PBDEs: 2.8 / 6.1 ng kg^{-1} day^{-1} ∑MeO-PBDEs: 0.49 / 0.99 ng kg^{-1} day^{-1} ∑OH-PBDEs: 22 / 26 pg kg^{-1} day^{-1} ∑BRPs: 8.8 / 0 pg kg^{-1} day^{-1}	• Mean concentration values (ng g^{-1} (w.w.)). OH-PBDEs, and BRPs (pg g^{-1}) • Dietary intake expressed as mean consumption • Risk was calculated for BDE-47,BDE-99 and BDE-153	[163]
France	Shellfish	dl-PCBs, PCBs, PCDD/F and PAHs	∑dl-PCBs: 0.193-0.334 ∑PCBs: 2700-4977 ∑PCDD/F: 0.199-0.272 ∑PAHs: 39-337	∑PCDD/F + dl-PCBs: 1.8 ng kg^{-1} bw day^{-1} ∑PAHs: 1.4 ng kg^{-1} bw day^{-1}	• Range of concentrations (pg g^{-1} (w.w.)) • Dietary intake expressed as mean consumption using WHO-TEQ values	[225]

Table 2. (Cont.).

Country	Matrix	Contaminant	Concentration	Dietary intake	Remarks	Ref.
Sweden	Fish	PCDD/F, dl-PCB, PCBs, PBDEs, DDTs and HBCD	PCDD/F: 0.180 dl-PCB: 0.260 PCBs: 5.151 PBDEs: 0.422 BDE47: 0.249 HBCD: 0.145 DDTs: 3.29	PCDD/F + dl-PCBs: 0.294 pg kg^{-1} bw day^{-1} PCBs: 3.136 ng kg^{-1} bw day^{-1} PBDEs: 0.266 ng kg^{-1} bw day^{-1} HBCD: 0.091 g kg^{-1} bw day^{-1} DDTs: $2.04 \cdot 10^{-3}$ µg kg^{-1} bw day^{-1}	• Mean concentration values (ng g^{-1} (w.w.)). PCDD/F and dl-PCB (pg WHO-TEQ g^{-1}) from 2005 survey • Dietary intake expressed as mean value using total dietary intake and % of fish consumption	[171]

Upper-bound (UB); middle-bound (MB); lower-bound (LB); non-detected (n.d.); lower than limit of quantification (<LOQ); body weight (bw); Catalan food consumption survey (ENCAT); Spanish food consumption survey (ENIDE)

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1.4. References

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CHAPTER 2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

The main objective of this doctoral thesis is to determine the presence of different families of emerging organic contaminants such as synthetic musk fragrances, brominated flame retardants and benzothiazoles in seafood that is widely consumed in Tarragona County and assess the risk that their ingestion through diet represents to humans. These compounds were selected because they are widely used in common daily products. To achieve this objective:

- Several analytical methods based on gas chromatography coupled to tandem mass spectrometry will be adapted or developed. The extraction techniques involved in these methods will be QuEChERS, PLE and SPME.

- The analytical methods will be applied to different seafood species.

- Dietary intake will be evaluated for all the compounds studied and the risk of those compounds which have toxicological information available will be assessed.

- The bioaccessibility of the most used synthetic musk fragrances, galaxolide, will be studied by reproducing an *in vitro* human digestion process which will show how it is distributed throughout the digestive system.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As discussed in the introduction to this doctoral thesis, most EOCs are not effectively removed during conventional treatments at WWTPs. Therefore, because of their lipophilic characteristics several EOCs are bioaccumulated in aquatic organisms when effluent water is discharged.

In fact, new chemical compounds are still found daily in environmental matrices because of industrial processes. These compounds are accumulated in the environment as a result of their physicochemical properties and their being potentially considered as future EOCs.

As mentioned above, this doctoral thesis studies the presence of different families of EOCs in seafood and evaluates their dietary intake and risk through seafood. The groups of compounds focused on are synthetic musk fragrances, brominated flame retardants and benzothiazoles. Therefore, since they are present in water and sediment matrices they may also be expected in aquatic biota. Nevertheless, their risk assessment has not been extensively reported and toxicological data are scarce. This doctoral thesis has been carried out in the Chromatography and Environmental Applications research group at the Rovira i Virgili University, which has extensive experience in the determination of EOCs, and in the Laboratory of Toxicology and Environmental Health at the Rovira i Virgili University, which has extensive experience in assessing the risk of chemical substances. Most of the results of this doctoral thesis are part of the European Union's Seventh Framework Programme (FP7/2007-2013) under the ECsafeSEAFOOD project (no. 311820).

This chapter includes the experimental part and results from the various studies that have been carried out in the course of this doctoral thesis. These results have already been published, or are in the process of being published, in international scientific journals. The following sections are organized in terms of the aforementioned objectives: the determination of EOCs in seafood and dietary intake and risk assessment (Section 3.1) and the bioaccessibility of synthetic musk fragrances (Section 3.2). The results are presented in paper format. Each section contains a brief introduction that establishes the context of the research and the most notable results are discussed after the papers. The list of the published papers resulting from this doctoral thesis is included in Appendix II.

In the first section, analytical methods for the determination of synthetic musk fragrances, brominated flame retardants and benzothiazoles are used to assess their presence in seafood samples, and the dietary intake and risk for human health through seafood consumption are calculated. GC-IT-MS/MS was used for all three studies and various extraction techniques were applied such as QuEChERS and PLE with organic solvent, and water followed by SPME. The methods were used to analyse seafood samples from local markets and supermarkets in Tarragona County. The method for determining benzothiazoles in seafood samples was developed for the first time because there is no literature on the presence of these compounds in seafood whereas the methods for determining synthetic musk fragrances and BFRs were adapted from previous studies.

In the second section, the *in vitro* bioaccessibility of HHCB in seafood is evaluated. This study was carried out in collaboration with the Portuguese Institute for Sea and Atmosphere (IPMA) from the Division of Aquaculture and Upgrading (DivAV).

3.1. Determination of emerging organic contaminants in seafood

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This section focuses on the determination of such groups of EOCs as synthetic musk fragrances, brominated flame retardants and benzothiazoles in seafood samples.

As mentioned in the introduction, these EOCs are widely used in therapeutic products, and daily products such as cosmetics and detergents, textiles and plastics, fungicides and herbicides, and they show hazardous properties that could be dangerous for the ecosystem and humanity. The EOCs included in this section are easily released into the environment and they have been detected in wastewater, surface water, river water and sediments [1–6]. For this reason, they can be expected to be present in seafood samples [7–9]. Therefore, the main objective of these studies is to determine the presence of the aforementioned compounds in seafood samples in Tarragona County and to evaluate the dietary intake and risk characterization for human health. As previously discussed in the introduction, several methods have already been developed for determining synthetic musk fragrances and brominated flame retardants by GC, but GC methods for the determination of benzothiazoles are very scarce, and particularly so for seafood samples.

Due to the different physicochemical characteristics that present these type of compounds, several methods will be used to determine them in seafood by GC-IT-MS/MS. A rapid, sensitive and accurate method will be adapted from Vallecillos *et* al [10] to determine polycyclic and nitro musk fragrances, and HHCB-Lactone, a degradation product of galaxolide. The extraction technique used will be QuEChERS extraction, a recent but well-established extraction methodology in the field of food analysis [11] that has increasingly been used in environmental analysis in recent years [12] because in comparison with instrumental extraction techniques it is quick, easy to use and cheap, and has minimal solvent requirements It can be very useful for monitoring studies. To efficiently extract the target compounds, several clean-up strategies for reducing the matrix effect (ME) will be tested (for example, the amount of florisil used as the clean-up sorbent, the addition of acid to disrupt compound-protein bindings and the use of both strong anion and cation exchange sorbents to retain charged impurities).

However, to determine PBDEs, MeO-PBDEs, HBB, DBDPE and PBEB, the Barón *et al* [13] method will be adapted using PLE as extraction technique, followed by SPE with Al-N cartridges as clean-up.

In contrast, a new method for determining five benzothiazoles in seafood that uses subcritical water extraction (SBWE) followed by SPME as preconcentration/clean-up technique will be performed for the first time. The PLE technique using water instead of organic solvents has become a powerful technique for extracting a wide range of polar organic compounds from environmental matrices [14]. In addition, water is more environmentally friendly than other organic solvents. One of the main advantages of using water as extraction solvent in PLE is that the SPME step can be applied directly with no need for the evaporation step when solvent exchange is necessary. To optimise the extraction process, on-cell clean-up and liquid-liquid extraction (LLE) will be tested in order to remove matrix interferences such as fats and oils from seafood samples.

A formula based on the combination of seafood consumption data and the concentration of these analytes will be used to assess the dietary intake of these compounds in the population living in Tarragona County through seafood consumption. In addition, the risk will be characterized using the NOAEL values (Non Observable Adverse Effect Levels) from the European Chemical Agency (ECHA) [15, 16] in the case of synthetic musk fragrances and benzothiazoles, whereas in the case of brominated flame retardants, the risk will be characterized using the MOE approach (Margin of Exposure) due to the limitation of NOAEL values for BFR compounds.

The results of these studies have been published in the *Environmental Research 143 (2015) 116-122, Food and Chemical Toxicology 104 (2017) 48-56* and *Analytical and Bioanalytical Chemistry 409 (2017) 5513-5522.*

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3.1.1. Exposure of the population of Catalonia (Spain) to musk fragrances through seafood consumption: Risk assessment

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EXPOSURE OF THE POPULATION OF CATALONIA (SPAIN) TO MUSK FRAGRANCES THROUGH SEAFOOD CONSUMPTION: RISK ASSESSMENT

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Abstract

The occurrence of ten synthetic musks in samples of 10 widely consumed fish and shellfish species from Tarragona (Catalonia, Spain) was determined. The most used nitro and polycyclic musks, as well as a well-known transformation product in tissues, were analysed. Furthermore, the human health risks derived from the musk exposure through seafood consumption were characterized. None of the nitro musks were detected in any of the analysed samples. In contrast, most of the polycyclic musks were found, being galaxolide (HHCB) and tonalide (AHTN) present in all the samples. HHCB was the greatest contributor, with maximum levels in sardine and mackerel (367 and 304 ng g^{-1} (d.w.) (dry weight), respectively). The highest exposure to individual musks was estimated for HHCB and HHCB-Lactone, with average values of 19.7 and 6.8 ng kg^{-1} bw day^{-1}, respectively, in adults. A notably lower mean exposure was calculated for AHTN, DPMI and ATII, being ranged between 1.1 and 3.7 ng kg^{-1} bw day^{-1}. The current concentrations of musks in fish and shellfish should not mean human health risks for the adult population living in Tarragona. However, a continuous monitoring would be desirable to assure that the exposure does not follow increasing temporal trends.

Keywords: *Synthetic musks; fish; chromatographic analysis; occurrence; dietary intake*

Abbreviations: *Dispersive solid-phase extraction, dSPE; dry weight, d.w.; emerging organic contaminants, EOCs; gas chromatography-ion trap-tandem mass spectrometry, GC-IT-MS/MS; Quick, Easy, Cheap, effective, Rugged and Safe, QuEChERS; lipid weight, l.w.; matrix effect, ME; method detection limits, MDLs; method quantification limits, MQLs; relative standard deviations, RSDs; sewage treatment plants, STPs; wet weight, w.w.*

1. Introduction

In recent years, the environmental fate of emerging organic contaminants (EOCs) has been an issue of increasing interest for the scientific community. EOCs may be released to the environment by a number of natural and anthropogenic sources, being potentially toxics to the humans and the ecosystems. Among the broad variety of EOCs, much attention has been paid to synthetic musks. These are cyclic EOCs used not only as fragrances in a wide variety of daily products such as personal care products (e.g., perfumes, skin creams, soaps) and household products (e.g., detergents, softeners), but also as food additives (Bester, 2009; Budd, 2013).

Synthetic musks also comprise a broad range of different compounds which can be divided according their chemical structure, including nitro, polycyclic, macrocyclic and alicyclic musks. Although nitro musks have been traditionally the predominant compounds in the commercial market, their use has been decreasing due to their bio-accumulative and toxicological properties. These are related to the presence of a nitro-aromatic group in their structures, as well as their high stability against chemical and biological degradation (Homem et al., 2013). In this respect, the European Union banned the use of some nitro musks (musk ambrette, musk moskene and musk tibetene) in cosmetic products, while others (musk xylene and musk ketone) have been restricted (Commission Directive 98/62/EEC; Regulation 1223/2009/EC). Contrasting with the decreasing use of nitro musks from 1990s, polycyclic musks have been increasingly utilised. Galaxolide and tonalide are the two predominant polycyclic musks, being also identified in the high production list of some environmental protection agencies. Although there is no consensus yet regarding the potential endocrine disrupting activity of polycyclic musks, the use of tonalide in the cosmetic industry has been recently regulated by the European Union (Commission Directive 2008/42/EC). Since they smell more intensely and degrade more easily in the environment, macrocyclic musks have emerged as a potentially good alternative to polycyclic compounds. Notwithstanding, their synthesis price is still too high to be considered as a viable option (Bester, 2009; Vallecillos et al., 2013). Finally, the use of alicyclic musks, as well as the investigations focused on their toxicological properties, is still very scarce. Although alicyclic musks have been suggested to biodegrade (Seyfried et al, 2014), more studies are necessary to confirm it.

The widespread use of synthetic musks in consumer products means that they are flushed down into sewer systems after their usage, entering sewage treatment plants (STPs). Although partial elimination in STPs has been reported for nitro as well as for polycyclic musks, little information is available for macrocyclic musks. Moreover, no data have been published for alicyclic musks (Ligon et al., 2008; Lopez-Nogueroles et al., 2013; Vallecillos et al., 2015). Moreover, some synthetic musks can be transformed in STPs as well as in biota, being

transformation products even more problematic than the parent musks (Rimkus et al., 1999), in terms of potential toxicity. Due to the lipophilic characteristics of synthetic musks and their slow biodegradation, a bioconcentration trend can be expected in surface water (Posada-Ureta et al., 2012), sludge (Vallecillos et al., 2012), sediments (Hu et al., 2011) and living species, such as fish (Subedi et al., 2011; Subedi et al., 2012; Zhang et al., 2013). Although dermal absorption from personal care products applications is reported as one of the major sources of exposure to musk fragrances, other exposure pathways such as dietary intake or inhalation cannot be disregarded (Roosens et al., 2007). Some studies have confirmed a relatively high presence of musks in fatty fish (Kannan et al., 2005). The consumption of seafood in Spain is notorious, contributing to the intake of up to 27 % of protein and 14% of polyunsaturated among adults (AECOSAN, 2014). Similarly to other environmental contaminants, such as persistent organic pollutants or heavy metals (Domingo et al., 2012a, b; Perelló et al., 2014), diet could be the most important route of exposure to musks. Unfortunately, there is a complete lack of knowledge regarding the dietary intake of musks, in front of other potential exposure pathways.

The European Chemical Agency (ECHA) provided human risk assessment reports for HHCB (galaxolide), AHTN (tonalide), MX (musk xylene) and MK (musk ketone), where respective Oral Non Observed Adverse Effect Levels (NOAEL) of 150, 5, 7.5 and 2.5 mg kg^{-1} bw day^{-1} were established from studies with rats (ECHA 2008a,b). The toxicological information concerning cashmeran, celestolide, phantolide, traseolide or HHCB-Lactone is sparse and non-harmonised by panels of scientific experts. Therefore, threshold values for risk characterization have not been established yet.

The main goal of this study was to determine the occurrence of ten synthetic musks in tissues from ten widely consumed fish and shellfish species in Tarragona region (Spain). The content of the most used nitro and polycyclic musks, as well as one well-known transformation product, was determined. This information was used to assess the dietary exposure of the general population to these fragrances through seafood consumption and to characterize the human health risks.

2. Materials and methods

2.1. Reagents and standards

The six polycyclic musks studied, supplied by Promochem Iberia (Barcelona, Spain), were the following: 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-tert-butyllindane (ADBI, celestolide), 6-acetyl-1,1,2,3,3,5-hexamethyllindane (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-tetrahydronaphtalene (AHTN, tonalide). Two 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} individual solutions in acetonitrile from Sigma-Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The musk fragance 4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (MK, musk ketone) was provided by Fluka (Buchs, Switzerland). International Flavors & Fragrances Inc. (Barcelona, Spain) supplied 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl cyclopenta-[g]-2-benzopyran^{-1}-one (HHCB-Lactone, galaxolidone), while the internal standard d15-musk xylene (d15-MX) came as a 100 µg mL^{-1} solution in acetone from Symta (Madrid, Spain).

Individual standard solutions of the synthetic musks were prepared in acetone at concentrations of 4000 µg mL^{-1}, for polycyclic musks, and 1000 µg mL^{-1}, for musk ketone and HHCB-Lactone. A working mixture solution of 100 µg mL^{-1} was prepared in methanol, containing all of the compounds except for MX, MM, and d_{15}-MX, as well as HHCB-Lactone. An individual working solution of HHCB-Lactone was prepared in methanol at the same concentration (100 µg mL^{-1}). Acetone and methanol were GC grade with purity >99.9% from Prolabo (VWR, Llinars del Vallès, Barcelona, Spain) and from SDS (Peypin, France), respectively. 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 chromatographic analysis (Carburos Metálicos, Tarragona, Spain).

2.2. Sampling and sample pre-treatment

Seafood species were selected among those most widely consumed by the Catalan population. Samples of hake (*Merluccius merluccius*), tuna (*Thunnus Thynnus*), sole (*Solea solea*), sardine (*Sardina pilchardus*), codfish (*Gadus morhua*), salmon (*Salmo salar*), mackerel (*Scomber scombrus*), squid (*Loligo vulgaris*), shrimp (*Aristeus antennatus*) and mussel (*Mytilus galloprovincialis*) were purchased from three different commercial establishments (supermarket, local market and fish store) in Tarragona, Spain. This practice ensured a different origin of the samples. After collection, the samples were immediately preserved in a refrigerator box. Lateral fillets were then dissected from the fish, and the shells of mussels and shrimps were taken off. Subsequently, they were homogenized and stored in a freezer until analysis. Frozen homogenized samples were lyophilized using the freeze-drying system (Labconco, Kansas City, MO, USA) and crushed using a mortar and pestle. In addition, mussel samples were also sieved through a 125 µm screen to homogenize the diameter of the particles. Then, a composite sample for each species was obtained by mixing equal amounts from the three commercial establishments.

2.3. *Analytical method*

For sample analysis, synthetic musks were first extracted by QuEChERS (Quick, Easy, Effective, Rugged and Safe)

extraction, and then determined by gas chromatography-ion trap-tandem mass spectrometry (GC-IT-MS/MS).

For QuEChERS extraction, 0.5 g (d.w.) of freeze-dried composite sample was weighed into 50 mL centrifuge tubes from Scharlab (Barcelona, Spain). Ten mL of ultrapure water was added to the tube, being shaken for 1 min. Then, 10 mL of acetonitrile (Prolabo) was added and followed by an extraction salt packet (Scharlab) according the Standard Method EN 15662 (Lehotay et al., 2010). This contained 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate tribasic dihydrate. Then, the mixture was vortexed for 3 min and centrifuged for 5 min at 7000 rpm (Hettich Universal 32R, Tuttlingen, Germany). The acetonitrile layer (supernatant) was removed and transferred to a 15 mL centrifuge tube containing 2 g of florisil (Sigma-Aldrich) for the dSPE (dispersive solid-phase extraction) clean-up. The tube was vortexed for 3 min and centrifuged again at the same conditions mentioned before. The supernatant was evaporated under a gentle stream of nitrogen to a final volume of, approximately, 1 mL. Then, the internal standard was added at a concentration of 0.1 μg mL^{-1} and the extract was reconstituted to 2 mL with ethyl acetate (Prolabo). Extracts were filtered with a 0.22 μm PTFE syringe filter and analysed by GC-IT-MS/MS.

The chromatographic 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) equipped with a 10 μL syringe of 23 gauge and point style 5 (Hamilton, Bonaduz, 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. The chromatographic separation was carried out on ZB-50 analytical column (50% phenyl-dimethylpolysiloxane, 30 m x 0.25 mm i.d.; 0.25 μm film thickness) from Micron Phenomenex (Torrance, California, USA). 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 and hold 3.4 min. The carrier gas employed was helium with 99.999% of purity at a constant flow rate of 1 mL min^{-1}. The target compounds were separated in 15 min. 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 rate of 50 mL min^{-1} the 1079 injector temperature was set at 70°C. 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, tandem mass spectrometry (MS/MS) mode was applied. Table 1 summarizes the retention time

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)	m/z range	Scan time (s/scans)
DPMI	7.58	191	107, **135**, 173	0.82	84.1	94-201	1.08
ADBI	8.68	229	131, **173**, 187	0.92	100	110-239	1.08
AHMI	9.14	229	131, 145, **187**	0.92	100	110-239	1.08
ATII	9.79	215	131, 171, **173**	0.88	94.7	104-225	1.03
HHCB [a]	10.00	243	171, **213**	0.96	122	132-253	0.53
AHTN [a]	10.06	243	**145**, 159, 187	0.96	103	113-253	0.53
MX [b]	10.67	282	**265**, 266, 281	1.08	124.2	134-292	0.34
MM [b]	10.74	263	187, 201, **211**	1.02	115.9	125-273	0.34
MK	12.53	279	**191**, 247, 280	1.07	122.9	132-289	1.05
HHCB-Lactone	15.58	257	183, 201, **239**	1.00	113.2	123-267	1.03
d15-MX [b]	10.49	294	170, **276**, 295	1.11	129.5	139-304	0.34

[a], [b] Compounds were separated using the Multiple Reaction Monitoring mode.
[c] Quantification ions (m/z) are shown in bold type.

and the optimal MS parameters for each compound.

2.4. Exposure assessment and risk characterization

The dietary exposure of the general population of Tarragona to musks was estimated by means of a deterministic method, combining consumption and pollution data, as it follows:

$$E_t = \sum_{f=1}^{p} C_f X_{t,f} \qquad \text{Eq. 1}$$

Where E_t is the global dietary exposure to the musk fragrance *t* in the general population (ng kg^{-1} bw day^{-1}), C_f is the mean consumption of the seafood species *f* by population (g kg^{-1} bw day^{-1}), and $X_{t,f}$ represents the concentration of musk *t* in the seafood species *f* (ng g^{-1}). The mean consumption was previously normalized, dividing the dietary intake with the mean body weight. Contamination data was implemented on a fresh weight basis. The concentration of non-detected samples was assumed to be one-half of the method detection limits.

The dietary risk was characterized by dividing the global dietary exposure by the provisional tolerable daily intake, estimated from the oral NOAEL, and considering an uncertainty factor of 100. The equation used to characterize the risk was:

$$R_t = \frac{E_t}{pTDI_t}\, x\, 100 \qquad \text{Eq. 2}$$

Where R_t is the health risk due to the dietary exposure to the musk *t*, E_t is the global dietary exposure to the musk fragrance *t*, and $pTDI_t$ is the provisional tolerable daily intake for the musk fragrance *t*, (ng kg^{-1} bw day^{-1}).

Consumption data were gathered from the Spanish Dietary Intake National Survey (ENIDE, acronym in Spanish) performed by the Food Safety and Nutrition Spanish Agency (AECOSAN, 2012). This survey was carried out between 2009 and 2010 with 3000 individuals (50 % males and females). The recruited participants were aged between 18 and 64, representing the whole Spanish geography, including the islands, and both, urban and rural populations. The consumption data was provided by means of a 3-day dietary record, 24 h record and a food frequency questionnaire. The seafood species were selected among those most consumed among the population. The consumption rates of the general population to each one of the analysed species (hake, tuna, squid, shrimps, sole, sardine, cod, salmon, mussels and mackerel) are summarized in Table 2.

3. Results and discussion

3.1. Development and validation of the analysis method

The method developed to determine the selected synthetic musks in fish and shellfish was based on a previous method (Vallecillos et al., 2015b).

Table 2. Mean consumption of a selection of ten seafood species by Spanish population (units in g day^{-1}).

Seafood species	Foodex1 code	Male	Female	Combined	
		Mean	*Mean*	*Mean*	*P99*
Hake	A.01.000895	15.02	13.10	14.02	125.00
Sole	A.01.000899	4.02	4.35	4.20	100.00
Cod	A.01.000894	4.26	2.78	3.48	76.67
Shrimps	A.01.000923	5.07	5.18	5.13	72.08
Squid	A.01.000928	5.61	5.01	5.30	100.00
Salmon	A.01.000883	2.81	2.95	2.88	68.33
Tuna	A.01.000891	7.16	6.11	6.61	50.00
Mackerel	A.01.000890	0.73	0.24	0.47	8.33
Sardine	A.01.000880	4.62	2.92	3.73	100.00
Mussels	A.01.000934	0.48	0.69	0.59	66.67

However, several clean-up strategies were tested in the present method in order to reduce the main drawback in the analysis of complex matrices by mass spectrometry, the so-called matrix effect. For each clean-up strategy tested, the matrix effect (ME) was calculated according to the following equation:

$$ME\% = ([(B-n)-A]/A) \times 100 \qquad \text{Eq. 3}$$

Where *B* is the peak area of the analyte from a mussel sample spiked after QuEChERS extraction, *n* is the peak area of the analyte present in the mussel sample, and *A* is the peak area of the analyte from a standard solution directly injected in the chromatographic system. According to it, ME<0 indicates ion suppression, ME=0 confirms there is no matrix effect, and ME>0 shows ion enhancement.

In QuEChERS extraction, clean-up is usually accomplished by using dSPE. Florisil was selected from a previous study as the best sorbent for this clean-up strategy (Vallecillos et al., 2015b), being tested different amounts (1, 2 and 3 g). All analytes showed signal enhancement, which was reduced for most of them by using 2 g of florisil. In turn, a higher amount (3 g) did not show any additional reduction. In

consequence, 2 g of florisil was selected as the dSPE sorbent. The matrix effect was thus reduced in comparison to 1 g of florisil, with ranges of 30-52% and 3-9% for polycyclic and nitro musks, respectively. Under final conditions, the compounds less affected by the matrix effect were AHTN and MK (ME%: 9 and 8, respectively), while those most affected were MX, HHCB, AHMI and MM (ME% 61, 50, 48 and 48, respectively).

Additional clean-up strategies were also tested. One of these was the addition of 2% formic acid into the acetonitrile layer. This practice is suggested to allow disrupting the compound-protein bindings more easily (Martínez Bueno et al., 2013), facilitating the reduction of the matrix effect. Moreover, a post-extraction clean-up with both strong anion and strong cation exchange sorbents was tested due to their affinity to retain charged impurities. Since those strategies were not successful here, they were discarded.

The method was validated by establishing the linear ranges, method detection limits (MDLs), method quantification limits (MQLs), intra-day and inter-day repeatability, and apparent recoveries. Although the matrix effect was substantially reduced by the clean-up step, matrix-matched calibration curves were used for quantification in order to obtain more accurate results. Moreover, species were classified into 3 groups (white fish, fatty fish, and mussels) according to their lipid content. A species from each group was selected to validate the method. Hake was selected as the representative species for white fish (sole, cod, shrimp and squid), while salmon was the representative of the fatty fish (tune, mackerel and sardine), respectively.

Linear ranges, MDLs and MQLs were obtained by spiking hake, salmon and mussel samples at different levels (d15-MX 0.1 μg mL^{-1}) (Table 3). Blank samples were also analysed, and peak areas of compounds detected were subtracted from the corresponding peak areas of each spiked sample. A good linearity for all the compounds (R^2>0.98) was achieved. MQLs, which were defined as the lowest calibration point, were lower for polycyclic musks than for nitro musks, independently of the matrix. This would indicate sensitivity differences. MDLs corresponded to a concentration that gives a signal/noise ratio equal to 3 for the compounds not present in the blank samples. While MDLs were estimated from recoveries and mass spectrometry responses for compounds present in the blank samples. MDLs agree with previous results (Vallecillos, 2015b), being also lower than those reported in the literature working with other extraction techniques combined with GC-IT-MS/MS (Mottaleb et al., 2009; Subedi et al., 2011). Intra-day and inter-day repeatability, expressed as relative standard deviation (RSD), were calculated by spiking (n=5) samples at 50 ng g^{-1} (d.w.) for all the compounds. They were lower than 21% for all compounds in each matrix. Apparent recoveries, which are referred to the overall method, were calculated by spiking (n=5) the three matrices at 50 ng g^{-1} (d.w.).

Table 3. Validation data.

Compounds	MDLs (ng g^{-1})			Linear range (ng g^{-1}) [a]			Apparent recovery (%) [b]		
	Hake	Salmon	Mussel	Hake	Salmon	Mussel	Hake	Salmon	Mussel
DPMI	2	4	4	5-100	10-100	10-250	68	95	47
ADBI	2	2	4	5-100	5-100	10-250	57	77	66
AHMI	2	2	4	5-100	5-100	10-250	82	80	71
ATII	2	2	2	5-100	5-100	5-250	61	67	55
HHCB	1	1	1	5-400	5-400	5-400	105	109	104
AHTN	1	1	1	5-400	5-400	5-400	99	98	64
MX	5	5	10	20-250	20-250	30-250	90	117	86
MM	5	5	10	20-250	20-250	30-250	103	113	67
MK	5	5	10	20-250	20-250	30-250	102	104	105
HHCB-Lactone	2	4	5	5-400	10-400	15-250	76	104	105

[a] MQLs (ng g^{-1}) were fixed as the lowest calibration point.
[b] Apparent recoveries (%), n=5, 50 ng g^{-1} (d.w.)
RSDs < 21%, n=5, 50 ng g^{-1} (d.w.)

Recoveries ranged from 47% to 117% (Table 3), and no significant differences between matrices were noted.

3.2. Occurrence of musk fragrances in commercial seafood

Samples of 10 species of fish and shellfish of wide consumption among the general population of Catalonia were here analysed. Composite samples for each species were used in order to avoid origin influence. The average concentrations of musk fragrances in these 10 commercial seafood species, expressed as dry weight (d.w.), are summarized in Table 4. As expected, none of the nitro musks were present in any of the samples analysed, probably due to current regulations. These data contrasts with results from previous investigations performed before nitro musks were subject to regulation (Rüdel et al., 2006; Schmid et al., 2007). Most polycyclic musks were identified in several samples. HHCB and AHTN were present in all the analysed samples. HHCB showed the highest concentrations, being sardine and mackerel the species with the greatest values (367 and 304 ng g^{-1} (d.w.), respectively). These results are consistent with the fact that HHCB and AHTN are the musk fragrances usually found at higher concentrations in sewage and superficial waters (Subedi et al., 2011; Posada-Ureta et al., 2012). The relatively high concentrations of HHCB also agree with reported results in fish tissue (Schmid et al., 2007). HHCB-Lactone, the main transformation product of HHCB, was also present in some samples of white and fatty fishes, at concentrations ranging from 20 to 91 ng g^{-1} (d.w.). DPMI and ATII were also found in most of the samples, being ranged between <MQL and 11 ng g^{-1} (d.w.). Finally, none of the samples contained ADBI and AHMI at detectable concentrations.

Hake was the species which showed the highest number of musk fragrances (five) present in seafood tissue. Concentrations in hake were ranged between 6.1 ng g^{-1}, for ATII, and 34.3 ng g^{-1} (d.w.), for HHCB. In turn, only two musk fragrances (HHCB and AHTN) were identified. However, the individual concentrations of musks in mackerel and sardine were more important, being notably important the level of HHCB. For comparative purposes, musk concentrations in aquatic species found in a number of countries are listed in Table 5.

3.3. Dietary intake of musks and risk characterization

The results from the current survey identified DPMI, ATII, HHCB, AHTN and HHCB-Lactone as the main musk congeners in seafood species. Because the remaining compounds were below the MDL or the MQL in the whole range of species, they were excluded from the exposure assessment study. Left-censored data of selected musks was managed by substitution of non-detected or non-quantified values by the half of limit of detection. Exposure and risk assessment estimates are summarized in Table 6, which shows the global exposure and the relative contribution of each species, for both

Table 4. Concentration of 10 musk fragrances (ng g^{-1} (d.w.)) in samples of 10 seafood species from Tarragona, Spain.

Compound	White fish					Fatty fish				Mussel
	Hake (*Merluccius merluccius*)	Sole (*Solea solea*)	Cod (*Gadus morhua*)	Shrimp (*Aristeus antennatus*)	Squid (*Loligo vulgaris*)	Salmon (*Salmo salar*)	Tune (*Thunnus thynnus*)	Mackerel (*Scomber scombrus*)	Sardine (*Sardina pilchardus*)	Mussel (*Mytilus galloprovincialis*)
DPMI	10.2	11.2	9.0	<MQL	n.d.	<MQL	n.d.	<MQL	<MQL	<MQL
ADBI	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AHMI	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATII	6.1	<MQL	<MQL	n.d.	n.d.	<MQL	<MQL	7.9	n.d.	<MQL
HHCB	34.3	18.8	146.7	15.8	41.6	17.1	38.4	303.9	367.3	49.1
AHTN	10.9	6.6	11.8	12.3	15.2	<MQL	5.5	16.0	31.3	n.d.
MX	n.d.	n.d.	n.d.	n.d.	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.	n.d.	n.d.	n.d.	n.d.
MK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HHCB-Lactone	33.8	n.d.	19.8	20.1	n.d.	n.d.	n.d.	61.2	91.1	n.d.

MQL: method quantification limit; n.d.: not detected.

Table 5. Levels of musk fragrances in aquatic species from United States and Canada.

Country	Musk fragrances	Aquatic species	Mean	Range	Units [a]	Reference
US	HHCB	Atlantic salmon		<1–3.2	ng g^{-1} ww	Kannan et al. 2005
	AHTN			<1-1.6	ng g^{-1} ww	
US	HHCB	Atlantic sharpnose shark	4.8	4.6–5.2	ng g^{-1} ww	Kannan et al. 2005
	AHTN		1.6	1.4–1.7	ng g^{-1} ww	
US	HHCB	Smallmouth bass	4.8	4.3–5.4	ng g^{-1} ww	Kannan et al. 2005
	AHTN		1.8	1.6–1.9	ng g^{-1} ww	
US	HHCB	Farmed Shrimps	198	48-683	ng g^{-1} lw	Sapozhnikova et al., 2010
		Wild Shrimps	334	66-762	ng g^{-1} lw	
	AHTN	Farmed Shrimps	185		ng g^{-1} lw	
		Wild Shrimps	384		ng g^{-1} lw	
Canada	HHCB	Lake trout	-	29-34	ng g^{-1} lw	Gatermann et al. 1999
		Mussels	1650	-	ng g^{-1} lw	
	AHTN	Lake trout		25-45	ng g^{-1} lw	
		Mussels	<MDL		ng g^{-1} lw	
	MX	Lake trout		2-4	ng g^{-1} lw	
		Mussels	<MDL		ng g^{-1} lw	
	MK	Lake trout		76-730	ng g^{-1} lw	
		Mussels	2200		ng g^{-1} lw	

[a] Units: ww: wet weight; lw: lipid weight

average and high consumers (percentile 99). The highest exposure was estimated for HHCB and HHCB-Lactone, with mean values of 19.7 and 6.8 ng kg^{-1} bw day^{-1}, respectively. Relatively lower values were calculated for AHTN, DPMI and ATII, the mean exposure was ranged between 1.1 and 3.7 ng kg^{-1} bw day^{-1}.

Despite the concentrations of musk were mostly higher in fatty fish than in white fish, the high consumption of the latest group (0.48 g kg^{-1} bw day^{-1} vs. 0.24 g kg^{-1} bw day^{-1}) led to a higher exposure of musks. The accumulated contribution of white fish to the global musk exposure was ranged between 55 and 84% for mean consumers. In case of fatty fish, its contribution was ranged between 11.5 and 49.5%. Hake, the most consumed fish in the country, was the main contributor to the global exposure of musk congeners, with the only exception of HHCB. Other relevant sources of HHCB-Lactone were sole, cod and shrimps. The high consumption of sardines also revealed a higher exposure of HHCB, HHCB-Lactone and AHTN, reaching contribution estimates between 14 and 31 %.

Due to the very limited data concerning the oral safety levels of musk, the risk characterization was only feasible for HHCB and AHTN. We used a provisional TDI (pTDI) estimated from the oral NOAEL of 150 and 5 mg kg^{-1} bw $day^{-1}$for HHCB and AHTN, respectively. These values were divided 100 as an uncertainty factor (ECHA, 2008a,b). Therefore, the pTDIs used in the present study were finally 1500 and 50 μg kg^{-1} bw day^{-1} for HHCB and AHTN, respectively. Considering the global exposure for mean and high consumers, the risk was estimated to be rather low for both compounds. Even under a worst-case scenario (p99), the exposure through seafood consumption was far from the pTDI, with percentages ranged 0.03-0.12%.

Some scientific studies suggest that the relevance of dermal exposure could be even higher than that of inhalation or oral exposure. Dermal exposure was estimated by Roosens et al. (2007), who reported daily amounts of 3.36 mg of HHCB (24000 μg day^{-1}, assuming a mean absorption rate of 14%). In turn, Correia et al. (2013) calculated a lower contribution of the dermal exposure in the total intake of HHCB, reaching 904 μg day^{-1}. Assuming 70 kg of body weight, this exposure would represent around 12914 ng kg^{-1} bw day^{-1}), apparently higher than the dietary exposure through seafood consumption estimated in the present study (19.7 ng kg^{-1} bw day^{-1}). However, the main drawback comes from the large uncertainty associated with the absorption and thus, the final fate of musk that reaches the systemic circulation. While gastrointestinal absorption is assumed to be around the 50%, dermal absorption has been estimated in the wide range between 0.1 and 16%. If we consider also the evaporation of musks on human skin (around 20 %), the final amount of fragrances reaching the systemic circulation through dermal exposure can be expected to be very low.

Our results agree with those from a biomonitoring study where plasmatic levels of HHCB were positively related

Table 6. Exposure estimates (mean and percentile 99) of DPMI, ATII, HHCB, AHTN and HHCB-Lactone from seafood consumption among Spanish population. Columns headed by C (%) represent the percentage of contribution of each seafood specie to the global exposure of each compound. Risk characterization was only estimated for HHCB and AHTN due to the lack of harmonized oral NOAEL levels for the rest of compounds. Units in ng kg^{-1} bw day^{-1}.

Seafood species	DPMI				ATII				HHCB				AHTN				HHCB-Lactone		
	Mean	C(%)	p99	C(%)	Mean	C(%)	p99	(%)	Mean	C(%)	p99	C(%)	Mean	C(%)	p99	(%)	Mean	C(%)	p99
Hake	1.00	45.9	10.18	28.7	0.59	55.3	6.07	39.3	3.33	16.9	34.11	8.6	1.06	28.6	10.89	18.2	3.30	48.8	33.75
Sole	0.39	17.9	9.00	25.4	0.06	5.8	1.43	9.3	0.65	3.3	15.00	3.8	0.23	6.1	5.29	8.8	0.06	0.9	1.43
Cod	0.26	12.1	5.48	15.4	0.05	4.9	1.10	7.1	4.29	21.8	89.70	22.7	0.35	9.3	7.23	12.0	0.58	8.6	12.16
Shrimps	0.07	3.3	1.03	2.9	0.07	6.8	1.03	6.7	0.64	3.2	9.06	2.3	0.49	13.2	7.00	11.7	0.81	12.0	11.53
Squid	0.11	5.2	1.43	4.0	0.11	10.5	1.43	9.3	2.60	13.2	33.14	8.4	0.95	25.6	12.14	20.2	0.11	1.7	1.43
Salmon	0.09	3.9	1.95	5.5	0.04	4.0	0.98	6.3	0.23	1.2	5.37	1.4	0.02	0.6	0.49	0.8	0.09	1.3	1.95
Tuna	0.05	2.1	1.43	4.0	0.02	2.2	0.71	4.6	0.29	1.5	8.93	2.3	0.04	1.1	1.29	2.1	0.05	0.7	1.43
Mackerel	0.01	0.7	0.24	0.7	0.02	1.7	0.31	2.0	0.71	3.6	11.80	3.0	0.04	1.0	0.62	1.0	0.14	2.1	2.37
Sardine	0.10	4.7	2.86	8.0	0.05	4.8	1.43	9.3	6.06	30.8	169.29	42.9	0.52	14.0	14.57	24.3	1.51	22.4	42.29
Mussels	0.09	4.1	1.90	5.4	0.05	4.2	0.95		0.86	4.4	18.29	4.6	0.02		0.48	0.8	0.11	1.7	2.38
Global exposure	2.17		35.49		1.07		15.43		19.67		394.69		3.73		59.99		6.77		110.71
% pTDI									0.001		0.026		0.007		0.120				

with fish consumption and use of body lotion and perfumes (Hutter et al., 2005). Anyhow, relevant parameters related with musk uptake, such as bioavailability, absorption or evaporation, are still required to provide accurate exposure estimates. Additionally, concentration values considered in the exposure assessment study were based on raw fish, so the effect of cooking was not considered. Since data related with the cooking effect on musk concentration are not available in the literature, this field still remains unexplored.

Conclusions

To the best of our knowledge, this is the first attempt focused on assessing the dietary exposure of human populations to musks by means of an integrated approach. Species of seafood of wide consumption among the population of Catalonia (Spain) were analysed. The results from the survey showed a lack of nitro musks in all the samples, with levels below de MDL/MQL. In contrast, most of the polycyclic musks showed detectable levels in all the samples. HHCB and AHTN were the most abundant musks, being especially relevant the levels of HHCB in sardine (367 ng g^{-1} (d.w.)) and mackerel (304 ng g^{-1} (d.w.)), respectively. The highest exposure was estimated for HHCB and HHCB-Lactone, with average intakes of 19.7 and 6.8 ng kg^{-1} bw day^{-1}, respectively. Contrastingly, the mean exposure to AHTN, DPMI and ATII was ranged between 1.1 and 3.7 ng kg^{-1} bw day^{-1}. Overall, the current intake of musks through seafood consumption should not mean a significant health risks for the population of Tarragona. Anyhow, a continuous monitoring is desirable to assure that the amount of musks in seafood as well as in other foodstuffs, does not follow an increasing trend. Furthermore, the role of other potentially important exposure routes, such as dermal absorption, should be studied in more detail.

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3.1.2. Human exposure to brominated flame retardants through the consumption of fish and shellfish in Tarragona County (Catalonia, Spain)

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HUMAN EXPOSURE TO BROMINATED FLAME RETARDANTS THROUGH THE CONSUMPTION OF FISH AND SHELLFISH IN TARRAGONA COUNTY (CATALONIA, SPAIN)

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Abstract

The concentrations of 19 brominated flame retardants (BFRs) (8 polybrominated diphenyl ethers (PBDEs), 8 methoxylated PBDEs (MeO-PBDEs) and 3 emerging flame retardants) were determined in 10 species of fish and shellfish widely consumed in Tarragona County (Catalonia, Spain), by pressurized liquid extraction followed by gas chromatography coupled to tandem mass spectrometry. A higher occurrence of PBDEs was found in all the analyzed samples, while MeO-PBDEs were only detected in a few ones and the levels of emerging pollutants were relatively low. In contrast, hexabromobenzene was found in almost all samples at concentrations ranging between non-detected and 0.2 ng g^{-1} wet weight (w.w.). Salmon, sole, hake, cod and tuna showed the highest concentrations of ΣPBDEs (>0.8 ng g^{-1} w.w.), while mussel was the species with the highest level of MeO-PBDEs (1.5 ng g^{-1} w.w.). The dietary exposure of BFRs through consumption of these 10 species of fish and shellfish by the population of Tarragona County was estimated for different subpopulations, classified according to age and gender. Furthermore, calculations were performed in upper-, middle- and lower-bound risk scenarios. According to our data, the current concentrations of BFRs in fish and shellfish suggest no significant health risks for the consumers.

Keywords: *brominated flame retardants*; *fish and shellfish*; *GC-MS, PLE*; *dietary intake*

1. Introduction

Consumption of fish and shellfish is an essential part of a healthy and well-balanced diet (Domingo et al., 2007). However, this food group may also contain potentially high/moderate levels of a number of environmental contaminants, whose consumption can pose a risk to human health (Domingo, 2004, 2012, 2016). Brominated flame retardants (BFRs) are mixtures of man-made chemicals that are intentionally added to a wide variety of commercial products, such as plastics, textiles, and electronic/electrical equipment (Mackintosh et al., 2015; Fromme et al., 2016.). Due to their lipophilic, bioaccumulative and persistent nature, as well as their ubiquitous distribution and toxicity, the use of certain BFRs, such as hexabromocyclododecane (HBCD) or some polybrominated diphenyl ethers (PBDEs), has been recently banned or restricted in the European Union and/or North America (EFSA, 2011; Yuan et al., 2016). However, there is still some concern on the potential risk of these substances for the public health. BFR-treated products, either in use or as waste, may release small amounts of chemicals to the environment, being able to contaminate air, soil, and water (Cruz et al., 2015). These pollutants may also reach humans through their diets, mostly via intake of foodstuffs of animal origin (Domingo et al., 2008).

PBDEs, which form one of the most traditional families of BFRs, have been used in large amounts for many years. However, as we have mentioned before, penta- and octa-BDEs mixtures are already banned in the EU and the US. Furthermore, the production and use of deca-BDE has dramatically decreased in recent years (Sutton et al., 2015). PBDEs are ubiquitously present in the environment, being detected in air and dust (Fulara et al, 2012), sludge (Barón et al., 2014; Gorga et al., 2013; Law et al., 2014), sediments (Barón et al., 2014; Law et al., 2014), water (Law et al., 2014), and biota (Barón et al., 2014; Law et al., 2014; Munschy et al., 2011). PBDEs can reach the human body through different exposure pathways (EFSA, 2011). However, diet has been estimated to be the main route of PBDE entrance, being fish and shellfish one of the foodstuffs with higher PBDEs content (Domingo, 2012; Linares et al., 2015). In recent years, an increasing amount of information has been generated regarding the toxicity of PBDEs (Blanco et al., 2012; Heredia et al., 2012; Reverte et al., 2014). Toxicological studies have highlighted liver as a target organ for PBDEs (Fromme et al., 2016), and neurobehavioral and endocrine disrupting effects are also reported (Linares et al., 2015; Messer, 2010).

In biota, some PBDEs can be biotransformed to methoxylated PBDEs (MeO-PBDEs) through metabolic pathways (Wang et al., 2014; Weijs et al., 2009), or transformed by methylation (Losada et al., 2010). Moreover, some MeO-PBDEs have been suggested to occur naturally in marine ecosystems (Rotander et al., 2012; Weijs et al., 2009), being this the reason why increasing attention has been paid to the presence of MeO-PBDEs in wildlife (Jaspers et al., 2013; Dahlgren et al.,

2016). Although toxicological information on these compounds is still limited, their structural similarity to PBDEs suggests that MeO-PBDEs may be also toxic for wildlife and humans (Ben Hassine et al., 2015).

In parallel to the restriction and ban of PBDEs, the usage of alternative BFRs, called emerging flame retardants, has been proposed (EFSA, 2012). Hexabromobenzene (HBB) is one of these compounds, with applications in the manufacture of paper and textiles. This chemical has been extensively used in Japan, while their production has not been reported in European countries. HBB is generated through the thermal degradation of deca-BDEs and other PBDEs. Recently, a number of studies have reported the wide occurrence of HBB in the environment (Munschy et al., 2011; Salamova et al., 2011; Gorga et al., 2013; Barón et al., 2014; Cruz et al., 2015). As for other emerging flame retardants, few data on the human toxicity of HBB are currently available, although a high exposure to HBB have been linked to liver effects (Feng et al., 2013). In any case, the production and use of HBB has not been regulated yet, while there is a lack of knowledge about the presence of this chemical in foodstuffs, and the potential role of the dietary intake as exposure pathway. In addition to HBB, decabromodiphenyl ethane (DBDPE) and pentabromoethyl benzene (PBEB) have been identified as other emerging BFRs of potential concern (EFSA, 2012). They were commercially introduced to replace PBDEs (Kierkegaard et al., 2004b; Liu et al., 2016), being currently detected in a wide range of environmental matrices (Barón et al., 2014; Egeback et al., 2012; Gorga et al., 2013; Santín et al., 2013). Despite the limited amount of human toxicity studies on emerging flame retardants (Nakari et al., 2010; Stieger et al., 2014), DBDPE is not expected to present a health risk for humans, at least considering data on ecotoxicological studies assessed in aquatic and sediment species (Hardy et al., 2012; Cruz et al., 2015).

The present study was aimed at determining the presence of 8 PBDEs, 8 MeO-PBDEs, as well as 3 emerging flame retardants (i.e., HBB, DBDPE and PBEB) in samples from 10 species of fish and shellfish widely consumed by the population of Tarragona County (Spain). The concentrations were used to evaluate the exposure to those compounds through the intake of fish and shellfish, as well as to characterize the human health risks.

2. Materials and methods

2.1. Reagents and materials

The concentrations of 8 different BDE congeners were determined in each sample. A standard stock solution with a mixture of 2,4,4'-tribromodiphenyl ether (BDE28), 2,2',4,4'-tetrabromo diphenyl ether (BDE47), 2,2',4,4',5-pentabromodiphenyl ether (BDE99), 2,2',4,4',6-pentabromodiphenyl ether (BDE100), 2,2',4,4',5,5'-hexabromo diphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), at a concentration of 1 µg mL^{-1}, and decabromodiphenyl ether (BDE209) at 10 µg mL^{-1}, in nonane, was

purchased from LGC Standards SLU (Barcelona, Spain). Similarly, for quantification by isotope dilution, a standard stock solution of ^{13}C-labelled PBDEs (^{13}C-BDE28, ^{13}C-BDE47, ^{13}C-BDE99, ^{13}C-BDE100, ^{13}C-BDE154, ^{13}C-BDE153, ^{13}C-BDE183 and ^{13}C-BDE209) at 1 µg mL^{-1}, and BDE209 at 10 µg mL^{-1}, in nonane, was also purchased from LGC Standards S.L.U (Barcelona, Spain). For analysis of MeO-PBDEs, a standard stock solution of native compounds containing 8 congeners (5-methoxy-2,2',4,4'-tetrabromodiphenyl ether (5-MBDE47), 6-methoxy-2,2',4,4'-tetra bromodiphenyl ether (6-MBDE47), 2,2',4',5-tetrabromo-4-methoxy diphenyl ether (4-MBDE49), 2'-methoxy-2,3',4,5'-tetrabromodiphenyl ether (2'-MBDE68), 5'-methoxy-2,2',4,4',5-pentabromodiphenyl ether (5'-MBDE99), 5-methoxy-2,2',4,4',6-pentabromodiphenyl ether (5-MBDE100), 4-methoxy-2,2',4',5,5'-pentabromodiphenyl ether (4-MBDE101), and 2,2',4',5,6'-penta bromo-4-methoxydiphenyl ether (4-MBDE103)), at a concentration of 5 µg mL^{-1} in nonane/toluene, was obtained from Wellington Laboratories Inc. (Guelph, ON, Canada). In addition, a standard stock solution with hexabromobenzene (HBB), decobromodiphenylethane (DBDPE) and pentabromoethylbenzene (PBEB) at levels of 50, 25 and 50 µg mL^{-1}, respectively, in toluene was also provided by Wellington Laboratories Inc.

Hexane, dichloromethane, toluene and sulfuric acid, all of them from J.T.Baker (Deventer, The Netherlands), were >99.9% grade purity. Diatomaceous earth was supplied by Thermo Scientific (Barcelona) and filters for the PLE cell came from Dionex Corporation (Sunnyvale, CA, USA). Ultrapure water was obtained by using a purification system from Veolia Water (Sant Cugat del Vallès, Barcelona), while the purity of helium gas (Carburos Metálicos, Tarragona, Spain) for the chromatographic analysis was 99.999%.

2.2. Sample preparation

Ten species of fish and shellfish were selected among the most consumed species by the Catalan population (ENCAT, 2003): sole (*Solea solea*), hake (*Merluccius merluccius*), sardine (*Sardina pilchardus*), tuna (*Thunnus thynnus*), codfish (*Gadus morhua*), shrimp (*Aristeus antennatus*), salmon (*Salmo salar*), mackerel (*Scomber scombrus*), squid (*Loligo vulgaris*), and mussel (*Mytilus galloprovincialis*). Samples of each species were purchased at various establishments (supermarkets, local markets and fish stores) from Tarragona County (Catalonia, Spain). After collection, samples were immediately preserved in a refrigerator box. Once in the laboratory, they were kept at -20ºC until their pre-treatment. Thus, lateral fillets were dissected from the fish, while the shells of mussels and shrimps were taken off. Subsequently, samples were homogenized, lyophilized by means of a freeze-drying system (Labconco, Kansas City, MO, USA), and finally grinded. In addition, mussels were also sieved through a 125 µm mesh screen to homogenize the diameter of the particles. Each analysed sample was, in fact, a composite sample

prepared by mixing equal amounts from each species purchased from the three commercial establishments.

2.3. Analytical method

A mixture solution in toluene containing all the ^{13}C-labelled PBDEs and ^{13}C-BDE209 at levels of 0.2 and 2 μg mL^{-1}, respectively, was prepared. A set of seven calibration standard solutions, containing a mixture of all congeners at concentrations ranging from 1 to 600 ng mL^{-1} for PBDEs, from 10 to 6000 ng mL^{-1} for BDE209, from 1 to 750 ng mL^{-1} for MeO-PBDEs, HBB and PBEB, and from 3 to 2250 ng mL^{-1} for DBDPE, was prepared by dilution of the corresponding standard stock solutions in toluene. In addition, an appropriate amount of surrogate mixture solution was added to the calibration standard solutions to obtain a concentration of 100 ng mL^{-1} of each ^{13}C-labelled PBDEs, excepting ^{13}C-BDE209, whose concentration was 1000 ng mL^{-1}.

A more detailed description of the method for extraction and clean-up has been previously given (Barón et al., 2014). Briefly, 1.5 g of lyophilized sample was fortified at 2 μg g^{-1} with a surrogate mixture standard (^{13}C-PBDEs), being kept in the fridge overnight to equilibrate. Then, a pressurized liquid extraction (PLE) (ASE 200, Dionex, Sunnyvale, CA, USA) with hexane/dichloromethane (1:1, v:v), followed by gravimetric determination of the lipid content, was done. The residue was dissolved again with 10 mL of hexane and subjected to solid phase extraction (SPE) with Al-N cartridges (5 g) (Symta, Madrid, Spain). The final extract was evaporated to dryness, re-dissolved with 40 μL of toluene, and analysed by gas chromatography-tandem mass spectrometry (GC-MS/MS).

The chromatographic analysis was performed by using a Varian ion trap GC-MS system (Varian, Walnut Creek, CA, USA), following an adaptation of a previously developed method (Barón et al., 2014). The system was equipped with a 3800 gas chromatograph, a 4000 ion trap mass detector, and a CombiPal autosampler (CTC, Analytics, Zwigen, Switzerland) equipped with a 10 μL syringe of 23 gauge and point style 5 (Hamilton, Bonaduz, Switzerland). The mass spectrometer was operated in the electron ionization (EI) mode (70 eV), being the whole system controlled by means of the Varian MS Workstation v.6.9 software. The chromatographic separation was carried out on a ZB-5 analytical column (5% phenyl 95% dimethylpolysiloxane, 15m x 0.25 mm i.d.; 0.1 μm film thickness) from Micron Phenomenex (Torrance, California, USA). The injected volume was 1 μL, using splitless injection mode for 1 min at 280 ºC. The oven initial temperature was 140ºC, being held for 2 min, and then raised again at 10ºC/min until 310ºC, which was kept for 10 min. Helium was used as a carrier gas, at a constant flow rate of 1 mL min^{-1}. The whole time for the separation of the target compounds was 21 min. Transfer line, manifold, and trap temperatures were 280°C, 50°C and 200°C, respectively. Tandem mass spectrometry (MS/MS) mode was applied for the quantitative analysis.

The retention time and the optimal MS parameters for each compound are summarized in Table 1.

2.4. Quality assurance and quality control

Quality control criteria used to ensure the correct identification of the target compounds consisted in the following: (1) the retention time should match that of the standard compound within ± 1s, (2) the signal-to-noise ratio (S/N) should be ≥ 3, and (3) the deviation of the two monitored ions intensities ratio should be within 15% of that of the standard compound.

Quantification of the target compounds was carried out by internal standard procedure with ^{13}C-labelled BFRs. As mentioned before, multi-level calibration curves were performed for the quantification and good linearity was achieved (R^2 > 0.998). The instrumental limits of detection (LODs) calculated as three times the signal-to-noise ratio, ranged from 0.3 to 16.7 μg L^{-1} for the analysed compounds. The instrumental limits of quantification (LOQs) were defined as the lowest calibration point and ranged from 10 to 50 μg L^{-1}. Intra-day and inter-day repeatability expressed as relative standard deviation (RSD) (n=5, 1 μg mL^{-1}), were lower than 21% for all compounds.

2.5. Exposure assessment and risk characterization

The dietary intake of the 19 BFRs by the population of Tarragona County was estimated by using a deterministic method, which combines consumption and concentration data. Human exposure was assessed by applying the following equation:

$$E_t = \sum_{f=1}^{p} C_f X_{t,f} \qquad \text{Eq. 1}$$

Where E_t is the dietary exposure to the BFR t (ng kg bw^{-1} day^{-1}), C_f is the mean consumption of the individual species of fish or shellfish f by the population (g kg bw^{-1} day^{-1}), and $X_{t,f}$ is the concentration of the BFR t in the fish or shellfish species f (ng g^{-1}). The mean consumption of each age/gender population subgroup was previously normalized by dividing the dietary intake by the mean body weight. Data on BFR levels in fish and shellfish were based on fresh weight.

Calculations, for those not detected or presented at concentrations lower than their limit of quantification, were conducted under 3 different scenarios (upper-, middle- and lower-bound; UB, MB, LB, respectively), according to their limit of quantification (LOQ) and limit of detection (LOD) (IPCS, 2009). The UB scenario was estimated by assuming the respective concentrations of LOD or LOQ. In the MB scenario, the concentrations were assumed to be one-half of the LOD or LOQ, and in the LB scenario a concentration of zero for non-detected BFRs and a concentration equal to the LOD for analytes with their levels below the LOQ were assumed. The intake of BFRs under these 3 scenarios was estimated for adolescents, adults and seniors (aged 10-19, 20-65 and >65, respectively)

Table 1. Retention times and MS conditions in the chromatographic analysis of BFRs in fish and shellfish.

Compound		Retention time (min)	Parent ion (m/z)	Product ions [a)] (m/z)	CID Amplitude (V)	CID Storage level (m/z)	m/z range	Scan time (s/scans)
PBDEs	BDE28 [b]	7.16	408	**248**, 246, 409	0.4	179.7	190-418	0.38
	BDE47	9.19	486	**326**, 328, 484	0.8	214.4	224-496	0.58
	BDE100 [e]	10.63	406	**405**, 323, 282	1.3	178.9	189-416	0.27
	BDE99 [e]	11.06	406	**405**, 403, 371	0.2	178.9	189-416	0.27
	BDE154 [g]	12.21	644	**483**, 643, 347	0.8	283.7	294-654	0.32
	BDE153 [g]	12.77	644	**643**, 536, 516	0.1	283.7	294-654	0.32
	BDE183	14.36	721	**548**, 631, 418	0.2	317.6	328-731	0.53
	BDE209	20.20	798	**616**, 461, 776	0.1	351.5	352-808	0.53
MeO-PBDEs	2-MBDE68 [d]	10.01	516	**515**, 517, 420	0.1	227.3	237-526	0.53
	6-MBDE47 [d]	10.32	516	**356**, 513, 424	0.3	227.3	237-526	0.53
	5-MBDE47 [e]	10.77	516	**358**, 479, 432	1.1	227.3	237-526	0.27
	4-MBDE49 [e]	10.85	516	**356**, 358, 500	0.3	227.3	237-526	0.27
	5-MBDE100 [f]	11.78	596	**497**, 419, 587	0.1	262.6	272-606	0.54
	4-MBDE103 [f]	11.90	596	**435**, 595, 435	0.6	262.6	272-606	0.54
	5-MBDE99 [g]	12.42	596	**558**, 381, 419	0.2	262.6	273-606	0.32
	4-MBDE101 [g]	12.52	596	**595**, 277, 463	0.7	262.6	273-606	0.32
Emerging BFRs	PBEB [b]	7.51	501	**499**, 420, 486	0.4	220.7	231-511	0.38

[a] Quantification ions (m/z) in bold.
[b-g] Compounds were separated using the Multiple Reaction Monitoring mode.

of both genders.

Currently, there is no international scientific consensus on the best system to estimate the risk assessment through food consumption, being several approaches commonly applied (COC, 2012; EFSA, 2005; Lachenmeier et al., 2012). In the present study, the health risks due to the intake of BFRs through fish and shellfish were calculated by using the MOE (Margin of Exposure) approach, according to the following equation:

$$MOE_t = \frac{BMDL_t}{E_t} \quad \text{Eq. 2}$$

Where MOE_t is the margin of exposure for the dietary exposure to the BFR *t*, E_t is the dietary intake of the BFR *t* (ng kg bw^{-1} day^{-1}), and *BMDL* (Benchmark dose confidence limit) is the point on the dose-response curve corresponding to a specific change due to the adverse response by the effect of BFR *t* (ng kg bw^{-1} day^{-1}). The BMDL estimates the dose that causes a low, but measurable response, typically chosen in the range of 1-10% incidence above the control (EFSA, 2011; USEPA, 1995).

2.6. Study population and data collection

Consumption data were collected from a nutritional survey conducted in Catalonia, Spain, between 2002 and 2003 (ENCAT, 2003). A food frequency questionnaire (FFQ) was carried out with 2160 individuals (54% female and 46% males) aged between 10 and 80, covering 83 towns representing the whole population in Catalonia. Of the selected individuals invited to participate in the study, 66% agreed to take part of it. Twenty-six trained interviewers visited cases and controls at home seven days per week to check the answers to the FFQ, and they clarified and helped participants to answer questions. Thus, the consumption data were provided by means of a 3-day dietary record, a 24 h record, and a food frequency questionnaire. Usual dietary intake was estimated from food frequencies and quantities reported by participants for the 15 months prior to the interview. Interviewers also obtained information via a structured interview on participants' medical history, lifetime smoking history, chronic disease history, nutritional supplement intake, healthy lifestyles, and social status, among others. Moreover, an anthropometric study was developed in order to control different human parameters. The seafood species were selected among those most consumed of the population. The consumption rates of the general population to each one of the analysed species (hake, cod, sole, squid, shrimp, mussel, tuna, mackerel, salmon and sardine) are summarized in Table 2.

3. Results and discussion

3.1. Occurrence of BFRs in commercial samples of fish and shellfish

The average concentrations of BFRs in each species of fish and shellfish, expressed in wet weight (w.w.), are shown in Table 3. For those not detected or present at concentration lower than

Table 2. Mean consumption (g day^{-1}) of the 10 species of fish and shellfish selected among those species most widely consumed by the population of Tarragona County, classified according to gender and age.

	Foodex1 code	Boys (10-19)	Adult men (20-65)	Senior men (>65)	Girls (10-19)	Adult women (20-65)	Senior women (>65)
Hake	A.01.000895	7.82	15.03	23.02	10.84	14.49	14.56
Sole	A.01.000899	6.22	4.84	3.65	2.44	5.28	5.17
Cod	A.01.000894	2.13	4.18	8.08	0.60	4.61	8.15
Shrimp	A.01.000923	2.71	2.83	2.42	2.94	3.44	1.68
Squid	A.01.000928	1.88	3.17	3.18	5.18	3.17	0.77
Salmon	A.01.000883	3.30	1.80	2.23	1.00	3.00	1.14
Tuna	A.01.000891	0.71	1.62	1.07	0	1.45	0.52
Mackerel	A.01.000890	0.36	1.13	0.50	0.32	1.27	2.86
Sardine	A.01.000880	0.99	2.92	2.60	2.08	2.69	4.70
Mussel	A.01.000934	1.26	0.97	2.06	0	1.84	0.67

their limit of quantification, specific values <LOD or <LOQ have been indicated, respectively. Moreover, values corresponding to one-half of the LOD or LOQ have been assumed to calculate total amount of each BFR's group. Among the 3 groups of analysed BFRs, PBDEs showed the highest concentrations. Quantifiable amounts of PBDEs were found in most samples, being BDE28 and BDE47 determined in all the analysed species. BDE47 was the congener with the highest contribution to the total level of PBDEs (ΣPBDEs). In contrast, BDE100, BDE183 and BDE209 were not detected in any sample, while BDE154 was identified only in mackerel at a concentration below its LOQ. The highest level of total PBDEs (ΣPBDEs) was found in salmon (1.3 ng g^{-1} (w.w.)), which is the species with the highest lipid content (25%). In addition, sole, tuna, cod and hake also presented relatively high concentrations of ΣPBDEs (1.2, 0.8, 0.8 and 0.9 ng g^{-1} (w.w.)). On the other hand, squid and shrimp, two species with low lipid content, showed the lowest values of ΣPBDEs (Table 3).

The levels of MeO-PBDEs were comparatively lower than those corresponding to PBDEs. Some MeO-PBDEs, such as 5-MBDE47, 4-MBDE49, 4-MBDE103, 5-MBDE99 and 4-MBDE101 were not detected in any of the samples. Mussels and tuna showed the highest concentration of total MeO-PBDEs (ΣMeO-PBDEs), with mean values of 1.5 and 1.0 ng g^{-1} (w.w.), respectively. In contrast, hake, cod and squid had values below the LOD/LOQ for all MeO-PBDEs.

Regarding emerging BFRs, neither traces of PBEB nor DBDPE were found in any of the analysed samples, which is in agreement with recent data from the scientific literature (Barón et al., 2014; Papachlimitzou et al., 2012). HBB was the only emerging pollutant with concentrations above its LOD. This compound was identified in most samples, showing concentrations of up

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Table 3 Concentration of 19 brominated flame retardants (in ng g^{-1} (w.w.)) in samples of 10 edible marine species widely consumed in Tarragona County (Catalonia, Spain).

		Hake (2%)	Sole (6%)	Cod (1%)	Shrimp (2%)	Squid (6%)	Salmon (25%)	Tuna (16%)	Mackerel (17%)	Sardine (14%)	Mussel (8%)
PBDEs	BDE28	0.2	0.3	0.1	0.1	0.1	0.3	0.2	0.1	0.1	0.2
	BDE47	0.5	0.7	0.5	0.3	0.3	0.7	0.5	0.5	0.3	0.3
	BDE100	<0.002	<0.002	<0.002	<0.002	<0.002	<0.001	<0.001	<0.001	<0.001	<0.002
	BDE99	0.1	0.1	0.1	0.1	0.1	0.2	0.1	<0.003	0.1	0.1
	BDE154	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.01	<0.04*	<0.01	<0.02
	BDE153	<0.03*	<0.03*	<0.03*	<0.03*	<0.03*	<0.02*	<0.02*	<0.02*	<0.02*	<0.02*
	BDE183	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	BDE209	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.01	<0.01	<0.01	<0.02
	ΣPBDEs	*0.9*	*1.2*	*0.8*	*0.6*	*0.6*	*1.3*	*0.8*	*0.7*	*0.4*	*0.6*
MeO-PBDEs	2-MBDE68	<0.01	0.1	<0.01	0.1	<0.01	0.1	0.8	0.1	<0.01*	0.7
	6-MBDE47	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2*	<0.1	<0.6
	5-MBDE47	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.01	<0.01	<0.01	<0.02
	4-MBDE49	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	5-MBDE100	<0.1*	<0.1*	<0.1*	<0.1*	<0.1*	<0.04*	<0.04*	<0.04*	0.2	<0.1*
	4-MBDE103	<0.002	<0.002	<0.002	<0.002	<0.002	<0.001	<0.001	<0.001	<0.001	<0.002
	5-MBDE99	<0.1	<0.1	<0.1	<0.1	<0.1	<0.04	<0.04	<0.04	<0.04	<0.04
	4-MBDE101	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	ΣMeO-PBDEs	*0.3*	*0.4*	*0.3*	*0.4*	*0.3*	*0.3*	*1.0*	*0.4*	*0.3*	*1.5*
Emerging BFRs	PBEB	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.01	<0.01	<0.01	<0.02
	HBB	0.2	0.2	0.2	0.2	<0.02	0.1	<0.01	0.1	0.1	0.2
	DBDPE	<0.01	<0.01	<0.01	<0.01	<0.01	<0.004	<0.004	<0.004	<0.004	<0.01

<LOD.
In parenthesis, percentage of lipid weight basis.

to 0.2 ng g^{-1} (w.w.), in 5 different species (Table 3). Only hake, cod, sole, mussel and shrimp, which are species with a low content of fat, showed the maximum level of HBB (0.20 ng g^{-1} (w.w.), also in accordance with previous results (Munschy et al., 2011).

The predominance of PBDEs in fish and shellfish with respect to other BFRs has been previously reported. Losada et al. (2010) also identified sole as the species with the highest concentration of ΣPBDEs (22.3 ng g^{-1} (l.w.)), and BDE47 as the main congener. In fact, the high contribution of BDE47 on the total concentration of PBDEs in fish and shellfish has been found in a number of investigations. When analysing BFRs in fish, Barón et al. (2014) observed that BDE47 was the most abundant congener among 19 brominated compounds. Similarly, in an investigation in which concentrations of BFRs in European farmed salmon were found to be higher than those from North and South America, Lyche et al. (2015) reported that BDE47 was the predominant compound in salmon. Although in recent years the occurrence of PBDEs has been investigated in a large number of aquatic species, mussels and salmon have been the most frequently studied, especially in dietary intake surveys (Cruz et al., 2015).

With respect to MeO-PBDEs, the concentrations in samples of fish and shellfish from Tarragona County are similar to those reported in the literature (Kierkegaard et al., 2004a; Losada et al., 2010). Furthermore, the same chemical profile has been observed, being 2-MBDE68 and 6-MBDE47 the predominant compounds. For comparison purposes, Losada et al. (2010) found that the levels of 6-MBE47 and 2-MBDE68 in samples of salmon from the Mediterranean Sea were 5.55 and 2.15 ng g^{-1} (l.w.)), respectively. However, in contrast to our results, no MeO-PBDEs could be quantified in mussels.

3.2. Dietary intake of BFRs and risk assessment

The total dietary exposure of BFRs through consumption of fish and shellfish by the population of Tarragona, as well as the dietary exposure of ΣPBDEs, ΣMeO-PBDEs, PBEB, HBB and DBDPE, are depicted in Fig. 1.

Human exposure was assessed for 6 population subgroups, based on age and gender, and under 3 scenarios: upper-, middle- and lower-bound intake. For all scenarios and subpopulations, the highest contribution to the intake of BFRs corresponded to ΣPBDEs (48%-68%), followed by ΣMeO-PBDEs, in the upper- and middle-bound scenarios, and HBB in the lower-bound scenario. In the upper- and middle-bound scenarios, senior men presented the highest exposure to BFRs (1.38 and 1.08 ng kg bw^{-1} day^{-1}), while adult women showed the highest exposure in the lower-bound scenario (0.83 ng kg bw^{-1} day^{-1}). Estimations were performed for the general adult population of Tarragona County. Among them, high fish consumers would be obviously the group with the highest intake of BFRs. However, as no current consumption data were available for this particular subpopulation group, no calculations

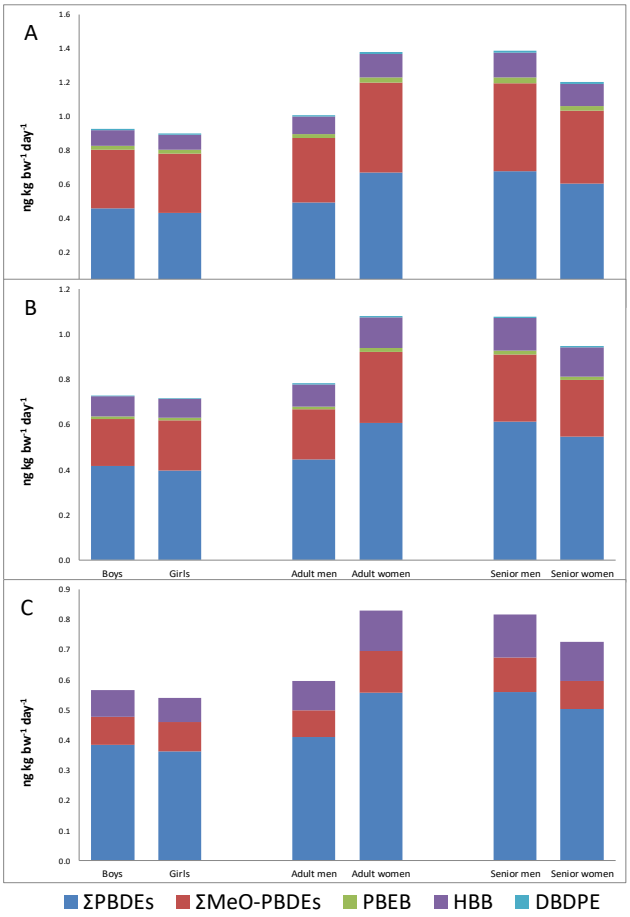

Fig. 1. Estimated dietary intake (ng kg^{-1} bw day^{-1}) of ∑PBDEs, ∑MeO-PBDEs, PBEB, HBB and DBDPE for the general population of Tarragona County (Spain) according to gender and age. A) Upper-bound scenario. B) Middle-bound scenario. C) Lower-bound scenario.

were conducted for them.

In recent years, there has been an increasing concern among scientists for the analysis of BFRs, mainly PBDEs, in fish and shellfish, as well as in other foodstuffs. Furthermore, these data have been frequently used in order to assess the dietary intake of these chemicals by the general population. Details and results of some of these investigations are summarized in Table 4. It must be highlighted that the

Table 4. A summary of studies on the concentrations and dietary intake of PBDEs.

Country	Matrix	Congeners analyzed	Concentration (in ng g^{-1} w.w.)	Dietary exposure (in ng day^{-1} or [ng $kg\ bw^{-1}\ day^{-1}$])	Remarks	References
Spain	Fish and shellfish from Catalonia, Spain	tetraBDE pentaBDE hexaBDE heptaBDE octaBDE	0.33, fish 0.32, shellfish	30.7	Predominance of the homologues tetra- and pentaBDEs, followed by hexaBDE.	Bocio et al., 2003
Spain	Fish and shellfish from Catalonia, Spain	tetraBDE pentaBDE hexaBDE heptaBDE octaBDE	0.56, fish	20.8	The highest levels of PBDEs were found in salmon. The highest and lowest levels corresponded to BDE47 and BDE183, respectively.	Domingo et al., 2006
Spain	Fish and shellfish from Catalonia, Spain	tetraBDE pentaBDE hexaBDE heptaBDE octaBDE	0.56, fish and shellfish	26.5	BDE47 was the congener with the highest concentration.	Domingo et al., 2008
Belgium	Fish and shellfish from Belgium	28, 47, 99, 100, 153 and 154	-	59.5 [0.85]	BDE47 was the congener with the highest concentration.	Sioen et al., 2008
Sweden	Fish and fish products from Sweden	28, 31, 47, 66, 99, 100, 138, 153, 154 and 183	0.397 0.249 only BDE47	19.3	Fish contributed 38% to the total PBDE intake	Törnkvist et al., 2011

Table 4. (Cont.).

Country	Matrix	Congeners analyzed	Concentration (in ng g^{-1} w.w.)	Dietary exposure (in ng day^{-1} or [ng kg bw^{-1} day^{-1}])	Remarks	References
China	Fish and shellfish from Shanghai	17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190 and 209	0.002-0.35, shellfish 0.003-1.25, fish	15 shellfish 41 fish	TetraBDE was the most abundant homologue. Bioaccessibility of PBDEs was studied.	Yu et al., 2011
Japan	Fish from South Korea	17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, 183, 184, 184, 191, 196, 197, 206, 207 and 209	0.06 - 6.25, shellfish	65.9	Predominant congeners were BDE47, 99 and 100.	Sunggyu et al., 2013
China	Carp from east-central china	28, 47, 99, 100, 153, 154 and 183	0.047	11.9 [0.199] BDE47 3.7 [0.061] BDE99 38.5 [0.642] BDE153	Standard adult of 60 kg body weight was used to calculate dietary exposure.	Gong et al., 2015
Italy	Fish and mollusk from local Italy market	7, 15, 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, 156, 183, 184 and 191	0.52, fish 0.49, mussels	20.3 [0.29] fish and mollusks	A high percentage of the BDE47, 99 and 100 congeners was observed.	Martellini et al, 2016
Spain	Fish and shellfish from Catalonia, Spain	28, 47, 99, 100, 153, 154, 183 and 209	0.8 fish and shellfish	[0.45]	Predominant PBDE congeners in fish and shellfish were BDE28 and BDE47.	This study

Calculation for dietary exposure was done by assuming that non-detected values were one-half of the limit of detection.
Results are given for a male adult of 70 kg body weight.
w.w. wet weight. b.w. body weight.

comparability between studies is difficult, and data interpretation must be performed with special caution. This is basically due to the potentially high number of different factors involved in a total diet study (e.g., analytical method, consumption data, exposure assessment model, food groups covered, congeners considered, etc.). In addition, concentration values refer to raw fish, disregarding the potential effect of cooking (Perelló et al., 2009; Domingo, 2016) and/or the bioavailability of these compounds (Yu et al., 2011).

Since 2000, our laboratory has been periodically performing a surveillance program to evaluate the dietary intake of chemical pollutants by the population living in Catalonia (Spain). In a first survey, total dietary exposure of ΣPBDEs was estimated in 97.3 ng day^{-1}, in a middle-bound scenario, and 81.9 ng day^{-1}, in a lower-bound scenario (Bocio et al., 2003). Fish and shellfish were the food group with the highest contribution (30% of the total), being the intake of ΣPBDEs through the consumption of 3 marine species (hake, sardine, and mussels) 30.7 ng day^{-1} (or 0.44 ng kg bw^{-1} day^{-1}). In the present study, the intake of ΣPBDEs through the consumption of 10 marine species was found to be 0.45 ng kg^{-1} bw day^{-1} (or 31.2 ng day^{-1}), a value very close to that firstly reported (Bocio et al., 2003). A more extensive study was subsequently performed. The occurrence of PBDEs was investigated in a more extensive number of species (Domingo et al., 2006). The dietary intake of ΣPBDEs through the ingestion of 14 edible marine species widely consumed by the Catalan population was calculated to be 20.8 ng day^{-1}, with tuna and salmon being the highest contributors (Domingo et al., 2006). In the last survey of the series, the intake of ΣPBDEs through food decreased 23% with respect to the first study, being the total dietary exposure 75.4 ng day^{-1} (Domingo et al., 2008). Considering only the group of fish and shellfish, the total intake was reduced to 26.5 ng day^{-1} (Domingo et al., 2008).

Also in Spain, Pardo et al. (2014) analysed the PBDE content in fish and shellfish marketed in the Region of Valencia over the period 2007–2012, estimating a daily intake of 0.137 ng kg bw^{-1} day^{-1} (9.59 ng day^{-1}) for the adult population. More recently, Aznar-Alemany et al. (submitted) analysed the levels of PBDEs, those of some emerging brominated flame retardants (PBEB, HBB and DBDPE), as well as MeO-PBDEs in commercial seafood samples from European countries. The dietary intake of some specific BDE congeners was of the same order of magnitude as that calculated for the inhabitants of Tarragona County. In fact, similar exposure levels have been also found in other European countries: 19.3 ng day^{-1} in Sweden (Tornkvist et al., 2011) and 20.3 ng day^{-1} in Italy (Martellini et al., 2016). Data corresponding to a number of Asian countries are also similar (Yu et al., 2011; Sunggyu et al., 2013; Gong et al., 2015), with mean exposure levels of 41 ng day^{-1} through fish consumption, and 15 ng day^{-1} through shellfish ingestion. With respect to MeO-PBDEs, information on their dietary intakes is

extremely limited. In one of the very few studies, Wang et al. (2011) studied the dietary exposure of MeO-PBDEs of Hong Kong residents through fish consumption, finding values in the range 0.5-4.3 ng kg bw^{-1} day^{-1}, well above the intake estimated for the population of Tarragona County (0.22 ng kg bw^{-1} day^{-1}). Covaci et al. (2007) investigated the occurrence of MeO-PBDEs in fish oil dietary supplement. They calculated an intake of these chemicals of 10 ng day^{-1} through the ingestion of these supplements, being the median intake of MeO-PBDEs between 3 and 6 times higher than the median intake of PBDEs.

Most studies regarding the dietary exposure of emerging BFRs have been conducted in China. Labunska et al. (2015) reported values of HBB in fish (0.05 ng kg bw $^{-1}day^{-1}$), as well as PBEB and DBDPE in shrimp (0.03 and 0.20 ng kg bw^{-1} day^{-1}, respectively). On the other hand, Peng et al. (2015) showed a dietary intake of PBEB (0.3 pg kg bw^{-1} day^{-1}), HBB (53 pg kg bw^{-1} day^{-1}), and DBDPE (640 pg kg bw^{-1} day^{-1}) through consumption of fish and shellfish in a Chinese production area of BFRs.

3.3. Risk assessment

Data on the human toxicity of BFRs is still quite limited. Moreover, most of the information refers to PBDEs, while there is a very notable lack of toxicological data on the potential hazard of other BFRs. Because of these limitations, in the current study the risk could be only assessed for a few BDE congeners. Values of BMDL of 309000, 12000, 83000 and 1700000 ng kg^{-1} bw were used for the risk characterization of BDE47, BDE99, BDE153 and BDE209, respectively (EFSA, 2011). Due to the NOAEL (no observed adverse effect level) limitations, described be Filipsson et al. (2003), BMDL has been pointed out as a viable alternative (EFSA, 2011). The MOEs to each one of these 4 congeners, for every population subgroup are shown in Table 5.

No health risks were associated to the intake of BFRs through fish and shellfish consumption, in any of the 3 exposure scenarios.

4. Conclusions

Nineteen BFRs, including MeO-PBDEs and 3 emerging compounds, were analysed in samples of 10 fish and shellfish species widely consumed in Tarragona County (Catalonia, Spain). BDE28 and BDE47 were the BDE congeners with the highest concentration. Salmon, sole and hake showed the greatest levels of ΣPBDEs, while mussels and tuna presented the highest values of ∑MeO-PBDEs. Moreover, 2-MBDE68, 6-MBDE47 and 5-MBDE100 were the most predominant congeners. Regarding the emerging compounds, HBB was identified in most samples, while PBEB and DBDPE were not detected in any sample. The daily intake of BFRs via ingestion of the 10 species of fish and shellfish was estimated under 3 different exposure scenarios. No health risks were associated to the intake of BFRs through the consumption of fish and shellfish. Furthermore, the current levels of exposure for the population living in Tarragona County are similar to those

Table 5. Risk characterization (MOE) of the exposure to 4 BDE congeners through consumption of fish and shellfish species by the population of Tarragona County.

		BDE47	BDE99	BDE153	BDE209
	BMDL (ng kg^{-1} b.w)	309000	12000	83000	1700000
Upper-bound	Boys	1.3E+06	2.4E+05	3.3E+06	7.6E+07
	Girls	1.4E+06	2.3E+05	3.1E+06	7.2E+07
	Adult men	1.2E+06	2.2E+05	2.9E+06	6.7E+07
	Adult women	8.8E+05	1.6E+05	2.1E+06	5.0E+07
	Senior men	8.8E+05	1.5E+05	2.1E+06	4.8E+07
	Senior women	9.5E+05	1.8E+05	2.4E+06	5.6E+07
Middle-bound	Boys	1.3E+06	2.4E+05	6.2E+06	1.5E+08
	Girls	1.4E+06	2.3E+05	5.7E+06	1.4E+08
	Adult men	1.2E+06	2.2E+05	5.5E+06	1.3E+08
	Adult women	8.8E+05	1.6E+05	4.0E+06	9.9E+07
	Senior men	8.8E+05	1.5E+05	3.9E+06	9.6E+07
	Senior women	9.5E+05	1.9E+05	4.6E+06	1.1E+08
Lower-bound	Boys	1.3E+06	2.4E+05	9.2E+06	n.c.
	Girls	1.4E+06	2.3E+05	8.6E+06	n.c.
	Adult men	1.2E+06	2.2E+05	8.2E+06	n.c.
	Adult women	8.8E+05	1.6E+05	6.0E+06	n.c.
	Senior men	8.8E+05	1.5E+05	5.8E+06	n.c.
	Senior women	9.5E+05	1.9E+05	6.8E+06	n.c.

n.c.: not calculated. The associated dietary exposure is 0. BMDLs were collected from the literature (EFSA, 2011).

reported in the scientific literature for a number of European and Asian countries.

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3.1.3. Determination of benzothiazoles in seafood species by subcritical water extraction followed by solid-phase microextraction-gas chromatography-tandem mass spectrometry. Estimating the dietary intake

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DETERMINATION OF BENZOTHIAZOLES IN SEAFOOD SPECIES BY SUBCRITICAL WATER EXTRACTION FOLLOWED BY SOLID-PHASE MICROEXTRACTION-GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. ESTIMATING THE DIETARY INTAKE

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Abstract

Benzothiazoles are high production volume chemicals widely used in many industrial and household applications. However, information on their occurrence in aquatic organisms is very limited, although a high level of bioaccumulation is expected. In this study and for the first time, a method was developed involving subcritical water extraction followed by solid-phase microextraction coupled to gas chromatography-ion trap-tandem mass spectrometry for the determination of five benzothiazoles in seafood. The repeatability and reproducibility of the method were under 21% (%RSD, n=5, 100 ng g^{-1} (d.w.)), while method detection limits and method quantification limits were between 0.5 and 10 ng g^{-1} (d.w.) and 1 and 50 ng g^{-1} (d.w.), respectively. Ten widely consumed fish and shellfish species from the county of Tarragona (Catalonia, Spain) were selected in order to estimate dietary exposure and to assess the human health risks. The most frequently determined compounds were benzothiazole and 2-(methylthio)-benzothiazole, with squid being the species which showed the highest level of benzothiazole (82 ng g^{-1} (d.w.)). In terms of human exposure, the current concentrations of benzothiazoles found in fish and shellfish could not be compared to threshold values because of the lack of toxicological data.

Keywords: *benzothiazoles, seafood, GC-MS/MS, dietary exposure, Tarragona, Spain*

1. Introduction

Benzothiazoles include a large class of high production volume chemicals with a very broad range of applications in industry and household products [1]. Due to the large amount of several chemicals currently used, high production may indicate high exposure, with inherent risks for health and the environment. Benzothiazoles are commercially manufactured for use as vulcanization accelerators in rubber production. In addition, they are used as biocides in paper and leather manufacture, corrosion inhibitors in antifreeze formulations [2], fungicides and herbicides [3], and ultraviolet light stabilizers in textiles and plastics [4]. Moreover, benzothiazoles comprise a class of therapeutic compounds that display anticancer [5], antimicrobial, antiviral and antidiabetic activity, among other benefits [6]. Their structure consists of a 5-membered 1,3-thiazole ring fused to a benzene ring. The nine atoms of the bicycle and the attached substituents are coplanar. Furthermore, they are structurally related to naturally occurring purines due to their interaction with charged biomolecules. This structural concordance may pose a human risk because there might be interactions between benzothiazoles and some proteins [5,7].

Benzothiazole (BT) is recognized as a safe substance, being used as a flavouring in food applications [8]. Despite the lack of toxicological studies [9], the European Food Safety Authority (EFSA) has set a limit in food of 0.05 mg kg^{-1}. However, some benzothiazoles have shown toxic effects in *in vivo* tests with fish cell cultures, including cell death but at concentrations higher than those reported in environmental samples [10]. Ginsberg et al. [9] indicated that BT may pose a high risk at sufficient exposure, exhibiting adverse effects on the liver and kidney, as well as dermatitis and respiratory problems [7,9,11]. However, the available information on the aquatic toxicology of benzothiazoles, and especially their effect on fish, is still very limited. Therefore, the knowledge of human risks due to exposure to benzothiazoles through seafood consumption is scarce. According to risk assessment data from the World Health Organization (WHO), an oral Non-Observed Adverse Effect Level (NOAEL) of 5.1 mg kg^{-1} bw day^{-1} has been set for BT, based on a 90-day dietary study on rats [12]. However, no threshold values have yet been established for other benzothiazoles.

Since benzothiazoles are easily released into the environment, it is not surprising that these compounds have been detected in a wide variety of matrices, such as wastewater [13–17], river water [2], sewage sludge [16–19], human urine [7,20], adipose tissue [21], house dust and indoor air [22], clothing textiles [4], and synthetic turf [9], among others. As a consequence of their presence in river water and sewage sludge, trace amounts of these compounds may be expected in aquatic organisms. Unfortunately, to the best of our knowledge, analytical methods to determine benzothiazoles in aquatic organisms have not been developed and validated yet. Nevertheless, there are a few methods that have been developed

to determine benzothiazoles in solid samples which use extraction techniques such as pressurized liquid extraction (PLE) [18], ultrasound-assisted solvent extraction (UASE) [4] and liquid-solid extraction (LSE) based on QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction [19]. Furthermore, sample pre-treatment is necessary before extraction, which usually includes a freeze-dry step to avoid the presence of water that could distort the results. The matrix effect becomes critical when complex matrices are analysed. The source of matrix effect in biological samples usually arises from the free fatty acids responsible for inducing an enhancement or suppression of the signal, or even changing the retention time. Hence, clean-up steps are necessary to improve the identification and quantification of target compounds. Moreover, some research on aquatic organisms have shown a significant positive correlation between the accumulation of chemicals and the lipid content of organisms [23]. For this reason, lipid determination in fish samples is commonly used in order to assess the bioaccumulation of the analytes.

The most widely used technique for the analysis of benzothiazoles is liquid chromatography (LC) and, to a lesser extent, gas chromatography (GC), both coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [2,4,14,16,20]. Despite the matrix effect being a major problem, electrospray ionization (ESI) is preferred when working with LC. To overcome this disadvantage, atmospheric pressure chemical ionization (APCI) is sometimes used as an alternative, since it is less vulnerable to matrix effects [17,24,25]. In contrast, the interaction of analytes and matrix components during sample preparation is the main matrix impact in the case of GC, rather than ionization [26]. In any case, little is known about analytical methods combining the determination of benzothiazoles by GC in solid samples, whether biological or otherwise [25]. Moreover, most of the few studies on benzothiazoles analysis by means of GC focus on liquid samples, such as water or urine [14,20,27].

The main goal of this study was to develop, for the very first time, a method based on gas chromatography-ion trap-tandem mass spectrometry (GC-IT-MS/MS) to determine five benzothiazoles in 10 species of seafood. Subcritical water extraction (SBWE) and solid-phase microextraction (SPME) were used to extract and preconcentrate the analytes. Concentration data were used to assess the dietary exposure to those compounds through seafood consumption, and to characterize the human health risks for the consumers in the county of Tarragona (Spain).

2. Experimental

2.1. Reagents and standards

The target benzothiazoles (benzothiazole (BT), 2-chlorobenzothiazole (ClBT), 2-aminobenzothiazole (NH_2BT), 2-hydroxybenzothiazole (OHBT), and 2-(methylthio)-benzothiazole (MeSBT)), were purchased from Sigma-Aldrich (St.

Louis, USA). Individual stock solutions were prepared in methanol at 1,000 mg L^{-1} and stored at -20ºC. A working mixture solution was also prepared in methanol at 10 mg L^{-1} and stored in a refrigerator until use.

Methanol, hexane and dichloromethane were GC grade with purity >99.9% from J.T. Baker (Deventer, the Netherlands). 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 chromatographic analysis (Abelló Linde, Barcelona, Spain).

2.2. Sample preparation

Seafood was obtained from 3 commercial establishments (supermarket, fish store and local market) in the county of Tarragona (Spain) to ensure samples from a different origin. Samples of cod (*Gadus morhua*), salmon (*Salmo salar*), sole (*Solea solea*), mackerel (*Scomber scombrus*), mussel (*Mytilus galloprovincialis*), hake (*Merluccius merluccius*), sardine (*Sardina pilchardus*), tuna (*Thunnus thynnus*), shrimp (*Aristeus antennatus*) and squid (*Loligo vulgaris*) were selected as representing the most widely consumed species in Catalonia (Spain) [28]. The edible part of the seafood species was removed and then preserved in a refrigerator until use. Frozen homogenized samples were freeze-dried using the freeze-drying system (Labconco, Kansas City, MO, USA) and crushed using a mortar and pestle. Additionally, mussels were sieved through a 125 μm screen to homogenize the diameter of the particles. Lastly, a composite sample for each species was obtained by mixing equal amounts from the three different commercial establishments.

Spiked samples were prepared by adding the stock mixture of standards in acetone at the volume required to cover the freeze-dried fish sample. 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. Extraction procedure

Subcritical water extraction was carried out using an ASE 350 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) with 11 mL stainless steel extraction cell. A glass fibre filter was placed at the bottom of the cell, then 1 g of diatomaceous earth (Thermo Scientific, Barcelona, Spain) was added, followed by 1 g of freeze-dried sample previously mixed with 1 g of diatomaceous earth, and then 1 g of diatomaceous earth, which filled up the cell. Prior to extraction, on-cell clean-up was performed with hexane to remove lipids following conditions adapted from a previous study [18]: temperature at 60 ºC, 2 cycles of 5 min each, 5 min static time, 1500 psi, flush volume of 80 % and a purge time of 300 s. Then, the extraction was carried out with one cycle of 5 min using ultrapure water as solvent extraction at 80 ºC and 1500 psi. The preheating time was 5 min, flush

volume was 60 % of cell volume and purge time was 120 s. After extraction, 10 mL of the PLE extract were taken to perform the SPME.

The suitability of two SPME fibres (PA 85 μm and PDMS/DVB 65 μm; both from Supelco (Bellefonte, PA USA)) was checked, being PDMS/DVB 65 μm selected to conduct the SPME extraction. The fibre was conditioned in line with the supplier's instructions, being inserted into the GC injector. 10 mL of SBWE aqueous extract was poured into a 20 mL SPME vial and immediately sealed tight with a Teflon septum and placed in the tray of the CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland), which allowed full automation of the SPME. After an equilibration time of 5 min, the PDMS/DVB 65 μm fibre was immersed in the water solution for 40 min at 80 ºC. During the extraction, the sample was magnetically stirred at 750 rpm. Afterwards, desorption took place at the GC injector at 270 ºC for 3 min, and the compounds were subsequently determined by GC-IT-MS/MS.

To prevent carry-over, the PDMS/DVB 65 μm fibre was cleaned by heating at 270 ºC for 10 min prior to every extraction and a blank test was performed to check for possible carry-over.

2.4. Chromatographic analysis

The GC-IT-MS/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-5 Plus analytical column (30 m x 0.25 mm i.d.; 0.25 μm film thickness) from Phenomenex (Torrance, CA, USA) was used for the chromatographic separation. Varian MS Workstation v.6.9 software was used for instrument control and data processing.

For the chromatographic analysis, the injector was operated in splitless mode at 270 ºC. The oven temperature was programmed as follows: 85 ºC held for 3.5 min, raised at 25 ºC min^{-1} to 200 ºC, then 5 ºC min^{-1} to 250 ºC and finally 10 ºC min^{-1} to 280 ºC and held for 2 min. The carrier gas employed was helium at a constant flow rate of 1 mL min^{-1}. The target compounds were separated in 10 min. Transfer line, manifold and trap temperatures were 280 ºC, 50 ºC and 200 ºC, respectively. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV. For quantitative analysis of the target compounds, tandem mass spectrometry (MS/MS) mode was applied. Table 1 summarizes the retention time and the optimal MS conditions for each compound.

2.5. Exposure assessment and risk characterization

The dietary exposure assessment combines food consumption data with the concentration of chemicals detected in food. As such, the resulting dietary exposure may be compared with values from databases on chemicals in food. The formula used to calculate the dietary exposure was:

$$E_t = \sum_{f=1}^{p} C_f X_{t,f} \quad \text{Eq. 1}$$

where E_t is the global dietary exposure to the benzothiazole *t* in the general population (ng kg bw^{-1} day^{-1}), C_f is the mean consumption of the seafood species *f* by population (g kg^{-1} bw day^{-1}), and $X_{t,f}$ represents the concentration of benzothiazole *t* in the seafood species *f* (ng g^{-1}). The mean consumption was previously normalized, dividing the dietary intake by the mean body weight (bw). Contamination data was implemented on a wet weight (w.w.) basis. The concentration of non-detected analytes and analytes below method quantification limits (MQL) were set as the upper bound (UB) intake, where the concentration of non-detected analytes was assumed as the corresponding method detection limit (MDL) values, and the concentration of analytes below MQL was assumed to be their MQL values [29,30]. Therefore, the dietary exposure was reported using the sum of exposure to benzothiazoles through seafood consumption, taking into account different subpopulation groups, classified according to age (10-19 years old, 20-65 and 65-80) and gender.

The dietary risk was characterized by dividing the global dietary exposure by the provisional tolerable daily intake, estimated from the oral NOAEL, and considering an uncertainty factor of 1000 [31], as a margin of safety. The equation used to characterize the risk was:

$$R_t = \frac{E_t}{pTDI_t} \times 100 \quad \text{Eq. 2}$$

where R_t is the health risk due to the dietary exposure to the benzothiazole *t*, E_t is the dietary exposure to the benzothiazole *t* (ng kg^{-1} bw day^{-1}), and $pTDI_t$ is the provisional tolerable daily intake for the benzothiazole *t*, (ng kg^{-1} bw day^{-1}).

Consumption data were collected from the Nutritional Survey of Catalonia [28], performed in 2002-2003 with 2160 individuals (54% female and 46% males). The volunteer participants were aged between 10 and 80, representing the whole Catalonia population. The consumption data was provided by means of 3-day dietary records, 24 h records and a food frequency questionnaire. The consumption rates of the general population of all the analysed species (hake, cod, sole, squid, shrimp, mussel, tuna, mackerel, salmon and sardine) are summarized in Table 2.

3. Results and discussion

3.1 Method optimization

3.1.1. GC-IT-MS/MS optimization

A mixed solution of 1 μg L^{-1} of the five benzothiazoles was prepared in methanol. One μL of this solution was injected directly into the GC-IT-MS/MS system, under full-scan acquisition mode. The target compounds were identified by their molecular ion and then the chromatographic separation was optimized. As mentioned above, the separation was performed in 10 min.

Table 1. Retention times and MS conditions

Compound	Retention time (min)	Parent ion (m/z)	Product ions [a] (m/z)	CID Amplitude (V)	CID Storage levels	m/z range	Scan time (s/scan)
BT	6.70	135	91, **108**, 136	0.50	59.2	69-145	0.5
ClBT	7.53	169	**108**, 134, 170	0.76	74.4	84-179	1
MeSBT	9.18	181	136, **182**	0.30	79.7	84-191	0.33
NH_2BT	9.18	150	**148**, 151	0.25	66.1	76-160	0.33
OHBT	9.18	151	96, 123, **149**	60.00	66.5	76-161	0.33

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

Table 2. Mean consumption (g day^{-1}) of 10 seafood species selected from those most widely consumed by the population of the county of Tarragona, classified according to gender and age [28]

	Foodex-1 code	Boys (10-19)*	Adult men (20-65)*	Senior men (>65)*	Girls (10-19)*	Adult women (20-65)*	Senior women (>65)*
Hake	A.01.000895	7.82	15.03	23.02	10.84	14.49	14.56
Sole	A.01.000899	6.22	4.84	3.65	2.44	5.28	5.17
Cod	A.01.000894	2.13	4.18	8.08	0.60	4.61	8.15
Shrimp	A.01.000923	2.71	2.83	2.42	2.94	3.44	1.68
Squid	A.01.000928	1.88	3.17	3.18	5.18	3.17	0.77
Salmon	A.01.000883	3.30	1.80	2.23	1.00	3.00	1.14
Tuna	A.01.000891	0.71	1.62	1.07	n.a.	1.45	0.52
Mackerel	A.01.000890	0.36	1.13	0.50	0.32	1.27	2.86
Sardine	A.01.000880	0.99	2.92	2.60	2.08	2.69	4.70
Mussel	A.01.000934	1.26	0.97	2.06	n.a.	1.84	0.67

*Age
n.a.: not available, and assumed to be zero

Although MeSBT, NH_2BT and OHBT coeluted, they could be quantified separately, due to the differences in their molecular and fragment ions. To achieve maximum sensitivity/selectivity, MS/MS was carried out selecting appropriated precursor/product ions and then optimizing the ion trap MS/MS parameters described in Table 1.

3.2.1. Extraction and clean-up optimization

Because low concentrations of benzothiazoles in seafood samples were expected, the SBWE extracts were preconcentrated by SPME. Therefore, SPME optimization was performed first and SBWE parameters were subsequently adjusted.

Firstly, the extraction efficiency of two fibres (PA 85 μm and PDMS-DVB 65 μm) was evaluated by comparison of the peak areas obtained in immersion mode under the working conditions described by Naccarato et al. [20], who determined a larger group of contaminants in aqueous matrices, including some of the same benzothiazoles here analysed. Then, 10 mL of ultrapure water spiked at 1 mg L^{-1} was extracted at 30 ºC for 40 min. Desorption was carried out for 3 min at 270 ºC and 290 ºC for PDMS/DVB 65 μm and PA 85 μm fibres, respectively. Because higher areas for almost all compounds were obtained with PDMS-DVB 65 μm (Fig. 1), this fibre was chosen to perform the SPME. Once the fibre was selected, various extraction temperatures (30, 50, 70 and 80 ºC) were tested in order to increase peak areas without sacrificing analysis time. The peak areas of BT, ClBT and MeSBT increased by 90% at 80 ºC. However, NH_2BT and OHBT showed no significant differences between all of the tested temperatures. Therefore, 80 ºC was selected as the optimal SPME extraction temperature. The other extraction parameters were kept at initial values because areas did not improve by varying them.

Then, SBWE was optimised and initial conditions were adapted from a previous study [18] in which a group of benzothiazoles, benzotriazoles and benzenesulphonamides were determined in sludge. The conditions were as follows: 1 cycle of 5 min, ultrapure water at 80 ºC, 1500 psi, preheating time of 5 min, flush volume of 60% and purge time of 120 s. Although there are several parameters of PLE extraction to optimize, solvent extraction, temperature and extraction time have the largest influence. In this case, only the extraction time was optimized because water was needed as the extraction solvent to facilitate the SPME step and the initial temperature was high enough with respect to the extraction solvent used. Therefore, 5 and 10 min were tested using 1 g of freeze-dried hake sample spiked at concentration of 10 mg kg^{-1} (d.w.). Benzothiazole was the compound that showed the highest differences between the two times tested, with a peak area 40% higher at 5 min. ClBT and OHBT also showed slightly higher peak

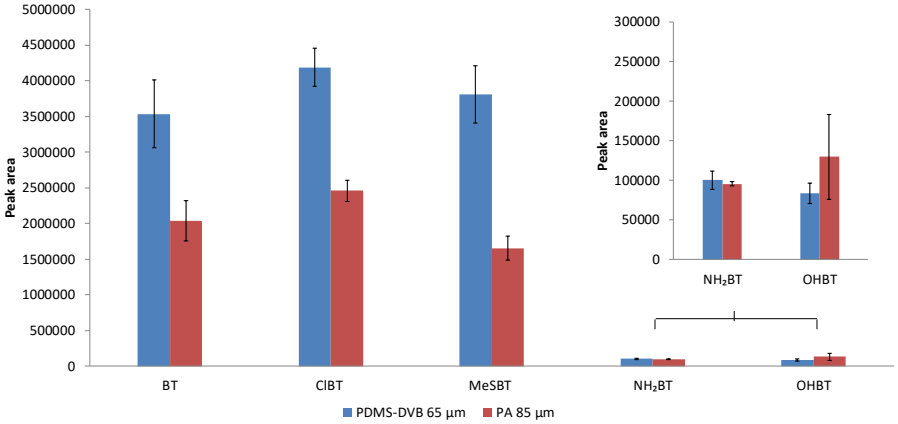

Fig. 1 Comparison of the chromatographic peak areas obtained with PDMS/DVB 65 mm and PA 85 mm SPME fibres under the same conditions (n=3). For other conditions, see text

areas at 5 min, while NH_2BT and MeSBT did not display any significant change. Thus, the extraction time selected was 5 min, as in the initial conditions.

Working with seafood samples, clean-up strategies are usually applied in order to remove interfering substances such as fat and oil, which may hinder the determination of trace levels [23]. In this study, on-cell clean-up and liquid-liquid extraction (LLE) were tested.

One gram of freeze-dried hake sample spiked at 10 mg kg^{-1} (d.w.) was used to test the different clean-up strategies. The on-cell clean-up was adapted from Hoff et al. [32], with the following conditions: hexane as the solvent extraction, temperature of 60 ºC, 2 cycles of 5 min each one, 5 min static time, 1500 psi, flush volume of 80% and purge time of 300 s. In the case of liquid-liquid extraction clean-up, it was performed with the SBWE extract which was poured into the separating funnel and then 5 mL of hexane was added. The process was repeated 5 times with fresh hexane, which allowed a cleaner aqueous solution to be achieved. Because an emulsion between organic and aqueous phase was observed, 3 g of sodium chloride was added.

Comparing all of the clean-up results, benzothiazoles showed peak areas 6 times higher with on-cell clean-up than with LLE. Furthermore, the addition of salt in LLE neither improved the extraction nor eliminated the emulsion. Moreover, the on-cell clean-up allowed peak areas between 80% and 95% times higher than those obtained when no clean-up was used, with BT, ClBT and MeSBT being the compounds that showed a larger increase. Since on-cell clean-up seemed to be a good clean-up strategy, the matrix effect (ME) was determined by analysing two representative species of low (hake, 2%) and high (salmon, 25%) lipid content, which was determined using 1.5 g of

lyophilized sample by PLE extraction with hexane:dichlorometane (1:1) followed by gravimetric determination [33]. The ME was calculated according to the following equation:

$$ME\% = ([(B-n)-A]/A)\cdot 100 \quad \text{Eq. 3}$$

where *B* is the peak area of the analyte from hake or salmon sample spiked after SBWE extraction, *n* is the peak area of the analyte present in blank sample, and *A* is the peak area of the analyte from standard solution directly injected in the chromatographic system. As such, ME<0 indicates ion suppression, while ME>0 indicates ion enhancement. ME=0 indicates that there is no matrix effect. When the on-cell clean-up strategy was applied, the ME was reduced, obtaining a range between -29% and 60% for hake and between -52% and -20% for salmon, in comparison to the results obtained without a clean-up step (from -54% to 100%, and from -99% to 120%, respectively). Thus, on-cell clean-up with the aforementioned conditions was applied.

3.2. Method validation

Before validating the method, the matrix effect was studied by statistically comparing the slopes of the calibration curves (α=0.05) for hake (low lipid content) and salmon (high lipid content). As expected, the matrix effect was observed for almost all of the compounds. Thus, both hake and salmon were used to validate the method as representative species of low and high lipid content, respectively. The method was validated by calculating the linear ranges, method detection limits (MDLs), method quantification limits (MQLs), apparent recoveries (R_{ap}) and intra-day and inter-day repeatabilities (Table 3).

The linear range was evaluated by matrix matched calibration by spiking hake and salmon at different concentrations. Non-spiked samples were also analysed to subtract the signal of the analytes present in the samples. The hake blank samples, corresponding to the white fish group, showed the presence of almost all the analytes. In turn, the salmon blank samples, corresponding to the fatty fish group, only showed the presence of BT and ClBT. To perform the matrix matched calibration curves, eight calibration points for both group of seafood samples were used and good linearity for all compounds was achieved (R^2 > 0.998) (Table 3).

MDLs corresponded to the concentration that caused a peak with a signal/noise ratio equal than 3 for the compounds that did not appear in the blanks. For the compounds present in the samples, they were estimated as the concentration that gave a signal average of three times higher than the standard deviation of the blank signal. Thus, MDLs were between 1 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.) for hake, and between 0.5 ng g^{-1} (d.w.) and 10 ng g^{-1} (d.w.) for salmon. MQLs were defined as the lowest points of the calibration curves, ranging from 5 ng g^{-1} (d.w.) to 50 ng g^{-1} (d.w.) for both seafood species. MDL and MQL values were consistent with those found in the literature for other solid environmental matrices, as there

Table 3. Validation data

Compound	MDLs (ng g^{-1} (d.w.))		Linear range (ng g^{-1} (d.w.)) [a]		Apparent recovery (%) [b]		Intra-day (%) [c]		Inter-day (%) [c]	
	Hake	Salmon	Hake	Salmon	Hake	Salmon	Hake	Salmon	Hake	Salmon
BT	1	1	5-250	10-250	132	86	2	14	7	10
ClBT	5	0.5	10-250	5-250	97	133	9	11	11	11
MeSBT	5	1	10-250	10-250	114	135	7	14	4	12
NH_2BT	5	10	10-250	50-400	104	120	10	19	9	20
OHBT	10	10	50-250	50-400	122	110	15	17	15	19

[a] MQLs were fixed as the lowest calibration point.
[b] Apparent recoveries (%), n=5, 100 ng g^{-1} (d.w.)
[c] Intra and inter-day repeatabilities (%), n=5, 50 ng g^{-1} (d.w.)

are no studies on the selected analytes in fish and shellfish. Stasinakis et al. [16] reported detection limits between 0.042 ng g^{-1} (d.w.) and 13 ng g^{-1} (d.w.) and quantification limits between 0.14 ng g^{-1} (d.w.) and 41 ng g^{-1} (d.w.) in the determination of BT, OHBT, MeSBT and NH_2BT in sludge by solid-liquid extraction (SLE) followed by solid-phase extraction (SPE) and liquid chromatography. In another recent study, detection and quantification limits from 0.25 ng g^{-1} (d.w.) to 25 ng g^{-1} (d.w.) and from 0.5 ng g^{-1} (d.w.) to 50 ng g^{-1} (d.w.) were recorded when working with SBWE and SPE extractions, respectively, and liquid chromatography, for the determination of BT, OHBT, MeSBT and NH_2BT in sludge [18].

Intra-day and inter-day repeatabilities (n=5), expressed as relative standard deviation (%RSD), were calculated using 1 g of spiked sample of each matrix (n=5) at concentrations of 50 ng g^{-1} (d.w.) and 100 ng g^{-1} (d.w.) and, since the obtained results were comparable, table 3 shows the values obtained at 50 ng g^{-1} (d.w.). They were lower than 21% for all of the compounds in each matrix. Apparent recoveries (R_{ap}) were obtained from 1 g of spiked sample of each matrix (n=5) at concentrations of 100 ng g^{-1} (d.w.) (Table 3). R_{ap} were calculated using the following formula:

$$R_{ap} = \frac{A-n}{B} \cdot 100 \qquad \text{Eq. 4}$$

where A is the peak area of the analyte from hake or salmon spiked before SBWE extraction, n is the peak area of the analyte present in blank sample, and B is the peak area of the analyte from standard solution directly injected in the chromatographic system.

They ranged from 97% to 132% and 86% to 135% for hake and salmon, respectively. As expected based on their lipid content, when comparing both matrices, higher R_{ap} values were reported for hake than for salmon.

3.3. Application to seafood samples

3.3.1 Benzothiazoles levels in commercial seafood

The SBWE SPME GC-IT-MS/MS method was applied to determine benzothiazoles in 10 species of fish and shellfish highly consumed in the county of Tarragona. Composite samples for each species were used in order to avoid origin influences. Furthermore, species were classified in 2 different groups (white fish and fatty fish) according to their lipid content (Table 4). To ensure the correct identification of the analytes, some criteria were taken into account. The retention time had to match that of the standard analyte within ± 1 s, the signal-to-noise ratio (S/N) had to be ≥3, and deviation of the two monitoring ion intensities ratio had to be within 15% of that of the standard analyte. As already described, two matrix-matched calibration curves were used to quantify white and fatty fish species. The average concentrations (n=5) of benzothiazoles in these ten

Table 4. Concentration of benzothiazoles (ng g^{-1} (d.w.)) in samples of 10 seafood species from the county of Tarragona, Spain

Compound	White fish						Fatty fish			
	Cod (1%)* (*Gadus morhua*)	Sole (6%)* (*Solea solea*)	Mussel (8%)* (*Mytilus galloprovincialis*)	Squid (6%)* (*Loligo vulgaris*)	Shrimp (2%)* (*Aristeus antennatus*)	Hake (2%)* (*Merluccius merluccius*)	Tuna (16%)* (*Thunnus thynnus*)	Mackerel (17%)* (*Scomber scombrus*)	Sardine (14%)* (*Sardina pilchardus*)	Salmon (25%)* (*Salmo salar*)
BT	18	49	58	82	47	13	n.d.	30	13	29
ClBT	n.d.	n.d.	19	<MQL	n.d.	n.d.	8	38	38	6
MeSBT	14	17	24	21	23	11	<MQL	11	17	n.d.
NH_2BT	16	27	26	38	70	11	n.d.	n.d.	<MQL	n.d.
OHBT	n.d.	n.d.	<MQL	n.d.	n.d.	<MQL	n.d.	n.d.	n.d.	n.d.

MQL: Method quantification limit; n.d.: not detected
* in brackets = (%) Percentage of lipid content

commercial seafood species, expressed as dry weight (d.w.), are summarized in Table 4.

BT was found in almost all of the samples, being the compound with the highest concentration. Squid was the species with the greatest value (82 ng g^{-1} (d.w.)), while the minimum BT level corresponded to tuna (n.d.). The second most abundant analyte in seafood was MeSBT, the concentration of which ranged between n.d. and 24 ng g^{-1} (d.w.), for salmon and mussel, respectively. Unlike NH_2BT, ClBT was more frequent in fatty fish samples than in white fish. Finally, OHBT was only found in two samples of white fish, hake and mussel, at concentrations <MQL. Mussel was the only species in which all benzothiazoles could be detected. The concentrations in mussels ranged between <MQL, for OHBT, and 58 ng g^{-1} (d.w.), for BT. Hake, squid and sardine showed four of five analysed benzothiazoles.

To the best of our knowledge, this is the first study reporting the presence of benzothiazoles in seafood. Consequently, our results cannot be compared with those from previous studies. However, data seem to be in accordance with those already published related to the presence of benzothiazoles in water. For instance, a review focusing on the occurrence of benzothiazoles, among other contaminants, in the environment [25], highlighted the presence of BT, OHBT, MeSBT and NH_2BT in effluent sewage water of different European countries and China, at low μg L^{-1} levels. Consequently, they can be present in superficial water and accumulate in biota.

3.3.2 Estimated dietary intake and risk evaluation

A summary of the estimated dietary exposure (ng kg^{-1} bw day^{-1}) to BT, ClBT, MeSBT, NH_2BT and OHBT through fish and shellfish consumption by the general population of Tarragona is depicted in Fig. 2. Human exposure was assessed for 6 population subgroups, based on age and gender, and assuming an upper-bound intake (U.B). Concentrations of benzothiazoles in seafood were recalculated and expressed on a wet weight (w.w.) basis for the human exposure assessment [34].

Although BT was the most frequent compound, the highest estimated intake corresponded to MeSBT (22 ng kg^{-1} bw day^{-1}) for senior women. It was followed by BT (11 ng kg^{-1} bw day^{-1}) in adult women. Overall, these two compounds were the main contributors to the total intake of benzothiazoles. Women, both senior and adult, showed the greatest intake of all benzothiazoles, which was estimated at 48 ng kg^{-1} bw day^{-1}. A linear increase of dietary intake with the age of the population was found (Fig. 2), with women displaying higher exposure than men. Due to the very limited data concerning the oral safety levels of benzothiazoles, the risk evaluation was only attainable for BT. A pTDI was used, estimated from an oral NOAEL of 5.1 mg kg^{-1} bw day^{-1} [12].

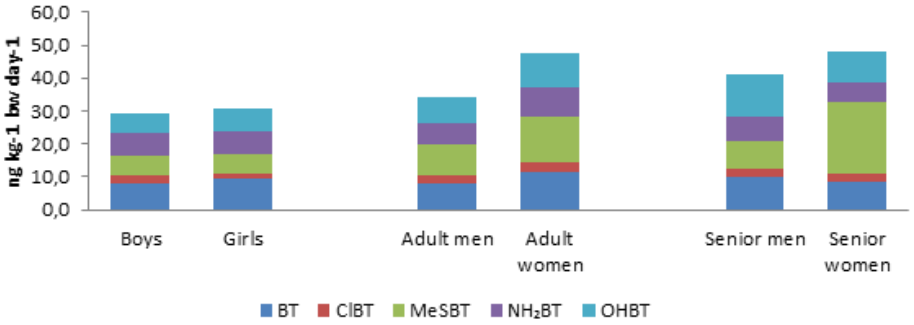

Fig. 2 Estimated dietary intake (ng kg^{-1} bw day^{-1}) of benzothiazoles for the general population of the county of Tarragona (Spain), classified by gender and age

This value was divided by 1000 as an uncertainty factor [31]. Thus, the pTDI used in the present study was 5100 ng kg^{-1} bw day^{-1}. In a worst-case scenario (UB intake), the risk was determined at 0.16% and 0.18% for boys and girls, 0.16% and 0.22% for adult men and adult women, and 0.19% and 0.16% for senior men and senior women, respectively. As there is no threshold value for risk characterization, these values could not be compared.

Recent literature shows a few studies about human exposure to benzothiazoles, with a lack of information regarding risk assessment. One of them studied dust ingestion as a route of human exposure in the USA and East Asian Countries. The results were categorized by age, reporting values of 2.871 and 0.452 ng kg^{-1} bw day^{-1} in children and adults in the USA, and values ranging from 0.520 to 4.221 ng kg^{-1} bw day^{-1} and 0.104 to 0.911 ng kg^{-1} bw day^{-1} in children and adults from Asian countries [35]. Overall, those intake values were 1-2 orders of magnitude lower than the values reported in the present study, thus highlighting the potential role of food as an exposure pathway for benzothiazoles. In turn, other investigations have focused on non-dietary sources. The inhalation pathway was studied by Wan et al. [22], who reported a 95th percentile exposure of 9.24 and 6.86 ng kg^{-1} bw day^{-1} in two population groups of different ages: 12-21 years old and ≥ 21 years old, respectively. These data would be in line with values of the present research. In any case, more information is needed to identify the contribution of dietary routes and non-dietary pathways for exposure to benzothiazoles.

4. Conclusions

An analytical method based on SBWE SPME followed by GC-IT-MS/MS has been developed, for the first time, to enable the determination of benzothiazoles in complex matrices,

such as seafood, at ng g^{-1} (d.w) levels. The method also provided good linearity, intra and inter-day repeatability and MDLs and MQLs at low nanogram per gram levels.

To the best of our knowledge, this is the first study that demonstrates the presence of five benzothiazoles in 10 commercial species of seafood, purchased in the county of Tarragona. Diet, and more specifically seafood consumption, was identified as a key pathway for exposure to benzothiazoles, although information on the contribution of other routes is still very limited. Our results showed detectable levels of BT and MeSBT in almost all of the samples, with squid being the species with the highest level of BT (82 ng g^{-1} (d.w.)). Moreover, mussel was the species which presented detectable amounts of all the benzothiazoles. Overall, the current intake of benzothiazoles through seafood consumption cannot be compared to threshold values because of the lack of reliable toxicological data.

Acknowledgments

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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3.1.4. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

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

GC-IT-MS/MS was successfully applied to determine the EOCs that are the focus of this doctoral thesis, even benzothiazoles which are normally determined using liquid chromatography. Various extraction techniques were used for each group of compounds, all of which used clean-up steps due to the complexity of the sample matrix.

QuEChERS extraction was adapted to determine polycyclic musks, nitro musks and HHCB-Lactone by Vallecillos *et al* [1]. For the efficient extraction of the target compounds, various clean-up strategies were evaluated, including dSPE being tested with different amounts of florisil as sorbent (1, 2 and 3 g), the addition of 2% of formic acid in the dSPE step of the acetonitrile layer and a post-extraction clean-up with both anion and cation exchange sorbents. The addition of formic acid and the post clean-up steps were not successful, and were therefore discarded. However, the matrix effect was reduced using 2 g of florisil because when dSPE was used with 1 or 3 g of florisil analytes showed either signal enhancement or no significant differences, respectively. Therefore, with 2 g of florisil as clean-up sorbent recovery values were similar (47%-117%) for hake, salmon and mussels, the seafood species selected to validate the method despite the differences in their lipid content. On the other hand, the method of Barón *et al* [2] was adapted to determine eight PBDEs, eight MeO-PBDEs and three emerging BFRs with PLE followed by an exhaustive clean-up step. The adaptation involved an acid treatment to remove lipid content from the samples, followed by an SPE clean-up with Al-N cartridges. The target compounds retained in the cartridges were eluted with hexane:dichloromethane, which was evaporated to dryness and then reconstituted in toluene. In contrast, a sensitive SBWE followed by SPME extraction was developed to determine five benzothiazoles in seafood for the very first time. In the optimization of the SPME process for seafood samples, two different SPME fibres were tested: PA 85 μm and PDMS-DVB 65 μm. PDMS-DVB 65 μm fibre gave the best results. Although the sorption and desorption process in SPME is affected by various parameters, the extraction temperature is one of the most important so it was optimised. The peak areas of the analytes were highest at 80 ºC. For SBWE, the extraction conditions were established from a previous study [3] and various extraction times were tested. Clean-up strategies were tested to remove matrix interferences. During the optimization of the clean-up strategies on-cell clean-up improved the extraction process. Peak areas were between 80%-95% and 60% higher than with no clean-up and LLE, respectively. Therefore, ME was evaluated using on-cell clean-up for two representative species of low (hake) and high lipid content (salmon). The results showed lower values of ME when on-cell clean-up was applied (between-29% and 60% for hake, and between -52% and -20% for salmon) than when no clean-up was used (between -54% and 100% for hake, and between -99% and 120% for salmon). Hence, on-cell clean-up was selected. Although ME was observed by comparing the results obtained with and without clean-up strategies, a t test (α=0.05) was performed to compare the slopes of the calibration curves for hake and salmon. As expected, the ME was confirmed

and both seafood species were used to validate the method for low lipid content species (hake) and for high lipid content species (salmon). The method provided good R_{ap} values between 97% and 132% for hake and between 86% and 135% for salmon.

Because it was not possible to remove the entire matrix effect from the samples, all methods included clean-up steps. Moreover, the seafood species were classified in different groups according to their lipid content. Matrix-matched calibration curves were used for quantification in order to obtain more reliable results, except for the method used to determine BFRs since it was adapted from Barón *et al* [2]. Overall, linearity (R^2<0,998) and inter-day and intra-day repeatabilities (RSD% < 21%) were good for all methods. All compounds were found at ppb levels.

The methods developed were applied to seafood species such as hake, sole, cod, shrimp, squid, salmon, tuna, mackerel, sardine and mussels purchased from three different establishments in Tarragona County. Overall, seafood samples showed five out of ten synthetic musk fragrances and seven out of nineteen BFRs while for benzothiazoles all target analytes were found in the various species (see Figure 1.3). HHCB was found in all seafood species (16-367 ng g^{-1} (d.w.)) whereas AHTN was found in all samples except mussels. These results are consistent with the fact that HHCB and AHTN are the synthetic musk fragrances generally found at higher concentrations in sediments and surface waters [4, 5] and also, the relatively high concentration of HHCB agrees with the results reported in fish tissues [6]. In the case of PBDEs and MeO-PBDEs, BDE28, BDE47 and 5-MBDE100 were found in all seafood species and BDE99 was found in all samples except mackerel. The higher concentrations of BDE47 (0.6-1.3 ng g^{-1} (d.w.)) found in seafood samples agree with some other scientific studies that have been reported [2, 7, 8]. Although 2-MBDE68 and 6-MBDE47 were not present in all samples, they showed a higher concentration of MeO-PBDEs which is in agreement with data from the scientific literature [9, 10]. Of the EFRs, only HBB was found in eight out of ten seafood samples (<0.04-0.4 ng g^{-1} (d.w.)) and no traces of PBEB and DBDPE were found in any of analysed samples, in accordance with the literature [2, 11]. Finally, only two benzothiazoles were found in almost all samples, BT and MeSBT being the most predominant. As this is the first study to report the presence of these EOCs in seafood samples, the results cannot be compared with those from previous studies. Some studies [12], however, report their presence in effluent water and sewage sludge, which suggests that they might be present in aquatic biota. Therefore, considering all 24 analytes (the sum of three EOC families), seafood species reported between 10 and 14 different sorts of analyte (40-60%), hake, mackerel, sardine and mussels being the species with 60% of the presence of these EOCs, followed by sole, code and shrimp (50%).

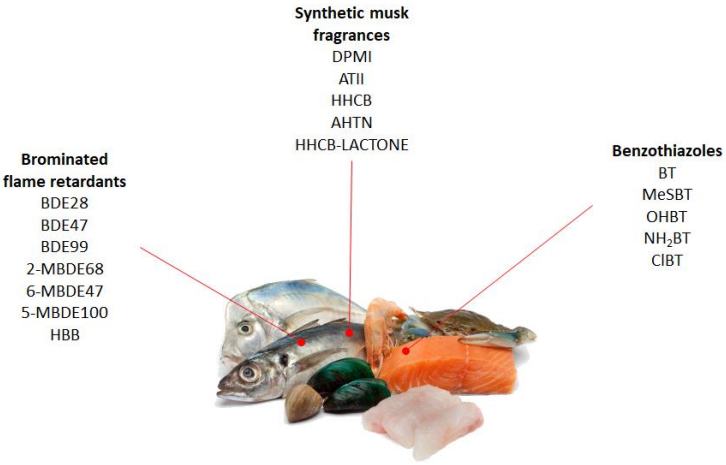

Figure 1.3. Synthetic musk fragrances, brominated flame retardants and benzothiazoles found in seafood from Tarragona County, Spain.

The dietary intake of synthetic musk fragrances was calculated using data from mean and higher consumer while BFRs and benzothiazoles used 6 population subgroups divided by age and gender. The study of BFRs used lower, middle and upper-bound scenarios (LB, MB and UB) in order to evaluate all the cases of exposure whereas the dietary intake of benzothiazoles was evaluated only in the worst-case scenario (UB). Also, in order to facilitate the assessment of dietary intake, wet weight basis (w.w.) was used to express the toxicological results [13]. The highest exposure related to synthetic musk fragrances was estimated for HHCB and HHCB-Lactone with mean values of 19.7 ng kg^{-1} bw day^{-1} and 6.8 ng kg^{-1} bw day^{-1}, respectively, and lower values of AHTN, DPMI and ATII. The accumulative contribution of white fish to overall exposure to synthetic musk fragrances varied from 55% to 84% for mean consumers and, in the case of fatty fish, from 11.5% to 49.5%. Although the concentrations of synthetic musk fragrances were higher in fatty fish, the high consumption of white fish led to greater exposure to musks. Hake, the most consumed fish in Tarragona County, was the main contributor to overall exposure to musk congeners. The major contributor to BFR intake was ∑PBDEs for all scenarios and subgroup populations with intakes ranging between 48%-68%, followed by ΣMeO-PBDEs, in the upper- and middle-bound scenario, and HBB in the lower-bound scenario. The main subpopulation that presented high exposure to BFRs was senior men in the upper- and middle-bound scenario (1.38 and 1.08 ng kg^{-1} bw day^{-1}), respectively, and adult women in the lower-bound scenario (0.83 ng kg^{-1} bw day^{-1}). The total intake of PBDEs (ΣPBDEs) through the consumption of seafood was found to be 0.45 ng kg^{-1} bw day^{-1}, comparable to the value obtained in the literature [14]. Moreover, similar results have been obtained in a recent study which analysed the same BFRs, among others, in seafood from European countries [15]. Indeed, the dietary intake of some BFRs in the European population was similar to that of the Tarragona population. Similar exposure values have been found in

other European countries such as Sweden [16] and Italy [17]. There is very little information about the dietary intake of MeO-PBDEs. These methoxylated PBDEs were studied in the Hong Kong population through fish consumption and the intake values ranged between 0.5-4.3 ng kg^{-1} bw day^{-1}. These levels of exposure were higher than those for the inhabitants of Tarragona County. Similarly, the dietary intake assessment of emerging BFRs found mainly in Asian countries such as China, which have more production areas of BFRs than other countries. The highest estimated intake of benzothiazoles corresponds to MeSBT with a value of 22 ng kg^{-1} bw day^{-1} for senior women followed by BT with a value of 11 ng kg^{-1} bw day^{-1} for adult women. Both adult and senior women showed the highest intake values of all benzothiazoles (48 ng kg^{-1} bw day^{-1}) and the linear trend between age and women was higher than between age and men.

There are very limited toxicological data available about the risk of these compounds. Therefore, risk characterization was only possible for HHCB, AHTN, BDE47, BDE99, BDE153, BDE209 and BT. The pTDI values used for synthetic musk fragrances and benzothiazoles were 1500000, 50000 and 5100000 ng kg^{-1} bw day^{-1} for HHCB, AHTN and BT, respectively, obtained from their corresponding NOAEL values. Whereas for BFRs, BMDL values of 309000, 12000, 83000 and 1700000 ng kg^{-1} bw day^{-1} were used for BDE47, BDE99, BDE153, BDE209, respectively.

Finally, the estimated risk of exposure to HHCB, AHTN and BDE congeners was far from threshold values.

However, the risk characterization for BT in the worst case scenario (UB) was 0.16% and 0.18% for boys and girls, 0.16% and 0.22% for adult men and women and 0.19% and 0.16% for senior men and women, respectively. As no risk threshold value exists for BT, these values could not be compared.

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3.2. Bioaccessibility of relevant synthetic musk fragrance in fish

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

The health benefits of a diet based on seafood have been widely reported because of the high levels of polyunsaturated n-3 fatty acids, essential elements and vitamins [1–3]. However, the accumulation of a wide range of EOCs in seafood causes some concerns for human health. Therefore, in order to characterise the risks for human health, dietary intake is generally evaluated on the basis of the concentrations in raw fish.

Although few studies have been made, Alves *et al* [4] and Cunha *et al* [5] demonstrated that both bioaccessibility and culinary treatments are factors that need to be taken into account by risk assessment studies because they affect the concentration of EOCs in raw fish, which will be different from the concentration ingested.

Synthetic musk fragrances, a wide range of different compounds including galaxolide (HHCB), have been extensively used in personal care products (PCPs). This widespread use in PCPs and other consumer products, and its ubiquity, means that HHCB is found in all analysed species.

This section presents for the first time a study that determines the bioaccessibility of galaxolide in fish samples and the effect of different culinary treatments on HHCB concentration.

The results of this study are being prepared to be published.

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3.2.1. Preliminary assessment of the bioaccessibility of Galaxolide in fish

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PRELIMINARY ASSESSMENT OF THE BIOACCESSIBILITY OF GALAXOLIDE IN FISH

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Abstract

Generally, dietary intake is assessed and risk characterized using contaminant concentration in raw fish. This can lead to overestimation because one of the essential issues for risk-benefit analysis is to determine the maximum amount of a contaminant that can be released from the seafood matrix and absorbed by the human body (bioaccessibility). Moreover, despite the fact that most seafood products are cooked before consumption, risk is still assessed in raw products, which is a disadvantage for public health guidelines. In the present study, the *in vitro* bioaccessibility of galaxolide (HHCB) in fish samples has been studied. Spiked samples of raw hake were *in vitro* digested and aliquots of each fraction of the digestion process were analysed. HHCB was quantitatively present in the bioaccessible fraction. Then, the effect of fish cooking on HHCB was evaluated in cod and mackerel samples. Results demonstrated that steaming and grilling led to a loss of between 50-70% of HHCB in fish.

Keywords: *galaxolide, bioaccessibility, fish, cooking effect.*

1. Introduction

The emerging organic contaminant (EOC) galaxolide (HHCB) is one of the most reported synthetic musk fragrances in recent years because of its presence in several environmental compartments [1]. It has been extensively used as a personal care product (PCP) and in daily consumer products. As a result of its considerable industrial production, it is released into the environment through wastewater treatment plants (WWTPs), since they do not have enough capacity to completely remove the input amount of HHCB.

Some of the characteristics of this polycyclic musk are its hydrophobicity and poor water solubility, so HHCB is expected to be adsorbed onto organic matter and lipids. In fact, HHCB was found in such environmental matrices as water and sediments [2–5] and in aquatic biota [6–9] such as fish and shellfish, after it was discharged from WWTPs due to its physicochemical properties. Although some studies have shown that assessment of HHCB does not necessarily indicate a risk for human health [10, 11], many studies have demonstrated its ubiquity in fish and shellfish at high concentration levels [1, 7, 8] and that aquatic organisms have toxic effects [12, 13]. For this reason, risk assessment for human health is one of the topics that has recently been attracting increasing interest. Although some studies have discussed the importance of dermal exposure [14] or inhalation through indoor dust [15] as routes by which PCPs enter the human body, the diet, and especially seafood consumption, is regarded as one of the main routes of exposure [7, 16]. Nevertheless, some studies have assessed the risk of fish consumption for HHCB [7].

To determine the amount of contaminant that is released from the seafood matrix into the human body, various factors need to be considered: for example, the kind of matrix, the physicochemical properties of the analyte, and the cooking process, all of which affect the bioaccessibility process (the fraction of a contaminant that goes from the matrix to the gastrointestinal tract during digestion) [17–20]. Although bioaccessibility studies on EOCs are scarce, recently Alves *et al* [18] performed a preliminary study of the bioaccessibility for such EOCs as perfluorinated compounds (PFCs) and brominated flame retardants (BFRs) in raw and cooked seafood. And Cunha *et al* [17] assessed the bioaccessibility of bisphenol A in canned seafood. Seafood samples are the main food matrix in this kind of study but Yu *et* al [21] assessed the bioaccessibility of polybrominated diphenyl ethers (PBDEs) in different foodstuffs purchased from China to determine the various dietary pathways of PBDEs and avoid obtaining misleading results about exposure assessment. Therefore, the analysis of bioaccessibility helps risk assessment provide more information and guidelines for seafood consumption to authorities, industry and consumers even though few studies have been made on this issue.

In most of the studies found in the scientific literature about the dietary

intake of environmental contaminants, it can be observed that the food analyses were carried out only on raw food products [22–24]. However, it is clear that a very large number of food products are consumed after being cooked. Moreover, the effect of cooking on seafood can be quite different for different contaminants [25] but little is known about these effects.

Therefore, the main aim of this study was to evaluate an *in vitro* digestion to determine the bioaccessibility of HHCB and to test different cooking processes to determine whether they affect the concentration of ingested HHCB.

2. Experimental

2.1. Reagents and materials

The standard 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-[g]-2-benzopyran (HHCB, galaxolide) was supplied by Promochem Iberia (Barcelona, Spain). The internal standard d_{15}-musk xylene (d_{15}-MX) came as a 100 mg L^{-1} solution in acetone from Symta (Madrid, Spain). The individual standard solution of HHCB was prepared in acetone at a concentration of 168 mg L^{-1} for the bioaccessibility experiments while for the study of the cooking effect, an individual standard solution of HHCB was prepared at a concentration of 4000 mg L^{-1} in acetone. The working solution of 1 mg L^{-1} was prepared in ethyl acetate (EtAc).

Acetone and EtAc were GC grade with purity > 99.9%, and acetonitrile (ACN) was supplied by Prolabo (VWR, Llinars del Vallès, Barcelona, Spain). Ultrapure water was obtained from an ultrapure water purification system (Veolia Water, Sant Cugat del Vallès, Barcelona, Spain). Helium gas with purity of 99.999% was used for chromatographic analysis (Abelló Linde, Barcelona, Spain).

2.2. Sampling and sample pretreatment

Three fish species were collected from various Portuguese markets: hake (*Merluccius merluccius*), cod (*Gadus morhua*) and mackerel (*Scomber scombrus*). For each species, muscle tissue was collected from fillets without skin and pooled. For each species, specimens had uniform commercial sizes and weights. To investigate the effect of cooking, cod and mackerel samples were divided into three portions, one of which was assessed raw while the others were cooked (steamed and grilled).

All raw and cooked samples were homogenised with a grinder (Retasch Grindomix GM2000, Germany) using polypropylene cups and stainless-steel knives at 5000 rpm until complete visual disruption of the tissues. Finally, the raw samples were kept at -20ºC until *in vitro* digestion, while cooked samples were lyophilised using a freeze-drying system (Labconco, Kansas City, MO, USA) and crushed using a mortar and pestle. Then, they were kept in a dry place until analysis. In addition, a portion of raw samples was also lyophilised and crushed before analysis in order to determine their HHCB content.

2.3. In vitro digestion protocol

HHCB bioaccessibility was assessed in raw hake using the static *in vitro* human

digestion model described in Alves *et al* [18]. The simulated gastro-intestinal digestion was performed at least in triplicate with four digestion fluids: salivary, gastric, duodenal and bile. Each digestion fluid contained several inorganic and organic components to simulate the three phases of human digestion: oral, stomach and intestinal (Fig. S1).

Since HHCB concentrations in raw hake are at low ng g^{-1} [7], the samples were spiked at 10 mg kg^{-1} (d.w., dry weight) (theoretical concentration) in order to avoid concentrations under the method quantification limit (MQL) in any of the digestion phases. Thus, two sets of spiked samples were prepared for practical reasons. A total of 357 μL of standard solution of HHCB at 168 mg L^{-1} in acetone was added to 6 g of homogenised hake sample (spike 1) and 476 μL of the same standard solution was added to 8 g of homogenised hake sample (spike 2). Both spiked samples (spike 1 and spike 2) were covered with acetone to facilitate the homogenous distribution of HHCB, and then they were stirred from time to time and left in the fridge for two days to ensure equilibration. Non-spiked hake samples (blank sample) were also analysed and their signal was subtracted from the corresponding signal of each spiked sample.

Several independent digestions were performed by using 1.5 g of spiked samples and stopped in each digestion phase. Thus, an initial set of digestions was stopped in the oral phase and a second set in the stomach phase by using spike 1 samples in triplicate. Then, a third set of complete digestions (oral, stomach and intestinal phases) was assessed by using spike 2 samples. In this case, 12 replicates were done to obtain a sufficient amount of pellet, also known as the non-bioaccessible fraction (NBIO), to be analysed by the analytical method used for solid samples (see section 2.5.2). For each set, digestion was stopped by placing the reaction tubes on ice, which were then centrifuged at 2750 g at 10ºC for 10 min to separate the liquid and solid phases. The liquid phases were analysed by the method described in section 2.5.3. The liquid phase of the complete digestion is also known as bioaccessible fraction (BIO).

2.4. Culinary treatments

To investigate the effect of the culinary treatment on HHCB concentration in fish, two different species, cod and mackerel, were selected as representative of low and high lipid content, respectively. Two of the most common culinary treatments for fish were tested in triplicate (steaming and grilling). Steaming was carried out at 105ºC for 15 min for fish wrapped in aluminium foil whereas for grilling, a kitchen gas was used to cook the fish for 10 min at 175ºC.

After cooking, samples were lyophilised and analysed by the method described in section 2.5.2.

2.5. Methods of analysis

In order to analyse the different kinds of sample during digestion, two different extraction methods were applied and then the extracts were analysed by GC-

IT-MS/MS. For solid samples, including raw and cooked fish and the non-bioaccessible fraction (NBIO) or pellet, the QuEChERS (Quick, Easy, Effective, Rugged and Safe) extraction method was applied [7]. In contrast, for liquid samples, including the oral, stomach and intestinal phase or the bioaccessible fraction (BIO), the extraction was carried out by a head-space solid-phase microextraction (HS-SMPE) method [26].

2.5.1. Control of blanks

It is well-known that when working with musk fragrances some cleaning precautions must be taken to avoid possible contaminations during the experimental process. Therefore, strict glass cleaning protocols are established, and restrictions must be placed on the personal care products used by laboratory personnel (creams, lotions, perfumes, deodorants, etc.) [27, 28]. Furthermore, in order to ensure that the analytical procedure is free of contamination, both blanks and samples are analysed. Although several different cleaning protocols have been suggested, in this study all the material was cleaned using tap water and then ultrapure water in a sonication bath for 20 min. Subsequently, it was cleaned in a sonication bath with isopropanol for 30 min. Blank analyses were performed to confirm the absence of HHCB.

2.5.2. QuEChERS extraction

Solid samples were extracted by QuEChERS using the method described by Trabalón *et al*. [7]. Briefly, 0.5 g dry weight (d.w.) of freeze-dried solid sample, 10 mL of ultrapure water and 10 mL of ACN were mixed. Then, in accordance with the Standard Method EN15662 [29] an extraction salt packet (Scharlab) was added and centrifuged. The acetonitrile layer (supernatant) was removed and transferred to a 15 mL centrifuge tube containing 2 g of florisil (Sigma-Aldrich) for the dSPE (dispersive solid-phase extraction) clean-up. The tube was centrifuged and the supernatant was evaporated under a gentle stream of nitrogen to a final volume of, approximately, 1 mL. The internal standard (d_{15}-MX) was added and the extract was reconstituted to 2 mL with EtAc. Extracts were filtered with a 0.22 mm PTFE syringe filter and 10 μL of the extracts was analysed by GC–IT-MS/MS.

2.5.3. HS-SPME extraction

Liquid samples were extracted by the HS-SPME method described by Vallecillos et *al*. [26]. The fibre was conditioned in line according to the supplier's instructions, and was inserted into the GC injector before the analysis started. Briefly, 10 mL of liquid sample was poured into a 20 mL HS vial and placed in a tray for SPME. When the temperature of the heat/stir accessory reached 60 °C, the vial was transported automatically, and the headspace was allowed to equilibrate with the sample at the extraction temperature for 5 min. The PDMS/DVB 65 μm fibre (Supelco, Bellefonte, PA, USA) was then introduced through the septum and kept in the headspace of the vial for 30 min at 60 °C. During the extraction, the sample was magnetically stirred at 750 rpm. Desorption was conducted at 250

°C for 3 min, and the analysis was done by GC-IT-MS/MS. To prevent carry-over, the PDMS/DVB 65 µm fibre was cleaned by heating to 250 ºC for 10 min prior to every extraction, and a blank test was performed to check for possible contaminations.

2.5.4 GC-IT-MS/MS analysis

The chromatographic analyses were performed using the method described by Trabalón *et al* [7] with 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 chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl-dimethylpolysiloxane, 30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Phenomenex (Torrance, California, USA). The oven temperature was programmed as follows: 70ºC hold for 3.5 min, raised at 50ºC min^{-1} to 200ºC, then 5ºC min^{-1} to 240ºC and finally 20ºC min^{-1} to 290ºC and hold for 3.4 min. The carrier gas used was helium at a constant flow rate of 1 mL min^{-1}. The target compound was separated in 10 min. 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. The transfer line, manifold and trap temperatures were 280 ºC, 50 ºC and 200 ºC, respectively. For quantitative analysis of the target compound, tandem mass spectrometry (MS/MS) mode was applied. Table S1 summarises the retention time and the optimal MS parameters for HHCB and d_{15}-MX.

For quantification purposes, matrix matched calibration curves were used because the HHCB response is matrix dependent. In the case of digestion fluids, enzymes were not used to avoid HHCB degradation.

3. Results and discussion

3.1 Selection of analyte and fish species

The polycyclic musk HHCB was selected because it is present at high concentration levels in all seafood samples analysed [7, 30] due to its lipophilic characteristic. Moreover, its bioaccessibility is unknown. For example, Trabalón *et al* [7] reported concentrations for ten musk compounds and HHCB was the musk congener that showed the highest concentrations, with sardine (367.3 ng g^{-1} (d.w.)), mackerel (303.9 ng g^{-1} (d.w.)) and cod (146.7 ng g^{-1} (d.w.)) being the main affected species. Moreover, Cunha *et* al [8] showed high concentrations of HHCB for mussels ranging between 8.68 and 34.52 ng g^{-1} (d.w.) and for clams (33.10 ng g^{-1} (d.w.)). In addition, Mottaleb *et* al [31] reported concentrations of HHCB between 234 and 970 ng g^{-1} expressed as wet weight (w.w.) for bluegill. These values were 10 times higher than those reported for the other contaminants studied.

Hake was the species selected to carry out the bioaccessibility study because its low lipid content facilitates the extraction procedure and analysis.

To study the effect of the culinary treatment on HHCB, cod and mackerel were selected as representative species with low and high lipid content, respectively, which showed higher concentrations of HHCB in the study done by Trabalón *et al* [7]. Therefore, steaming and grilling, the most common cooking processes for fish [18, 25, 32], were applied to determine whether they affect the concentration of ingested HHCB.

3.2. Bioaccessibility study

First of all, levels of HHCB in non-spiked and spiked hake samples were determined. The average concentrations, expressed as dry weight (d.w.), are shown in Table 1, as are the corresponding relative standard deviations (%RSD) and the average amounts (ng) of HHCB. For non-spiked hake samples (blank samples), the concentrations were in line with previous results [7], and these values were subtracted from those obtained for the spiked samples. Both spike 1 and spike 2 showed concentrations closer to the theoretical one (10 mg kg^{-1} (d.w.)), although spike 2 showed lower precision. However, RSDs around 20% are acceptable values taking into account the complexity of both, the analytical method and the matrix [30].

Spike 1 and spike 2 samples were submitted to the *in vitro* digestion as it has been described in section 2.3. Table 2 collects the average amount (ng) of HHCB in all analysed digestion phases, the percentage (%) of HHCB in each digestion phase and their %RSDs.

Although HHCB was not expected in any of the digestion fluid blanks, salivary fluid contained small amounts which were subtracted from the amount of oral phase. The RSD value was highest for the intestinal phase, probably because it was the last step of the *in vitro* digestion and, consequently, sample manipulation was higher. As can be seen in Table 2, HHCB was quantitatively extracted by the intestinal phase while oral and stomach phases did not significantly affect it. Figure 1 shows the bioaccessible fraction (BIO) versus the non-bioaccessible fraction (NBIO). Although the concentration of HHCB found in the pellet (NBIO) was approximated because 12 replicates were used to obtain a sufficient amount of sample due to the method limitations, results demonstrated that HHCB was highly bioaccessible (around 100%) in raw hake. Because no studies have been made of HHCB bioaccessibility, our results cannot be compared.

However, Alves *et al* [18] reported bioaccessibility values for different EOCs and showed that raw mackerel provided high bioaccessibility (75% and 89.6%) for perfluoroundecanoic acid (PFUnA) and for hexabromocyclododecane (α-HBCD), respectively. In addition, polybrominated diphenyl ethers (PBDEs) showed low bioaccessibility (18-26%) in mackerel. Cunha *et al* [17] also reported bioaccessibility values between 81% and 99% in canned tuna and canned sardine for bisphenol A.

Table 1. Average concentrations (n=3) of HHCB in spiked and non-spiked raw freeze-dried hake prior to *in vitro* digestion.

Sample	HHCB		
	ng g^{-1}(d.w.)	ng in sample	%RSD
Blank sample	22.2	11.1	10
Spike 1	11899.4	5953.0	15*
Spike 2	13581.0	6795.4	18*

(*) spiked at 10 mg kg^{-1} (d.w.)

Table 2. Average amount (ng) of HHCB in samples from *in vitro* digestion and the digestion fluids' blank.

Phase	Sample	HHCB		
		ng in sample	%	%RSD
ORAL	Blank of salivary fluid (n=1)	5.1		-
	oral phase (n=3)	37.6	0.6	15
STOMACH	Blank of gastric fluid (n=1)	n.d.		-
	stomach phase (n=3)	103.0	1.7	9
INTESTINAL	Blank of duodenal:biliary fluid (n=1)	n.d.		-
	intestinal phase (n=12)	9197.1	135	20
	Pellet (12 pooled digestions) (n=1)	1089.4		-
	Pellet (estimation for 1 digestion)	90.78	1.3	-

n.d.: not detected.

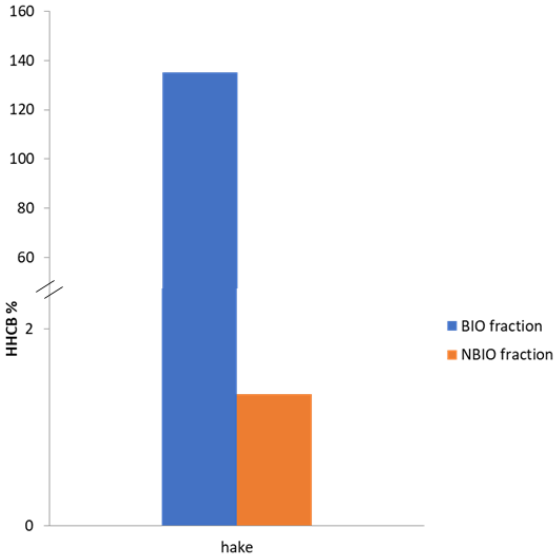

Figure 1. Bioaccessible and non-bioaccessible fraction.

3.3. Effect of cooking

The effect of steaming and grilling on HHCB concentration was investigated in non-spiked cod and mackerel samples. These species were selected as representative of low and high lipid content, respectively, in order to asses not only the effect of the culinary procedure but also the effect of lipids on HHCB degradation. Since previous studies showed the presence of HHCB at higher levels in these species, non-spiked samples were used. For each species, pooled samples were divided into three groups (raw, grilled and steamed) and each group into three subgroups, which were analysed in triplicate. The results obtained, expressed as ng g^{-1} (d.w.), are summarised in Table 3. In order to quantify the effect of the cooking process on HHCB concentration, recoveries were calculated by the following equation 1:

$$R = \frac{[\text{cooked fish}] \cdot 100}{[\text{raw fish}]} \quad (1)$$

where *R* is the recovery of the HHCB in each culinary treatment for different fish species, *[cooked fish]* means the concentration of HHCB in cooked fish samples (ng g^{-1}) and *[raw fish]* means the concentration (ng g^{-1}) of HHCB in raw fish samples. Recoveries are also included in Table 3.

Raw cod samples showed an average concentration of HHCB of 76.9 ng g^{-1} (d.w.), while raw mackerel samples showed a higher average value, 103.7 ng g^{-1} (d.w.). These values are in

accordance with previous studies [7] and corroborate that the lipophilic properties of HHCB cause this compound to be at higher concentrations in high lipid content species. Both steamed and grilled cooking processes reduced HHCB levels in both raw species. Although grilling was expected to have a greater influence on HHCB degradation because it is a more aggressive cooking procedure, no significant differences were found for the two species. To confirm this, the two culinary treatments were studied using statistical tests ($\alpha = 0.05$) for cod and mackerel samples. The values obtained showed no significant differences. The presence of HHCB after both cooking procedures was reduced by around 50% in cod and 70% in mackerel. Although RSD values are acceptable, mackerel showed higher values than cod probably because of its greater matrix complexity caused by the presence of lipids [33].

However, HHCB reduction was greater in mackerel, a high lipid content species, than in cod, a lower lipid content species, with a difference of about 20%. These results agree with Domingo *et al* [25] in the sense that cooking processes that remove fat from fish tend to reduce levels of contaminant concentration in cooked fish.

The preliminary findings of the present study highlight the importance of performing further research on the dietary exposure of HHCB focusing on more realistic data, such as the effect of cooking procedures and bioaccessibility.

4. Conclusions

This study focuses on HHCB bioaccessibility through fish consumption as well as the concentration of HHCB in cooked fish in Portugal. In general, the preliminary results revealed that the intestinal phase showed the highest concentration of HHCB, while the oral and stomach phases showed insignificant concentrations. As a result, HHCB was around 100% bioaccessible in the *in vitro* digestion procedure for raw fish samples. Moreover, steaming and grilling procedures caused the HHCB content to decrease between 50% and 70% in cod and mackerel, respectively. The results indicate that exposure should be assessed and health risk characterized in cooked fish samples because the bioaccessibility results obtained in raw fish samples may be misleading.

Acknowledgments

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP/2007-2013) under grant agreement nº 311820.

Table 3. Concentrations and recoveries of HHCB in cod and mackerel (raw, steamed and grilled) in ng g^{-1} (d.w.).

	Cooking process	ng g^{-1} (d.w.)	%RSD	Average ng g^{-1} (d.w.)	%Recovery
Cod	Raw	78.4	8	76.9	100
		77.6	5		
		74.7	6		
	Steamed	47	12	41.6	54
		41	21		
		35	14		
	Grilled	39.7	7	37.5	49
		33.1	10		
		39.7	5		
Mackerel	Raw	98	16	103.7	100
		107	21		
		104	17		
	Steamed	23	19	28.8	27
		31	18		
		30	17		
	Grilled	28	15	31.9	31
		36	15		
		30	20		

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Supplementary information

UNIVERSITAT ROVIRA I VIRGILI
SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

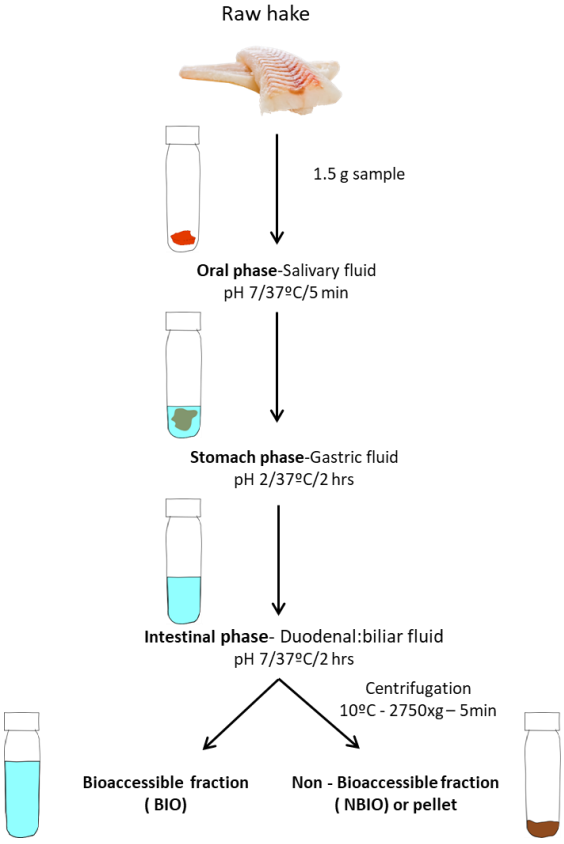

Figure S1. *In vitro* digestion scheme used to assess HHCB bioaccessibility [18].

Table S1. Retention times and MS conditions.

Compound	Retention time (min)	Ions (m/z)	Product ions [a] (m/z)	Ionization storage level (m/z)	CID amplitude (V)	CID storage level (m/z)	m/z range	Scan time (s/scan)
HHCB	8.99	243	171, **213**	70	0.96	107	117-253	0.53
d_{15}-MX	9.40	295	170. **276**, 295	70	1.11	129	139-305	0.34

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

3.2.2. Discussion of results

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In this study, an *in vitro* human digestion process was performed for the first time to determine the bioaccessibility of galaxolide (HHCB) and the effect of cooking fish on HHCB was evaluated.

HHCB was selected because of its high presence in fish samples and its lipophilic characteristics. Also, its bioaccessibility is unknown.

Two analytical methods and the *in vitro* digestion protocol [1] were successfully applied. For solid samples, the QuEChERS extraction method described by Trabalón *et al* [2] was used while for liquid samples the HS-SPME method was used [3].

To assess the bioaccessibility study, spiked raw hake was used to ensure the concentrations obtained were above the method quantification limits (MQLs) in each digestion phase since the concentration of HHCB in raw hake is at low ng g^{-1}. The *in vitro* digestion process was divided into three different liquid phases – oral, stomach and intestinal or the bioaccessible fraction (BIO) – and, one solid phase, known as the pellet or non-bioaccessible fraction (NBIO). Regarding the effect of steaming and grilling, raw cod and mackerel were collected as representative species for white and fatty fish, respectively. Pooled samples were divided into three groups (raw, grilled and steamed) and each group was then subdivided into three subgroups, which were analysed by GC-IT-MS/MS.

Overall, the bioaccessibility study reported that all the HHCB content was almost 100% bioaccessible for the human body because the intestinal phase showed a concentration that was close to the initial spiked one, while the oral and stomach phase did not affect the digestion process. Moreover, the presence of HHCB was not observed before the intestinal phase probably because the digestion conditions cause the release of HHCB from fish in the intestinal phase. The results obtained from cooked fish agreed with the literature [4–6] since both steaming and grilling decreased the concentration of HHCB after the fish had been cooked. Steaming decreased it around 50% while grilling decreased it around 70%. In addition, it seems that fish which has a high lipid content, such as mackerel, tends to lose a greater concentration of HHCB after cooking because HHCB shows lipophilic behaviour [4].

Therefore, the results show that bioaccessibility studies and cooked samples should be used in studies on exposure assessment and human risk characterisation to avoid drawing misleading conclusions.

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CHAPTER 4. CONCLUSIONS

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SEAFOOD AS A DIETARY SOURCE OF EMERGING ORGANIC CONTAMINANTS. A CASE-STUDY IN TARRAGONA COUNTY, SPAIN.
Laura Trabalón Escoda

The main conclusions drawn from the studies presented in this doctoral thesis can be summarized as follows:

1. PLE with hexane/dichloromethane as extracting solvent successfully extracted brominated flame retardants from seafood samples. In addition, SBWE was useful for extracting benzothiazoles, because it did not require the use of organic solvents and allowed SPME to be applied directly. Moreover, QuEChERS extraction with the Standard Method EN 15662 was successfully applied to extract synthetic musk fragrances from seafood samples.

2. A clean-up step was mandatory because the matrix effect was high. The most effective strategies depended on the extraction technique used and the type of analyte: dSPE was used with florisil as the clean-up sorbent when synthetic musk fragrances were extracted by QuEChERS, SPE was used with Al-N cartridges when brominated flame retardants were extracted by PLE and on-cell clean-up was used with hexane followed by SPME with PDMS/DVB 65 μm fibre when benzothiazoles were extracted by SBWE. In the latter case, SPME also made it possible to preconcentrate the extract.

3. GC-IT-MS/MS was successfully used to determine all the EOCs studied, including benzothiazoles, which are usually determined by LC.

4. The lipid content of seafood samples had a strong influence on the validation parameters. Thus, validation was carried out for low, medium and high lipid content species.

5. The methods that were adapted or developed to determine synthetic musk fragrances, brominated flame retardants and benzothiazoles have limits of quantification at ng g^{-1} levels for their application in seafood samples.

6. The synthetic musk fragrances HHCB and AHTN; the brominated flame retardants, BDE28, BDE47, 2-MBDE68, 6-MBDE47, 5-MBDE100 and HBB; and the benzothiazoles, BT and MeSBT, were the most frequently determined contaminants in seafood samples at ng g^{-1} levels.

7. Nitro musk and the flame retardants PBEB and DBDPE were not found in any of the samples analysed.

8. Benzothiazoles were determined for the first time in seafood samples at ng g^{-1} levels.

9. The seafood species with the highest content of the EOCs studied in this doctoral thesis were hake, mackerel, sardine and mussels.

10. For all age and gender groups, the risk characterization of synthetic musk fragrances and brominated flame retardants was lower than the reported threshold values. However, there are no threshold values for benzothiazole so no comparison is possible.

11. HHCB proved to be completely bioaccessible according to the *in vitro* digestion tested using spiked raw hake.

12. Steaming and grilling decreased the concentration of HHCB by around 50% and 70%, respectively, in comparison to raw fish. No significant differences were observed in cod and mackerel.

APPENDIX

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Appendix I. Abbreviations used in this doctoral thesis.

$[M]^+$	Molecular ion
2-MBDE68	2',3,4',5-Tetrabromo-2-methoxydiphenyl ether
4-MBDE101	2,2',4,5,5'-Pentabromo-4'-methoxydiphenyl ether
4-MBDE103	2,2',4',5,6'-Pentabromo-4-methoxydiphenyl ether
4-MBDE49	2,2',4',5-Tetrabromo-4-methoxydiphenyl ether
5-MBDE100	2,2',4,4',6'-Pentabromo-5-methoxydiphenyl ether
5-MBDE47	2,2',4,4'-Tetrabromo-5-methoxydiphenyl ether
5-MBDE99	2,2',4,4',5-Pentabromo-5'-methoxydiphenyl ether
6-MBDE47	2,2',4,4'-Tetrabromo-6-methoxydiphenyl ether
ABS	Acrylonitrile-butadiene-styrene
ADBI	Celestolide
ADI	Acceptable daily intake
ADME	Adsorption, distribution, metabolism and excretion
AHMI	Phantolide
AHTN	Tonalide
APCI	Atmospheric pressure chemical ionisation
ASE	Accelerated solvent extraction
ATII	Traseolide
A-ZIF-8	Acidified zeolitic imidazole framework-8
BDE100	2,2',4,4',6-Pentabromodiphenyl ether
BDE153	2,2',4,4',5,5'-Hexabromodiphenyl ether
BDE154	2,2',4,4',5,6'-Hexabromodiphenyl ether
BDE183	2,2',3,4,4',5',6-Heptabromodiphenyl ether
BDE209	Decabromodiphenyl ether
BDE28	2,4,4'-Tribromodiphenyl ether
BDE47	2,2',4,4'-Tetrabromodiphenyl ether
BDE99	2,2',4,4',5-Pentabromodiphenyl ether
BFR	Brominated flame retardant
BIO	bioaccessible fraction
BMD	Benchmark dose
BSTFA	[bis (trimethylsilyl) trifluoroacetamide

BT	Benzothiazole
BTs	Benzothiazole derivates
bw	Body weight
CEN	European Committee for Standardization
CI	Chemical ionization
ClBT	2-chlorobenzothiazole
d.w.	Dry weight
d_4-BTR	Deuterated 1-H-benzotriazole
DBDPE	Decabromodiphenyl ethane
DHS	Disodium hydrogencitratesesquihydrate
DPMI	Cashmeran
dSPE	Dispersive-SPE
ECHA	European Chemical Agency
ECNI	Electron capture negative ionisation
EFR	Emerging flame retardants
EFSA	European Food Safety Authority
EI	Electron impact
EOC	Emerging organic contaminant
EPA	Environmental Protection Agency
EU	European Union
FFQ	Food frequency questionnaire
FR	Flame retardant
GC	Gas chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
GCB	Graphitised carbon black
GPC	Gel permeation chromatography
HBB	Hexabromobenzene
HBCD	Hexabromocyclododecane
HHCB	Galaxolide
HHCB-Lactone	Galaxolidone
HPLC	High performance liquid chromatography
HPSE	High pressure solvent extraction

HPV	High production volume
HS	Headspace
I.S	Internal standard
IT	Ion trap
l.w.	Lipid weight
LB	Lower-bound scenario
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOAEL	Lowest-observable adverse effect level
LOD	Limit of detection
LOQ	Limit of quantification
LVI	Large-volume-injection
MA	Musk ambrette
MAE	Microwave-assisted extraction
MB	Middle-bound scenario
MDL	Method detection limits
ME	Matrix effect
MeO-PBDE	Methoxylated polybrominated diphenyl ester
MeSBT	2-(methylthio)-benzothiazole
$MgSO_4$	Anhydrous magnesium sulphate
MK	Musk ketone
MM	Musk moskene
MOE	Margin of exposure
MQL	Method quantification limits
MRM	Multiple-reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid-phase dispersion
MT	Musk tibetene
MW	Molecular weight
MX	Musk xylene

n.a.	Not available
n.d.	Not detected
NaOAc	Sodium acetate
NBIO	non-bioaccessible fraction
ND	Non-detected
NH_2BT	2-aminobenzothiazole
NMR	Nuclear magnetic resonance
NOAEL	No-observable adverse effect level
NQ	Non-quantified
OECD	Organisation for economic co-operation and development
OHBT	2-hydroxybenzothiazole
OH-PBDE	Hydroxylated polybrominated diphenyl ester
PA	Polyacrylate
PAH	Polycyclic aromatic hydrocarbons
PBB	Ppolybromobiphenyl
PBDE	Polybrominated diphenyl ester
PBEB	Pentabromoethylbenzene
PCB	Polychlorinated biphenyl
PCP	Personal care product
PDMS	Polydimethylsiloxane
PDMS/CAR	Polydimethylsiloxane/carboxen
PDMS/CAR WR	Polydimethylsiloxane/carbon
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
PFE	Pressurised fluid extraction
PHSE	Pressurised hot solvent extraction
PHWE	Pressurised hot water extraction
PLE	Pressurized liquid extraction
POP	Persistent organic pollutant
PSA	Primary-secondary amine
pTDI	Provisional tolerable daily intake
p-TSA-d_4	Deuterated Para-toluenesulfonamide

PTV	Programmed temperature vaporisation
PVC	Polyvinyl chloride
Q	Quadrupole
QqQ	Triple-quadrupole
Q-TOF	Quadrupole-time-of-flight
QuEChERS	Quick, Easy, Cheap, Rugged and Safe
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
RfD	Reference dose
RSD	Relative standard deviation
R_t	Risk factor
SBWE	Subcritical water extraction
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected-reaction monitoring
SSE	Subcritical solvent extraction
TBBPA	Tetrabromobisphenol A
TMSH	Trimethylsulfonium hydroxide
TNT	Trinitrotoluene
TSCD	Trisodium citrate dehydrate
UAE	Ultrasound-assisted extraction
UB	Upper-bound scenario
UF	Uncertainty factor
UHPLC	Ultra-high-performance liquid chromatography
US	United States
UV	Ultraviolet
UVAE	Ultrasonication and vacuum assisted extraction
VOC	Volatile organic carbon
w.w.	Wet weight
WHO	World Health Organization
WWTP	Wastewater treatment plant

Appendix II. List of publications.

List of publications derived from this Doctoral Thesis:

- Trabalón L, Alves RN, Nadal, M, Borrull F, Pocurull E, Marques A (2017) Preliminary assessment of the bioaccessibility of Galaxolide in fish (to be published).

- Trabalón L, Nadal M, Borrull F, Pocurull E (2017) Determination of benzothiazoles in seafood species by subcritical water extraction followed by solid-phase microextraction-gas chromatography-tandem mass spectrometry: estimating the dietary intake. Anal Bioanal Chem. 409:5513-5522.

- Trabalón L, Vilavert L, Domingo JL, Pocurull E, Borrull F, Nadal M (2017) Human exposure to brominated flame retardants through the consumption of fish and shellfish in Tarragona County (Catalonia, Spain). Food Chem Toxicol 104:48–56.

- Trabalón L, Cano-Sancho G, Pocurull E, Nadal M, Domingo JL, Borrull F (2015) Exposure of the population of Catalonia (Spain) to musk fragrances through seafood consumption: Risk assessment. Environ Res 143:116–122.

Complementary environmental research:

- Aznar-Alemany Ò, Trabalón L, Jacobs S, Barbosa VL, Tejedor MF, Granby K, Kwadijk C, Cunha SC, Ferrari F, Vandermeersch G, Sioen I, Verbeke W, Vilavert L, Domingo JL, Eljarrat E, Barceló D (2017) Occurrence of halogenated flame retardants in commercial seafood species available in European markets. Food Chem Toxicol 104:35–47.

- Domínguez-Morueco N, Augusto S, Trabalón L, Pocurull E, Borrull F, Schuhmacher M, Domingo JL, Nadal M (2017) Monitoring PAHs in the petrochemical area of Tarragona County, Spain: comparing passive air samplers with lichen transplants. Environ Sci Pollut Res 24:11890–11900.

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