SEAFOOD AS A LINK BETWEEN HIGH PRODUCTION VOLUME CHEMICALS CONTAMINATION AND POPULATION HEALTH

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Seafood as a link between high production volume chemicals contamination and population health

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Doctoral Thesis

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WE STATE:

That the present study, entitled "SEAFOOD AS A LINK BETWEEN HIGH PRODUCTION VOLUME CHEMICALS CONTAMINATION AND POPULATION HEALTH", presented by ÓSCAR CASTRO SERRANO for the award of degree of Doctor, has been carried out under our supervision at the Department of Analytical Chemistry and Organic Chemistry of this university.

Tarragona, February 17th 2023

Doctoral Thesis Supervisors

Prof. Eva Pocurull Aixalà

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Suposo que, com a la gran majoria de primers paràgrafs d'uns agraïments de tesi, esteu esperant llegir que content estic d'haver acabat i tot l'esforç i dedicació que m'ha suposat escriure aquest totxo. I si, tot això hi és, però poca gent en parla del sentiment de tristesa i enyorança que et deixa acomiadar un lloc i unes persones que s'havien convertit en casa teva. Deixo enrere una etapa que, encara d'haver costat sang, suor i llàgrimes (literalment de les tres), la puc considerar una de les etapes més emocionants de la meva vida. He après, he rigut, he conegut, he madurat, he canviat i, fins i tot, m'he quedat gairebé calb. No totes les emocions han sigut bones, però, pel camí també hi han aparegut l'estrès, la por i la marxa de persones importants a la meva vida. Tot això queda ja al passat, i només em queda mirar enrere per recordar, riure, aprendre i agafar empenta per continuar endavant, no sense abans donar les gràcies a totes aquelles persones amb qui he creuat camins.

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"Ain't about how fast I get there,
Ain't about what's waitin' on the other side,
It's the climb"

Miley Cyrus

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ABSTRACT

[ENG] High production volume chemicals (HPVs) are produced or manufactured in more than 1,000 tonnes per year in the European Economic Area. Synthetic musk fragrances, phthalate esters, organophosphate esters, benzothiazoles, benzotriazoles and benzenesulfonamides are just some of the compound families labeled as HPVs. Their extended use in both industrial and consumer products has led to a widespread ubiquity of these compounds, reaching the aquatic environment and in consequence, the organisms inhabiting. HPVs bioaccumulation in seafood has become a matter of concern due to its possible dietary intake via seafood consumption. The negative health effects associated to these compounds have awakened the necessity of monitoring their presence in these specimens. To do so, reliable and sensitive analytical methods are needed.

The present Doctoral Thesis focuses on the development and application of analytical methodologies for the determination of various HPV families in seafood samples using novel extraction procedures and gas chromatography-tandem mass spectrometry. Thus, methods involving novel extraction techniques or greener, easier and faster alternatives to previously described methodologies have been developed on this purpose. The methods have been applied to several seafood samples including the most consumed species of Catalonia and the Canary Islands, to provide useful data on the occurrence of HPVs in seafood of common consumption. Exposure and risk assessments have been performed to elucidate the possible risk associated to the consumption of seafood. Moreover, a preliminary study on the bioaccessibility of relevant HPVs in raw and cooked fish has been performed.

[CAT] Els productes químics d'alt volum de producció (AVPs) es produeixen i/o fabriquen en més de 1000 tones a l'any a l'Espai Econòmic Europeu. Les fragàncies sintètiques, els èsters de l'àcid fosfòric, els èsters de l'àcid ftàlic, les benzotiazoles, les benzotriazoles i les benzosulfonamides són només algunes de les famílies incloses al grup del AVPs. El seu ús estès tant en productes industrials com de consum ha donat lloc a una ubiqüitat generalitzada d'aquests compostos, arribant al medi aquàtic i, en conseqüència, als organismes que hi habiten. La

bioacumulació dels AVPs en peix i marisc s'ha convertit en un motiu de preocupació a causa de la seva possible ingesta dietètica a través del consum d'aquests aliments. Els efectes negatius per a la salut associats a aquests compostos han despertat la necessitat de controlar la seva presència en aquests espècimens. Per poder dur a terme aquest control es necessiten mètodes analítics robustos i sensibles.

La present tesi doctoral se centra en el desenvolupament i aplicació de metodologies analítiques per a la determinació de diferents famílies de AVPs en mostres de peix i marisc utilitzant noves tècniques d'extracció seguit de cromatografia de gasos - espectrometria de masses en tàndem. Per tant, s'han desenvolupat mètodes que impliquen noves tècniques d'extracció o alternatives més verdes, més fàcils i més ràpides que les metodologies descrites anteriorment. Els mètodes s'han aplicat a diverses mostres de peix i marisc, incloent les espècies més consumides de Catalunya i les Illes Canàries, per proporcionar dades útils sobre la presència d' AVPs en peix i marisc. També s'han realitzat avaluacions d'exposició i risc per a aclarir el possible risc associat al consum de peix i marisc. D'altra banda, s'ha dut a terme un estudi preliminar sobre la bioaccessibilitat dels AVPs més rellevants en peix cru i cuinat.

[ESP] Las sustancias químicas de alto volumen de producción (AVPs) se producen o fabrican en más de 1000 toneladas al año en el Espacio Económico Europeo. Las fragancias sintéticas de almizcle, los ésteres de ftalato, los ésteres organofosforados, los benzotiazoles, los benzotriazoles y las benceno-sulfonamidas son sólo algunas de las familias de compuestos etiquetados como AVPs. Su uso extendido tanto en productos industriales como de consumo ha dado lugar a una ubicuidad generalizada de estos compuestos, que llegan al medio acuático y, en consecuencia, a los organismos que lo habitan. La bioacumulación de los AVPs en el pescado y el marisco se ha convertido en motivo de preocupación debido a su posible ingesta alimentaria a través del consumo de estos alimentos. Los efectos negativos para la salud asociados a estos compuestos han despertado la necesidad de monitorizar su presencia en estos especímenes. Para ello, se necesitan métodos analíticos fiables y sensibles.

La presente Tesis Doctoral se centra en el desarrollo y aplicación de metodologías analíticas para la determinación de diferentes familias de AVP en muestras de pescado y marisco utilizando procedimientos novedosos de

extracción seguido de cromatografía de gases-espectrometría de masas en tándem. Para ello se han desarrollado métodos que implican técnicas de extracción novedosas o alternativas más ecológicas, fáciles y rápidas a las metodologías descritas anteriormente. Los métodos se han aplicado a varias muestras de pescado y marisco, incluyendo las especies más consumidas de Cataluña y Canarias, para proporcionar datos útiles sobre la presencia de AVPs en pescado y marisco de consumo habitual. Se han realizado evaluaciones de exposición y riesgo para dilucidar el posible riesgo asociado al consumo de estos alimentos. Además, se ha llevado a cabo un estudio preliminar sobre la bioaccesibilidad de los AVPs más relevantes en pescado crudo y cocinado.

CHAPTER 1. INTRODUCTION

1.1. High Production Volume Chemicals

Mankind is continuously surrounded by chemical compounds. The current way of life has led society to produce massive amounts of chemicals to satisfy both industrial and consumer necessities. Their widespread release into the environment may become a source of exposure to the population, thus being a matter of concern due to the possible health adverse effects. Some of these compounds are produced in large amounts, which automatically leads to a higher chance of the population being exposed to them. It is by this means that highly produced compounds are known and classified as High Production Volume Chemicals (HPVs).

The use of term High Production Volume Chemical refers to compounds with a minimum production or importation per year. The value set for its inclusion varies between countries or regions. Hence, the Organisation for Economic Cooperation and Development (OECD) and the European Union list compounds which are produced or imported in quantities higher than 1,000 tonnes per year in the region or at least one member country [1], whereas the United Estates Environmental Protection Agency (EPA) fixed a minimum of 500 tonnes per year as a requirement to be included. The classification of these compounds was aimed to prioritise its study to assess the potential risk associated with these compounds for the environment and the population.

Listing of HPVs began back in the mid-1980s when the US National Research Council concluded that just 22% of the HPVs produced in the United States had available toxicity data. At the same time, the OECD member countries established the necessity of investigating existing chemicals, thus leading to 1991's decision to start the investigation focusing on HPVs [2]. The focus on HPVs had its premise on the correlation between a higher production being a surrogate for data on consumer, occupational and environmental exposure. Hence, the HPV chemicals Programme started cooperatively assessing the listed chemicals, with each of the member countries agreeing to sponsor part of the HPV chemicals assessment and sharing the work. Later, in 1998, the International Council of Chemical Associations (ICCA), joined forces with the OECD HPV Program to speed up the results, aiming to provide hazard assessments for up to 1000 substances. In order to collectively assess the chemicals in a similar way, all members (countries and

industries inside the ICCA) agreed on a minimum set of information to be assessed for each chemical, which was called Screening Information Data Set (SIDS) [3].

SIDS are used as a cross-country harmonized data template on the minimum information needed to assess the hazard associated with a chemical. Member countries along with industries select a chemical to sponsor, thus providing the research needed to complete the information included in the SIDS. Information includes chemical identification, chemical and physical properties, fate and behaviour in the environment and ecotoxicological and toxicological information (Figure 1) [4]. SIDS can be described as preliminary risk assessment tools, which are then yearly discussed between experts from member countries, industry and other non-governmental organisations to evaluate the need of undergoing further investigation into chemicals predicted to be a hazard to health [5].

Final assessment reports as well as the status of all the chemicals under assessment in the Program can be consulted in the OECD Existing Chemical Database [6]. The website also includes an overall status report with detailed information on the status of chemicals in the assessment process including data on the number of chemicals for which information is being gathered, the ones with an assessment report prepared or the chemicals provisionally set aside, among other categories. Figure 2 shows a screenshot of the current overall status. To date, more than 1500 chemicals have already been assessed and have a published report in the database.

There have been other national and international HPV programs aimed to regulate these compounds, mostly with the intention of giving transparency about chemical properties and assessing their possible exposure and hazard. The US HPV Challenge Program started in 1999 by the EPA, the US-based American Chemistry Council, the American Petroleum Institute and the US-based Environmental Defense Fund invited chemical companies to voluntarily collect basic hazard information of highly produced and manufactured chemicals. Based on the OECD SIDS guidelines, companies successfully gathered data on HPV chemicals in the US market [2]. Other relevant programs included Canada's Canadian Environment Protection Act (CEPA) and the Japanese HPV Challenge. In either case, including the US program, data collection was based on the OECD's guidelines, as these fulfil the requirements of each of the commented programs.

Screening Information Data Set (SIDS)

Chemical Identity

- CAS number
- Chemical Name

Exposure information

- Production Volume
- Use pattern
- Sources of exposure

Physical Chemical Data

- Melting point
- Boiling point
- Vapour pressure
- Water solubility
- Octanol-water partition coefficient
- Dissociation constant

Environmental Fate and Pathways

- Photodegradation
- Abiotic degradation
- Biodegradation
- Transport and distribution in the environment

Ecotoxicological data

- Acute toxicity to fish
- Acute toxicity to invertebrates
- Toxicity to algae
- Long-term toxicity to aquatic organisms
- Toxicity to terrestrial organisms

Toxicological Data

- Acute toxicity
- Repeated dose toxicity
- Genetic toxicity

Figure 1. Screening Information Data Set (SIDS) minimum data to gather by the sponsors [2].

Hence, companies, manufacturers, or countries could contribute and decide whether to submit the results to any of the existing programs, thus avoiding duplication and contributing to an overall objective [4,7].

Programs and regulations of chemicals have evolved since their implementation back in the 1990s, the initial OECD HPV Program is now known as the OECD Chemicals Assessment Programme (CoCAP). The evolution appeared as a response to the worldwide increase of comprehensive chemical assessment programs, aiming to save resources and avoid duplication while including new approaches to testing chemicals. The use of in silico methods has been included as an excellent tool to fill data gaps or support experimental data. In this sense, OECD has released the (Quantitative) Structure-Activity Relationships toolbox, aimed to predict chemical properties from the chemical's molecular structure. This new program is integrated with regulation frameworks such as the European Union REACH Regulation (Registration, Evaluation and Authorization of Chemicals) or the US Toxic Substances Control Act (TSCA) [2,7–9].

Status of Chemicals in the assessment process		Detailed number of chemicals	
		All Chemicals	From ICCA Initiative
Information gathering and data review		256	72
Assessment Report prepared for Assesment Meeting		-	-
Assessment Report discussed but not agreed		23	4
Conclusions published but Assessment Report not yet available	Assessment Report not received	249	232
	Assessment Report received	12	9
	Awaiting national publication	-	-
Conclusions and Assessment Report published		1084	707
Report published and further work underway		-	-
Provisionally set aside		7	-
	Total (Assessments)	1631	1024
	Total Chemicals	1596	1006

Figure 2. Status of the OECD Chemical Assessment Program. Extracted from the OECD Existing Chemicals Database webpage (Accessed in November 2022).

All in all, the production and manufacture of compounds are closely linked to the exposure to population, thus becoming a powerful tool to prioritise their study. Over 1500 chemicals have been already assessed, which does not even cover the 50% of compounds listed in the OECD 2004 HPVs list (4843 compounds). The continuous progress and adaptations as well as the constant development of new compounds enlighten

the urge of assessing highly produced chemicals way faster, with the focus on the application of restrictions such as the ones applied by the European Union under the bottom-line no data-no market. Under this premise, ECHA (European Chemicals Agency) REACH regulation does not allow the production and manufacture of substances produced in more than 1 tonne per year without being registered [10]. That means new highly produced substances manufacturers are required to include data on the chemical substance's safety.

Due to their extensive production, the use and applications for which HPVs are manufactured cover many areas. Thus, compounds included in the HPVs list compile a broad range of physical and chemical properties. The present thesis focuses on the evaluation of an enclosed group of chemical compounds included in the HPV list as well as some of their derivates or related substances. These include synthetic musk fragrances, phthalate esters, organophosphate esters, benzothiazoles, benzotriazoles and benzenesulfonamides. The following sections will briefly summarize the most relevant characteristic of each of the aforementioned groups of compounds, including their structure, chemical and physical properties, uses and manufacture as well as data on the proved or suspected health adverse effects.

1.1.1. Synthetic musk fragrances

The pleasant odour emitted by perfumes, cosmetics, household products, or air fresheners arises from man-made chemicals included in the prior mentioned products as part of their composition. Synthetic musk fragrances with enhanced and persistent scents were created as alternatives to the previously used natural musks to reduce costs. These compounds are part of the so-called Emerging Organic Compounds (EOCs), included as part of personal care products (PCPs). These are found in daily commodities, including washing and cleaning products, perfumes and fragrances, polishes and waxes and also in biocides and air care products, always used as scent agents. From the first apparition of nitro-musks to the vastly used polycyclic musks, the journey of these compounds has evolved, with their structures and properties being adapted to new regulations and environment protection requisites.

Based on their structure, the most relevant synthetic fragrances are classified as nitro-musks and polycyclic musks. Figure 3 comprises the synthetic musk fragrances studied in the present doctoral thesis. Nitro-musks are synthetic alkylated nitro benzenes with low octanol-water coefficients and lipophilic characteristics. Musk xylene (MX) and musk ketone (MK) are two of the most important compounds in this group, with MX being very toxic for aquatic life with long lasting effects and both being under assessment for suspected carcinogenic behaviour [11,12]. European regulations prohibited the use of MX and restricted the use of MK, all due to the negative health effects linked to these substances. Nowadays, MX is not manufactured or produced in Europe due to its ban, while MK is produced at rates between 100 and 1,000 tonnes per year. These negative effects led to the necessity of developing new synthetic fragrances, with polycyclic musks emerging as possible replacements.

Polycyclic musks comprise methylated and acetylated tetralin, pyran and indane compounds being cashmeran (DPMI), celestolide (ADBI), traseolide (ATII), phantolide (AHMI), tonalide (AHTN) and galaxolide (HHCB) the main representatives. Characteristics such as high chemical stability, non-biodegradability, or poor water solubility are mutual for this group of compounds. The chirality of some of these compounds is responsible for their characteristic scent. For instance, just two of the four stereoisomers of HHCB (4S7R and 4S7S) emit distinctive musk odour [13]. Given their extended use, their production and manufacture in Europe reach rates of production from >1 - <10 tonnes per year for AHMI [14], ATTI [15], and ADBI [16], between >100 - <1,000 tonnes per year for DPMI [17] and between >1,000 - <10,000 tonnes per year for HHCB [18] and AHTN [19].

Despite their widespread use and production, some of these compounds have lately been put in the spotlight due to their believed negative effects. All the previously commented polycyclic musks are classified as very toxic to aquatic life with long lasting effects. Regarding human toxicity, the EU ECHA identifies DPMI as skin sensitising [17] as well as keeps HHCB [18] and AHTN [19] under assessment for endocrine disrupting behaviour. Some studies have reported the oestrogenic and anti-oestrogenic of AHTN and HHCB [20] as well as effects on

pregnancy development [21]. Moreover, HHCB is also being assessed as persistent, bioaccumulative and toxic.

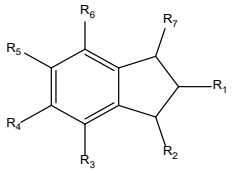
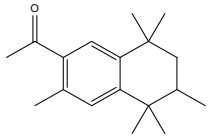
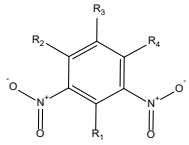
General Structure	Abbreviation	Name	M.W. (g mol^{-1})
	DPMI	6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (Cashmeran)	206.32
	ADBI	4-Acetyl-6-tert-butyl-1,1-dimethylindane (Celestolide)	244.37
	AHMI	6-acetyl-1,1,2,3,3,5-hexamethylindane (Phantolide)	244.37
	ATTI	5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (Traseolide)	258.40
	HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran (Galaxolide)	258.40
	HHCB-lac	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran-1-one (HHCB-lactone)	272.40
	AHTN	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide)	258.40
	MX	2,4,6-trinitro-1,3-dimethyl-5-tert-butylbenzene (Musk xylene)	297.26
	MK	4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (Musk ketone)	294.31

Figure 3. General structure, abbreviation, name and molecular weight of the synthetic musk fragrances studied in the present doctoral thesis.

Due to all these negative effects, new synthetic fragrances are being created, which is the case for macrocyclic musks and alicyclic musks. The first is still scarcely used due to the elevated cost, however, alicyclic musks, even though also scarcely used, are believed to be the fourth generation of musk due to their higher biodegradability and lower price [22].

The OECD and EPA HPV lists included compounds such as AHTN, HHCB, MX and MK due to their high production in Europe and the United States result of their wide use in many personal care products. These compounds were rapidly studied as the result of the sponsor provided by The Netherlands, further obtaining their corresponding SIDS. While the last OECD 2004 list still included musk xylene and musk ketone as HPVs, their presence in the list at the present

time is excluded. This follows the application of European Regulations on the use of some synthetic fragrances in daily life products. Following the ban of some nitro musk fragrances by the International Fragrance Association (IFRA) [23], the European Union permanently banned the use of musk xylene (EU 143/2011 [24]) as well as restricted the use of musk ketone and AHTN (EU Commission Directive 2008/42/EC [25]). As these were prohibited or restricted, their production in Europe decreased, leading to the loss of their category as HPVs in the case of musk xylene and musk ketone, or the reduction of their production like AHTN. Their presence in the EPA's HPVs list remains, as no prohibitions have been performed for their use in the United States.

1.1.2. Phthalate esters

Obtained from the reaction between the phthalic anhydride and an alcohol, phthalates esters (PAEs) consist of a benzene ring with two ester functional groups in consecutive carbons on the ring. Their structure depends on the kind of alcohol used for the reaction, thus obtaining compounds with straight or branched chains in the ester moiety, a fact that confers their different properties [26]. Hence, these compounds are typically divided between low molecular weight (3-6 carbon atoms chains) and high molecular weight (7-13 carbon atoms chains) phthalates. While low molecular weight phthalates like di-iso-butyl phthalate (DiBP) can be used in adhesives, coatings, or paints, along with higher molecular weight ones are mostly used as plasticisers. Phthalate esters are closely linked to polymers like polyvinyl chloride (PVC), being included in its manufacture to increase its flexibility, durability, or softness, among other characteristics. Given the massive use of PVC and other plastic polymers, phthalates are expected to occur in a wide variety of plasticised products: wires and cables, toys, adhesive labels, coated fabrics, roofing, flooring, electronics, automotive applications, etc. Bis(2-ehtylhexyl) adipate (DEHA), being and ester of adipic acid in this case, is also a heavily used plasticiser. Other compounds such as dimethyl phthalate (DMP) or diethyl phthalate (DEP), not included in the previous stated categories due to their short carbon atom chains, are not used with the same purpose. These are usually used as fixatives and solvents in some fragrances, as well as additives in

cosmetics or household and personal care products. The studied PAEs in the present doctoral thesis are comprised in Figure 4.

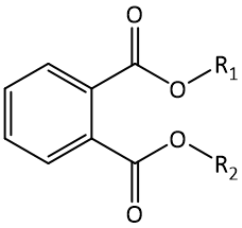
General structure	Abbreviation	Name	M.W. (g mol^{-1})
	DEP	diethyl phthalate	222.24
	DMP	dimethyl phthalate	194.18
	DEHA	bis(2-ethylhexyl) adipate	370.57
	DiBP	di-iso-butyl phthalate	278.34
	DEHP	bis(2-ethylhexyl) phthalate	390.56
	DnOP	di-n-octyl phthalate	390.56

Figure 4. General structure, abbreviation, name and molecular weight of the phthalate esters studied in the present doctoral thesis.

The manufacture of these compounds is large, as expected due to their extended use. Their production or importation in Europe as stated by the ECHA's REACH is led by DEHP and DEHA, those achieving rates between >10,000 – 100,000 tonnes per year [27,28], followed by DMP with >10,000 tonnes per year [29] and DEP with >1,000 tonnes per year [30]. Compounds such as DnOP or DiBP are produced to a lesser extent, with amounts ranging between 1-10 tonnes per year [31,32].

Even though heavily used, several concerns have arisen over the possible negative health effects that some of these compounds could cause. For instance, some PAEs are classified as endocrine disruptors (DEP, DiBP and DEHP) as well as display negative effects on fertility. Studies on the exposure of mammals to phthalates esters showed effects on the male reproductive system caused by DEP and DEHP [33], premature ovarian failure [34] and development impairment via thyroid and growth hormone axes [35]. Not only reproductive and fertility effects have been described, but some authors have also linked prenatal exposure to neurobehavior and cognition effects on children [36] as well as head circumference decrease [37]. Moreover, some studies have also pointed out the possible cumulative effects of some phthalates, thus becoming dose-additive (individual doses of each of the compounds can be added up as one) [33].

Due to these negative effects, DEHP, DiBP and DnOP have been classified as substances of very high concern (SVHC) and require a previous authorisation before their use [27,31,32]. Moreover, DiBP has also been classified as possible persistent, bioaccumulative and toxic compound [32]. Some regulations have been established in the European Union regarding the use of these compounds in some articles. Thus, DEHP, DiBP, benzyl butyl phthalate (BBP) and di-n-butyl-phthalate (DnBP) shall not be used as substances or mixtures (either alone or in combination) in concentrations equal to or greater than the 0.1% (by weight) of plastic materials, toys or childcare articles (some exceptions apply for exclusively industrial or agricultural use, aircraft or motor vehicles, among others) [38]. Due to their presence in food wrapping and packaging materials, the European Food Safety Agency (EFSA) has also established a group tolerable daily intake (TDI) threshold value set at 50 μg kg^{-1} bw^{-1} for the collective ingestion of DBP, BBP, DEHP and di-isononyl phthalate (DiNP) [39].

Regarding their presence in the HPV lists, all the aforementioned compounds appear in the OECD list as well as the EPA list, with only DEHA and DEHP being sponsored by the United States and Sweden, respectively, and having a final SIDS report [40,41].

1.1.3. Organophosphate esters

Organophosphate esters (OPEs) increasing use started upon the urge to replace brominated flame retardants (BFRs) such as hexabromocyclodecane (HBCD) or polybrominated diphenyl ethers (PBDEs) due to their persistence, bioaccumulation and toxicity [42]. These compounds, which structure relies on a phosphoric acid with aryl, alkyl, or halogenated substituents, appeared as a possible alternative to act as flame retardants. As said, their use mainly focuses on their incorporation into the composition of the material as additives to prevent or delay its combustion [43]. Even though mostly known for their flame retardant use, not all OPEs are intended to prevent fire ignition. Their use as plasticisers present in furniture, plastics, electronics and textiles is also widely found [44]. Other applications include anti-foaming agents and lubricants [43,45]. The most

usual OPEs found in the literature which have been studied in the present thesis are summarized in Figure 5.

Most of the studied compounds are manufactured or imported in Europe in quantities between 1,000 – 10,000 tonnes per year (TEHP, TBP, EHDPP, TTP and TiBP) [46–50] or even higher >10,000 – 100,000 tonnes per year for TEP [51] and TCPP [52]. TPP is the exception, with a lower production of up to 100 tonnes per year [53].

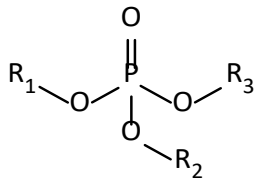
General structure	Abbreviation	Name	M.W. (g mol^{-1})
	TPP	triphenyl phosphate	326.28
	TEP	triethyl phosphate	182.15
	TEHP	tris(2-ethylhexyl)phosphate	434.63
	TBP	tributyl phosphate	266.31
	EHDPP	2-ethylhexyl diphenyl phosphate	362.40
	TCPP	tris(2-chloroisopropyl)phosphate	327.57
	TCEP	tris(2-chloroethyl)phosphate	285.49
	TTP	tritolyl phosphate	368.36
	TiBP	tri-iso-butylphosphate	266.31

Figure 5. General structure, abbreviation, name and molecular weight of the organophosphate esters studied in the present doctoral thesis.

As regards the negative health effects, both TBP and TCEP are currently under assessment as possible carcinogens [47,54]. Moreover, TCEP is also classified as toxic for reproduction and TPP as an endocrine disruptor [53]. On its part, TEP may be harmful when swallowed and could cause serious eye irritation [51]. TEHP has also been linked to serious eye and skin irritation [46], whereas TiBP may cause allergic skin reactions [50]. OPEs have also been proven to be neurotoxic [55] as well as responsible for developmental, endocrine and reproductive negative effects [56]. Hu et al. [57] also correlated the exposure to TPP and EHDPP with alteration of the progesterone synthesis in the placenta, affecting both female reproduction and fetal development.

As for some PAEs, TCEP has been included in the substances of very high concern list due to its proven negative health effects, meaning its use needs previous authorisation [54].

Even though presenting negative health effects, legislation on its use and limitations of their presence is still scarce. Due to its carcinogenicity, the use of TCEP has been restricted in toys by the 2009/49/EC EU Directive in Toys safety, which indicates TCEP content must not exceed 5 mg/kg [58]. Years later, in 2014, the European Union extended this measure for TCPP and tris[2-chloro-1-(chloromethyl) ethyl] phosphate (TDCP) [59]. On its part, several US organisations have recently requested the US EPA further testing and assessment on TCEP, TCPP and TDCP given their possible toxicity and persistence.

As for their classification as HPVs, all the studied OPEs are listed in the OECD and EPA's HPV lists, most of them being already sponsored by Germany (TPP [60], TEP [61] and TCEP [62]), Ireland (TCPP [63]) and the US (TBP [64]) and with available final SIDS reports.

1.1.4. Benzothiazoles, benzotriazoles and benzenesulfonamides

Benzothiazoles, benzotriazoles and benzenesulfonamides could be classified as the less-known and studied families of all the presented families until this point. Its presence in literature is scarce, especially for benzenesulfonamides. However, their extended production and described negative health effects deserve some attention. The structures, names and molecular weight of the benzo-compounds studied in the present thesis are summarized in Figure 6.

Benzothiazoles (BTs) are aromatic heterocyclic compounds formed by a benzene ring fused to a 1,3-thiazole ring. The different substitutions of the free carbon atom of the thiazole confer the derivates obtained from this substance. Benzothiazole derivates are found in natural products like vitamin B, enzymes, genetic material and other complex structures [65]. The use of benzothiazole as a precursor agent for the development of new derivatives has generated a huge attraction in the pharmacological field, as these exhibit a wide range of biological

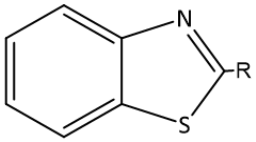
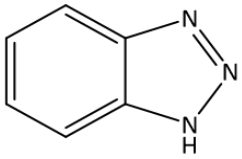
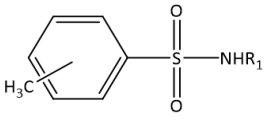
General structure	Abbreviation	Name	M.W. (g mol^{-1})
	BT	Benzothiazole	135.19
	ClBT	2-chlorobenzothiazole	169.63
	NH2BT	2-aminobenzothiazole	150.20
	OHBT	2-hydroxybenzothiazole	151.19
	MeSBT	2-(methylthio)-benzothiazole	181.30
	BTR	1-H-benzotriazole	119.12
	4TTR	4-methyl-1-H-benzotriazole	133.15
	5TTR	5-methyl-1-H-benzotriazole	133.15
	XTR	5,6-dimethyl-1-H-benzotriazole	147.18
	BSA	Benzenesulfonamide	157.19
	o-TSA	ortho-toluenesulfonamide	171.22
	p-TSA	para-toluenesulfonamide	171.22
	Me-p-TSA	N-methyl-p-toluenesulfonamide	185.24
	Et-p-TSA	N-ethyl-p-toluenesulfonamide	199.27

Figure 6. General structure, abbreviation, name and molecular weight of the benzothiazoles, benzotriazoles and benzenesulfonamides studied in the present doctoral thesis.

activities as anticancer, antimicrobial, antiviral or analgesic agents, among others [66]. The anthropogenic 2-substituted benzothiazoles are compounds with high thermal stability usually applied in the rubber industry as vulcanization accelerators, but also in other applications as antifreeze agents, ultraviolet stabilizers in textiles and plastics, fungicides and herbicides and biocides in paper and leather [67].

Current production of benzothiazoles as stated by the ECHA's REACH indicates that approximately between 10-100 tonnes per year of BT [68] and 1-10 tonnes per year of MeSBT [69] and NH_2BT [70] are being manufactured/imported in the European Economic Area. No data regarding the manufacture of OHBT or ClBT is currently available.

Despite some natural products and medical drugs exhibiting beneficial effects, not all benzothiazole derivates behave in the same way. In vitro studies conducted by Hornung et al. [71] indicated the possible endocrine disruption effects of NH_2BT and BT, disrupting the thyroid hormone. Ye et al. [72] reported the cytotoxicity of BT and OHBT in human gastric and lung carcinoma cells, as well as the genotoxicity of NH_2BT, OHBT and MeSBT. Other negative health effects include severe dermatitis and irritation of the skin and sensory organs, produced by the intake or contact with benzothiazoles [67].

Benzotriazoles (BTRs) structure consists of two fused rings one being a benzene and the other a five-membered ring with three nitrogen atoms. Due to their ability to coordinate with metal compounds, these are manly used as corrosion inhibitors, found in aircraft anti-icing and de-icing fluids for aircraft or dishwashing detergents, powders and tablets [73]. These can also act as biocides and can be included in textiles and plastics as UV-stabilizers and as biocides [42].

1-H-benzotriazole (BTR) production/manufacture in Europe is comprised of between 1,000 – 10,000 tonnes per year. On the other hand, 4 and 5-methyl-1-H-benzotriazole (4TTR, 5TTR) as well as 5,6-dimethyl-1-H-benzotriazole (XTR) are produced at volumes between 1 -10 tonnes per year [74–76].

Endocrine disrupting effects have been linked to BTR [77], authors like Liang et al. [78] observed the degeneration of the ovary and the spermatogenesis stimulation in rare minnows specimens. Liang et al. [79] reported the dysregulation of molecular responses in the liver of Chinese rare minnows exposed to BTR, thus suggesting the possible hepatotoxicity of this compound. Fent et al. [80] observed transcriptional alterations, even though little, in BTR-exposed zebrafish specimens, indicating its likely genotoxicity. The Health Committee of the Netherlands has also included BTR as a possible carcinogen [81]. Other BTR derivates such as tolyltriazole have been identified as phytotoxic and mutagenic in bacterial systems [42,82].

Lastly, benzenesulfonamides (BSAs) structure is based on a benzene ring with a sulfonamide substituent. Derivates can form upon the introduction of a methyl moiety on the ring or the introduction of a group in the N position of the sulfonamide [83]. Their use mostly concentrates on applications such as dyes

synthesis, disinfectants or photochemical products [84]. P-TSA has been used as plasticiser and intermediate product in the synthesis of pesticides and drugs, while o-TSA takes part in the production of saccharin [85]. ECHA's REACH registers report production/manufacture of o-TSA and p-TSA between 10 -100 tonnes per year [86,87], while N-Et-p-TSA and Me-p-TSA go from 1-10 tonnes per year [88,89]. Negative health effects related to BSAs are still scarcely found in the literature with p-TSA toxicity classified as moderate, this being slightly toxic to algae but not to fished or daphnids [82]. Further evaluation of BSAs toxicity is needed.

The labelling of these compounds as HPVs is not extended to all the previously commented substances. For benzothiazoles, only benzothiazole itself is considered an HPV chemical by the OECD and the EPA [90]. Something similar occurs for benzotriazoles, with only 1-H-benzotriazole and tolyltriazole (TTR) being considered [91]. No sponsor for any of these compounds is known to date, meaning no SIDS exists for these HPVs. As for benzenesulfonamides, BSA, p-TSA and o-TSA already have SIDS result from the sponsor provided by Japan [92–94]. However, only o-TSA remains considered as HPV by the OECD, despite all three being included in the 2004 HPV list. On the other hand, US EPA still considers p-TSA and o-TSA as HPVs. Even though most of their derivates are not HPVs, we believe their relevance is important to the field due to the negative effects these may present. Hence, we decided to include them in the present thesis to evaluate their impact.

1.1.5. High production volume chemicals pathway towards seafood

The extended use of these compounds has led to their widespread ubiquity. Several studies have reported the presence of most of the aforementioned HPV chemicals in many environmental and biological fates. For instance, compounds like organophosphate esters, phthalate esters and musk fragrances have been reported in air, both indoor [95,96] and outdoor [97], dust [84,96], water [43,98–102] and even biota [45,103,104]. Benzotriazoles, benzenesulfonamides and benzothiazoles have also been determined in outdoor air [97,105,106] as well as in house dust [84], wastewater influents and effluents [67,83] and even surface

water [107]. Some studies have also depicted the presence of HPV chemicals in human biological samples, e.g. urine [108–111], blood [108,112,113], breast milk [114–116], hair [117,118] and nails [117,119], being organophosphate esters and phthalate esters the families with the highest number of matrices where these are reported.

With seafood being the main object of study of the present thesis, it is necessary to briefly illustrate the reason why some of these compounds end up being bioaccumulated in these organisms, focusing on their possible release sources as well as their environmental path leading to our table dishes. Synthetic musk fragrances, being mostly used in personal care products, are mainly released as household discharges [101]. Their presence in soaps, cosmetics, detergents, or perfumes among other daily commodities, end up being washed away from people's bodies or textiles, leading to domestic discharges packed with some of these compounds [120]. On the other hand, compounds such as phthalate and organophosphate esters are not chemically bonded to the materials these are placed at, enabling their release via volatilization, abrasion or even dissolution [121,122]. Benzotriazoles, benzothiazoles and benzene-sulfonamides are mostly released as industrial or domestic discharges [67,123].

All in all, discharges containing HPVs are intended to be treated in wastewater treatment plants (WWTPs) prior to their release into the environment. Despite working for other contaminants and biological residues, WWTP removal efficiency of HPVs like the ones comprised in this thesis is low, meaning compounds such as OPEs, BSAs, BTRs, BTs and PAEs are often not conveniently removed from the influents and are further released to the aquatic environment [83,124–126].

Once released as WWTP runoffs, HPV chemicals follow their path towards rivers, seas and oceans, where these may accumulate in sediments or diffusively move through waters. At this point, HPVs are accessible to organisms inhabiting the aquatic environment, becoming available for their accumulation in fish and further being introduced in their trophic chain, with the possibility of trophic biomagnification over the levels. Figure 7 graphically summarizes the pathway followed by HPVs to reach seafood.

Figure 7. Schematic pathway of HPVs towards seafood.

1.2. Determination and occurrence of HPVs in seafood

The presence of high production volume chemicals in seafood samples, as stated before, has been reported by many authors throughout recent history, gaining attention every day. Their presence in foodstuffs such as fish, mussels, shrimps and other seafood commodities together with the believed negative effects that some of these compounds could cause on the population's health, has led to an increasing urge of developing analytical strategies capable of determining them.

Seafood analysis represents a continuous challenge due to the huge amounts of endogenous compounds present in these organisms. Vitamins, fats, proteins or minerals are just some of the chemicals present in these species, which combined with a broad spectrum of chemical compounds susceptible to being accumulated, turns seafood into a high-complexity matrix. Methodologies involved in the extraction of contaminants from these specimens are therefore complex and usually require an extensive clean-up to successfully determine them. Moreover, the wide range of physical and chemical properties covered by HPVs adds another level of complexity, as the use of a certain extraction method, solvent or clean-up may not be suitable for the extraction of all the target HPVs. To fulfil this purpose, authors have developed methods involving different extraction techniques such as solid-phase microextraction (SPME), solid-liquid extraction (SLE), microwave-assisted extraction (MAE), ultrasound assisted extraction (USE), quick, easy, cheap, effective, rugged and safe extraction (QuEChERS) or pressurized liquid extraction (PLE), among others. Due to the prior mentioned physical and chemical properties, chromatographic separation has also been reported to work with both liquid and gas chromatography, coupled with different detectors including ultraviolet detectors (UV), nitrogen-phosphorous detectors (NPD) or mass spectrometry detectors (MS), among others.

The following section compiles an overview of the most used analytical procedures for the determination of high production volume chemicals in seafood samples. Literature regarding the determination and occurrence of phthalate esters, organophosphate esters, benzothiazoles and benzotriazoles in seafood is compiled and discussed in a review article published in Trends of Analytical Chemistry, whereas the determination and occurrence of synthetic musk fragrances and benzenesulfonamides will be briefly commented afterward.

1.2.1. High production volume chemicals in seafood: A review of analytical methods, occurrence and population risk

HIGH PRODUCTION VOLUME CHEMICALS IN SEAFOOD: A REVIEW OF ANALYTICAL METHODS, OCCURRENCE AND POPULATION RISK

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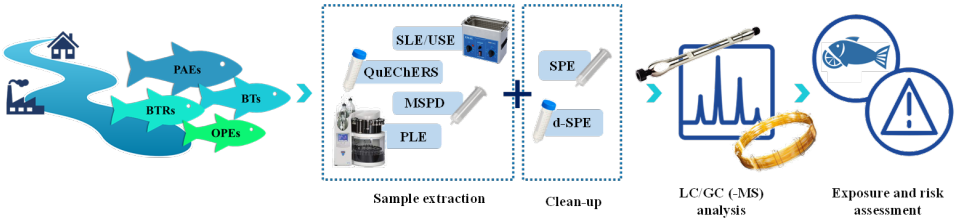
Abstract



The extended use of chemicals in both consumer and industrial products has led to a widespread ubiquity of compounds listed as high production volume chemicals (HPVs). Organophosphate esters, phthalate esters, benzothiazoles and benzotriazoles are part of this list, intended to prioritise their study to evaluate population's safety. Their fate throughout the environment reaches seafood, thus becoming accessible to the population via dietary intake. The reported negative effects led to the development of selective and sensitive methods capable of determining these compounds. The present review compiles the most used analytical methods for the determination of HPVs in seafood, mainly based on solid-liquid extraction, QuEChERS or pressurised liquid extraction, as extraction techniques, among others. The utility of efficient clean-up strategies is also discussed, aiming for reliable determinations while maintaining the instruments efficiency. Finally, an overview of the occurrence of these compounds in seafood and the exposure and risk associated to their ingestion is also discussed.

Highlights

- Widespread ubiquity of HPVs in seafood has been recently put on the spotlight.
- QuEChERS and d-SPE are efficient seafood extraction and clean-up strategies.
- HPVs dietary intake via seafood represents a minor risk for population.
- Future perspectives rely on greener seafood analysis methodologies.

1. Introduction

The continuous progress and adaptations to keep our current way of life have led society to become a major producer of many chemicals for both consumer and industrial products. This massive production of chemicals, whose impact on health is nowadays a concern, led the Organisation for Economic Co-operation and Development (OECD) to create a list of the chemicals with a production of over 1000 tones/year in at least one of the member countries [1]. In the same way, the US Environmental Protection Agency (EPA), also gathered the compounds produced in a minimum of 500 tonnes per year. The chemicals listed receive the name of High Production Volume Chemicals (HPV). The list is intended to prioritize chemicals in terms of the creation of data concerning screening information datasets (SIDS) valuable to define risk assessments. It is, therefore, necessary to develop strategies and methods to control how these compounds are spreading worldwide and which are the implications of it. Organophosphate esters (OPEs), phthalate esters (PAEs), benzothiazoles (BTs), and benzotriazoles (BTRs) are only a few of the compound families which are included in this list, in which we can also find families such as benzotriazole UV light stabilizers (BUVS) [2,3] or synthetic phenolic antioxidants (SPAs) [4,5] Their characteristics enable the use of these compounds as fire ignition preventors (OPEs), plasticisers (PAEs and OPEs) and even corrosion inhibitors, ultraviolet light stabilizers, or antifungal agents (BTRs and BTs) (Figure 1). The extended use of these compounds in everyday commodities has led to widespread contamination, with these being reported in many environmental fates such as air [6], dust [7], water [7,8] and even biota [9,10]. Their release through domestic and industrial discharges and the fact that these compounds cannot be totally removed at WWTP becomes an issue due to their path reaching aquatic environments. There, species inhabiting are directly exposed to these contaminants [11–13]. Amongst organisms susceptible to incorporate these contaminants into their organisms, seafood represents a major deal due to its ingestion by population via dietary intake [14–17]. The toxicity related to these group of compounds is nowadays a concern. Compounds such as DBP and DEHP are confirmed to have development and reproductive adverse effects in laboratory animals [18], whereas compounds like DnBP or BBP have been linked to steroid-hormone reduction [19]. OPEs exposure may lead to potential adverse effects with TCEP and TCPP being suspected as mutagenic, carcinogenic and even endocrine disruptors [20]. As regards BTs and BTRs, their toxicity has been reported by several studies [7,21–25], concluding that these compounds have shown

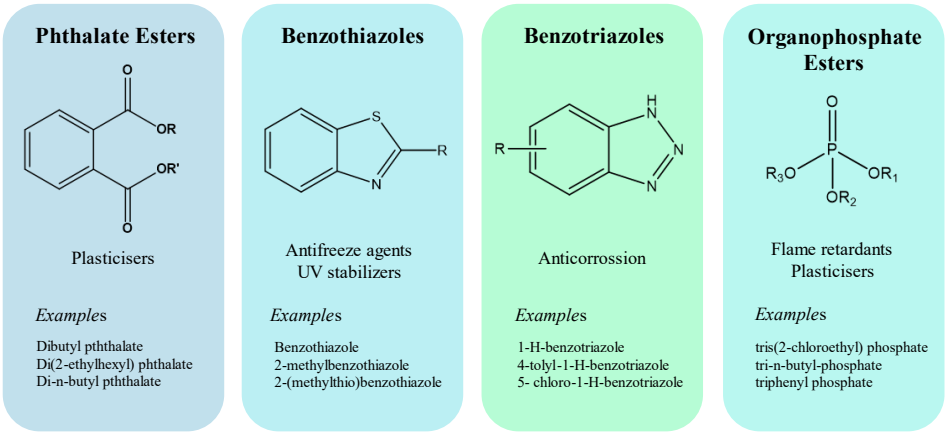


Figure 1. Structure, principal applications, and examples of the considered high production volume chemicals families.

respiratory irritant effects as well as dermal sensibilitation.

The ubiquitous presence of these compounds in seafood, along with the reported negative health effects, leads to an increasing urge of developing new analytical methods capable of providing useful data for the exposure and risk assessments associated with the consumption of those via dietary intake. This review is intended to comprise the most recently used analytical methodologies for the determination of these compounds in seafood samples, focusing on their extraction and clean-up strategies. Moreover, an overview of the occurrence of these compounds in different studies around the world will be presented, along with the exposure and risk assessment calculations performed by several studies regarding the effects that the consumption of those via fish intake can imply on human health.

2. Analytical Methodologies

Data concerning the risk associated with the ingestion of these HPV chemicals via fish and seafood intake is the result of applying several toxicity models to the exposure levels calculated. At the same time, these exposure values are based on the experimental data found by the analysis of seafood samples. It is therefore necessary to develop sensitive and selective methods for their determination. To fulfil these requirements, efficient extractions, and clean-ups, as well as sensitive instrumental techniques to reach the required levels are usually needed. A summary of the extraction techniques used as well as clean-ups and

determination techniques can be found in Table 1.

2.1. Extraction procedures

Seafood can be analysed both as the whole organism or divided into the different parts or organs that those are constituted of, e.g., muscle, liver, brain, plasma, etc. As the intention of the review is to cover the analytical methods susceptible to proving data necessary to perform exposure and risk assessment calculations of the ingestion of these compounds via dietary intake, only the common edible parts of the seafood will be considered. Thus, the analytical methods regarding fish muscle and other seafood which are usually served as the whole organism like shrimps or mussels will be discussed in this and the upcoming sections.

Before the sample extraction, a sample pre-treatment is usually performed to obtain a suitable part of the organism to be analysed. Therefore, fish muscle fillets are dissected from the organism. For other seafood species containing shells, these are usually removed and only the soft part is kept. Once the edible parts are extracted from the organism, these can be analysed wet [26–28] or lyophilised [29–31]. Dry weight analysis enables the interpretation of the data without considering the humidity percentage of the sample, whereas wet weight values are essential for the later exposure and toxicity assessment calculations. The process of lyophilizing the sample can be performed in different ways: it can be dried using a freeze-drying system [32], or, as an alternative, water can be removed using dehydrating substances such as sodium sulfate (Na_2SO_4) [33]. Independently of the sample being treated wet or dry, a grinding and homogenization step is required before analysis.

2.1.1. Solid-Liquid Extraction

The use of simple solid-liquid extraction has been successfully applied for the determination of HPVs in seafood. A good partitioning of the compounds between the sample and the extraction solvent can be achieved with mechanical shaking either using a shaking table [34–37] or vortex [38–40]. However, most of the methods incorporate sonication for better penetration of the solvent into the seafood sample, thus using ultrasonic-assisted extraction (USE) [14,16,26,32,33,41–55]. Parameters such as extraction solvent, solvent volume, and sonication time are considered key factors for a successful extraction. Regarding extraction solvent, mixtures of Hex/Acetone (1:1) are mostly used for the extraction of PAEs and OPEs from seafood, yielding recoveries between 45 and 123%. As for BTs and BTRs, more apolar solvent

Table 1. Compilation of methods involving the determination of HPVs in seafood samples.

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Shrimp, Fish	**7 PAEs**	SLE (3-20 g d.w. sample) 3x Hex/Acetone (1:1)/ mix 30 min	LLE Hex/ACN	GC-MS DB-5MS	56 - 101%	2 -10 ng g^{-1} w.w. a	[34]
Fish	**8 PAEs**	SLE (5-20 g w.w. sample) 20 mL Hex/Acetone (1:1)/ mix 30 min	GPC styrene - divinylbenzene	GC-MS DB-XLB	93-100%	0.25 - 80 µg kg^{-1} w.w. (5-100 µg kg^{-1} l.w.)	[35]
Fish, Crustaceans	**8 PAEs**	SLE (5-20 g w.w. sample) 20 mL Hex/Acetone (1:1)/ mix 30 min	GPC styrene - divinylbenzene	GC-MS DB-XLB	88-104%	5-145 µg kg^{-1} l.w.	[36]
Fish, shrimp, oyster	**5 PAEs**	SLE (2 g w.w. sample) 10 mL EtOH/Water (8:2)/ mix 1 min	d-SPE PSA	GC-MS DB-5MS	80-91%	2.53 - 9.61 µg L^{-1}	[38]
Fish	**10 PAEs**	SLE (5-20 g w.w. sample) 20 mL Hex/Acetone (1:1)/ mix 30 min	GPC styrene - divinylbenzene	LC-APCI-QqQ BEH C18	89-100%	0.5 - 70 µg kg^{-1} w.w.	[86]
Fish	**3 PAEs**	SLE (w.w. sample) 2 x 3 mL DCM	-	LC-ESI-QqQ HSS C18	-	-	[100]
Shrimp	**5 PAEs**	SLE (0.5 g w.w. sample) 2 x(0.5 mL n-pentane + 3 mL n-pentane/MeOH (1:4)) /mix 1 min	GPC	LC-ESI(+)-QqQ	98-100%	40 ng g^{-1} w.w.	[39]
Fish, shellfish	**5 OPEs**	SLE (0.5 g d.w. sample) 5 mL ACN/ mix 1 min	d-SPE Z-sep SPE Florisil	GC-MS HT-8	53-71%	0.15 - 1 ng g^{-1} w.w.	[40]
Fish, mussel, oyster	**14 OPEs**	SLE (0.3 g d.w. sample) 5 mL ACN/ mix 1 min	d-SPE PSA + C18 SPE Florisil SPE APC	GC-QqQ ZB-5	74-130%	0.001 - 0.33 ng g^{-1} w.w.	[83]
Fish	**8 OPEs**	USE (0.5 g w.w. sample) 2 x 5 mL Hex/Acetone (3:1) x 20 min	SPE Florisil	GC-MS HT-8	-	-	[33]
Fish	**16 OPEs**	USE (0.25 g d.w. sample) 2x 15 mL Hex/Acetone (1:1) x 15 min	SPE Alumina + C18	LC-ESI(+)-QqQ Purosphere RP-18	45-115%	0.34 - 51.6 ng g^{-1} l.w.	[51]
Fish	**2 OPEs**	USE (1 g d.w. sample) 2x 10 mL EtAc/Acetone (1:1) x 15 min	Acid attack Sulfuric Acid SPE Bond Elut ENV	LC-UV Hypersil GOLD	58-98%	0.04 - 0.17 µg g^{-1} w.w.	[52]
Fish, mussel, shrimp	**14 OPEs**	USE (0.1 g d.w. sample) 2 x 4 mL EtAc x 1 h	SPE NH_2	LC-ESI (+)-QqQ ACQUITY BEH-C18	48 - 80%	0.001 - 0.14 ng g^{-1} w.w.	[53]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish, oyster, shrimp	**9 OPEs**	USE (5 g d.w. sample) 5 mL ACN/Toluene (9:1) x 1 h	SPE FL-PR Florisil d-SPE Z-sep + DSC-18	LC-ESI (+/-)-QqQ Synergi Fusion-RP	60 - 96%	0.004 - 0.33 ng g^{-1} w.w.	[54]
Fish, shrimp, lobster	**15 OPEs**	USE (1 g w.w. sample) 5 mL ACN (5% Formic acid) x 2 h	d-SPE PSA + C18 SPE Oasis HLB	LC-ESI (+)-QqQ Betasil C18	63 - 121%	0.01 - 0.17 ng g^{-1} w.w.	[16]
Mussel	**7 OPEs**	USE (0.5 g d.w. sample) 2 x 15 mL Hex/Acetone (1:1) x 15 min	TurboFlow CycloneTM-P + C18-XL	LC-HESI-QqQ Purosphere RP-18	47 - 98%	0.19 - 19.3 ng g^{-1} l.w.	[55]
Fish	**14 OPEs**	USE (0.5 g d.w. sample) 2 x 15 mL Hex/Acetone (1:1) x 15 min	TurboFlow CycloneTM-P + C18-XL	LC-HESI-QqQ Purosphere RP-18	47 - 98%	0.19 - 19.3 ng g^{-1} l.w.	[41]
Fish	**9 OPEs**	USE (1 g w.w. sample) 3 x 10 mL MeOH x 15 min	SPE Strata X	LC-ESI (+)-QqQ Kinetex XB-C18	47 - 123%	0.02 - 0.30 ng g^{-1} w.w.	[42]
Fish, shrimp, mussel, bivalve	**7 OPEs**	USE (0.2 g d.w. sample) 3 x 10 mL ACN x 20 min	SPE GCB + NH_2	LC-ESI (+)-QqQ BEH-C18	95 - 115%	0.046 - 0.306 ng g^{-1} w.w.	[43]
Fish	**6 OPEs**	USE (0.5 g d.w. sample) 2 x 15 mL Hex/Acetone (1:1) x 15 min	TurboFlow CycloneTM-P + C18-XL	LC-ESI-QqQ Purosphere RP-18	48 - 102%	0.2 - 19.3 ng g^{-1} l.w.	[44]
Fish	**19 PAEs**	USE (1 g d.w. sample) 2 x 5 mL ACN x 20 min	d-SPE PSA + C18 + GCB	GC-QqQ DB-5MS	73 - 114%	0.23 - 0.96 ng g^{-1} d.w.	[45]
Fish	**14 PAEs**	USE (1 g d.w. sample) 2x 20 mL MeOH/DCM (3:7, v/v) x 20 min	SPE Florisil + Silica	GC-MS DB-5	89 - 118%	0.17 - 0.53 ng g^{-1} d.w.	[46]
Fish	**4 PAEs**	USE (2 g w.w. sample) 10 mL DCM x 30 min	SPE Oasis HLB	GC-MS HP-5	88-117%	2.4 - 4.1 ng g^{-1} w.w.	[47]
Mussel	**4 PAEs**	USE (1 g d.w. sample) 1 x 10 mL DCM/Hex + 2 x 10 mL Hex/acetone x 20 min	SPE Florisil	GC-MS HP-5	78 - 101%	4 - 71 ng g^{-1} d.w.	[48]
Fish	**5 PAEs**	USE (2 g d.w. sample) 2 x 20 mL Hex/Acetone (8:2) x 20 min	SPE Florisil	GC-MS HP-5	56 - 101%	2 - 10 ng g^{-1} w.w.	[101]
Fish	**6 PAEs**	USE (5 g f.w. sample) 3 x 10 mL Hexane x 10 min	SPE Florisil	GC-MS HP-5	69-97%	0.003 - 0.018 ng g^{-1} w.w.	[49]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish	**7 PAEs**	USE (2 g d.w. sample) 2 x 20 mL Hex/Acetone (8:2) x 20 min	SPE Florisil	GC-MS ZB-5	57 - 87%	0.4 - 10 ng g^{-1} w.w.	[78]
Fish, squid, bivalve, shrimp	**6 PAEs**	USE (1 g d.w. sample) 5 mL Hexane + 1mL H_20 20%NaCl x 5 min	-	GC-MS ZB-5HT	53 - 94%	6.8 - 10 ng g^{-1} w.w.	[32]
Fish	**5 PAEs**	USE (2 g w.w. sample) 5 mL Acetone/Hexane (1:1,v/v) x 10 min	-	LC-ESI (+)-QqQ Accucore C18 aQ	70 - 120%	0.1 - 0.5 ng g-1 w.w.	[14]
Fish, shrimp, crab, shellfish	**15 PAEs**	USE (2 g f.w. sample) 2 x 10 mL Hex/ DCM (1:1, v/v) x 30 min	SPE Cleanert PAE	GC-QqQ HP-35MS	70 - 117%	0.15 - 0.78 ng g^{-1} w.w.	[26]
Mollusc	**4 BTRs** 4 **BTs**	USE (0.1 g d.w. sample) 2 x 5 mL Hex/DCM (1:1) x 20 min	SPE Oasis HLB	GC-QqQ (BTs) TG-5MS LC-ESI(+)-QqQ (BTRs) CSH Phenyl Hexyl	51 - 120%	0.607 - 5.12 ng g^{-1} d.w.	[50]
Fish	**13 OPE**	MSPD (0.5 g d.w. sample) sample + 1.2 g activated silica + 3 g deactivated florisil /E: 10 mL ACN	-	GC-NPD DB-5	65 - 104%	0.2 - 3.1 ng g^{-1} w.w.	[71]
Mussel	**13 OPEs**	MSPD (0.5 g f.w. sample) sample + 1 g anhydrous Na_2SO_4 + 2 g florisil + 1 g alumina/ W:5 mL Hex/DCM (1:1) /E: 10 mL Hex/Acetone (6:4)		LC-ESI(+)-QqQ Purospher STAR Luna C18	69 - 122%	0.06 - 5 ng g^{-1} d.w.	[28]
Fish	**6 PAEs**	MSPD (2 g d.w. sample) sample alone/ W: 6 mL EtAc/E: 6 mL EtAc	-	GC-MS DB-5MS	79 - 109%	6 - 11 ng g^{-1} d.w.	[72]
Fish, shrimp, oyster, scallop	**4 PAEs**	MSPD (1 g f.w. sample) sample + 2 g florisil + 0.1 g GCB + 2 g anhydrous Na_2SO_4 /E: 20 mL EtAc	-	LC-UV VP-ODS	85 - 106%	0.682 - 1.892 ng	[73]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Bivalve	**1 PAE**	MSPD (0.1 g d.w. sample) sample + 0.4 g C18 + 0.1 g Florisil / E: 1.2 mL ACN + 2.6 mL H_2O	In-tube SPME GC TRB-5	GC-MS DB-5MS	-	10 µg L^{-1} (measured concentration)	[74]
Fish, shellfish	**6 PAEs**	MSPD (0.1 g d.w. sample) sample + C18 + florisil /E: 5.75 mL MeOH/DCM		GC-MS DB-5MS	59 - 96%	20 - 50 ng g^{-1} w.w.	[75]
Mussel	**3 PAEs**	MSPD (0.1 g w.w. sample) sample + 0.5 g Florisil + 0.5 g Na2SO4 + 0.2g sea sand /E:9 mL MeOH/ACN (3:7)		LC-DAD ACE 5 C18-PFP	89.1-104%	0.42 - 1.65 ng g^{-1} w.w.	[76]
Fish	**2 BTs** **5 BTRs**	USE-MSPD (0.5 g d.w. sample) sample + 1 g florisil + 10 mL H2O + USE x 10 min		LC-ESI(+)-QTOF Poroshell 120 EC-C18	BTS: 73-91% BTRs: 70-93%	BTs: 0.05 - 0.1 ng g^{-1} BTRs: 0.05-0.5 ng g^{-1}	[77]
Fish	**7 PAEs**	PLE (0.5 g d.w. sample) 3 cycles (5 min, 100ºC) Hex/DCM (1:1) + Hex/Acetone (1:1)	SPE Florisil	GC-QqQ ZB-SemiVolatiles	89 - 170%	1.29 - 11.5 ng g^{-1} d.w.	[61]
Fish, mussel	**10 OPEs**	PLE (3 - 6 g d.w. sample) 2 cycles (100ºC) EtAc/Cyclohexane (5:2) + 1 cycle (100ºC) Cyclohexane/Diethyl Ether (9:1)	GPC Biobeads	GC-HRMS DB-5	64 - 132%	0.05 - 23 ng g^{-1} l.w.	[59]
Fish	**8 OPEs**	PLE + SPME (1 g w.w. sample) 1 cycle (150ºC, 5 min,) H_2O (10% ACN) 10 mL extract in SPME vial + 3 g NaCl + immersion SPME (PDMS/DVB) x 40 min	In-cell Acidified silica	GC-FPD HP-5		0.01 - 0.208 ng g^{-1} w.w.	[56]
Fish	**11 OPEs**	PLE (0.5 - 2 g w.w. sample) 1 cycle DCM/Hex (1:1)	SPE Aminopropyl silica	LC-ESI(+)-QqQ Waters symmetry C18	-	0.02 - 0.16 ng g^{-1} w.w.	[79]
Fish	**10 OPEs**	PLE (whole organism d.w.) 3 cycles (70ºC) DCM/Acetone (1:1)	SPE NH2	LC-ESI-QqQ Luna C18	43 - 134 %	0.2 - 29 ng g^{-1} w.w.	[80]
Fish	**10 OPEs**	PLE (d.w. sample equivalent to 0.25 mg lipid) 1 cycle DCM/Acetone (1:1)	GPC Silica gel SPE HybridSPE	LC-ESI-QqQ Luna C18	-	-	[57]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish	**3 OPEs**	PLE (1 g d.w. sample) 1 cycle (100ºC, 5min) EtAc	In-cell Hex/DCM GPC Alumina+Silica+Florisil+PSA	GC-MS DB-5 MS	58 - 107%	0.06 - 1.35 ng g^{-1} w.w.	[58]
Fish, shrimp	**17 OPEs**	PLE (1 g d.w. sample) 1 cycle (100ºC, 5min) EtAc	In-cell Hex/DCM GPC Alumina+Silica+Florisil+PSA	GC-MS DB-5 MS	77-97%	0.06 - 1.29 ng g^{-1} w.w.	[31]
Fish	**5 BTs**	PLE (1 g d.w. sample) 1 cycle H_2O (80ºC, 5min, 1500 psi) SPME 10 mL extract PDMS/DVB, immersion 40 min, 80ºC	In-cell Hexane	GC-IonTrap ZB-5	86- 135%	0.5 - 10 ng g^{-1} d.w.	[17]
Fish	**3 BTRs**	PLE (1 g d.w. sample) 2 cycles EtAc/DCM (1:1) (100ºC, 5 min)	In-cell Florisil SPE C18	LC-ESI(+)-QqQ Purospher STAR HR R-18	-	0.33 - 5.91 ng g^{-1} d.w.	[60]
Fish	**10 OPEs**	QuEChERS (1g f.w. sample) 4 mL ACN (5% F.A.)/400 mg NaCl + 400 mg MgSO4	d-SPE PSA + C18 + Alumina	LC-ESI(+)-QqQ Waters BEH C18	73 - 106%	0.05 - 0.42 ng g^{-1} w.w.	[9]
Fish	**13 OPEs**	QuEChERS (4 g f.w. sample) 4 mL ACN / 2 g $MgSO_4$/NaCl (4:1)	Online SPE PSA, C18, Carbon X	GC-QqQ DB-5MS LC-ESI-QTRAP/Q-Orbitrap Nano Acquity UPLC + BEH C18	69 - 122%	0.5 - 1 ng g^{-1} w.w.	[66]
Fish	**9 OPEs**	QuEChERS (0.5 g d.w. sample,) 10 mL H_2O + 10 mL ACN / + 4 g MgSO4 + 1 g NaCl + 1 g sodiumcitrate + 0.5 g disodium citrate sesquihydrate	LipiFiltr	GC-QqQ HP-5MS	67 - 116%	0.05 - 2 ng g^{-1} d.w.	[10]
Fish	**13 PAEs**	QuEChERS (5 g f.w. sample) 5 mL ACN (1% F.A.)/ 2 g NaCl	d-SPE PSA	GC-QqQ HP-5MS	71 - 116 %	0.01 - 10 ng g^{-1} w.w.	[64]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish, shrimp, mussel	**5 PAEs**	QuEChERS (2 g f.w.sample) 2 mL H_2O + 10 mL ACN/ 1 g NaCl	SPE PSA	GC-QqQ HP-5MS	53 - 120%	0.019 - 2.2 ng g^{-1} w.w.	[27]
Fish, mussel, shrimp	**5 PAEs**	QuEChERS (2 g f.w.sample) 2 mL H_2O + 10 mL ACN/ 1 g NaCl	SPE PSA	GC-QqQ HP-5MS	60 - 120%	0.034 - 2.2 ng g^{-1} w.w.	[67]
Fish, mussel	**7 PAEs** **9 OPEs**	QuEChERS (0.5 g d.w. sample) 10 mL DCM/EtAc (1:1) + vortex 1 min + sonicated 10 min	d-SPE PSA + C18 d-SPE Florisil + Alumina	GC-MS HP-5MS	52 - 91 %	0.001 - 0.06 ng g^{-1} d.w.	[29]
Fish	**6 PAEs** **6 mPAEs**	QuEChERS (1 g d.w. sample,) 10 mL H_2O pH 2 + 10 mL ACN / + 4 g MgSO4 + 1 g NaCl + 1 g sodiumcitrate + 0.5 g disodium citrate sesquihydrate	PAEs: d-SPE LipiFliltr mPAEs: d-SPE C18	LC-(ESI +/-)-HRMS (Orbitrap) Ascentis Express C18	PAEs: 14 - 66% mPAEs: 13 - 77%	PAEs: 2.5 - 75 ng g^{-1} d.w. mPAEs: 1 - 25 ng g^{-1} d.w.	[69]
Fish	**2 BTRs** **3 OPEs**	QuEChERS (0.5 g d.w. sample) H_2O + ACN (V_{ACN}/V_{H2O} = 2:1) / 4 g MgSO4 + 1 g NaCl	d-SPE PSA + C18	LC-ESI(+)-QTRAP ACQUITY BEH-C18	34 - 101 %	0.02 - 0.50 ng g^{-1} d.w.	[68]
Mussel, razor shell. Oyster, cockle	**1 BTR**	QuEChERS (1 g d.w. sample) 5 mL H_2O + 15 mL ACN / 4 g $MgSO_4$ + 1 g NaCl + 1 g sodiumcitrate + 0.5 g disodium citrate sesquihydrate	d-SPE PSA + C18 SPE Ostro™ Phospholipid removal	LC-ESI(+)-HRMS STAR RP-18	62 - 148 %	0.10 ng g^{-1} d.w.	[30]
Shellfish	**1 BTR**	QuEChERS (1 g d.w. sample) 5 mL H_2O + 10 mL ACN / 4 g $MgSO_4$ + 1 g NaCl + 1 g sodiumcitrate + 0.5 g disodium citrate sesquihydrate	d-SPE PSA + C18 SPE Ostro™ Phospholipid removal	LC-ESI(+)-HRMS STAR RP-18	78-98%	0.10-0.50 ng g^{-1} d.w.	[84]
Fish	**4 BTRs**	QuEChERS (2 g f.w. sample) 5 mL H_2O + 10 mL ACN (1% Acetic acid) / 6 g $MgSO_4$ + 1.5 g sodium acetate	d-SPE PSA + C18	LC-ESI(+)-QqQ Zorbax SB-C18	51 - 120%	0.02 - 0.11 ng g^{-1} w.w.	[82]
Fish	**9 PAEs**	Soxhlet (2-3g d.w. sample) 120 mL Acetone/DCM/Hex (1:1:1) x 18 h	SPE Activated copper + florisil	GC-MS HP-5MS	73 -116 %	5 ng g^{-1} d.w.	[94]

Table 1. (Cont.)

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish	**3 PAEs**	Soxhlet (5 g d.w. sample) 120 mL DCM x 6-8 h	SPE Alumina + Na_2SO_4	LC-UV (254 nm) C18	-		[102]
Fish	**6 PAEs**	ASE (2g d.w. sample) 6 mL EtAc at 9 mL/min x 10 min + 2 mL/min x 50 min	-	GC-MS DP-5MS	102%		[103]
Fish	**11 OPEs**	Soxhlet (10 g d.w. sample) 80 mL Hex/Acetone (1:1) x 1 h	GPC SPE Florisil	GC-MS DB-5 MS	58 - 103 %	0.005 - 0.641 ng g-1 w.w.	[87]
Fish	**11 OPEs**	Soxhlet (1 g d.w. sample) Hex/Acetone (1:1) x 24 h	SPE Florisil	LC-ESI(+)-QTOF ACQUITY BEH-C18	73 - 109 %	0.16 - 2 ng g^{-1} w.w.	[92]
Shrimp, crab, oyster	**11 OPEs**	Soxhlet (1 g d.w. sample) 150 mL Hex/Acetone (1:1) x 48 h	SPE Envi - Florisil	GC-QqQ HP-5 MS	75 - 110%	0.016 - 0.484 ng g^{-1} d.w.	[104]
Fish	**14 OPEs**	MAE (1 g d.w. sample) 0 mL Hex/Acetone (1:1)/ 30 min/ 130ºC	GPC Biobeads SPE Deactivated Silica	GC-MS DB-5MS	39 - 105 %	0.006 - 0.021 ng g^{-1} l.w.	[105]
Fish	**8 OPEs**	UVAE (2.5 g d.w. sample) 5 mL ACN/Toluene (9:1) / USE 1h / MSPD with 1 g $MgSO_4$	SPE Florisil d.SPE Z-SEP + C18 SPE Aminopropyl Silica	GC-MS HT-8 ()	67 - 111 %	1.4 - 3.7 ng g^{-1} d.w.	[106]
Fish	**5 PAEs**	DI-SPME Fiber preconditioning with phosphate buffer with agitation during 3 h + introduction of the fiber in the fish fillet for 25 min + removal of the fiber + wash in a vial with 1 mL H2O + desorption with 80 uL MeOH/H2O (80;20)	-	LC-ESI (+)-QqQ Accucore C18 aQ		0.2-0.3 ng g^{-1} d.w.	[14]

mixtures such as Hex/DCM are used, with recoveries comprised between 51-120% [50]. Greener approaches have also been described using mixtures of EtOH/H_2O (8:2) for the extraction of PAEs from fish, oyster, and shrimp samples with recoveries of 80-91%. Concerning the extraction solvent, Lorenzo et al. [42] denoted the higher ability of solvent mixtures of extracting non-polar compounds, thus leading to higher matrix effects and more critical clean-up steps and suggested the use of MeOH on its own instead of mixtures when extracting seafood samples. With respect to the solvent volume used for the extraction, total volumes of 5 – 40 mL are used for sample weights between 0.1 – 5 g. Assuring quantitative extractions also involves the amount of time the sonication is performed, for HPVs and seafood samples, this time varies between 5 min and 2 h being the longest time for the extraction of OPEs from fish, oyster, shrimps and lobsters [16,54].

Albeit using larger extraction solvent volumes, SLE can be considered a great option for the extraction of HPVs from seafood samples given its simplicity, effectivity, and relatively low cost. Considering time demand, sonication can be pointed out as the bottleneck, enlarging the time needed to perform the extraction, especially given the fact that most of the samples need to be reextracted at least once to achieve satisfactory recoveries. Moreover, centrifugation is usually necessary to properly separate the extract, thus incorporating another analytical step. It should be pointed out that a tendency for the reduction of sample weight is seen throughout the years, thus leading to a reduction of the volume of solvent used and hence a greener approach.

2.1.2. Pressurized Liquid Extraction

The lower solvent consumption, as well as its fast and semi-automated procedure, has turned PLE into a highly attractive option. Its presence in the extraction of HPVs from seafood is usually found in literature, with methods involving all the families comprised in this review. For the number of cycles, temperature, and static time, common values are found for most of the methods described to date, then being 1 cycle [17,31,56–58], 100ºC [31,58–60] and 5 min [17,31,58–61], yielding recoveries within 43-135% for sample weights between 0.5 – 6 g (d.w.). Several extraction solvents have been used for this purpose, mainly being DCM mixtures with hexane or acetone (DCM/Hex or DCM/Acetone), ethyl acetate on its own, or even water. Most of the solvents prior listed can be used for successful extraction of PAEs and OPEs whereas compounds such as BTRs have been proved to be successfully extracted from fish muscle samples using a mixture of EtAc/DCM (1:1) and 2

cycles (100ºC, 5 min) [60]. Even though methods involving water for the extraction of OPEs and BTs are described in the literature, authors such as Hidalgo-Serrano et al. [62] have described the apparition of foam in the collection vessel which interrupted the process and they have linked this problem with the use of water and the high lipid content present in some fish species.

Methods involving PLE have been proven to be useful for the determination a wide range of HPVs in seafood, which emphasizes the elevated grade of extraction potential that this technique offers but also the loss of selectivity during the extraction process [58,60,63].Even though being a low selectivity extraction technique could lead to the development of multi-residue methods capable of determining a wide range of analytes from different families at the same time, it also represents a problem when extracting samples from a complex matrix. In the case of seafood, using PLE as the extraction method leads to a higher percentage of matrix interferents such as lipids being extracted along with the target analytes, thus hindering the determination and making clean-up steps more critical [31,56]. Different alternatives for the obtention of a cleaner extract directly from PLE have been studied and will be discussed later on in the clean-up strategies section.

2.1.3. QuEChERS

Far from its initial purpose, the use of QuEChERS has expanded to the determination of other compound families, in which HPVs are also included. The composition of the extraction salts or the solvent volumes and ratios are parameters usually modified to adapt for each of the compound families and studied commodities. Regarding the solvent, I remains the most used extraction solvent with volumes ranging from 4 – 15 mL for sample weights between 0.5 – 2 g (d.w.)/ 1 – 5 g (w.w.) with recoveries within 51 – 148%. The addition of water is also a common procedure, especially when working with lyophilised samples. It is usually added in a 1:1 (v/v) ratio for OPEs, whereas compounds such as PAEs and BTRs tend to make use of higher I ratios for their extraction (from 1:2 to 1:5). The addition of pH modifiers into I such as 1-5% formic acid [9,64] or 1% acetic acid [65] have also been proven to provide satisfactory results in the determination of these compounds, mainly increasing their recovery (71-116% for OPEs and PAEs). Concerning the extraction salts used to increase the partitioning of the compounds between water and I, most of the methods described for HPVs determination in seafood make use of the original (OR) [9,27,64,66–68] or the EN 15662 (EN) [10,69,70] methods, which usually contains mixtures of NaCl and $MgSO_4$ for

the first or NaCl, $MgSO_4$ and a citrate buffer (sodium citrate + disodium citrate sesquihydrate) for the later. Even though methods determining BTRs in different seafood species using the same salts are found in the literature, a study conducted by Yao et al. [65] demonstrated the higher efficiency of the AOAC method for the determination of BTR, 5TTR, ClBTR, and XTR from fish muscle samples. The authors did point out the negative effect that acidic pH of the extraction solvent had on the recoveries of BTRs, concluding that acidic phases led to increased hydrolysis of BTRs, thus diminishing their recovery.

The use of QuEChERS in the determination of HPV chemicals is growing on account of the good results obtained as well as their easy implementation given the lack of expensive materials or instruments. A problem currently found when using QuEChERS is its low enrichment factor. This could be solved by either increasing the sample weight or reducing the final extract volume. However, both options lead to higher co-extracted matrix concentrations, thus leading to more critical clean-up steps. Nonetheless, the wide range of compounds that can be extracted using this technique leads to the development of multi-residue methods,as well as when using pressurized liquid extraction, but with the advantage of extracting less interferences. Moreover, the described methods include the following clean-up which can be performed directly with the obtained extract (d-SPE), enabling an easy and effective procedure.

2.1.4. Matrix Solid-Phase Dispersion

The possibility of performing both the extraction and the clean-up in a single step has led matrix solid-phase dispersion (MSPD) to become a useful methodology for the extraction of HPVs compounds from seafood samples. Sample weights between 0.1 – 2 g are usually packed into an SPE cartridge mixed with a dispersant and a co-sorbent. Sorbents such as florisil [28,71–76] and activated silica [28] have been successfully used as dispersants mixed with the sample. Among them, florisil stands out as the most used sorbent for the determination of OPEs and PAEs in seafood. Even though florisil can behave both as dispersant and co-sorbent due to its capacity of retaining lipids and other interferences, additional co-sorbents such as alumina [71], GCB [73] or C18 [74,75] have been used for further clean-up of the extracts. Regarding elution, solvents such as I [28,74], Hex/Acetone [71], EtAc [72,73], MeOH/DCM [75] and MeOH/I [76] have been used with satisfactory results (recoveries comprised between 59 - 122%). The mixture of MeOH/I along with the use of florisil as dispersant yielded the highest recoveries (89 – 104%) for the determination of PAEs

from bivalves. Campone et al. [71] also denoted the importance of the solvent election and its role in the washing step, indicating that a mixture of Hex/DCM (1:1) could lead to an efficient lipid removal while avoiding a substantial analyte loss.

Even though not extensively used due to its laborious procedure, MSPD can be seen as an alternative with clear advantages such as reduced sample size, fast procedure, reduced solvent volume, and lack of required equipment. However, attention must be paid to the fact that MSPD uses the sample matrix as the extraction core itself, it becoming an issue in terms of reproducibility, not only since every sample is different but also because of the differences produced in the confection of the MSPD itself every time.

2.1.5. Extraction methods comparison

Given the literature to date previously discussed, HPVs can be extracted from seafood in many different ways. Comparing different extraction methods can be quite tedious as many parameters can be involved. Regarding the recoveries, most of the methods comprise their recoveries in a wide range, mainly going from the low 30-40% (usually related to the most volatile compounds) to the surroundings of 120%. As the methods described make use of different determination techniques as well as different analysers, the reported recoveries, which usually are difficult to differentiate between relative or apparent, may also include the effects caused by the matrix. Thus, given this and the fact that there are no remarkable differences, using recoveries as a parameter to select the best extraction technique may not be the best option. Similarities are found when talking about method limits of detection. It is obvious that the methods involving triple quadrupole mass spectrometers or HRMS may reach lower values (reaching 0.001 ng g^{-1} (w.w.) for some of the compounds). However, methods such as standard SLE or USE require higher sample weights to reach those levels (up to 5 g of sample), whereas other techniques such as PLE or QuEChERS use smaller amounts, which later translates to fewer matrix coextractants. Regarding their extraction capacity and selectivity, the different properties of the studied compounds demand the use of methods with low selectivity such as QuEChERS (partially limited to low-midpolar compounds) and PLE. Despite the lack of selectivity being an advantage, the highly exhaustive extraction of PLE may lead to the over-extraction of many matrix endogenous compounds such as lipids. These compounds could act as interferences, especially those from species with higher lipidic

content,hindering the determination of the target compounds and making the clean-up more difficult.. Another topic that usually becomes a concern when determining PAEs and OPEs is the contamination of the samples throughout the process due to the use of plastic material that may contain some of the compounds. Authors such as Muñoz-Ortuño et al. [74] tested the use of glass instead of polypropylene cartridges for the extraction of DEHP from bivalves using MSPD, concluding that similar signals were observed. On its part, Jimenez et al. [29] performed experiments to evaluate the presence of PAEs in PP tubes used for QuEChERS extraction, finding concentrations of DEHP 2 to 36 times higher than when using glass ones. Avoiding this issue can be easy for extraction techniques not directly linked to the use of plastic such as USE and can be addressed for extraction methods where plastic can be easily substituted by glass homologs as in QuEChERS (glass tubes instead of PP tubes) or MSPD (glass cartridges instead of plastic). Concerning this, authors such as Chen et al. [77] have developed alternative strategies to MSPD combining vortex mixing and sonication avoiding the use of mortar and SPE cartridges, thus preventing the possibility of PAE contamination. Keeping the eyes on the progress towards greener analytical chemistry, parameters such as organic solvent, energy and time consumption, generated laboratory waste, reusability, and automatization are key. Methods such as USE do not align with these principles due to their high usage of organic solvent, whereas this issue is minimized for methodologies such as PLE or QuEChERS. However, the demand of energy counterparts for PLE, while the laboratory waste generated by QuEChERS may be an important issue. The use of glass tubes as well as their miniaturization (reduction of the sample weight and solvent consumption) could help minimizing this issue, as well as make QuEChERS partially reusable. With microextraction being one of the greenest options, methods such as the developed by Panio et al. [14] for the determination of PAEs in fish fillets using direct immersion SPME are excellent references for future method development. Despite being an excellent alternative, the fiber must be cleansed once the extraction is performed, in this case, the authors reported no loses when ultrapure water was used. However, due to higher polarity of some of the HPVs such as benzothiazoles and benzotriazoles, further investigation should be considered to evaluate possible loses on this step. All in all, QuEChERS extraction seems to align with most of the important parameters such as good recoveries, easy procedure, and minimal sample weight. Moreover, it can be considered a relatively green approach if used with reusable material

and in a miniaturized way. Its applicability manages to extract a wide range of compounds while the amount of matrix coextractants is generally lower than when using other techniques such as PLE, thus leading to multitarget analysis as well as reducing the necessity of incorporating exhaustive clean-ups. A sum-up of the most used conditions of each of the techniques along with some of the advantages and disadvantages of each of them can be found in Figure 2.

2.2. Clean-up strategies

Seafood samples are considered high complexity matrix samples due to the huge amount of co-extractive substances that are usually found when these are analysed. From matrix endogenous highly polar compounds to ionic species and lipids, it represents an analytical challenge to selectively determine certain compounds without getting rid of these other substances that may act as interferents and cause signal enhancement or suppression. Clean-ups are intended to eliminate or at least diminish the presence of these interferences in the obtained extracts, thus leading to a more efficient determination but also preventing the instrument from losing efficiency. The following sections will discuss the different approaches followed by the methods described in the literature concerning this topic. Table 1 compiles the clean-up methodologies used for each of the listed methods.

2.2.1. Solid-Phase Extraction (SPE)

Solid-phase extraction is the most used clean-up technique when working with seafood samples. Both the sample and the properties of the compounds must be taken into account when selecting the most convenient sorbent. Sorbents such as florisil, PSA, HLB, or aminopropyl-silica (APC) are described in the literature as efficient clean-up sorbents for HPVs in seafood extracts. Among these, florisil appears as the most used by authors for the determination of PAEs [27,48,49,61,78], whereas OPEs seem to be better extracted when using APC cartridges [53,79,80]. Regarding the elution solvent, most of the methods described make use of DCM even though other solvents like acetone, diethyl ether or ACN can be also successfully applied. Authors such as Gu et al. [81] pointed out the importance of the election of the elution solvent. In their study, prawn, fish, and mollusc extracts clean-ups were tested, comparing the elution solvent between hexane and ACN. Even though hexane yielded higher recoveries, authors chose I as fewer interferences were observed in the chromatograms. Regarding BTRs and BTs, Oasis HLB cartridges have been used for the clean-up of molluscs extracts performing a washing step

Figure 2. Comparison of the most used extraction and clean-up techniques for the determination of HPVs in seafood samples.

using H_2O with a 10% MeOH and finally eluting with pure MeOH [50]. The use of a single SPE procedure is sometimes not enough for an efficient clean-up, thus leading to the incorporation of subsequent SPE procedures to enhance its results. Authors like Santín et al. [51] studied the efficiency of florisil, alumina, and silica SPE cartridges as a clean-up for USE extracts of barbels, carps, and trouts in search of 16 OPEs and denoted that its standalone applicability resulted in high matrix effects and low recoveries. They observed that the problem could be solved if cartridges of C18 were paired along with the previously stated. Thus, the combination of basic alumina + C18 and elution using I resulted in recoveries yielding between 48 – 113% and RSD values below 10%.

All in all, the extended use of SPE is sustained by the good results observed for this clean-up strategy. It also allows easier solvent exchange as well as higher preconcentration of the extract avoiding evaporation if eluted with low solvent volumes. However, some drawbacks should be assessed for SPE. The use of highly lipidic extracts in this technique could lead to the clogging of the cartridges, thus diminishing the extraction efficiency and leading to unreliable results. Moreover, SPE cartridges are usually made of plastic, which could be an interference source when analysing PAEs as previously stated for MSPD.

2.2.2. Dispersive Solid-Phase Extraction (d-SPE)

Clean-ups are usually found tedious due to the incorporation of another analytical step into the procedure. Strategies such as SPE need some preconditioning of the cartridge, and subsequent sample loading, cleaning, and elution steps, which can be found quite laborious as well as extend the overall method time demand. Dispersive solid-phase extraction (d-SPE) has been an extremely useful tool due to its high efficiency as well as simplicity. Usually used after QuEChERS extraction, d-SPE uses sorbents dispersed in the obtained extracts to adsorb the interferences. Methods involving the use of d-SPE for extracts of seafood samples mainly concur on the use of sorbents like primary-secondary amine (PSA), C18, and their mixtures. Xu et al. [64] evaluated the use of d-SPE for the determination of PAEs from QuEChERS extracts of fish samples from Shanghai markets. PSA, C18, PSA + C18, and neutral alumina were evaluated in terms of apparent recovery, being PSA the sorbent that yielded higher recoveries. Jakismka et al. [68] also tested the efficiency of d-SPE using PSA along with other alternatives such as PSA + C18, PSA + C18 + GCB, and C18 + GCB. In this case, BTR, TTR, TBOEP, and TCPP achieved higher recoveries when

C18 was incorporated along with PSA in the d-SPE. The results of these studies match with PSA [38,64] and PSA+C18 [68,82] being the most used sorbents to perform d-SPE for the determination of PAEs, BTRs, and OPEs in seafood samples. This can be explained due to the ability of PSA binding nonpolar lipids via hydrogen bonds and C18 retaining long-chain fatty compounds. Even though d-SPE achieves great recoveries while diminishing the matrix effect of fish and seafood samples, sometimes compounds such as OPEs and BTRs seem to be more affected by matrix co-extractants, thus leading to more intensive clean-up procedures. In this sense, additional steps are incorporated into the method, them being either an additional d-SPE or an SPE clean-up. Examples of that can be found in the methods described by Castro-Jiménez et al. [29] or Poma et al. [83] where an additional d-SPE with Florisil + alumina and two SPE using Florisil and Aminopropyl silica (APC) were used respectively. For BTRs in bivalves and fish, Álvarez-Muñoz et al [30,84] incorporated an additional clean-up after the PSA + C18 d-SPE focused on the phospholipid removal using an Ostro SPE plate.

The fast, inexpensive, and easy-to-use procedure that is d-SPE leads to this technique being one of the most extended clean-ups nowadays. Nonetheless, attention must be paid to the fact that a filtration or centrifugation step needs to be performed afterward. Moreover, higher sample amounts, as well as species with higher lipid contents, may need further clean-up once a single d-SPE is performed.

2.2.3. Chromatographic related techniques

The use of chromatography fundamentals can achieve a successful role in cleaning the extracts obtained from seafood samples. Techniques such as gel permeation chromatography (GPC) enable a more efficient determination due to the obtention of cleaner extracts. It is therefore widely used in the clean-up of extracts where target analytes need to be separated from high molecular weight compounds such as pigments, resins, or lipids [85]. Studies such as the ones conducted by Fierens et al. [35,36], Sakhi et al. [86], or Sundkvist et al. [59] used GPC with styrene/divinylbenzene columns for the removal of lipids from the extracts for the determination of PAEs and OPEs. The elution of the compounds was carried out with DCM for PAEs while OPEs were eluted using a mixture of cyclohexane/EtAc (3:1). Even though GPC can be used as a stand-alone clean-up strategy, some authors have coupled this technique with others to achieve better results. Thus, combinations of GPC with SPE clean-ups [57,87,88] have

been described in the literature. Aznar-Alemany et al. [89] and Giulivo et al. [41] both also described the use of online clean-up coupled with LC for the determination of OPEs in mussel and fish samples, respectively. A combination of two columns was selected for this purpose, CycloneTM-P followed by C18-XL yielding recoveries between 47-98%.

Using GPC as a clean-up strategy can be useful when working with samples with a high lipidic content such as seafood. Nevertheless, GPC can be tedious due to the long waiting times as well as the higher solvent consumption. On the other hand, online coupling with clean-up columns greatly improves the method's overall time demand, as the elevated automatization of the process leads to its reduction. However, specific instruments are needed to fulfill this purpose, thus increasing the cost.

2.2.4. Clean-up comparison

Clean-up procedures are usually mandatory when working with seafood samples due to their high complexity. Thus, special attention must be paid when selecting the most appropriate clean-up. Regarding the efficiency of the clean-up procedures, it needs to be pointed out that most of the methods described in the literature do not show matrix effects values or compare how the clean-up step reduces its presence. This could be linked to the use of surrogate standards before sample extraction, usually neglecting the importance of a convenient clean-up. Even though this strategy is faster and easier to implement, a minimal clean-up should be performed specially for seafood samples to lengthen the instrument's efficiency. Strategies such as SPE and d-SPE are the most extended due to their easy application and good results. As observed, florisil appears as a great sorbent when using both of them. Its combination with other sorbents such as C18 or PSA could even enhance its effectiveness. The combination of these sorbents can be easier to achieve when working with d-SPE, as bulk sorbents can be mixed in a single tube. Despite strategies such as GPC having good results, its large solvent consumption, the long extraction times as well as the need for additional instrumentation, may not be an affordable and ecological alternative for most laboratories. As regards the use of novel devices such as Cleanert PAE or LipiFiltr, the application of selective cartridges like Cleanert PAE restricts the range of compounds to determine. On the contrary, selective sorption of lipids as the one given by LipiFiltr provides good results in a non-restrictive way, however, due to its novelty, the price of these cartridges may lead to an overall method price rising, especially taking into account the single use of these.

All in all, its easy applicability after QuEChERS extraction, the possibility of incorporating more than one sorbent at a time, and the good results observed in the literature, takes d-SPE one of the most competitive strategies to perform as a clean-up for the determination of HPVs in seafood.

2.3. Determination techniques

Once the extraction is performed, HPVs should be analysed to determine their presence in the extracts. The preferred techniques for this purpose are gas and liquid chromatography, mainly coupled to mass spectrometry, either simple or in tandem. As per the preference for gas or liquid chromatography, PAEs are mostly determined using GC due to their low polarity and high volatility. For OPEs, these can be determined either by gas or liquid chromatography. Finally, the higher polarity inherent in BTs and BTRs turn these compound families more suitable to be determined using LC. The use of GC instead of LC allows easier separations of some of the isomeric compounds derived from benzotriazole such as tolyltriazole isomers (4 and 5-tolyltriazole, 4/5TTR). The selection of the chromatographic technic can also be influenced by the sample. For seafood, as high complexity samples, the matrix effect can be something to take into consideration. Mechanisms involved in the signal enhancement or suppression in hyphenated chromatographic systems are quite different when presented for gas and liquid chromatography. As per liquid chromatography and its soft ionization (mainly when using ESI), a competition between the analyte and the interferences for the proton transfer could lead to a modified signal, whereas the higher ionization energy (EI) used in GC-MS systems is supposed to overcome this problem. Nonetheless, adsorption of matrix compounds in free active sites present in the column or injector of the GC systems could cause some disparity when comparing the signals of neat and matrix-containing standards [90]. Those mechanisms could be used in favor of the determination of HPVs in seafood samples, as the use of GC could reduce the matrix effect, thus leading to the development of easier and more reliable methods. However, this can only be applied to the determination of compounds with GC amenable properties. Most of the presented compound families, as seen in Table 1, can be analysed via GC or LC, however, analytes such as benzotriazoles yield low sensitivities when using GC systems due to their higher polarity. Strategies like higher enrichment factors or injection volumes as well as derivatization could overcome this problem. Still, further investigation is needed in this field as, to date, literature dealing with the use of GC for the

determination of benzotriazoles in samples such as seafood is scarce.

Regarding the columns used for GC analysis, most of the methods use capillary columns with low polarity properties, being 5% phenyl 95% dimethylpolysiloxane (e.g. ZB-5, HP-5, DB-5) the most common. For column length, inner diameter, and film thickness, the standard 30 m x 0.25 µm x 0.25 mm is presented as the best option. LC systems mainly use columns based on C18 stationary phases (e.g. Poroshell C18, Purosphere C18, Acquity BEH C18) with lengths ranging between 50 – 150 mm. Concerning the analyser, mass spectrometry is currently the most used technique either single or in tandem. Due to the higher instrumental signal of PAEs, low limits of detection can be achieved using single quadrupole systems combined with GC [46,47,72]. Some methods take advantage of QqQ for the determination of these compounds in seafood, especially when using LC [39,86]. For OPEs, QqQ is the preferred option [43,45,51,53,66,83], even though literature contains studies using other mass spectrometry systems such as QTRAP [66], HRMS [91] or QTOF [92], and even other detectors like nitrogen-phosphorous detector (NPD) [71], flame photometric detector (FPD) [56] or UV [52]. Methods involving the determination of benzothiazoles and benzotriazoles in fish mainly use tandem mass spectrometry or high-resolution mass spectrometry [30,50,63,77]. While electron ionisation (EI) is the main used source for GC-MS, electrospray ionization (ESI) in positive mode is extensively used for the determination of these compound families when working with LC-MS(/MS).

3. Occurrence, human exposure, and risk assessment

3.1. Phthalate esters

The occurrence of phthalate esters has been widely studied and several articles describe their widespread presence in many environmental fates, including seafood. A recap study published in 2014 by Serrano et al. [93] placed seafood as a varied phthalate concentration food group, denoting concentrations between 13 µg kg^{-1} and 928.6 µg kg^{-1} (w.w.) in samples throughout the world. In the same way, the authors denoted the minimal contribution of seafood ingestion (0.1 – 0.6 %) to the total dietary intake of DEHP for the population of the US, being young infants (1-2 years) the most affected group with daily intakes of 0.05 µg kg^{-1} day^{-1}. Opposite to this study, He et al. [15] constated that seafood had the highest PAEs concentrations out of all the analysed food groups for the population of Yanjji (China), with concentrations between 658 – 1610 ng g^{-1} w.w. (DEHP and DBP being the

highest contributors), and EDIs up to 2530 ng kg^{-1} bw day^{-1} for DEHP, still representing low exposure. The presence of PAEs in different seafood samples was also denoted by Gu et al. [67], expressing concentrations (Σ_5PAEs) between 5 – 46.3 ng g^{-1} w.w. in fish, 3.3 – 219.3 ng g^{-1} w.w. in mollusc and 5.0 – 57.3 ng g^{-1} w.w. in prawn. Hu et al. [27] analysed fish samples from the Yangtze River Delta, China. DEHP and DBP were the compounds with higher detection frequency with mean concentrations of 1941 ng g^{-1} w.w. and 78.7 ng g^{-1} w.w. respectively. A study conducted by Cheng et al. [94] on the bioaccessibility of phthalate esters present in the twenty most consumed species from Hong Kong concluded that percentages between 2.44 – 45.5% of the raw PAEs concentrations were bioavailable. Given the bioavailable concentrations (0.20 – 1.23 µg g^{-1} w.w.) and after performing risk calculations, fish consumption was considered safe by the authors. Most of the studies conclude that DEHP is the compound with the highest concentrations as well as highest detection frequency in seafood samples. Due to its elevated concentrations and its negative health effects, experiments focused on the determination and behavior of this compound are usually found in the literature. Guerranti et al. [95] evaluated the exposure and risk of the Italian population to the intake of DEHP when ingesting Atlantic bluefin tuna fillets, concluding that the estimated ingestion was very far from being any risk for the Italian population. Regarding the sources from which these compounds reach the seafood, Sakhi et al. [86] proposed plastic food packaging as one of the possible sources of phthalate contamination, while Cheng et al. [94] stated that fish can accumulate DEHP from water and ingestion of contaminated sediments and food.

3.2. Organophosphate esters

The occurrence of OPEs in fish has been recently put in the spotlight due to their presence in most of the analysed samples. Sundkvist et al. [59] started reporting their appearance back in 2010 when comparing fish samples collected in sites with potential sources of OPEs with background locations. OPEs profiles from background locations (Swedish lakes) showed similarities, being TCPP and TPP the most abundant, which led to the belief of OPEs diffusive sources. Nonetheless, TBOEP and TDCPP appeared in samples collected after a WWTP while higher concentrations of TBP, which is usually a component of aircraft hydraulic fluids, were found in specimens collected from an effluent receiving water from a nearby airport. High concentrations were found in samples of catfish (*C. fuscus*) and grass carp (*C. idellus*) from Pearl River, China [88]. There, TnBP, TCEP, TCPP, and

TBOEP were found in all the analysed samples at concentrations ranging from 43.9 – 2946 ng g^{-1} l.w. for TnBP, 11.7 – 281 ng g^{-1} l.w. for TCEP, 62.7 – 883 ng g^{-1} l.w. for TCPP and 164 – 8842 ng g^{-1} l.w. for TBOEP. The presence of chlorinated OPEs is heavily sustained in the literature, Gao et al. [96] analysed samples of different fish species (grass carp, croaker, crucian and perk) from a Nanjing supermarket (China), being TCEP and TDCPP the only two OPEs present in all the samples. These compounds were also found in estuarine food webs of the Western Scheldt, Netherlands, where Brandsma et al. [80] determined those in both benthic and pelagic species. The studies conducted by Malarvannan et al. [33] on the levels and profiles of OPEs in European eels (Anguilla Anguilla) from a highly industrialised Flanders region showed differences from the previous studies in terms of compounds proportion in samples, being the following for the Belgian samples: TDCPP (64%) > TPP (17%) > EHDPP (12%) > TBOEP (5%) > TCEP (1%) > TDCPP (1%). The differences in the concentrations of OPEs found between the river and marine fish species were stated by Giulivo et al. [41] stating that river fish species had higher concentrations (55.5 – 646 ng g^{-1} l.w., Σ_{14}OPEs) than the marine ones (14.6 – 15.8 ng g^{-1} l.w.), with TBP as the most abundant. This effect could be explained due to the discharges from industries, WWTPs, and other local sources in rivers. Reports of these compounds being present in fish led to the apparition of studies focused on their presence in consumer foodstuffs. Studies on the Swedish population intake of OPEs through food performed by Poma et al. [40] revealed that fish in the market baskets of the population had mean concentrations of TCEP, TPP, EHDPP, TDCPP, and TCPP of 0.10, 0.46, 2.46, 0.29 and 0.08 ng g^{-1} w.w., respectively. Data concerning the distribution of the compounds in the different food categories considered fish the group with percentages between 2- 8% of the total OPEs. Later on, Poma et al. [83] continued the study on food groups, this time for Belgian foodstuffs, reinforcing the low contribution of fish and seafood to the total OPEs distribution (6%). Even though the previous studies placed fish and seafood as a minor contributor to the total OPEs intake in market baskets, a study conducted in Southeast Queensland, Australia placed fish and seafood behind plant-based foods (vegetable, cereal, and fruit), but on top of the animal products with median concentrations of 1.8 ng g^{-1} w.w. (Σ_9OPEs) over dairy products (1.4 ng g^{-1} w.w.), meat (1.0 ng g^{-1} w.w.) and eggs (1.0 ng g^{-1} w.w.) [54]. The higher presence of OPEs in seafood instead of meat has been reported by other studies conducted in the USA [16,66] where median concentrations of seafood were 7.1 and 8.7 ng g^{-1} w.w.

versus 6.7 and 6.2 ng g^{-1} w.w. in meat, respectively. It is also observed that, like most of the commented studies, chlorinated OPEs appear on top in detection frequency. Some studies have also conducted exposure and risk assessment calculations based on the concentrations found in the different analysed species. A previously commented study by Sundkvist et al. [91] stated that everyday consumption of eelpout would result in a total exposure of 180 ng g^{-1} day^{-1} (Σ_8OPES), which is far below the 40 µg g^{-1} day^{-1} suggested by the guideline. Other studies such as the ones conducted by Kim et al. [97] regarding fish species from Manila Bay (Philippines) estimated a dietary intake of 5.9 µg g^{-1} day^{-1} (Σ_9OPES), while dietary intakes associated with the consumption of eels by local fishermen from a region of Flanders ranged between 0.18 (TBOEP) – 1.0 (TCPP) ng kg^{-1} day^{-1}. These studies, together with the ones by Poma et al. [40,83] at Sweeden and Belgium regarding consumer baskets, all conclude that the risk associated with the ingestion via seafood intake is minor, with values even being up to several orders of magnitude below the reference doses (RfD).

3.3. Benzotriazoles

The presence of benzotriazoles in fish was first studied by Cancilla et al. [98] when proving the environmental impact of anti-icing fluids for aircrafts present in an effluent receiving those compounds from an airport runoff. Fathead minnow specimens accumulated 4TTR and 5TTR after their exposure to a stream located after the outfalls of the airport. Jakimska et al. [68] also reported the presence of 4/5TTR in fish samples present in rivers in Spain. Particularly, the appearance of triazoles was noted in fish samples of two locations heavily influenced by external factors such as a city with an important industrial input (Llobregat river, 10.18 ng g^{-1} d.w.) and the outfalls of a WWTP (Ebro River, 1.25 ng $^{-1}$ d.w.). Following the line of the effects of WWTP Yao et al. [82] remarked the high detection frequency (100%) of BTR, 4/5TTR, and ClBTR (concentrations from <0.25 ng g^{-1} w.w. for ClBTR to 3.88 ng g^{-1} w.w. for BTR) in fish samples from the Dondjing River, which receives domestic wastewater discharges from the nearest city. Later on, Yao et al. [99] further confirmed the presence of BTR and 5TTR in fish samples from Pearl River and Yangtze River in China with concentrations ranging between 0.27 – 0.90 and 0.30 – 0.40 ng g^{-1} w.w. for each river, respectively. Greek Evrotas River endemic chub specimens were analysed by Díaz-Cruz et al. [60], confirming the presence of 5TTR in concentrations comprised between 3.5 and 6.2 ng g^{-1} d.w. The study also reported the strong tendency that this compound has to bioaccumulate in fish tissues. Specimens

of tilapia (*Tilapia aurea*), striped bass (*Morone saxatilis*), grouper (*Epinephelinae*), and billfish (*Makaira nigricans*) were collected from local fish stores at Chung-Li City, Thailand, and analysed in search of triazoles [77]. Results found in those samples agreed with the previous studies in terms of the congeners found in the samples as well as their distribution. Thus, BTR appeared in concentrations from 42.5 – 71.3 ng g^{-1} d.w. followed by ClBTR (3.7 – 4.2 ng g^{-1} d.w.) and 4/5TTR (0.4-0.9 ng g^{-1} d.w.). Jia et al. [50] found a shift in that tendency when analysing molluscs from the Bohai Sea, China. In contrast to the other studies, XTR appeared at concentrations twice what was found for BTR in molluscs (GM of 14.4 ng g^{-1} d.w. for XTR vs. GM of 6.47 ng g^{-1} d.w. for BTR) and a 100% detection frequency. Related to these results, estimated daily intakes (EDI) were calculated for BTR ingestion via mollusc consumption, with values comprised between 5.34 and 8.71 ng kg^{-1} bw day^{-1} were estimated for Σ_5BTRs, assessing that children and teenagers were more exposed to triazoles. Previously commented studies such as the one conducted by Yao et al. [99] also calculated the risk associated with the ingestion of BTR through fish consumption resulting in a maximum hazard quotient (HQ) value ranging between 1.19E-07 and 7.87E-06, which were far below 1, meaning that the health risk was minimum.

3.4. Benzothiazoles

Data concerning the occurrence of benzothiazoles in fish samples is highly scarce in the literature. To date, few articles have proven the presence of these compounds in fish and seafood. Trabalón et al. [17] studied the presence of BT, ClBT, MeSBT, NH2BT, and OHBT in samples of the ten most consumed seafood species from Tarragona, Spain. Results showed that BT was present in most of the analysed samples with the highest concentrations in a range between 13 and 82 ng g^{-1} d.w. followed by NH_2BT (11 – 70 ng g^{-1} d.w.), ClBT (6 – 38 ng g^{-1} d.w.) and MeSBT (11-24 ng g^{-1} d.w.). Squid was the species with the highest concentration of a single compound (BT at 82 ng g^{-1} d.w.) whereas mussel appeared as the species with the most present benzothiazoles. As with benzotriazoles, Jia et al. [50] also described the presence of BT, MeSBT, OHBT, NH_2BT, SCNMeSBT, MeBT in molluscs from nine industrial coastal cities. BT, SCNMeSBT, MeBT, and MeSBT were detected in all the analysed samples (n=166), being BT the compound with the highest concentration in a range between 132 – 13400 ng g^{-1} d.w. (GM: 595 ng g^{-1} d.w.), followed by SCNMeSBT (GM: 67.1 ng g^{-1} d.w.), MeBT (GM: 24.2 ng g^{-1} d.w.), OHBT (GM: 20.1 ng g^{-1} d.w.), MeSBT (GM: 14.2 ng g^{-1} d.w.) and finally NH_2BT (GM: 0.165 ng g^{-1} d.w.). In contrast with the previously stated by Trabalón et al.

[17], OHBT was more present in molluscs despite it not being found in any of the fish species from Tarragona. On the other hand, NH_2BT, which was found in fish samples, was present at much lower concentrations in molluscs. BT concentrations appear at way higher rates in mollusc compared with the fish samples from Tarragona which may indicate higher bioaccumulation. The presence of OHBT in fish samples was also denoted by Chen et al. [77], whose study found concentrations ranging from 15.4 to 26.1 ng g^{-1} d.w. in samples of striped bass, billfish, tilapia, and grouper from Chung-li city (Taiwan) local markets. Regarding exposure and risk assessment, both Trabalón et al. [17] and Jia et al. [50] conducted calculations for the exposure and risk associated with the ingestion of benzothiazoles via dietary intake. In both cases, BT appeared as one of the major congeners with EDI values between 22 – 94.9 ng kg^{-1} bw day^{-1}. The existence of repeated dose oral expose values such as NOAEL for benzothiazoles is only available for benzothiazole (BT). Thus, calculations related to toxicity can only be performed for this compound. In this sense, and with a reference value (RfD) of 5000 ng kg^{-1} bw day^{-1}, both studies concluded that minor risk was associated with the ingestion of BT via dietary intake, with levels reaching up to 2-3 orders of magnitude lower than reference BT RfD value for molluscs.

3.5. Overall HPVs occurrence and toxicity

The presence of PAEs in seafood is the most notable in terms of higher concentrations and detection frequency, especially for DEHP, with variable concentrations reaching the high µg g^{-1} w.w. Being the most known compound family, data on their occurrence as well as investigation on their potential bioaccessibility are easily found in the literature. The occurrence of OPEs in seafood is, as observed, an emerging topic gaining more attraction every day. Believed to be widespread by diffusive sources, their presence in fish from locations all over the world has been widely proven. Their study can lead to an onsite interpretation of OPEs contamination, as local sources represent a highly important indicator of the different OPEs used by a certain location, as well as the impact of surrounding industries or the appearance of unwanted leakages. As regards levels and distribution, concentrations surrounding the low ng g^{-1} w.w. are mostly found, whereas chlorinated OPEs, represent the most frequently detected congeners. Most of the data found in the literature constrain the concentrations of benzotriazoles and benzothiazoles found in seafood at low ng g^{-1}, with slightly higher levels for the latter. As per their concentration distribution on the different fish species, no significant

Table 2. Estimated Daily Intakes (EDI, ng/kg body weight /day) of different HPVs in seafood.

Compounds	Population	Species	EDI	Most affected group	Top contributors	Ref.
Σ_{11}OPEs	China	Shrimp, oyster, crab	24	Young males (6-18 years)	TCEP, TCPP	[107]
Σ_{1}PAE	Taiwan	Shrimp	0.07	Adult males	DEHP	[39]
Σ_{6}BTs	China	Mollusc	95	Children and teenager males	BT	[50]
Σ_{5}BTRs	China	Mollusc	8.7	Children and teenager females	BTR, XTR	[50]
Σ_{5}BTs	Spain	Fish, mussel, squid	48	Adult women, Senior women	BT	[17]
Σ_{1}PAE	US	Fish	0.6	Females of reproductive age	DEHP	[93]
Σ_{6}PAEs	China	Fish	1500	General population	DEHP	[15]
Σ_{6}PAEs	China	Fish	85	General population	DEHP	[34]
Σ_{11}OPEs	US	Fish	1.8	Toddlers (1 to <6 years)	TBOEP, TnBP, TCPP	[16]
Σ_{9}OPEs	Philippines	Fish	5.9	General population	TEHP, TEP, TnBP	[97]
Σ_{5}OPEs	Sweden	Fish	20	Adults	TCPP, TBOEP, EHDPP	[59]
Σ_{7}OPEs	Belgium	Fish	300	Adults	EHDPP, TPhP, TCPP	[83]

differences are noticeable given the available data. It is however highly proven that industrial and domestic discharges are the main sources of the widespread occurrence of these contaminants in seafood. Nevertheless, data concerning the concentration of these compounds in commercially available fish and seafood species is scarce.

The exposure and risk associated with the presence of HPVs in seafood seem to be far from being a problem, with most of the studies placing its dietary intake far below the reference dose (RfD) This chronic risk (long-term) is usually obtained as the result of the comparison between the estimated daily intake (EDI) and a reference dose value. These RfD values are established upon the NOAEL values (non-observed-adverse-effect-level), which are found experimentally by means of assays involving rodents' dose-response effects upon the repeated administration of known concentrations of the studied compound. Organisations like the Environmental Protection Agency (EPA) or the European Food Safety Authority (EFSA) provide reference dose values for some of the compounds, especially for the most well-known like phthalates and some organophosphates. However, data regarding the reference doses for benzothiazoles and benzotriazoles are still uncertain. Table 2 compiles some of

the results involving the estimated daily intakes (EDIs) of OPEs, PAEs, BTs and BTRs present in seafood along with the population group with the highest impact and the compounds with the highest contribution. As observed in the table, PAEs EDI values are greater when compared to the other families, a fact directly linked with the presence of DEHP, which always appear as the highest contributor. Regarding OPEs, EDIs values are comprised in a wide range between 1.8 – 300 ng kg^{-1} body weight day^{-1}, which can associate with the different concentrations found in the samples along with the number of congeners included in the total sum. For BTRs and BTs, slightly higher results are found for the latter, also agreeing with the concentration levels typically found in the samples. As for the most affected groups, differences are found between the studies, mainly based on the seafood intake, which can be linked with the geographic and cultural differences of the populations where the studies were conducted.

It is also important to mention that none of the previously described studies reported EDI values higher than the reference doses (RfD) for any of the studied compounds nor a hazard quotient (HQ) which indicated a risk to the population when consuming seafood. Nonetheless, synergic effects and alternative and complementary exposure pathways such as inhalation, dermal exposition, or ingestion of particulate matter or other foodstuffs are not included in the previous values, thus only giving a partial view of how the presence of HPVs could lead to a real menace towards population health.

4. Conclusions

High production volume chemicals presence in seafood is nowadays attracting more attention. Its ability to give information on both the environmental status and the exposure and risk associated due to the ingestion of contaminated seafood turns its study into a fundamental strategy. The progress towards the development of effective, sensitive, and greener methods has led to the use of QuEChERS as an excellent alternative to determine these compounds. Its easy procedure, along with the wide selectivity and proven good results suggest that this technique should be extended to even more HPVs family's determination in seafood. Regarding one of the most critical steps, the clean-up, strategies such as dispersive solid phase extraction using sorbents like florisil, C18, and PSA yield good results in terms of apparent recovery as well as reduction of matrix effect. Focus should be pointed towards florisil application as clean-up sorbent, as most of the methods involving its use convey on its effectiveness. Other alternative clean-up strategies such as LipiFitlr are quite promising. Even

though methods for most of the compounds are found in the literature, families such as benzothiazoles and benzotriazoles are yet to be further investigated, both in terms of development of alternative determination methodologies as well as monitorization of their occurrence in seafood samples. Further progress is expected in this field in terms of miniaturization, greener approaches, and novel methodologies capable of overcoming challenging and crucial steps of the determination of HPVs in seafood. Future research should also focus on the determination of the possible metabolites and degradation products that may be present in the samples as a result of both their incorporation from external sources and their metabolic degradation once ingested. The ubiquitous presence of most of these compounds in several seafood samples should lead to the development of replacement compounds with lesser or ideally no negative effects on the population, thus reducing their presence in seafood or any other fate susceptible of being accessible to the population. Regarding population's exposure and toxicity risk via dietary intake, values of estimated daily intake (EDI) were found under the reference dose (RfD) for those compounds with an available value, suggesting chronic toxicity due repeated dose intake through fish consumption is minor. Albeit toxicity values being low, no studies regarding the synergic and metabolic effects, or even the bioaccessibility of some of the compounds have been portrayed, thus diminishing the certainty of these risk values being correct. Future research on this topic is needed so as to enhance the data to portray more accurate calculations and provide better risk assessment reports.

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1.2.2. Synthetic musk fragrances in seafood: determination and occurrence

Determination of synthetic musk fragrances in seafood has been a relevant issue in recent times due to the believed negative health effects that these compounds could pose for the population over their intake via seafood consumption. If focused on the last decade, several methods have been reported for their determination in these samples. Table 1 compiles a selection of the most relevant chromatographic methods for this purpose, compiling different extraction techniques and clean-up strategies.

SLE and USE, being effective and easy-to-handle methods, have been successfully used for the determination of compounds such as AHTN, DPMI, MX or HHCB in fish samples with recoveries ranging between 77-114% [127–129]. Acetonitrile and acetone appear as good extraction solvents, with volumes between 10 – 40 mL. Regarding sample weights, these are found in the low weight range, with values comprised between 0.5 and 5 g. These techniques, as previously stated in the review, only have the disadvantage of requiring a high solvent consumption and exhibiting low selectivity.

MSPD has also been used for the extraction of HHCB and AHTN in fish and mussel samples. Florisil, C18, and activated/deactivated silica have demonstrated high efficiency as dispersants/clean-up sorbents for this kind of extraction. Comparing the recoveries obtained by Ocaña-Ríos et al [130] and Ziarrusta et al. [131] for the determination of these compounds in fish and mussel samples, respectively, it can be seen that AHTN recovery for mussels was found lower (45 vs 79%), probably due to the fact that mussels, being filtrating organisms, may present higher co-extractants, thus hindering the efficient recovery of this compound. On this same topic, Ziarrusta et al. [131] also evaluated the effect of the co-dispersant/clean-up sorbent on the extraction efficiency, observing HHCB had similar recoveries when using Envicarb, florisil, silica, diatomaceous earth or zeolite, except for the use of C18 on its own, which yielded much higher recoveries, probably linked to an enhanced matrix effect due to inefficient matrix removal.

QuEChERS could be defined as the most used technique for the determination of the highest range of musk fragrances among all the reported methods, also including the HHCB-lactone. Thus, several authors have reported its use recently, with most of them using the EN 15662 method salts combination (sodium citrate, disodium citrate sesquihydrate, sodium chloride and magnesium sulfate) for the process [132–136], yielding recoveries of 46 -120%. Despite these salts being widely used, methods using the original [137,138] and the AOAC [139,140] methods salts have also been reported with similar results. As regards the sample weight needed, these methods mostly take less than 1 g for the extraction (0.2-0.5g).

Table 1. Selection of relevant chromatographic methods for the determination of synthetic musk fragrances in seafood samples.

Matrix	Compounds	Extraction	Clean-up	Instrumental analysis/Column	R_{app} (%)	LODs	Ref.
Fish	AHTN	SLE (0.5 g w.w.sample) ACN/Isopropanol (1:1)/ mix 10 min	-	LC-HRMS Hypersil Phenyl	77%	2.4 ng g^{-1} w.w.	[127]
Fish	DPMI, MX	USE (5 g w.w.sample) 4 x 10 mL ACN x 15 min	SPE ENV+	GC-MS TG-5SIL	99 - 104%	0.0063 – 0.081 ng g^{-1} w.w.	[128]
Fish	HHCB, AHTN, MX	USE (1 g w.w.sample) 10 mL Acetone x 15 min	SPE Silica	GC-MS VF-5MS	95 - 114%	-	[129]
Fish	HHCB, AHTN	MSPD (0.2 g d.w. sample) sample + 1 g florisil + 0.5g C18 E: 5 mL ACN	-	GC-MS Elite-5MS	79 - 108%	6 – 8 ng g^{-1} w.w.	[130]
Mussel	HHCB, AHTN	MSPD (0.3 g d.w. sample) sample + 0.6g deactivated silica + 4g of activated silica / E: 25 mL DCM	-	GC-MS/MS (QqQ) HP-5MS	45 - 123 %	4.1 – 6.3 ng g^{-1} w.w.	[131]
Fish	DPMI, ADBI, AHMI, ATTI, HHCB, AHTN, MX, MK, HHCB-lac	PLE (0.5 g d.w. sample) 1 cycle (5 min, 60ºC) DCM	In-cell Florisil	GC-MS/MS (IT) ZB-50	45 – 109%	0.25 - 5 ng g^{-1} d.w.	[134]
Fish	HHCB, AHTN, MX, MK	QuEChERS (2 g w.w. sample) 5 mL H_2O + 10 mL ACN (1% Acetic acid) / 6 g $MgSO_4$ + 1.5 g sodium acetate	d-SPE PSA + C18	GC-MS DB-5MS	74 - 112%	0.10 - 0.64 ng g^{-1} w.w.	[139]
Fish	HHCB, AHTN, MX, MK, ADBI, AHMI, ATTI	QuEChERS (0.2 g d.w. sample,) 1 mL ACN / + 670 mg MgSO4 + 170 mg NaCl + 170 mg sodium citrate + 80 mg disodium citrate sesquihydrate	d-SPE PSA + C18	GC-MS HP-5MS	46 - 120%	0.001 – 1.94 ng g^{-1} w.w.	[135]
Fish, mussel	DPMI, ADBI, AHMI, ATTI, HHCB, AHTN, MX, MK, HHCB-lac	QuEChERS (0.5 g d.w. sample,) 10 mL H_2O + 10 mL ACN / + 4 g MgSO4 + 1 g NaCl + 1 g sodium citrate + 0.5 g disodium citrate sesquihydrate	d-SPE Florisil	GC-MS/MS(IT) ZB-50	24 - 110%	0.25 - 10 ng g^{-1} d.w.	[134]
Fish, mussel	DPMI, ADBI, HHCB, AHTN	QuEChERS (0.5 g d.w. sample,) 4.5 mL H_2O + 5 mL ACN / + 2 g MgSO4 + 0.5 g NaCl	d-SPE EMR-Lipid DLLME CCl_4	GC-MS ZB-XLB	70 - 118 %	0.5 - 50 ng g^{-1} w.w.	[138]

Despite QuEChERS extraction being extensively used, authors such as Vallecillos et al. [141] compared the use of PLE and QuEChERS extraction on the determination of musk fragrances in fish and mussel samples. Results showed that PLE yielded overall better method quality parameters, with higher recoveries and lower method detection limits. Even though effectively used by other authors like Díaz-Cruz et al. [142] and Cunha et al. [143], its use is mainly punctual, probably due to the need for specific instrumentation.

Techniques such as Soxhlet extraction have also been applied. For instance, Zhang et al. [144] used a mixture of hexane/acetone (1:1, v/v) to successfully extract AHMI, ADBI, ATII, HHCB, AHTN, MX and MK from fish specimens from Taihu Lake, China. Recoveries exhibited an average value of 72%, with the disadvantage of needing between 65-185 mL of solvent to reach those values.

Regarding clean-up strategies, SPE and d-SPE still represent the most used clean-up strategies also for these HPVs. Sorbents such as florisil [134,145] and PSA+C18 [135,139] exhibit efficient matrix removal, leading to reliable and effective determinations.

Novel methodologies are being developed nowadays, mostly aiming at the use of greener alternatives. Ocaña-Ríos et al. [146] recently reported the use of in-vivo SPME for the determination of HHCB and AHTN in rainbow trout specimens. The C18 SPME fiber is introduced into the previously anesthetized fish and left for 20 min while the fish is placed in the water again. The fiber is then desorbed with 600 µL of ACN and a solvent exchange combined with a derivatization agent is performed. The presented method achieved detection limits of 15 and 20 ng g^{-1} w.w. for HHCB and AHTN, respectively. The present thesis includes a novel method for the determination of musk fragrances in fish samples, being an alternative greener option to the previously stated methods. Fish samples are lyophilised and subsequently determined using headspace SPME Arrow, offering a solventless and totally automatic method, both being characteristics closely linked to green chemistry. The development of the method is included later on in the experimental section.

Concerning their determination, synthetic musk fragrances are mostly determined using GC due to their high volatility properties [128–131,134,135,138,139]. MS, usually with a single quadrupole [128–130,139,147], is the most used analyser, with some methods using other alternatives such as MS/MS with ion trap [134,145] to reach lower detection limits. Low polarity columns are highly used, with stationary phases mostly being (5% phenyl)-dimethylpolysiloxane [128,129,131]. Some authors have also reported good separations when working with higher polarity phases such as (50% phenyl)-dimethylpolysiloxane [134,145].

All in all, these compounds do not heavily differentiate from the previously commented HPVs in terms of preferred extraction techniques or clean-up strategies.

Literature reporting the occurrence of musk fragrances in seafood has been increasing over the last decade. Similar profiles are found for most of the analysed specimens. The presence of HHCB and AHTN is ubiquitous, always exhibiting high detection frequencies and concentrations between <MQL – 414.4 ng g^{-1} w.w. [101,129–132,135,137–141,143,144,148–150] and <MQL – 81 ng g^{-1} w.w. [101,129–132,135,137–141,143,144,148–151], respectively. Generally, AHTN concentrations are found lower than those for HHCB, which can be linked to a lower use of AHTN as reported in the yearly production/manufacture. These compounds have been reported in nearly all the species analysed, including fish from different species, mussels, oysters and other seafood from worldwide locations.

ATII is rarely found in seafood samples, some studies have reported its presence at low concentrations. That's the case for seafood of common consumption samples from Tarragona, where ATII was found in concentrations between n.d. – 6.1 ng g^{-1} d.w., mainly in mackerel and hake samples [145] or seafood from different European spots with ranges between <MDL – 1.45 ng g^{-1} w.w. [147]. The highest reported concentration of this compound was found in commercially available crab samples from Portugal, with concentrations up to 39.6 ng g^{-1} d.w. [152]. Regarding other polycyclic musks, the presence of DPMI has been reported in some specimens at concentrations ranging between n.d. – 33.53 ng g^{-1} [143,149,153,154], being sheatfish from Tarragona markets the species with the highest concentration value. AHMI, despite being detected at low detection frequencies, has also been reported by some authors [135,141,143], at concentrations up to 12.51 ng g^{-1} d.w. (carp from Tarragona).

One of the things to be outlined is the presence of nitro musks in fish throughout the world. The European Union's restriction of their use led to a reduction of their presence in fish samples from Europe, whereas places such as China and India, still use some of them. It can be observed that studies such as the ones carried out by Vallecillos et al. [134], Trabalón et al. [153] or Cunha et al. [133] report the non-detection of these compounds in all of the analysed samples or, as in the case of Saraiva et al. [147], concentrations are found at really low levels (<MDL – 0.01 / <MDL – 0.14 ng $^{-1}$ w.w. for MX and MK, respectively). This agrees with the origin of the studied samples, as all of them come from European countries including Portugal, Spain or France, among others. On the other hand, musk xylene and musk ketone have been determined in fish samples from diverse Chinese Rivers and in fish samples from India. Zhang et al. [144] reported concentrations and detection frequencies of <LOD-51 ng g^{-1}, 19% and <LOD -470 ng g^{-1},

25% for MX and MK, respectively, in fish samples from the Taihu Lake, China. Yao et al. [139] also reported the presence of MK at a median concentration between 2.40 – 9.96 ng g^{-1} w.w. in samples from the Dongjing River, China. In India, Vimalkumar et al. [101] observed the presence of MK at concentrations ranging between 8 – 33 ng g^{-1} w.w. in fish.

The presence in seafood of the HHCB main transformation product, HHCB-lactone, is not widely found in the literature. Only a few methods report its presence, with values ranging between n.d. – 63.51 ng g^{-1} d.w., n.d. – 17.94 ng g^{-1} d.w., n.d. – 91.1 ng g^{-1} d.w., n.d. – 228 ng g^{-1} d.w., and n.d. – 11.06 ng g^{-1} w.w., in samples from European hotspots [143], Tarragona [134,145], European seas/Atlantic Sea [149] and Portugal [150], respectively. As can be seen, most of the analysed samples showed concentrations under 100 ng g^{-1}, except for the samples from Portugal, which showed concentrations up to 228 ng g^{-1}. The biotransformation of HHCB into HHCB-lactone was also studied by Cunha et al. [152], suggesting that the biotransformation was closely linked to the species as the results of comparing the HHCB/HHCB-lactone ratio of several seafood species.

Species-specific distribution of musk fragrances has also been studied, with results showing a correlation between high lipid content species and musk fragrances concentration levels. This lipophilic character has been confirmed by several authors, especially for HHCB and AHTN. Trabalón et al. [145] reported higher concentrations found in species such as sardine and mackerel, those having a higher lipid content than the species with a lower percentage. Lyu et al. [132] obtained similar results, concluding that HHCB and AHTN concentrations were related to lipid content when studying fish samples from Chaohu Lake, China. This effect can be also observed in specimens of the same species, as stated by Ocaña-Ríos et al. [130] whose studies on the determination of HHCB and AHTN in rainbow trout, showed a correlation between the lipid content of the specimen and the musk fragrances concentration. The same topic also described the possible trophic magnification of DPMI and HHCB, as well as the trophic dilution of AHTN, which has also been reported by Zhang et al. [144].

As a summary, concentrations and profiles of musk fragrances in seafood usually trend as HHCB> AHTN > other polycyclic musks > MK, MK, with concentrations found in the low ng g^{-1} w.w. levels, with HHCB occasionally reaching higher values (Table 2). Samples from European countries tend to be nitromusks free, whereas samples from other origins where nitro musks are not restricted are keener to accumulate compounds such as MK or MX. Regarding HHCB-lactone, its presence may be linked to the analysed species. Hence, synthetic musk fragrances, especially HHCB, are expected to be present in most seafood as a result of the widespread use of these chemicals in many daily commodities.

Table 2. Occurrence of synthetic musk fragrances in seafood samples (ng g^{-1})

Species	Location	DPMI	ADBI	AHMI	ATTI	HHCB	AHTN	MX	MK	HHCB-lact	Ref.
Oysters	Taiwan	-	-	-	-	-	0.4 - 2.7	-	-	-	[155]
Fish	China	-	-	-	-	<MDL - 52.9	<MDL -7.5	<MDL51	<MDL - 470	-	[144]
Fish	US	-	-	-	-	0.16 - 0.89	0.068 - 0.904	-	n.d.	-	[129]
Mussel, clam, fish	European Hotspots	n.d. - 9.11	n.d. - <MQL	n.d. - <MQL	n.d.	<MQL - 34.52	<MQL - 12.99	-	-	n.d. - 63.51	[143]
Fish, mussel	Tarragona, Spain	n.d. - 33.53	n.d. - 8.26	n.d. - 12.51	n.d.	2.97 - 18.04	1.17 - 8.42	n.d.	n.d.	n.d. - 17.94	[134]
Oysters	Colombia/Nicaragua	-	-	-	-	42	81	-	-	-	[131]
Fish, mussel	Tarragona, Spain	n.d. - 11.2	n.d.	n.d.	n.d. - 7.9	15.8 - 367.3	n.d. - 31.3	n.d.	n.d.	n.d. - 91.1	[136]
Oysters, fish, mussel	Spain/Portugal/France	-	<MDL - 0.13	<MDL - 0.007	<MDL - 1.45	<MDL - 9.9	0.42 - 6.85	<MDL - 0.01	<MDL - 0.14	-	[135]
Fish	China	-	-	-	-	5.11 - 13.5	1.15 - 4.6	-	2.40 - 10.1	-	[139]
Fish	China	-	-	-	-	19.8 - 43.8	7.35 - 23.7	-	-	-	[140]
Fish	European seas/Atlantic	<MQL - 14.9	<MQL	<MQL - 2.23	<MQL - 39.6	2.5 -414.4	n.d. - 14.1	n.d.	n.d.	n.d. - 228	[133]
Fish	India	-	-	-	-	1-350	2-9	-	8-33	-	[101]
Shrimp	Portugal	-	-	-	-	3.16 - 7.55	n.d. - 2.97	-	-	n.d. - 11.06	[150]
Fish	China	<MQL - 7.65	<MQL	<MQL - 2.23	-	<MQL - 28.9	<MQL- 22.9	-	<MQL	-	[154]
Fish	Portugal	-	-	-	-	n.d. - 143.8	n.d. - 30.5	-	-	-	[137]
Fish, mussel	Portugal	-	-	-	-	n.d. - 306.3	n.d. - 10.9	-	-	-	[138]

1.2.3. Benzenesulfonamides occurrence

Contrary to what was previously stated for synthetic musk fragrances, benzenesulfonamides are still out of the public eye, meaning literature regarding their presence in environmental fates is extremely scarce, let alone the report of their presence in seafood. Even though no methods for their detection nor information about their presence are described for seafood, it is not wild to theorize about their possible apparition in these species given the available information.

Some authors have studied the presence of these compounds mainly in water bodies, with waste-water treatment plants influents and effluents as the main object of study. Back in 2007, Richter et al. [155] started studying the presence of p-TSA, o-TSA and BSA in Berlin's water cycle, reporting the ubiquitous presence of them in wastewater, surface water, groundwater and drinking water samples. Mostly, the presence of p-TSA, o-TSA and BSA was found in the low µg/L levels, being their presence in drinking water the lowest (<0.05 – 0.08 µg/L). The study reported the great efficiency of the WWTP on the removal of p-TSA, with values surrounding the 90% of reduction. Opposite to this effect, o-TSA and BSA concentrations were found higher in the outfalls of the treatment plants, suggesting a possible bioconversion of higher molecular benzensulfonamides to o-TSA and BSA. This theory was later confirmed by the same authors [156], with the analysis of several samples from different treatment plants in Berlin. The depletion of 90% of p-TSA as well as the 4 to 6 times enhancement of BSA was further confirmed, whereas o-TSA seemed to modify its behaviour, suggesting a possible random effect on the concentration of o-TSA caused by the treatments of the plant, being possible an increase, a decrease, or a stable value of the concentration. Further on, Jover et al. [157] used SPME followed by comprehensive two-dimensional gas chromatography (SPME-GCxGC-TOF-MS) to characterize benzothiazoles, benzotriazoles and benzenesulfonamides in aqueous matrixes. Water samples from the Besós River and different Barcelona area WWTPs were analysed, with results showing the presence of Et-p-TSA in all the analysed samples ranging from 85 – 165 ng/L, being the concentrations of the effluent waters higher than the influents, thus suggesting a similar behaviour for Et-p-TSA as for BSA or o-TSA. The highest concentrations were found on samples collected at the effluent of a WWTP receiving mostly industrial wastewater, reaching values up to 2091 ng/L. Herrero et al. [85] corroborated the increase of

BSA after the WWTP treatments along with the presence of p-TSA, o-TSA, Et-p-TSA and Me-p-TSA in influent and effluent samples from different sewage treatment plants (STPs) from Catalonia.

Surface water was also analysed by Speltini et al. [158], reporting levels of 1.0 -1.2 µg/L of BSA and p-TSA in samples from the Ticino River in Italy. In 2019, Xu et al. [159] also reported values between 4.95 – 199.26 ng/L of BSA in samples from different rivers in China. More recently, a suspect and nontarget screening in the nearshore marine environment of Puget Sound in the US conducted by Tian et al. [160] showed the presence of Et-p-TSA in 28% of the analysed samples.

Water samples are not the only available fates where these compounds have been detected. Herrero et al. [161] reported the presence of BSA and TSA in sludge samples from different sewage treatment plants. The concentrations ranged between n.d. – 75 and between n.d. – 83.9 ng/g (d.w.) for BSA and TSA, respectively. Table 3 compiles the concentrations found for different benzenesulfonamides in the abovementioned environmental fates as a summary.

As seen in the literature, the presence of compounds such as p-TSA, o-TSA, BSA, Me-p-TSA, or Et-p-TSA in those environmental fates suggests a possible widespread ubiquity of these compounds throughout the environment. Their release into the environment clearly shows a generalised worldwide use, as studies from different world locations have reported their presence, mainly in water samples. It is also a fact that WWTP's role in benzenesulfonamides releases into the aquatic environment is key, as it is responsible for a possible bioconversion/transformation of higher molecular weight compounds in BSA, TSA or even Et-p-TSA, thus becoming a direct source of emission. It is then clear that most of the fates which conclude in the arrival to the sea or oceans, like rivers or effluents, do contain, even though small, certain amounts of these compounds. As these compounds reach these seas or oceans, the possibility of them being accumulated in the organisms inhabiting them should be at least contemplated. For this purpose, the present thesis aims to answer this concern, developing useful methodologies for their determination in seafood and proving their occurrence.

Table 3. Compilation of the concentration of benzenesulfonamides in different environmental fates (µg L^{-1} for liquids, ng g^{-1} for sludge)

Compound	Influent	Effluent	Drinking water	Surface water	Sludge	Ref
BSA	0.05	0.35	0.05	0.52	-	[155]
	<0.05 - 0.64	0.25 - 0.49	<0.05 - 0.08	<0.05 - 0.42	-	[156]
	<LOQ - 0.06	n.d. - 0.03	-	0.006-0.01	-	[85]
	-	-	-	-	n.d. - 75	[161]
	-	-	-	<MDL - 1.2	-	[158]
		-	-	0.005 - 0.2	-	[159]
p-TSA	5-8	0.5 - 1	0.27	1.15	-	[155]
	2-15	0.15 - 2.34	<0.05 - 0.54	<0.05	-	[156]
	n.d.	n.d.	-	0.26	-	[156]
	0.16 - 0.35	n.d. - 0.43	-	0.03-0.07	-	[85]
	-	-	-	-	n.d. - 83.9	[161]
	-	-	-	<MDL - 1.0	-	[158]
o-TSA	<0.5	0.5 - 3.50	0.09	0.80	-	[155]
	0.11 - 8	0.14 - 4	<0.05 - 0.24	<0.05 - 0.59	-	[156]
	0.05 - 0.09	n.d. - 0.08	-	0.008- 0.02	-	[85]
Et-p-TSA	0.09	0.12	-	0.16	-	[156]
	0.03 - 0.07	0.002-0.07	-	0.002-0.03	-	[85]
					n.d. - 39.8	[161]
Me-p-TSA	<LOQ - 0.004	0.002-0.005	-	n.d.	-	[85]
	-	-	-	-	n.d.	[161]

1.3. Exposure and risk assessment

Seafood consumption has been closely linked to balanced and healthy diets. Several studies have reported the health benefits of many intrinsic components of seafood, with minerals, vitamins or polyunsaturated fatty acids being some of the most relevant [162–165]. As stated before, not only health-beneficial compounds are present, as many pollutants can also bioaccumulate in these organisms, turning frequent seafood consumption into an issue of concern as the intake of these bioaccumulated contaminants could then pose a risk to the population. It is, therefore, necessary to conveniently assess the risk associated with the intake of compounds with known or suspected negative health effects via seafood consumption. To do so, guidelines and toolkits regarding the procedures involved to fulfil this purpose have been reported by worldwide organisations such as the World Health Organization (WHO) [166], the OECD [167] or the EPA [168]. The procedures involved in the characterization and assessment of this risk are commonly known as risk analysis/assessment. The following section briefly synthetizes the principles of risk analysis with an emphasis on its application in the present thesis.

The World Health Organization along with the Food and Agricultural Organization of the United Nations (FAO) described *Risk analysis* as the combination of processes aimed to identify, estimate, implement measures and communicate the risks to human safety and health [169]. Even though the term risk analysis has lost its use in recent times, the process for which it stands for is still very much needed. This process can be divided into three main components: risk assessment, risk management and risk communication, each of these focused on a different area. Although this fact, the three components are perfectly integrated within the discipline. Risk assessment could be described as the scientifically based component, consisting at the same time of the following processes: hazard identification, hazard characterization/dose-response assessment, exposure assessment and risk characterization. Risk management closely works with data obtained from the risk assessment along with the economic, social, ethical and cultural factors to conveniently select the preferred risk management actions. As per risk communication, its relevance lies in the interactive and effective exchange of information between the risk analysis processes and all the interested parties (risk managers, risk assessors, industry, consumers, the academic community, etc.) [166]. Given that the present thesis

belongs to a research-based scientific area, risk assessment will be the focal point.

Risk assessment could be defined as the process to estimate the risk associated with the exposure to a particular agent for a target organism, system, or population. In this process, both the agent to which the exposure is given as well as the exposed organism characteristics are considered. In this case, the agents involved are chemicals, specifically high production volume chemicals. These exposure assessments can be performed for any chemical present in any product or fate (air, soil, food, water, etc.) and can be retrospective (related to past or current exposures) or prospective (potential future exposures). In broad terms, risk depends on the amount of chemical present in the product, the extent of the contact between the person and the product containing the chemical, how the body responds to the chemical, and the chemical's toxicity [166]. The US Environmental Protection Agency [168] and the OECD [167] have released handbooks/toolkits aimed to guide the process of evaluating risk assessment. The guides provide information on the different stages involved in the assessment: hazard identification, dose-response assessment, exposure assessment and risk characterization, which will be briefly explained below and are summarized in Figure 8.

1.3.1. Hazard identification

This first step includes the identification of adverse effects (type and nature) that might result from the exposure to a certain chemical. The quality and weight of evidence sustaining the possible adverse effects of the given chemical should also be characterized in this step to check the possibility of this chemical increasing the incidence of a specific adverse effect or even the likeliness of occurring in humans. To do so, available scientific data is examined when existent. In the case of chemicals with no available data, studies using animals are usually performed to assess the potential hazard to humans of a certain chemical. The use of animals for these studies covers the inability of conducting controlled clinical studies on humans due to ethical concerns, however creating uncertainties related to the differences between the results from animal subjects and humans.

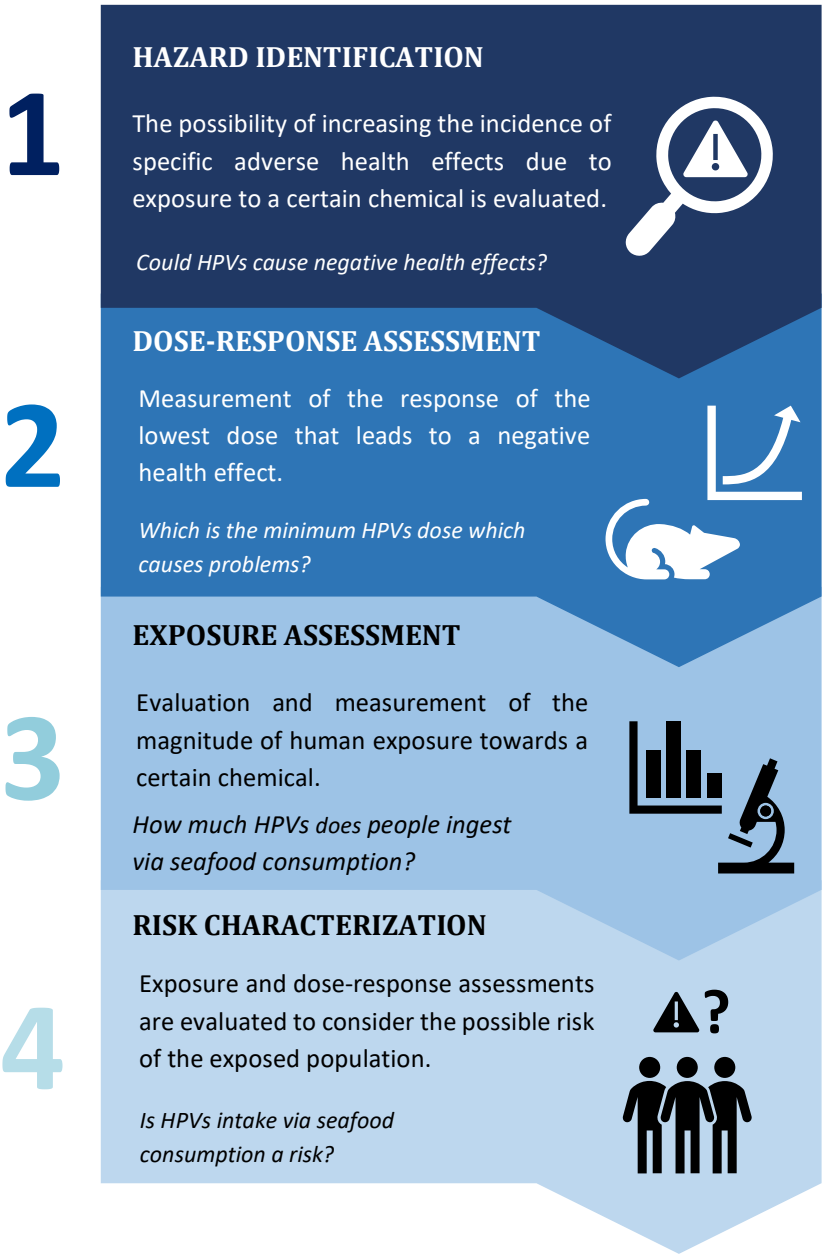

Figure 8. Steps involved in the assessment: hazard identification, dose-response assessment, exposure assessment and risk characterization.

Disciplines such as toxicokinetics or toxicodynamics are usually used for the obtention of data regarding hazard identification. Toxicokinetics focuses on the ability of the human body of absorbing, distributing or even eliminating a certain chemical, while toxicodynamics uses models to describe the possible impact of a chemical on human health [170].

Data on the identification, hazardous properties, and toxicities of a huge number of chemicals are usually compiled in databases. The world health organisation compiled the most relevant and important databases in its last report [166], including the ECHA substance evaluation reports (ECHA REACH database), the International Chemical Safety Cards (ICSCs), or even the previously commented Screening Information Dataset for High Production Volume Chemicals (SIDS), among others. All the listed databases contain resources and detailed information on chemicals identified in scientific investigations or their classification according to international organisations' regulations.

In this sense, most of the high production volume chemicals studied in the present doctoral thesis have been assessed and appear in these databases, usually indicating their scientific properties as well as their hazard classification and labelling. The negative health effects associated with each of these compounds have been previously listed in section 1. 1..

1.3.2. Dose-response assessment

Once the chemicals have been identified and the possible adverse health effects have been assessed, it is necessary to quantify the potential of causing those adverse effects due to exposure. To do so, the most used technique is dose-response assessments, for which a quantitative relationship is searched between exposure (dose) and response, usually performing animal toxicity experiments [171]. Hence, the main objective of dose-response models is to obtain a numeric value to be used along with an exposure value to determine whether a risk exists or not. For instance, a dose could be defined as the amount of chemical, in this case, an HPV chemical, that enters the organism crossing an external exposure route. Generally, as the doses increase, an increase in the response is observed. However, doses and response rates can very much vary based on the type of pollutant, the exposure route or the individual. In the same sense, the response's

shapes are also linked to the kind of adverse effects tested, which has led to limiting the range of adverse effects susceptible to being tested. As stated for hazard identification, dose-response models in humans are also scarce, meaning the use of animals is usually required. However, given the differences between both species, some extrapolations need to be performed to conveniently apply the data obtained from these models.

Once a dose-response curve is established, a point of departure should be selected prior to its extrapolation into a reference value. Points of departure (PODs) are defined as the point in the curve corresponding to an estimated no-effect or low-effect level. There are different PODs to use, being the no-observed-adverse-effect level (NOAEL), the lowest-observed-adverse-effect level (LOAEL) and the statistical benchmark dose lower-confidence limit (BMDL) the most common. NOAEL is described as the highest exposure level at which no significant increases in the severity or frequency of an adverse effect between the control group and the exposed group are observed. In cases where the NOAEL values are not experimentally available, the lowest dose tested (LOAEL) is used. BMDL on its part is the lowest limit of the BMD, which can be defined as the level that corresponds to an increase in the probability of a certain adverse response compared with a background with zero exposure [2]. Figure 9 shows a dose-response curve model with different points of departure.

Once the convenient POD is selected, the value is divided by the corresponding uncertainty factor (UF), which are modifying factors used to address the possible differences between the experimental data and the human situation. These factors take into account the interspecies or intraspecies differences, the duration of the exposure or the quality of the data. The values obtained from the division are used to define the maximum amount of a chemical that can be incorporated into the organism and receive different names depending on the field. Thus, we can find the Acceptable Daily Intake (ADI), used for chemicals susceptible to being ingested and the Tolerable Daily Intake (TDI), more usually used for pesticide residues and food contaminants. US EPA uses a term that includes both previously stated: reference dose (RfD) [172].

Selection of the most convenient POD is a critical step in the risk assessment process. Different values can be found for the same compound, usually regarding

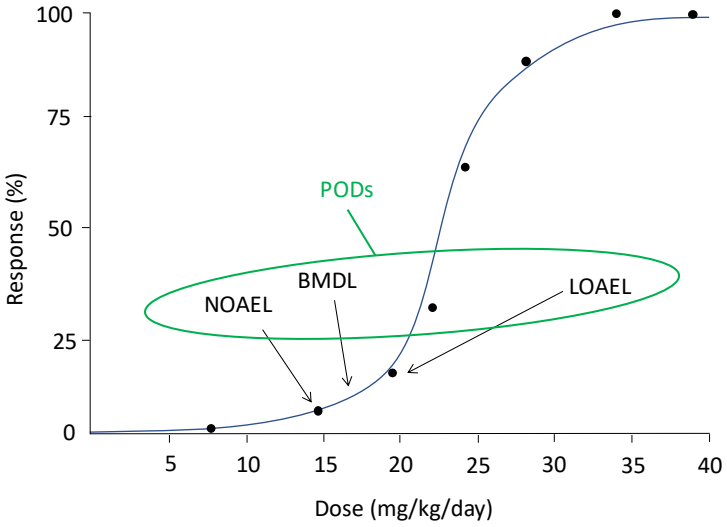

Figure 9. Dose-response curve model.

different adverse effects, doses or experimental designs. Mainly, NOAEL/LOAEL values are set for acute, sub-chronic or chronic effects, and are linked to different exposure routes. Hence, the selected value should be in accordance with the data used for the assessment. In our case, given the fact that the HPV chemicals are present in seafood, values regarding a chronic effect (based on a repeated dose) and exposed through the oral route are used for the assessment. Discussion between the use of BMD and NOAEL is also a matter of concern. Despite being not as widely used, BMD is the preferred value for the EPA and its use is also recommended by the European Food Safety Authority. Its advantages over the NOAEL rely on the non-restriction of BMD to experimental dose levels, and its higher level of confidence since it takes into account the statistical limitations better than NOAEL or the use of all the data from the dose-response curve, among others [173]. All in all, BMD should be used when available or in combination with NOAEL as suggested by the EFSA. However, currently investigated compounds do not have available NOAEL or BMDL values to use. Data on this kind of threshold values is slowly being generated, meaning some of the risk assessments are limited to the available toxicologic data.

1.3.3. Exposure assessment

Exposure assessment could be defined as the step aimed to determine and quantify the possible contact between a chemical and the population. Aspects like the route of exposure, the amount of contact or the media for which the contact was made trough are relevant. For human assessments, these exposure routes are mainly inhalation, ingestion and dermal exposure [166]. For inhalation, compounds should be present in the air, even though it must be pointed out that some compounds could volatilize from other media such as soil, water or consumer products. For ingestion, compounds are required to be present in food, soil, water or particulate matter present in the air. Finally, dermal exposure requires contact between the skin and a chemical, process that could happen with water, soil, consumer products or even air with high concentrations. Figure 10 summarizes the types of human exposure routes based on their exposure media.

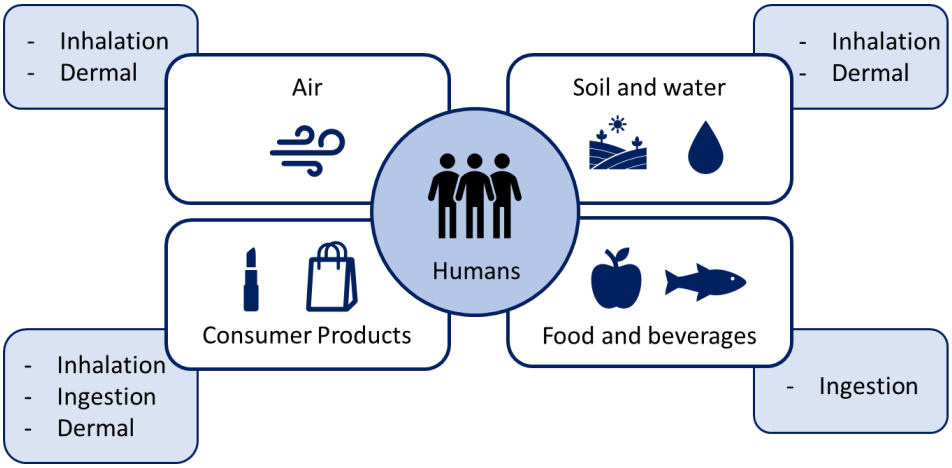

Figure 10. Human exposure routes based on their exposure media.

Data on the exposure is generally given as a concentration or rate. Thus, exposure is expressed as the concentration of a chemical (in this case HPV) in the exposure medium (seafood) over a specific duration. In the case of the present

thesis, the exposure values are obtained by applying the following equation (Eq. 1):

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

The dietary exposure (E_t) is the result of the sum obtained by multiplying the concentration of a compound *t* found in a fish species *f* ($X_{t,f}$, ng g^{-1}) and the mean consumption of the individual seafood species *f* (C_f, g kg bw day^{-1}). Therefore, data on the concentration of each of the studied compounds in each of the analysed species, as well as data on the consumption rate of the same seafood species is needed to perform the assessment.

For instance, the present thesis has relied on the use of available data on the consumption of seafood at regional (Catalonia) and national (Spain) levels. Data on the most consumed seafood species from Catalonia was obtained from the *Enquesta de l'Estat Nutricional de Catalunya* (ENCAT 2003) survey [174], for which data was segregated by age and gender, thus providing information about dietary intake (g/day) of boys and girls (10-19), adult men and women (20-65) and senior men and women (>65). On the other hand, data on the consumption of certain fish species was also acquired from the *Infome del Consumo Alimentario 2021* [175]. In this case, data regarding the seafood intake was segregated into months and geographical zones, other demographical criteria (population size, social class, number of individuals per home, presence of kids, etc.) and acquisition channels.

The chemical concentrations have been obtained from self-made chromatographic methods for the determination of HPVs in seafood samples. Regarding the non-quantified compounds approach, values for compounds found under the methods limit of detection (LOD) and quantification (LOQ) were treated by the substitution method as suggested in the EFSA report on *Management of left-censored data in dietary exposure assessment* [176] and the WHO *Principles and Methods for the Risk Assessment of Chemicals in Food* [172] for which three different scenarios are given: lower-bound scenario (LB), middle-bound scenario (MB) and upper-bound scenario (UB). For the LB, non-detected and under LOQ compounds are assigned a concentration of zero or LOD value, respectively. In the case of the MB, one-half of the LOD or LOQ concentrations are assumed for

non-detected or under the LOQ compounds, whereas for the UB, concentrations equal to the LOD and LOQ are assumed.

1.3.4. Risk characterization

This last step of the risk assessment process could be described as the quantitative comparison between the data obtained in the dose-response assessment and the exposure assessment.

Different approaches can be performed for the characterisation of this risk. The first approach is intended to characterise chemicals with potential non-cancer effects. In this case, the ratio between the exposure rate/concentration and the health-based value obtained from dose-response extrapolated values (ADI, TDI, RfD) is calculated. Equation 2 can be used for this purpose.

$$R_t = (E_t / ADI_t)*100 \qquad \text{Eq. 2}$$

In this case, the risk factor (R_t) for a compound *t* is the ratio between the global dietary intake (E_t) and the acceptable daily intake (ADI_t). Ratios lower than 1 indicate that the risk associated with the intake of the specific compound is less than the reference concentration, thus indicating the possibility of resulting in an adverse effect is unlikely. On the contrary, ratios greater than 1 mean the reference values are exceeded, and further evaluation is needed [166].

Another approach usually destinated to the evaluation of chemicals with genotoxic and cancer effects is the Margin of Exposure (MOE). This unitless value provides data on how close the obtained exposure is to the values anticipated to cause a measurable effect. This approach is mostly based on the application of BMD values, for which the ratio is calculated following Equation 3.

$$MOE_t = BMD_t / E_t \qquad \text{Eq. 3}$$

The margin of exposure to a chemical *t* (MOE_t), is described as the ratio between the BMD and the global dietary intake (E_t). In this sense, obtained values lower than 10,000 are associated with a low level of concern [177].

Even though the current tendency in occurrence studies is to include a dietary intake risk assessment, risk characterization may be missing due to the lack of data available in the literature regarding the guideline's threshold values. Thus, experimentation and studies focused on the production of data on this topic are heavily encouraged and represent a future challenge.

1.4. References

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CHAPTER 2. OBJECTIVES

The present doctoral thesis aims to provide useful analytical methodologies to determine the concentrations of several high production volume chemicals in the most consumed seafood and further evaluate their impact on population health caused by dietary intake. Compound families such as musk fragrances, organophosphate esters, phthalate esters, organophosphate esters, benzothiazoles, benzotriazoles and benzenesulfonamides were evaluated due to its wide use in daily commodities as well as their industrial usage. Different goals have been set to reach the main objective, being them summarized as follows:

- Development of gas chromatography coupled to mass spectrometry based methods involving extraction techniques such as solid phase microextraction Arrow (SPME Arrow) and QuEChERS. Special attention on seafood matrix effect evaluation and correction.
- Monitorization of the presence of these compounds in the most consumed seafood species from Tarragona, Spain.
- Comparison of the risk associated to the intake of high production volume chemicals via seafood consumption from Catalonia and the Canary Islands.
- Exposure and risk assessment of compounds with available toxicological information.
- Bioaccessibility of relevant high production volume chemicals in raw and cooked fish samples using *in vitro* human digestion.

CHAPTER 3. EXPERIMENTAL PART, RESULTS AND DISCUSSION

As previously discussed in the introduction, the widespread ubiquity of high production volume chemicals throughout the environment is the result of the massive production of chemicals over the last decades. Fates such as air, dust, water and even biota are susceptible to becoming contaminated with these compounds, thus leading to a potential risk not only for the environment but for mankind. Compound families included in the HPVs such as musk fragrances, PAEs, OPEs, BTs, BTRs and BSAs have been determined in numerous matrices, including seafood, which as part of the diet, becomes an alternative exposure route for the population. Even though the presence in seafood of some of these compounds like musk fragrances or PAEs has been studied in recent times, other novel families such as OPEs have been recently put on the spotlight, while BTs, BTRs and BSAs are still quite unknown. Methods involving the determination of HPVs for matrices such as seafood are scarce for most families, thus disabling the possibility of evaluating the status of the environment as well as the health risk to the population. Hence, the present doctoral thesis is focused on the development of novel chromatographic methods for the determination of HPVs in seafood aimed to become useful tools to evaluate the exposure and risk for the population associated with the dietary intake of these compounds.

The following chapter describes the experimental part, results and discussion of the studies developed in the course of the present doctoral thesis. The sections have been arranged according to the aforementioned objectives: the development of new chromatographic methods for the determination of HPVs in seafood samples (section 3.1), the monitoring of these HPVs in commercially available seafood samples (section 3.2) and the preliminary study of the effects of cooking and the bioaccessibility of some HPVs present in seafood (section 3.3). Each section includes a brief introduction of the problematic as well as a following discussion of the most relevant findings. The results presented in the sections have been or are in the process of being published in international peer-reviewed journals. A list of the articles derived from the studies carried out during the doctoral thesis can be found in Appendix II.

This doctoral thesis and its experimental part have been carried out within the Chromatography, Environmental Applications research group (“Cromatografia, Aplicacions Mediambientals”, CROMA) at Universitat Rovira i Virgili (URV) from Tarragona, Spain. The study of the bioaccessibility of HPVs present in seafood has

been conducted during the PhD stay at the Division of Aquaculture and Upgrading (DivAV) from the Portuguese Institute for Sea and Atmosphere ("Instituto Português do Mar e da Atmosfera", IPMA). The research reported in the present doctoral thesis has been financially supported by the Ministerio de Economía y Competitividad, the Agencia Estatal de Investigación (AEI), the European Regional Development Fund (ERDF) (CTQ2017-84373- R), the Ministerio de Ciencia e Innovación (PID2020-114587GB-I00) and the Universitat Rovira i Virgili (2018-PMF-PIPF- 15).

3.1. Determination of high production volume chemicals in seafood

High production volume chemicals comprise several compound families with a wide variety of physical and chemical properties. From highly volatile apolar compounds such as synthetic fragrances to mid-polar compounds like benzotriazoles. Hence, methods involving a wide selectivity extraction are usually needed to conveniently determine the maximum compound families in a single extraction. Seafood can be considered a high-complexity matrix due to the high amount of co-extractive compounds found when performing extractions. It is for that reason that determinations in this matrix can be tedious and complicated, as the clean-up of the extracts must be exhaustive in order to achieve reliable values. Thus, the objective is to develop methods that selectively determine the target HPVs, at the same time that clean the extract enough to avoid or at least minimise the matrix effects and the effects on the instrument's efficiency.

All the methods developed in the conducted studies employ gas chromatography coupled with tandem mass spectrometry for the determination of the target compounds. The use of GC as the preferred determination instrument can be linked to different factors. On one hand, volatile compounds like synthetic fragrances and some phthalate esters are easier to determine using GC due to their intrinsic properties. However, low signal issues can become a problem for benzothiazoles, benzotriazoles, benzenesulfonamides and some organophosphate esters due to their lower volatility and their interaction with the system. Strategies such as sample preconcentration or the increase of injection volume can partially solve this problem. On the other hand, seafood complex matrix and its presence in the final extract could lead to what is called "matrix-effect". This effect, typically linked to the use of liquid chromatography-mass spectrometry ionisation sources like ESI or APCI, is described as the enhancement or suppression of the signal due to the competition between the mobile phase/interferences and the analyte of becoming charged during the ionisation process. In GC, the effect is minimised as the carrier gas is not susceptible to being charged, and the preferred ionisation, electron ionisation, is considered a high fragmentation source. However, other mechanisms act as enhancement and suppression causes for GC. The adsorption of matrix components in free active sites of the inlet liner or column leads to a differentiation between clean and matrix-containing extracts. Even though the matrix effect is also found in GC, the effects can be considered minimal when

compared with the effects produced for LC-MS. Strategies such as liquid-liquid extraction, dispersive solid-liquid extraction or the use of novel selective clean-up devices are typically used to reduce the matrix-effect and perform more reliable determinations. Regarding the use of mass spectrometry, the low concentrations in which these compounds are usually found in seafood require the use of instruments with high sensitivity, selectivity and identification power, being tandem mass spectrometry the best option, either using ion trap or triple quadrupole analysers.

Previous studies performed in the research group have successfully determined musk fragrances, benzothiazoles and other high production volume chemicals in seafood samples using extraction techniques like SPME [1], QuEChERS [2,3] or PLE [1,3]. However, new methodologies have been developed in the present thesis to go further in terms of greener alternatives, lower detection limits or the introduction of new target compounds.

SPME Arrow appeared as an enhanced combination of stir-bar sorptive extraction (SBSE) and conventional SPME, with a substantial increase of sorbent and a more robust device with a metallic tip resembling an arrow (thus the adopted name). Thanks to a collaboration with CTC Analytics in Switzerland, we were able to test the potential of this novel device. To do so, several modifications of the autosampler (CombiPAL from CTC Analytics) and the GC-MS/MS system (Varian 4000 Ion trap) were needed. Regarding the autosampler, an additional conditioning module was acquired, aimed to clean and condition the SPME Arrow fibers prior to their use. Moreover, the main module (conventional fiber holder) was changed to accommodate the new SPME Arrow fiber holder. As for the GC-MS/MS system itself, the injector port had to be modified to fit the wider diameter of the novel fiber. Given the previous experience of the group on the determination of synthetic musk fragrances and the ongoing focus on seafood analysis, these were selected as perfect candidates to check the efficiency of the SPME Arrow. Thus, we aimed to develop a method for the determination of synthetic musk fragrances in seafood samples using SPME Arrow. The combination of conventional SPME with the higher coating of SBSE in a single device promised a substantial increase in the available adsorption phase, thus increasing the amount of analyte being extracted and therefore so, sensitivity. The study describes the optimisation of the procedure in terms of coating

selection, extraction time, extraction temperature and the effect of water addition. The developed method was then compared with conventional SPME and further applied to successfully determinate eight synthetic fragrances in samples of cod, hake and sole.

Even though the results obtained from the SPME Arrow study were promising, the acquisition of an Agilent 8890 GC system coupled with an Agilent 7000D triple quadrupole mass spectrometer and a PAL RSI 120 autosampler by the group shifted the initial plans of pursuing the development of SPME Arrow based methods. The newly acquired system was not adapted to the use of the novel device, thus making it difficult to couple the SPME Arrow to the triple quadrupole analyser.

The new GC-MS/MS system opened the door to the determination of lower concentrations of chemicals due to the enhanced sensitivity offered by the triple quadrupole analyser. The higher sensitivity and selectivity of the system were clearly an advantage for the determination of other high production volume chemicals in complex matrices such as seafood. As we faced the impossibility of using SPME Arrow, it was decided to use another well-known extraction technique: QuEChERS. Its easy and effective use was already assured by previous studies in the group, thus providing a good starting point to further use the technique on the extraction of compounds like OPEs, BTs, BTRs or BSAs, among others.

Once the newly arrived system was installed and QuEChERS was selected as the preferred extraction technique, the development of the new methodologies began. One of the most problematic concerns when extracting seafood samples is the possibility of the matrix causing signal enhancement or suppression. Most of the reported methods to that date required extensive clean-ups to cover that issue, thus complicating the overall extraction procedure. Hence, our goal was to focus on that and test easier and novel strategies to reduce the matrix effect. Starting with OPEs and later BTs, BTRs and BSAs, two methods for their determination were developed based on a method previously described by Trabalón et al. [2]. Parameters such as the type of QuEChERS salts used or the amount of sample were tested. Several strategies including liquid-liquid extraction with hexane, dispersive solid phase extraction with different sorbents

(PSA, C18, charcoal, activated carbon, etc.) and a novel selective lipid removal push-through device, LipiFiltr, were used as clean-up. Analytical issues regarding low instrumental signal were also addressed, especially for benzothiazoles, benzotriazoles and benzenesulfonamides, suggesting injection volumes up to 25 µL as a solution to solve the problem. These volumes were achieved thanks to the solvent vent mode of the injector of the newly installed system (multi-mode injector). The application of both methods to real samples displayed the presence of some of the target HPVs at low concentrations. Moreover, exposure and risk calculations were performed to elucidate the possible negative impact on the population that the ingestion of these compounds may cause due to dietary intake.

All the developed methods were tested, and the quality parameters were evaluated in terms of apparent recoveries (%R_{app}), method limits of detection (MDL), method limits of quantification (MQL), linear range, R^2, repeatability (intra-day, %RSD) and reproducibility (inter-day, %RSD).

The articles derived from the studies of the following sections have been published in Journal of Chromatography A (sections 3.1.1. and 3.1.2.) or are currently submitted to the same journal (section 3.1.3.).

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3.1.1. Solid phase microextraction Arrow for the determination of synthetic musk fragrances in fish samples

SOLID PHASE MICROEXTRACTION ARROW FOR THE DETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN FISH SAMPLES

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Abstract

A novel solid phase microextraction Arrow (SPME Arrow) system has been applied for the first time to determine synthetic musk fragrances in fish samples. The lack of regulation concerning the concentration of musk fragrances in fish along with the risk associated to these compounds has led to an increased development of analytical methods on this topic. This study applies SPME Arrow followed by gas chromatography coupled to tandem mass spectrometry (ion trap) to determine nine musk fragrances and compares this novel technique with its predecessor (SPME). Parameters such as type of coating, extraction time and temperature as well as water addition were optimized to achieve higher sensitivity. Results show that detection limits ranging between 0.5 ng g^{-1} (for cashmeran, celestolide, phantolide, tonalide and musk ketone) and 2.5 ng g^{-1} (dry weight) (for musk xylene) when SPME Arrow is used instead of a conventional fibre, with an up to ten-fold increase in sensitivity. Moreover, commercial fish samples were analysed using the method developed and galaxolide and tonalide were quantified at concentrations ranging from 6.5 ng g^{-1} to 17.5 ng g^{-1} (d.w.) and 2.9 ng g^{-1} and 5.1 ng g^{-1} (d.w.) in all the species analysed.

Highlights

- A novel SPME Arrow system was successfully applied for fragrances determination.
- SPME Arrow-GC–MS/MS presents sensitivity 10x higher than conventional SPME.
- Low ng g^{-1} of HHCB and AHTN were determined in commercial fish samples.

1. Introduction

In recent years, a considerable number of microorganic contaminants has been found worldwide. Among them, there is a group known as "contaminants of emerging concern" (CECs). These are natural or synthetic substances, which have not necessarily been discovered only recently, that are not generally monitored but are believed to have an undesirable impact on both ecosystems and society [1–3]. Of these CECs, personal care products (PCPs) are of particular concern because of their toxic effects on aquatic biota. They enter the environment as the result of the widespread use of daily products such as lotions, toothpaste, cosmetics or food that contain high quantities of, for example, disinfectants (triclosan and triclocarban), synthetic musk fragrances (nitro musks and poly-cyclic musks), UV filters (UV-9, UV-234, UV-320, etc.), preservatives (parabens) and stabilizers (benzotriazoles) [4]. This study focuses on the determination of musk fragrances as they are present in everyday products. Synthetic musk fragrances include synthetic man-made chemicals produced in large amounts and mostly used in household products, air fresheners, perfumes, cosmetics, and personal care products. These are responsible for the enhanced and persistent scent of the products and were first created to replace natural musks obtained from natural sources in order to reduce the cost of production. The widespread use of musk fragrances in everyday life products increases the concentration of these compounds in household wastewater, which makes house- hold effluents one of the major sources of contamination. These effluents head for the wastewater treatment plants (WWTP) where they are treated. Studies such as the ones carried out by Homem et al. [5] or Vallecillos et al. [6] show that WWTP effluents still contain concentrations of musk fragrances at ng L^{-1} levels, which shows that these plants are not very effective at eliminating fragrances. Hence, treated WWTP effluent water still contains musk fragrances and contaminates the aquatic environments into which they are introduced. Once musk fragrances reach the aquatic environment, they are liable to become part of the food chain of aquatic species, with fish being the most affected. Studies have shown the bio-accumulation of musk fragrances in a variety of fish species and mussels. Fussell et al. [7] demonstrated the presence of cashmeran, celestolide, galaxolide and tonalide in fish at low ng g^{-1}. Vallecillos et al. [8] also demonstrated the presence of musk fragrances in both river and sea fish as well as in mussel. Therefore, even though cutaneous exposure is the main source of exposure to musk fragrances, fish intake is an alternative, albeit lesser, source.

Determination of musk fragrances in fish samples as well as population risk assessment has become an issue of the greatest importance because of the lack of European regulations on the maximum levels permitted. Even though their effects on the population have yet to be confirmed, as well as their toxicity, musk fragrances are believed to act as endocrine disruptors [9].

Methodologies for determining musk fragrances in fish samples differ mainly in the extraction technique used. To date, the methods that have been reported include Soxhlet extraction [10], solid liquid extraction (SLE) [11], solid phase microextraction (SPME) [12], microwave assisted extraction (MAE) [13], focused- ultrasound solid liquid extraction (FUSLE) [13], pressurized liquid extraction (PLE) [14] and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure [15]. The main drawbacks of some of those techniques is that they require high amounts of organic solvent (Soxhlet) or specific instrumentation (MAE, FUSLE and PLE). However, techniques such as SPME require no specific instrumentation other than the fibres. New procedures such as QuEChERS are cheaper and use less solvent with similar results even though handling times are longer. As for the separation and detection, gas chromatography coupled to mass spectrometry (GC–MS) is the most suitable technique for determining musk fragrances because they are highly volatile even though methods using liquid chromatography (LC) or capillary electrophoresis (CE) have been developed [16–18].

Recently, a new SPME-based device has been developed: SPME Arrow. It combines the large sorption phase volumes used in stir bar sorptive extraction (SBSE) with the main advantages of conventional SPME. The device consists of a steel rod coated with a sorbent material protected by an outer tube, which together with the arrow shaped tip forms the needle. Classical SPME coatings are commercially available for SPME Arrow. Even though larger volumes of sorption phase are used, and the diameter of the fibre is increased, a specific thermal desorption port is not needed as conventional injection ports can be adapted to fit the SPME Arrow, which enables full automation of the process unlike SBSE. The features commented above suggest that SPME Arrow enhances sensitivity because the sorption phases are larger and the device, and hence the whole technique, is more robust. As this novel technique has appeared only recently, only a few methods have been reported in the literature that demonstrate that it is suitable for analysing different kinds of compound and samples such as amines in wastewater [19], organic compounds

and polycyclic aromatic hydrocarbons (PAHs) in water [20,21] and biogenic volatile organic compounds in the atmosphere [22].

To the best of our knowledge, this is the first study to focus on proving the suitability of SPME Arrow for the determination of musk fragrances in fish samples. Moreover, conventional SPME fibres are compared with those studied in order to prove their performance.

2. Experimental part

2.1. Materials and reagents

The studied polycyclic musk fragrances were purchased at Promochem Iberia (Barcelona, Spain): 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5 H)-indanone (cashmeran, DPMI), 4-acetyl-6-tert-butyl-1,1dimethylindane (celestolide, ADBI), 6-acetyl-1,1,2,3,3,5-hexamethylindane (phantolide, AHMI), 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (traseolide, ATTI) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (tonalide, AHTN). The polycyclic musk 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8 hexamethylcyclopenta-(g)-2-benzopyran (galaxolide, HHCB) was supplied by Sigma-Aldrich (St. Louis, USA). The nitro musk fragrance 2,4,6-trinitro-1,3-dimethyl-5-tert butylbenzene (musk xylene, MX) was purchased as a 100 µg mL^{-1} individual solution in acetonitrile from Sigma- Aldrich (St. Louis, USA) and the remaining nitro musk fragrance 4-aceto-3,5-dimethyl-2,6-dinitro tertbutylbenzene (musk ketone, MK) was provided by Fluka (Buchs, Switzerland). The compound 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2 benzopyran-1-one (galaxolidone, HHCB-lactone) was supplied by International Flavours & Fragrances Inc. (Barcelona, Spain).

Individual stock solutions of the solid compounds were pre- pared in acetone at concentration of 4000 mg L^{-1} for all the compounds except for AHMI (100 mg L^{-1}) and for HHCB-lactone (1000 mg L^{-1}). A working mixture solution of 1 mg L^{-1} was prepared in ethyl acetate, containing all the compounds except for HHCB-lactone. An individual working solution was prepared in ethyl acetate for HHCB-lactone at the same concentration (1 mg L^{-1}). Acetone and ethyl acetate were GC grade with purity >99.9% from J.T. Baker (Deventer, The Netherlands). Helium gas with a purity of 99.999% for chromatographic analysis was purchased at Carburos Metálicos (Tarragona, Spain).

Three SPME Arrows were supplied by CTC Analytics AG (Zwingen, Switzerland). Divinylbenzene/Polydimethylsiloxane (DVB/PDMS) arrow had a 120 µm sorbent film thickness and a film length of 20 mm. The other two: Polydimethylsiloxane (PDMS) and Poly-

acrylate (PA) arrows both were 100 µm and 20 mm. An additional SPME conventional fibre of PDMS of 100 µm and 10 mm was purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Gas chromatography - mass spectrometry

The chromatographic analysis was performed using a Varian 4000 GC–MS/MS with Ion trap system from Varian (Walnut Creek, USA). The system consisted of a 3800 gas chromatograph coupled to a 4000 ion trap mass spectrometer and a 1079 PTV (Programmable Temperature Vaporization) injector. The system also incorporates a CombiPal auto sampler from CTC Analytics (Zwingen, Switzerland) containing a SPME Arrow holder for automatic extraction, a PAL Stirrer module, and a PAL SPME Arrow conditioning module.

Chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl-50% dimethylpolysiloxane, 30 m 0.25 mm i.d.; 0.25 µm film thickness) provided by Phenomenex (Torrance, USA). The GC–MS conditions were adapted from Trabalón et al. [23]. The carrier gas used was helium at a constant flow rate of 1 mL min^{-1}. Most of the compounds (except for AHTN and HHCB which had different ions) were chromatographically separated in 20 min using a temperature program of the oven as follows: 70 °C hold for 3.5 min, raised to 200 °C at 50 °C min^{-1}, then to 240 °C at 5 °C min^{-1} and finally to 290 °C at 20 °C min^{-1} and held for 3.4 min. The mass spectrometer operated in electron ionization (EI) mode (70 eV). The trap, manifold and transfer line temperatures were 200 °C, 50 °C and 280 °C, respectively. Tandem mass spectrometry mode (MS/MS) was used to quantify the compounds. Retention times and MS optimized parameters for all the compounds are summarized in Table S1.

2.3. Sample treatment and extraction

Seafood species such as codfish (Gadus morhua), sole (Solea, solea) and hake (Merluccius merluccius) were purchased at local commercial establishments (local market, supermarket and fish market). Samples were stored in a refrigerator before their analysis. Fish samples were dissected, and the lateral fillets were then homogenised and stored in a freezer. Frozen homogenised samples were lyophilized using the miVac Duo with Speed Trap freeze-drying system from Genevac (Ipswich, United Kingdom) and then ground using a coffee grinder from Moulinex (Alencon, France). Samples were also sieved through a 500 µm mesh in order to homogenise particle diameter. Spiked samples were prepared by adding the stock mixture of standards to freeze-dried fish samples

covered with acetone. 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 overnight.

A portion of 0.25 g of lyophilized fish sample was weighed in a 20 mL headspace SPME glass vial and placed in a tray. Prior to extraction, PDMS SPME Arrow was conditioned at 100 °C for 15 min in the conditioning port. Once the heat/stir module reached 100 °C, the vial was automatically transported to the module and equilibrated for 1 min. Afterwards, the arrow was exposed for 45 min in the vial headspace. During the extraction, the sample was magnetically stirred at 750 rpm. After 45 min, the desorption of the arrow was conducted in the injection port at 250 °C for 3 min. To prevent carry-over the SPME Arrow was baked for 15 min at 250 °C at the conditioning port after every extraction.

For the conventional SPME fibre the same procedure was performed with different extraction temperatures and times (80 °C (Fig. S1) and 60 min, respectively).

3. Results and discussion

In order to set up the optimal extraction conditions, various extraction experiments were performed using SPME Arrow in headspace mode (HS). The experiments were carried out using 0.25 g of lyophilized cod (*Gadus morhua*) spiked at a concentration of 1 mg kg^{-1} dry weight (d.w.) of all the musk fragrances except for HHCB-lactone as it is a degradation product of HHCB. The presence of both HHCB and HHCB-lactone could make results difficult to understand if they are both spiked together. Therefore, HHCB- lactone extraction was not optimized and was included in the method later. Cod fish was chosen for the extraction optimization as it has a lower lipid content, which interferes less in the analysis. Blank samples were analysed in order to subtract the blank signal of the compounds present in the fish.

The parameters that can be optimised for SPME Arrow are the same as for conventional SPME: type of coating, extraction time and temperature, and other parameters such as water addition or desorption temperature and time. They were optimised using a univariate approach for which an initial parameter was set and the rest were optimised one by one. The optimal values obtained are discussed and described in detail in the sections below.

3.1. Sorbent comparison

Three SPME Arrow sorbents were compared for their ability to extract musk fragrances from the fish samples.

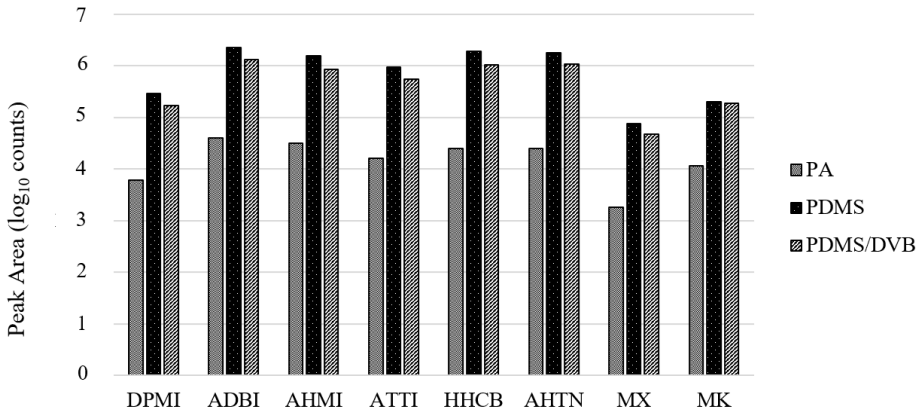

Fig. 1. Comparison of different SPME Arrow coatings for the extraction of musk fragrances from cod samples. 0.25 g of sample spiked at 1 mg kg^{-1} with the compounds studied. n = 3, RSD < 11%. The results are represented in logarithmic scale.

Two absorbent type coatings (polyacrylate, PA and polydimethylsiloxane, PDMS) and one adsorbent type (divinylbenzene-polydimethylsiloxane desorption conditions were the same for all the SPME Arrows. Portions of 0.25 g of cod were extracted at 80 °C for 45 min while stirred at 750 rpm and desorbed at 250 °C for 3 min. Peak area values were considered to select which type of sorbent was the best (Fig. 1).

The results show that PDMS and DVB/PDMS coatings were the best for the extraction of musk fragrances while PA was fairly below. This agrees with the fact that PDMS and DVB/PDMS coatings are non-polar phases designed to extract compounds with low polarity and mid-high volatilities. On the other hand, PA coatings have a moderate polarity and are preferred for mid-high polar compounds. Since musk fragrances are volatile and nonpolar com- pounds, PDMS and DVB/PDMS could be the most suitable coatings. PDMS had slightly better results than DVB/PDMS so it was chosen as the preferred coating for the SPME Arrow extraction.

3.2. Extraction conditions

The section below describes the optimization of the most significant

parameters related to the extraction procedure itself: extraction time and temperature and water addition. The sample amount was also tested using weights of 0.25, 0.5 and 1 g of lyophilised fish but no differences were observed. Therefore, sample weight was kept at the lowest value (0.25 g) to reduce the possible effect of the matrix and the consumption of the sample.

3.2.1. Extraction temperature

Increasing the extraction temperature may affect the extraction efficiency. Thus, extraction temperatures ranging from 60 to 120 °C were tested. All experiments were carried out with the same extraction time (45 min) and desorption conditions (250 °C for 3 min). The results are represented in Fig. 2A.

Increasing the temperature affects SPME collection as it increases the partitioning of compounds into the headspace of the vial. However, at the same time the partitioning into the sorbent can decrease. As observed in the figure, all the compounds were affected by the temperature changes, mostly enhancing the extraction efficiency while increasing the temperature. For DPMI, ADBI and AHMI the effect is the opposite. These three compounds are the most volatile of the musk fragrances studied, so they are more likely to decrease the partitioning into the sorbent as the temperature increases, which could be the reason why efficiency decreases with temperature.

Even though the maximum extraction efficiency of these three compounds is at 80 °C, the other compounds show better results when heated to 100 °C. Therefore, the selected temperature for the extraction was 100 °C.

3.2.2. Extraction time

After selecting the optimal temperature for the extraction (100 °C) experiments with different extraction times ranged between 20 and 80 min were performed to construct the extraction time profiles. The desorption conditions were the same as the ones used for the temperature optimization, 250 °C for 3 min. The extraction time profiles obtained are represented in Fig. 2B. All the compounds reached their maximum extraction efficiency at 45 min except for the most volatile compound, DPMI, the optimal extraction time of which was between30 and 40 min. Consequently, extraction time was set at 45 min.

3.2.3. Water addition

The addition of small volumes of water was tested to check if extraction efficiency was enhanced as previous

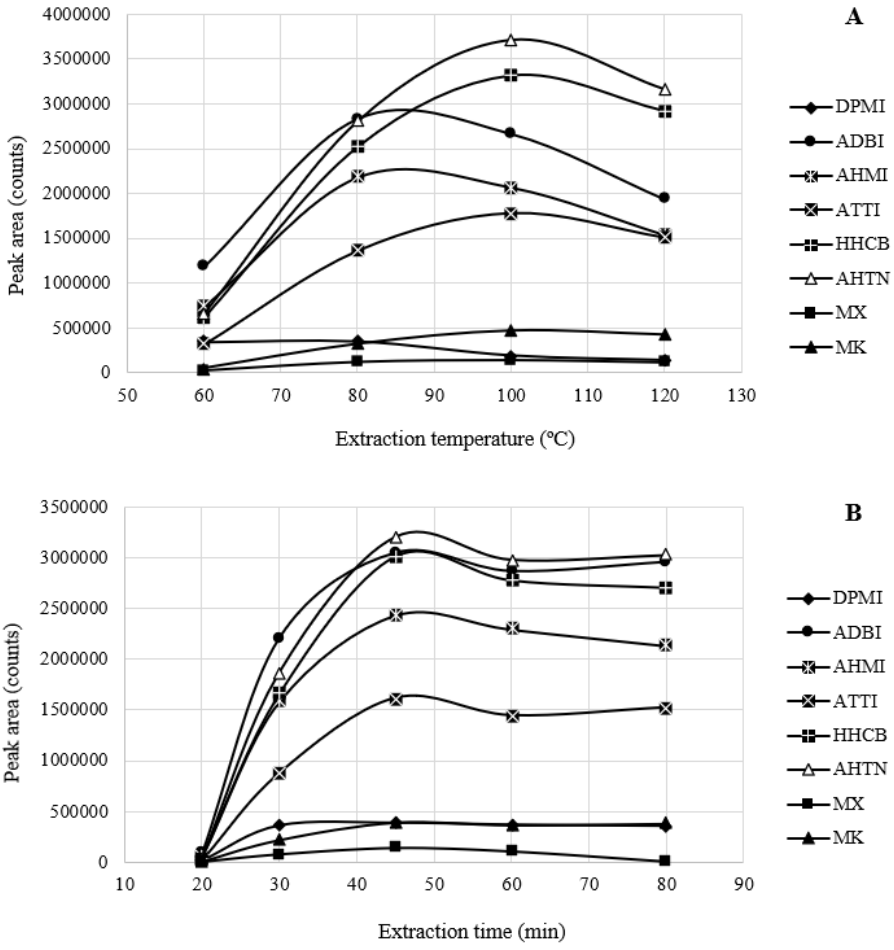

Fig. 2. The effect of extraction temperature (A) and time (B) on the extraction efficiency using PDMS SPME Arrow. Extraction conditions: 0.25 g of sample spiked at 1 mg kg^{-1}.

reported methods demonstrated this behaviour (Vallecillos et al. [24]). Water volumes of 0.5 and 1 mL of ultrapure water were added to the SPME vial. Experiments were performed under optimised extraction conditions: 100 °C for 45 min while stirred at 750 rpm and desorbed at 250 °C for 3 min.

Results show that the addition of water reduces the extraction efficiency. As the volume of water added to the vial increases, the extraction efficiency decreases. This may be related to the humidity formed during the extraction. The sealed vial along with the high temperatures used for the extraction cause water to vaporize, which increases the humidity formed in the headspace of the vial during the extraction process. This water vapour could be condensed and trapped onto the sorbent, thus reducing the extraction efficiency of the SPME Arrow. Authors such as Helin et al. [19] and Feijó et al. [22] also reported a decrease in extraction efficiency when the humidity increased using SPME Arrows. Therefore, no water was added to the vial for the extraction.

3.3. Desorption conditions

Desorption conditions were tested but no improvement was observed when the temperature was changed (250 °C, 300 °C) or the desorption time increased (tested range between 1 and 5 min). Thus, initial conditions (250 °C for 3 min) were selected, and no carryover was observed.

3.4. Method characterization

3.4.1. Comparison with conventional SPME

The coating sorbent used for conventional SPME was the same as the one for SPME Arrow so that they could be compared. Thus, a 100 µm PDMS conventional fibre was selected and its main parameters were optimised (extraction temperature and extraction time) by using cod samples. The initial extraction conditions for the conventional fibre were the ones selected for the SPME Arrow. Then, further experiments were carried out in order to find the optimal temperature of the extraction. Extraction temperatures ranging from 40 to 120 °C were tested. Triplicates of the experiments were carried out with the same extraction time (45 min) and desorption conditions (250 °C for 3 min). The results obtained are shown in Supplementary Material (Fig. S1). They show that, with the exception of DPMI, the most volatile compound, the optimal extraction temperature was 80 °C. The fact that DPMI showed a lower optimal temperature, 60 °C, could be explained for the same reasons stated in the discussion of temperature extraction for SPME Arrow.

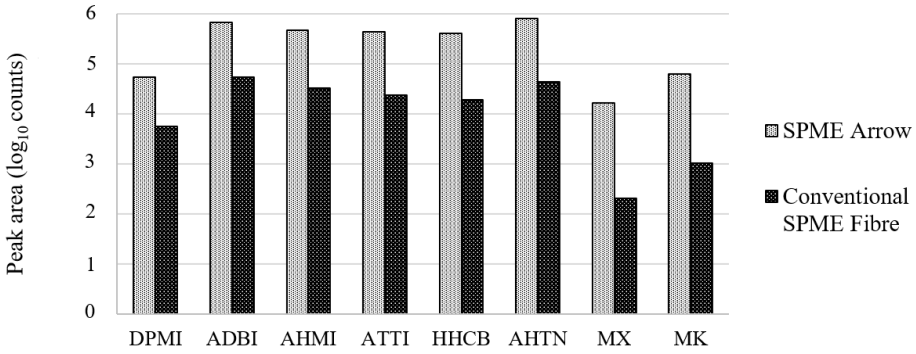

Fig. 3. Comparison of the use of a conventional PDMS SPME Fibre and a PDMS SPME Arrow for the extraction of musk fragrances from cod samples. Samples were spiked at 1 mg kg^{-1} with the compounds studied. n = 3, RSD < 23%.

Once the optimal temperature had been selected, experiments with extraction times between 10 and 80 min were performed to construct the extraction time profiles of the compounds for the conventional fibre. The time ranges tested were lower than the SPME Arrow ones as conventional fibres take less time to reach equilibrium [22]. The results obtained are shown in Supplementary Material (Fig. S2). The extraction time profiles obtained for the com- pounds showed that the extraction efficiency is highest at 60 min, when most of the compounds reach equilibrium. DPMI reached equilibrium after 30 min, before all the other compounds because of its high volatility. Therefore, 60 min was chosen as the optimal extraction time. Samples of cod (0.25 g) spiked at the same concentration (1 mg kg^{-1}) were analysed by both techniques in optimal conditions. The results obtained are represented in logarithmic scale in Fig. 3. As seen in the figure, for most of the compounds the extraction efficiencies of SPME Arrow were up to 10 times higher than when conventional SPME fibres were used. Therefore, the method was more sensitive, so the detection limits were lower when SPME Arrow was used instead of a conventional fibre. These experiments demonstrated the advantages of SPME Arrow over conventional fibres.

3.4.2. Method quality parameters

The analytical performance of the HS-SPME Arrow method was inves- tigated under optimal conditions by establishing the linear ranges, method detection limits (MDLs), method

quantification limits (MQLs), repeatability (intra-day) and reproducibility (inter-day). HHCB-lactone was added to the compound list for the validation process.

Matrix matched calibration was selected because of the extraction technique used (SPME) and because the compounds interacted with matrix. The linear range was evaluated by constructing matrix-matched calibration curves and spiking cod samples at concentrations between 2.5 to 500 ng g^{-1} (d.w.). Non-spiked samples were analysed so that the signal of the compounds present in the samples could be subtracted. All the compounds showed good linearity at low ng g^{-1} (ranging between 2.5 – 250 ng g^{-1}) with $R^2 > 0.992$. Results are shown at Table 1.

Method detection limits (MDLs) for compounds present in the blanks were estimated as the average signal of the blanks plus three times the standard deviation of the blank samples. For the compounds that were not present in the blank sample, the MDL corresponded to the concentration that provided a signal/noise ratio equal to 3. Hence, MDLs ranged between 0.5 ng g^{-1} (d.w.) and 2.5 ng g^{-1} (d.w.) for cod samples. Method quantification limits (MQLs) were fixed as the lowest points of the calibration curves and ranged from 2.5 ng g^{-1} (d.w.) to 5 ng g^{-1} (d.w.). There are no reported methods for the

Table 1 Method quality parameters.

Compound	MDL (ng g^{-1})	MQL (ng g^{-1})	Linear range (ng g^{-1})	r^2	Repeatability[a]		Reproducibility[a]	
					10 ng g^{-1}	100 ng g^{-1}	10 ng g^{-1}	100 ng g^{-1}
DPMI	0.5	5	5 - 250	0.9920	4	8	11	14
ADBI	0.5	2.5	2.5 - 75	0.9996	4	5	12	10
AHMI	0.5	5	5 - 250	0.9998	5	4	10	14
ATTI	1	5	5-75	0.9977	5	4	13	18
HHCB	1	5	5 - 75	0.9997	15	4	16	11
AHTN	0.5	2.5	2.5 - 75	0.9995	2	2	9	20
MX	2.5	5	5-50	0.9989	15	6	23	20
MK	0.5	2.5	2.5 - 25	0.9992	6	3	12	10
HHCB-Lactone	1	2.5	2.5 - 100	0.9999	8	11	9	6

[a] Expressed as Relative Standard Deviation (%RSD); n = 5.

determination of musk fragrances in fish using SPME Arrow or conventional SPME. Thus, the MDLs obtained for this method cannot be compared with other data obtained with the same extraction technique. However, authors like Vallecillos el at. [8] or Trabalón et al. [23] developed methods using pressurized liquid extraction (PLE) and QuEChERS followed by GC-IT-MS/MS with MDLs ranging between 0.25 and 5 ng g^{-1} (d.w.) and 1 and 5 ng g^{-1} (d.w.), respectively, when 0.5 g of fish sample was used. Therefore, the method developed achieves similar or even lower MDLs using a technique that requires less sample handling and does not require specific instrumentation for the extraction procedure apart from the SPME Arrow.

Repeatability and reproducibility, expressed as relative standard deviation (%RSD), were calculated by spiking (n = 5) cod samples at 10 and 100 ng g^{-1} (d.w.) for all the compounds, with values below 23% which were similar to those obtained by the methods mentioned above. The results of the method validation are summarized in Table 1.

3.5. Application to commercial fish samples

The nine musk fragrances in fish samples were determined using the optimised and validated method. Three fish species were analysed: cod (*Gadus*

Table 2. Musk fragrance concentrations (ng g^{-1}) and ion ratios (in brackets) determined in fish samples by HS-SPME Arrow-GC-IT-MS/MS.

Compound	**COD** (*Gadus morhua*)	**SOLE** (*Solea solea*)	**HAKE** (*Merluccius merluccius*)
	ng g^{-1}	ng g^{-1}	ng g^{-1}
DPMI	n.d.	<MQL	n.d.
ADBI	n.d.	n.d.	n.d.
AHMI	n.d.	n.d.	n.d.
ATTI	<MQL	n.d.	n.d.
HHCB	17.5	6.5	12.6
AHTN	5.1	2.9	3.1
MX	n.d.	n.d.	n.d.
MK	n.d.	n.d.	n.d.
HHCB-lactone	n.d.	<MQL	n.d.

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

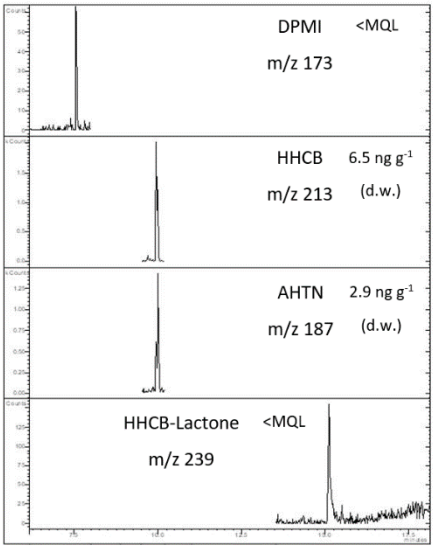

Fig. 4 XIC obtained from HS-SPME arrow and GC-IT-MS/MS analysis of a sole fish sample. The extraction conditions were: 0.25 g of sample, 100 °C, 45 min using PDMS SPME Arrow and desorption at 250 °C for 3 min.

morhua), sole (*Solea solea*) and hake (*Merluccius merluccius*). All the samples pertained to fish species with low lipid content.

In order to confirm the presence of the compounds in the sample, the following values must be the same as those obtained with a standard: retention time (min), presence of the quantifier and qualifier ions and qualifier/quantifier ratio. The ion ratios were confirmed using the guidelines of European Directive 2002/657/EC [25]. The ions ratios and the concentrations obtained from the analysis of triplicates using the developed method as well as the ion ratios are summarized in Table 2. HHCB and AHTN were found in all the samples at concentrations ranging from 6.5 and 17.5 ng g^{-1} (d.w.) and 2.9 and 5.1 ng g^{-1} (d.w.), respectively. This agrees with the fact that they are the most used polycyclic musk fragrances worldwide. However, the fact that AHTN was found at lower concentrations than HHCB may be explained by the recent European Directive 2008/42/EC, 2008 [26] that regulated its use in cosmetics. HHCB-lactone was only detected in sole and its concentration was found below the method quantification limit. The concentration ranges for cod and hake agree with the ones reported by Cunha et al. [27] for the same species: 15.3 to 18.5 ng g^{-1} (d.w.) and 6.3 to 7.6 ng g^{-1} (d.w.) for HHCB and AHTN in cod samples and 14.7 to 29.3 ng g^{-1} (d.w.) and 5.7 to 6.6 ng g^{-1} (d.w.) in hake samples. As for the other polycyclic musks, DPMI was also found in sole, while ATTI was found in cod. All the reported compounds found in the samples fulfil the requirements of retention time and quantifier/qualifier ratios. As an example, Fig. 4 shows the XIC chromatogram of the detected compounds in a sole sample.

The results show that cod had the greatest concentration of musk fragrances followed by hake and sole. These results are in agreement with those reported by Trabalón et al. [23] where the overall concentration of musk fragrances had the same pattern as the obtained in this study. However, concentrations reported for cod samples were higher, and hake was the species in which the most compounds were detected.

Finally, none of the nitro musk fragrances (MX and MK) were detected in any of the samples analysed. This was expected as the use of these compounds in cosmetics was regulated or prohibited by the European Regulation No 1223/2009, 2009 [28].

4. Conclusions

The novel SPME Arrow was successfully applied for the first time to determine musk fragrances in fish samples. PDMS SPME Arrow provided

an extraction efficiency up to 10 times higher than conventional SPME fibre extraction, mainly due to its larger sorbent volume. Thus, the developed method based on HS-SPME Arrow combined with GC-IT-MS/MS allowed LODs at low ng g^{-1} levels, which are comparable with those described in literature by using other extraction techniques such as PLE or QuEChERS coupled to GC–MS. Trace levels of musk fragrances were determined in various commercial fish samples with concentrations levels of low ng g^{-1} of HHCB and AHTN.

To sum up, the results of this study show that SPME Arrow represents a promising novel device for future studies focused on environmental contaminants.

Acknowledgements

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Supplementary Material

Solid phase microextraction Arrow for the determination of synthetic musk fragrances in fish samples

CONTENTS

Table S1. Retention times and MS conditions.

Compound	Retention Time (min)	Precursor Ion (m/z)	Product Ion[a] (m/z)	CID Amplitude (V)	m/z Range	Scan time (s/scans)
DPMI	7.5	191	107 (29), 135 (78), **173**	0.82	94 - 201	1.08
ADBI	8.6	229	131 (8), **173,** 187 (17)	0.92	110 - 239	1.08
AHMI	9.1	229	131 (6), 145 (7), **187**	0.93	110 - 239	1.08
ATTI	9.7	215	131 (3), 171 (32), **173**	0.88	104 - 225	1.03
HHCB[b]	9.9	243	171(3), **213**	0.96	132 - 253	0.53
AHTN[b]	9.9	243	145 (24), 159 (16), **187**	0.96	113 - 253	0.53
MX	10.6	282	**265,** 266 (12), 280 (33)	1.08	134 - 292	0.34
MK	12.5	279	**191**, 247 (3), 280 (10)	1.07	132 - 289	1.05
HHCB-Lactone	15.1	257	183 (14), 201 (89), **239**	1.00	123 - 267	1.03

[a] Quantification ions (m/z) are shown in bold type. Qualifier/quantifier ratio is in % in brackets.

[b] Compounds were separated using Multiple Reaction Monitoring mode.

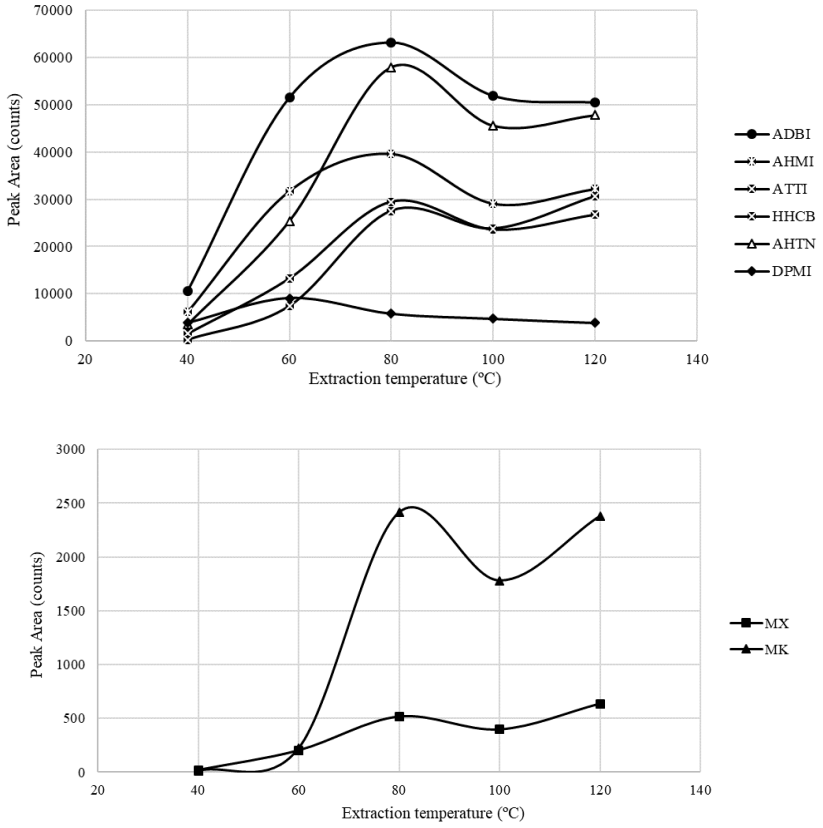

Fig. S1. The effect of extraction temperature on the extraction efficiency using a PDMS SPME conventional fibre. Extraction conditions: 0.25g of sample spiked at 1 mg kg^{-1}, extraction time of 45 min.

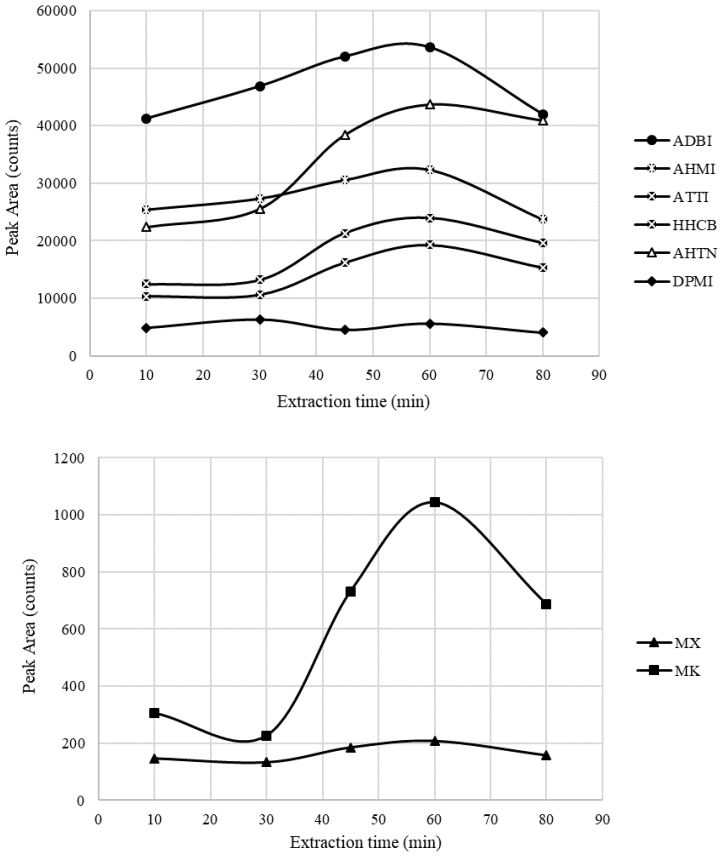

Fig. S2. The effect of extraction time on the extraction efficiency using a PDMS SPME conventional fibre. Extraction conditions: 0.25g of sample spiked at 1 mg kg^{-1}, extraction temperature of 80 °C.

3.1.2. Determination of organophosphate ester flame retardants and plasticisers in fish samples by QuEChERS followed by gas chromatography-tandem mass spectrometry. Exposure and risk assessment through fish consumption

DETERMINATION OF ORGANOPHOSPHATE ESTER FLAME RETARDANTS AND PLASTICISERS IN FISH SAMPLES BY QUECHERS FOLLOWED BY GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. EXPOSURE AND RISK ASSESSMENT THROUGH FISH CONSUMPTION

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Abstract

The presence of organophosphate esters (OPEs) in everyday commodities such as furniture, household appliances and baby toys have rendered these contaminants ubiquitous in environmental fates such as air, water, soils and biota. Their presence in food-related species suggests that an additional route of exposure to these esters for the general population is fish intake through diet. Their incipient toxicity and carcinogenetic behaviour make it essential to develop methods for determining OPEs in fish samples. In this paper we have developed a new method for determining 9 OPEs based on the QuEChERS extraction method followed by a simple clean-up using a novel device for selective lipid removal (LipiFiltr) and GC–MS/MS to extract these compounds from fish samples regardless of lipid content. QuEChERS salt packet optimisation and clean-up strategies such as liquid-liquid extraction, dispersive-solid phase ex- traction and LipiFiltr were tested. Our results showed that EN 15662 method salts and Lipifiltr were the best combination to produce efficient analyte apparent recovery (67–116%) and negligible matrix effects (<10%). Limits of detection ranged from 0.05 ng g^{-1} (dry weight) for TiBP and TBP to 2.00 ng g^{-1} (dry weight) for TCEP. Fish samples from four fish species were determined with a median concentration of ΣOPEs 5.31 ng g^{-1} on a wet weight basis, with TBP, TiBP and TCPP as the main contenders. Estimates of exposure and risk associated with consuming these compounds via dietary intake showed low levels of concern for the population of Tarragona.

Highlights

- A new QuEChERS- GC-MS/MS method for OPEs in fish has been successfully developed.
- Clean-up using the novel LipiFiltr greatly reduced lipidic fish matrix.
- Median concentration of ΣOPEs found at the fish species was 5.31 ng g^{-1} (w.w).
- Estimates of exposure and risk via dietary intake showed low levels of concern.

1. Introduction

Due to ever-increasing health concerns, organophosphate ester (OPE) flame retardants and plasticisers have recently appeared as alternatives to widely used brominated flame retardants (BFRs) such as poly-brominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) [1]. As flame retardants, OPEs are chemical additives integrated into combustible materials to prevent or de-lay the start of fire. They can also be added to materials to modify their plastic properties. Their extended use in everyday commodities such as furniture, electronics, plastics, and construction mate- rials has led some of these compounds to be catalogued as high production volume chemicals (HPV).

Since no chemical bonding exists between the polymer material and the additives, OPEs can be easily discharged into the environment by abrasion, volatilisation, leaching or dissolution [2,3]. Their widespread dispersion means they have become ubiquitous in environmental fates such as outdoor and indoor air [4–6], dust [7,8], surface and wastewater [9,10], soils [11], sediments [12,13] and biota species such as cetaceans [14], birds [15] and fish, chicken and cattle [16] that may be components of human diet. Although most OPE-containing foodstuffs, such as meat, cereals and milk, are believed to become contaminated during their production or storage steps, food such as fish, which is usually consumed fresh, is believed to bioaccumulate OPEs in the aquatic environment through ingestion or via the gill membranes [3]. The fish present in the human diet therefore becomes a source of exposure to these com- pounds via dietary intake. High concentrations of OPEs may cause adverse health effects such as neurotoxicity and reproductive toxi- city and have carcinogenic, mutagenic, and even endocrine disruptor effects [17–19]. It is important, therefore, to develop methods of determining low levels of OPEs in fish matrices in order to monitor their presence and prevent the most contaminated fish from being ingested.

Although some studies have recently focused on the presence of these compounds in this kind of matrix, information on the occurrence of organophosphate esters in fish samples is still limited and methods for determining OPEs in fish samples are scarce. Cur- rent methods are usually based on a two-step procedure. First, an initial solid-liquid extraction procedure is conducted to extract the compounds of interest from fresh and lyophilised fish samples and transfer them to a solvent. These procedures comprise numerous extraction techniques ranging from solid-liquid extraction

(SLE) [20,21], ultrasound-assisted extraction (UAE) [22–24] and Soxhlet extraction [16] to more complex techniques such as pressurised liquid extraction (PLE) [25–29]. Although SLE, UAE and Soxhlet are easy techniques to use when it comes to required extraction procedures and instrumentation, their disadvantages include the need for large volumes of organic solvents and the long extraction times and low recoveries. Although PLE has appeared as an upgraded and automatic SLE, its main drawback is its requirement for specific instrumentation that may not be affordable for most routine laboratories. In addition to these financial issues, using PLE in fish analysis may cause greater matrix effect problems since this extraction technique is not highly selective and therefore requires a more thorough clean-up procedure [30].

Once extraction is performed, all methods include a clean-up step such as gel permeation chromatography (GPC) [26,31,32], solid phase extraction (SPE) [22–24,28–30,32,33] or solid phase microextraction (SPME) [27]. Despite their good performance overall, the main disadvantage with these techniques is that they are usually more time-consuming – either because of the need to elute different fractions (GPC) or conduct a loading, clean-up and elution process (SPE) or because of the extraction time required for equilibration (SPME). Faster and easier-to-handle techniques such as dispersive solid phase extraction (dSPE) may therefore provide efficient alternative clean-up procedures [20]. Moreover, novel specific lipid-removal push-through cartridges such as those commercialised under the name LipiFiltr® by UTC promises excellent matrix clean-up and good recoveries simply by passing the extract through a syringe-disposable cartridge. Studies involving this type of clean-up should therefore be conducted to test their utility in fish-matrix removal. The need for an easy-to-handle and low-cost method for determining OPEs in fish samples led us to use QuEChERS extraction followed by a simple clean-up procedure. To the best of our knowledge, this is the first study to optimise a QuEChERS method for determining organophosphate esters in fish samples

The best instrumentation techniques for determining such compounds are usually liquid chromatography (LC) and gas chromatography (GC). The non-polar quality of most compounds usu- ally makes gas chromatography with non-polar stationary phases (usually 5% phenyl methyl-polysiloxane) the preferred technique, while liquid chromatography tends to be more suitable for lower-volatility compounds such as TEHP and EHDPP. Chromatography coupled to mass spectrometry detectors is usually

the most reliable technique for accurately and reliably determining organophosphate esters [34].

As we stated earlier, organophosphate esters contain compounds with several toxic and carcinogenic characteristics that may negatively affect human health. Since they are shown to be present in edible fish samples, the possibility of incorporating them into the human body and their possible adverse effects are a concern. Studies into exposure levels and toxicological effects should therefore be conducted to assess the risk to humans associated with consuming fish samples that may be contaminated with these compounds.

For all these reasons, in this study we develop a more effective, easier and less time-consuming method for determining OPEs in fish samples using QuEChERS, a simple clean-up, and GC-QqQ- MS/MS. We then use our results to estimate exposure and risk to the population of Tarragona, Spain, associated with these chemical additives through dietary intake.

2. Materials and methods

2.1. Chemicals and reagents

The standards used were nine organophosphate esters (OPEs): tributyl phosphate (TBP), triethyl phosphate (TEP), tri-iso-butyl phosphate (TiBP), triphenyl phosphate (TPP), tris (2-ethylhexyl)- phosphate (TEHP), 2-ethylhexyl-diphenyl-phosphate (EHDP), tris(2- chloroethyl)-phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP) and tritolyl phosphate (TTP). The deuterated tributyl phosphate (TBP-d27) was used as internal standard. All compounds were purchased from Sigma Aldrich (St. Louis, USA).

Stock solutions of the individual compounds were prepared in ethyl acetate at a concentration of 1000 mg L^{-1}. A working mixture solution of 100 mg L^{-1} was also prepared in ethyl acetate for further dilutions. Stock and working solutions were stored at −20 °C until use.

The ethyl acetate, acetone and hexane used during the study were GC grade with purity > 99.9% from JT Baker (Deventer, The Netherlands). The acetonitrile used for the extraction procedure was gradient grade also supplied by JT Baker, while ultrapure water was obtained from a Synergy purification system from Millipore (Massachusetts, USA). Helium gas at a purity of 99.999% for the chromatographic system and the nitrogen used for the MS system were supplied by Carburos Metálicos (Tarragona, Spain). The different QuEChERS salt packets (Original, AOAC and EN15662) were purchased from

Scharlab (Barcelona, Spain), while the sorbents used for the dispersive solid phase extraction (PSA, coco and activated charcoal and florisil) were purchased from Sigma Aldrich (St. Louis, USA). LipiFiltr was supplied by Carlo Erba (Barcelona, Spain).

2.2. Sample preparation

Samples of several fish species, i.e., cod (Gadus morhua), hake (Merluccius merluccius), mackerel (Scomber vincialis) and salmon (Salmo salar), were purchased from local markets. To evaluate the effect of different lipid percentages, these species were divided into two groups: low lipid content species (cod and hake) and high lipid content species (salmon and mackerel). Lateral fillets from the samples were dissected from the fish and freeze-dried using a miVac Duo freeze-drying system from Genevac (Ipswich, United Kingdom). The freeze-dried samples were then homogenised and ground. The samples were also sieved through a 500 µm mesh to obtain a homogenised particle size. The moisture contents (%) of the fish species were calculated from the differences in weight before and after lyophilisation.

2.3. QuEChERS extraction method

Fish samples were extracted by QuEChERS using a method adapted from Trabalón et al. [35], by which synthetic musk fragrances were determined in fish samples. A 0.5 g portion of the lyophilised fish sample was weighed into a 50 mL centrifuge tube. Aliquots of 10 mL of ultrapure water and 10 mL of acetonitrile were added to the tube, which was then vortexed for 1 min. A QuEChERS extraction salt packet (Standard Method EN15662) containing 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium hydrogencitrate and 1 g of sodium citrate was then added to the tube. The tube was then vortexed for 3 min and centrifuged for 5 min at 7000 rpm using a Hettich Universal 32R centrifuge (Tuttlingen, Germany). The supernatant (acetonitrile layer) was removed and transferred to a 10 mL disposable plastic syringe connected to a LipiFiltr® push-through cartridge. The extract obtained was collected in a 20 mL glass vial and then evaporated under gentle nitrogen steam to a final volume of approximately 1 mL. Finally, the extract was spiked with the internal standard at a concentration of 10 µg L^{-1} and reconstituted to a final volume of 2 mL with ethyl acetate.

2.4. Gas chromatography-tandem mass spectrometry

The analyses were performed using an Agilent 7890A GC system coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). A ZB-5MSPlus cap-

illary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA) was used for the chromatographic separation of the target compounds using the following oven temperature programme: the temperature was initially set at 50 °C (held for 3 min) and then raised at a rate of 15 °C/min to 290 °C (held for 5 min). The total run time was 24 min with a 4-minute solvent delay. Helium was used as the carrier gas at a constant rate of 2.0 mL/min. The injection volume was 5 µL and an Agilent multimode inlet (MMI) was used in solvent vent mode at an initial temperature of 50 °C (held for 0.15 min) and then raised at a rate of 600 °C/min to 300 °C (held for 3 min). The triple quadrupole mass spectrometer operated in electron ionisation mode at 70 eV. Temperatures for the ion source,

Table 1. GC-QqQ-MS/MS parameters.

	Rt (min)	MRM Q[a]	CE[b] (eV)	MRM q[c]	CE[b] (eV)
TEP	7.87	155 → 99	5	99 → 63	40
				99 → 81	25
TiBP	11.53	99 → 81	25	99 → 63	25
				99 → 47	45
TBP	12.62	99 → 81	20	99 → 63	45
				99 → 47	50
TCEP	13.55	249 → 125	10	249 → 63	25
				249 → 187	5
TCPP	13.76	125 → 99	10	124 → 81	25
				99 → 81	20
TPP	17.61	326 → 233	15	326 → 170	20
				77 → 51	15
EHDPP	17.71	251 → 77	20	251 → 152	20
				251 → 175	20
TEHP	17.82	99 → 81	25	99 → 81	45
				113 → 57	10
TTP	19.06	368 → 165	30	368 → 91	35
				368 → 243	30
TBP-d27	12.48	103 → 83	25	103 → 63	45
				147 → 62	20

quadrupole 1 and quadrupole 2 were set at 230 °C, 150 °C, and 150 °C, respectively. Multiple reaction monitoring (MRM) mode was employed for the quantification using one quantifier transition (Q) and one or two qualifier transitions (q) to identify each compound. The triple quadrupole mass spectrometer parameters are shown in Table 1.

2.5. Exposure assessment and risk characterization

The exposure associated with the dietary intake of the 9 organophosphate esters by the population of Tarragona was assessed using a method that combines fish consumption with the concentrations found in each species. Human exposure values (E_t) were obtained by applying Eq. (1), which considers the mean fish consumption of the individual species *f* (C_f, g kg bw^{-1} day^{-1}) and the concentration of each contaminant *t* for the species *f* ($X_{t,f}$, ng g^{-1}). The sum of the contributions of each fish species provides the global human exposure for contaminant *t*.

$$E_t = \sum_{f=1}^{p} C_f X_{t,f} \quad (1)$$

The mean consumption values were previously normalised by dividing mean dietary intake by mean body weight for each gender/age population subgroup. The concentrations of the contaminants found in the fish samples were applied on a wet weight (w.w.) basis.

Three scenarios were estimated by assuming various concentrations based on the method detection limit (LOD) and method quantification limit (LOQ) for non-detected or below-LOQ compounds [36]. The upper-bound scenario (UB) was estimated by assuming the concentrations of the respective LOD or LOQ for the non-detected and below-LOQ compounds, respectively. For the middle-bound scenario (MB), half of the LOD and LOQ concentrations were estimated. The lower-bound scenario (LB) was assessed by assuming the LOD value for the compounds present at concentrations below the LOQ and reduced to zero for the non-detected compounds.

There is no consensus within the scientific community on which risk assessment methodology is most suitable. We therefore used the approaches suggested by the European Safety Authority (EFSA) and the Environmental Protection Agency (EPA), which are those most frequently found in the literature, to perform our calculations. A first approach using NOAEL (*non-observed-adverse-effect-level*) va-lues was applied for non-genotoxic and non-carcinogenic compounds following Eq. (2), where R_t, E_t and ADI_t are the risk factor, the global dietary exposure, and the acceptable

daily intake, respect-tively, associated with compound *t*. Acceptable daily intake values were obtained by dividing the oral NOAEL factors of each compound by an uncertainty factor of 100.

$$R_t = (E_t / ADI_t)*100 \quad (2)$$

Finally, for genotoxic and carcinogenic compounds, risk was calculated using the MOE (margin of exposure) approach in accordance with Eq. (3), where MOE_t is the margin of exposure to compound *t*, BMD_t is the benchmark dose (estimated as the dose that causes a measurable response in a 5–10% range above the control) for compound *t* and E_t is the global dietary exposure to compound *t*.

$$MOE_t = BMD_t/E_t \quad (3)$$

2.6. Study of population and data collection

Our data on the consumption of the fish species in Tarragona was extracted from a nutritional survey conducted in Catalonia be- tween 2002 and 2003 (ENCAT, 2003) [37]. The study, based on a representative sample of the population of Catalonia, comprised 2160 individuals aged 10–80 (996 men and 1164 women). The survey involved 24-hour recall, a general questionnaire on physical activity, knowledge and opinions on nutrition, and an 80-item food frequency questionnaire. Body weight, height, and waist circumference were also measured. The data were collected by previously trained dietitians at volunteers' homes via personal interviews con- ducted on any day of the week. Table 2 shows the mean consumption of the species determined in the present study (mackerel, hake, salmon, and cod) by the population of Tarragona.

3. Results and discussion

3.1. GC-MS conditions

Organophosphate esters did not display high sensitivity when low amounts (1 µL) of standard solutions were injected, so large-volume injections of 5 µL were tested to achieve lower limits of detection. The injections were performed using the solvent vent mode of the GC system in a multi-mode inlet. Optimisation of the solvent vent parameters, such as the solvent elimination rate, inlet temperature gradient rate and inlet vent time, were established using the Solvent Elimination Wizard software included in the MassHunter acquisition software for GC systems. Optimal conditions with regard to gas chromatography and mass spectrometry are discussed in Section 2.4.

3.2. QuEChERS procedure optimisation

Our method for extracting the fish samples was adapted from that used by Trabalón et al. [38] to extract musk fragrances from fish samples. That method's initial conditions were maintained and conditions such as the QuEChERS salt packets and clean-up were optimised. Therefore, 0.5 g of sample was used and the times for the extraction procedure were maintained at 1 min for the first solid-liquid step and 3 min for the salt solvation process. Centrifugation conditions (i.e. 5 min and 7500 rpm) were also maintained. Cod was used for the QuEChERS salt packet optimisation. Species such as salmon and cod were used for the optimisation of the clean-up procedure. These species were chosen to represent low lipid content (cod) and high lipid content (salmon) in order to observe differences in extract clean-up efficiency due to lipid content.

3.2.1. Salts packet optimization

The content of the salt packets commercially available for the QuEChERS procedure mainly differ in the type of salts and the ratio between them. These are commonly known by the standard method which they are used for. Therefore, three different salts packets can be found commercially. The first of these, which follows the original

Table 2. Mean consumption (g day^{-1}) by the population of Tarragona of the determined fish species.

	FoodEx2 code	Boys (10-19)	Adult men (20-65)	Senior men (>65)	Girls (10-19)	Adult women (20-65)	Senior women (>65)
Mackerel	A02CV	0.36	1.13	0.50	0.32	1.27	2.86
Hake	A02CB	7.82	15.03	23.02	10.84	14.49	14.56
Salmon	A028P	3.30	1.80	2.23	1.00	3.00	1.14
Cod	A02BV	2.13	4.18	8.08	0.60	4.61	8.15

method by Anastassiades et al. [39], contains 4 g of magnesium sulfate and 1 g of sodium chloride. The second improves on the original method by introducing certain species that act as buffering salts. This contains 6 g of magnesium sulfate and 1.5 g of anhydrous sodium acetate and is known as the AOAC method [40]. Thirdly, the European Committee for Standardisation released an official standard method, the EN 15662 [41], with the following variations in the composition of the salts: 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium hydrogencitrate sesquihydrate, and 1 g of sodium citrate. These three packets were tested to achieve the highest recoveries of the analytes present in the fish samples. For the experiments, 0.5 g cod samples that had previously been spiked at 2.5 μg g^{-1} (d.w.) were placed in a 50 mL centrifuge tube and 10 mL of ultrapure water and 10 mL of acetonitrile were added. The tube was mixed for 1 min and one of the three QuEChERS salt packets was added. Once the contents of the packet were inside the tube, they were mixed for 3 min and then centrifuged at 7500 rpm for 5 min. Finally, the organic layer (supernatant) was collected in a 20 mL glass vial and evaporated to approximately 1 mL to finally add the internal standard and reconstitute the extract to 2 mL using ethyl acetate. During the process to optimise the type of salts, no clean-up was performed so as to prevent losses

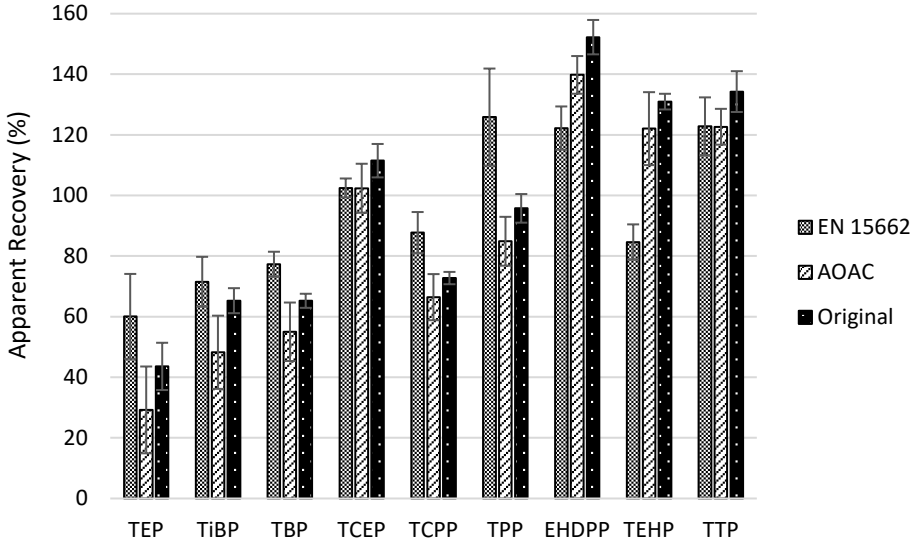

Fig.1. Apparent recoveries (%) of the organophosphate compounds from cod samples when using different QuEChERS extraction salts packets. From left to right, compounds ordered in elution order.

caused by adding further steps. The extraction process was performed in triplicate to compare the results for each salt type. Fig. 1 shows the apparent recoveries of the organophosphate compounds from fish samples with different QuEChERS extraction salt packets.

This figure shows that most compounds showed higher recoveries when the EN 15662 method was used. It also shows that the higher mass compounds (EHDPP, TEHP and TTP) showed recoveries of over 120% when the AOAC method and original method were used. This could be due to matrix interference, which enhances the signal of the compounds in the detection system (the matrix enhancement effect). We therefore preferred to lower the interference by using the EN15662 method. Our results agree with previous studies conducted by Trabalón et al. [42], which showed that this method is also suitable for extracting musk fragrances from fish samples. We therefore selected QuEChERS extraction using EN 15662 packet salts as the preferred method.

3.2.2. Clean-up selection

Matrices such as fish are expected to contain large amounts of diverse compounds that can interfere in the analysis of target compounds by increasing or decreasing the signals obtained with the instrument. A clean-up step should therefore be included in the extraction procedure in order to reduce interference. Specifically, the lipid content of fish is one of the most difficult analytical challenges when working with this kind of matrix. High lipid contents can interfere with extraction efficiency, causing low recoveries or even enhancing or suppressing the signal once the extract is injected into the GC–MS system. We therefore conducted experiments on extract clean-up for species with high and low lipid contents. For species with a high lipid content, we chose salmon (*Salmo salar*), whose lipid content is approximately 25%. For species with a low lipid content, we chose cod (*Gadus morhua*), whose lipid content is below 1%.

We tested three clean-up methods liquid-liquid extraction (LLE), dispersive solid-phase extraction (dSPE), and LipiFiltr® and compared apparent recoveries with each method. Blank fish samples of cod and salmon were spiked at a concentration of 2.5 mg kg^{-1} (d.w.) and extracted with and without the suggested clean-ups. Each clean-up strategy was performed in triplicate and the results were evaluated (see Fig. 2).

For LLE, after the QuEChERS salt packet was added and the organic and aqueous phases separated, the supernatant (organic layer) was

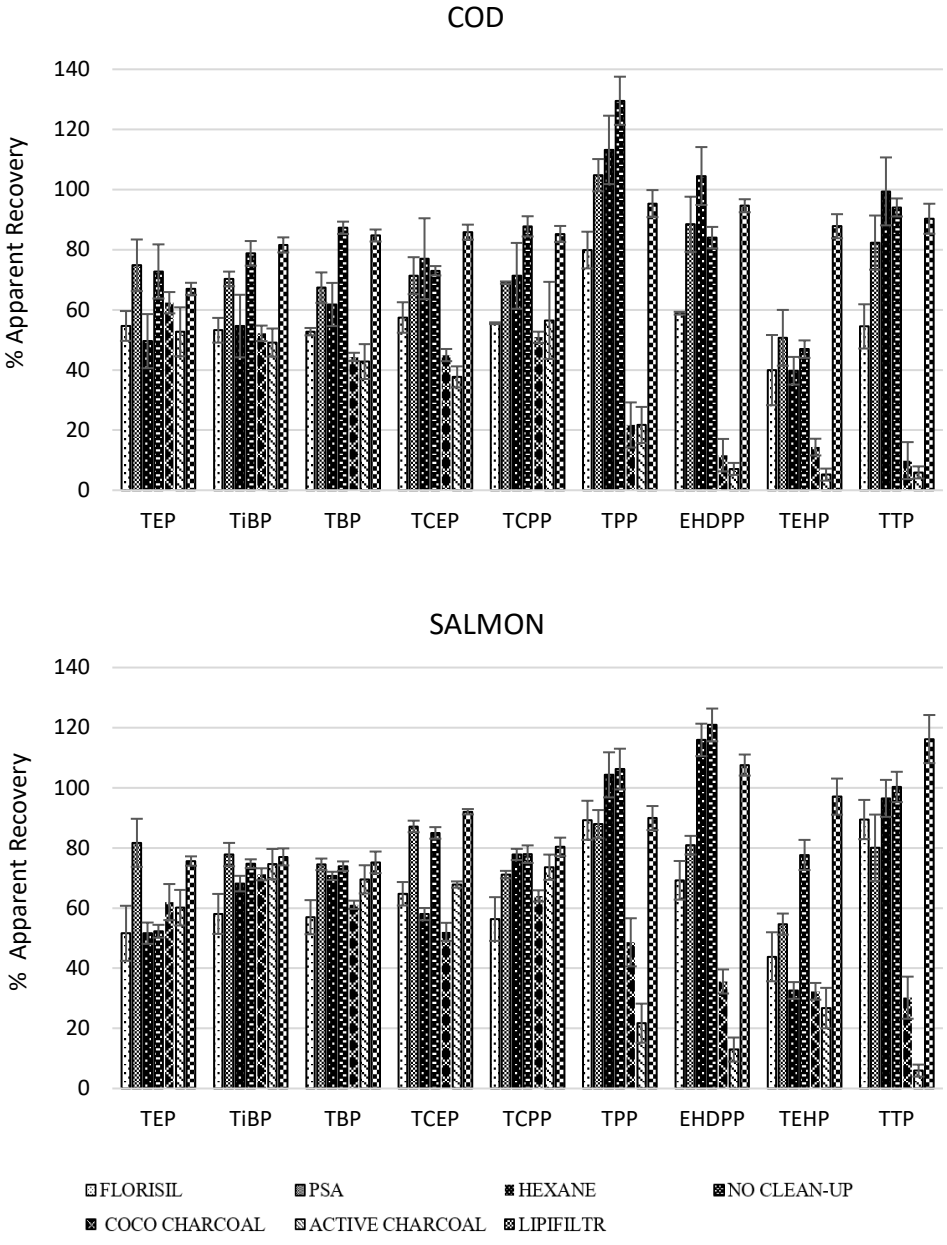

Fig. 2. Apparent recoveries (%) of the organophosphate compounds from cod and salmon samples when using different clean-up strategies.

transferred to a 50 mL centrifuge tube. Then, 2 mL of hexane was added and subsequently vortexed for 3 min. Once the two phases were separated, the acetonitrile phase (lower layer) was cautiously separated and transferred to a 20 mL glass vial so as to continue the procedure described above. Following previous studies by Trabalón et al. [43], which demonstrated its effectivity for lipid clean-up when analysing fish samples to determine benzothiazoles, hexane was selected as the clean-up solvent. However, as Fig. 2 shows, when hexane was used for the clean-up, apparent recoveries with the method ranged from 23% to 116% for salmon and from 40% to 113% for cod, which were unsatisfactory. Similar trends were observed for both salmon and cod, i.e. recoveries did not differ substantially from those obtained when no clean-up was applied. Consequently, we discarded clean-up using hexane as LLE solvent.

We then tested dSPE using different sorbents as dispersive agents, conducting experiments with florisil, primary-secondary amine (PSA), coconut and activated charcoal. Our selection of dispersive agents comprised some of the more typical sorbents (florisil and PSA) and some of the more retaining ones (activated and coconut charcoal). The procedure for the dSPE was similar to that previously described for LLE. The QuEChERS organic extract was separated and placed in a 50 mL centrifuge tube containing either 2 g of florisil, 200 mg of PSA, 200 mg of coconut charcoal or 200 mg of activated charcoal. After the extract was placed in the tube with the dispersive agent, it was vortexed for 3 min and the liquid layer was separated. The procedure then continued as previously described. Fig. 2 shows that florisil did not work well for most compounds in either matrix, with recoveries ranging from 40 to 89%. PSA, on the other hand, demonstrated slightly better behaviour for the lighter compounds in salmon, though its effectivity decreased with the heavier ones. The opposite effect occurred in cod, where heavier compounds displayed better recoveries when PSA was used, with TEHP being the only exception. On the other hand, as the mass of the compound increased, apparent recoveries with activated or coconut charcoal decreased in both cod and salmon. This effect can be explained by the retention capacity of these sorbents. As the compound's mass increases, it is more likely to be retained in the sorbent and therefore be extracted from the organic layer kept during the process. However, although PSA seemed to work well for most compounds in both matrices, recoveries of the method with no clean-up were still higher in most cases.

Finally, we tested a novel sorbent commercially known as LipiFiltr®, which

is intended for trapping lipids and fats. The procedure involved transferring the supernatant obtained after adding the QuEChERS salt packet to a 10 mL plastic syringe before passing it through a LipiFiltr® cartridge to obtain a clear, clean extract. The extract was then evaporated and reconstituted using the procedure described earlier. Our results showed high recoveries for both high and low lipid content species and for lighter and heavier compounds, with values ranging from 75 to 116% for salmon and from 67 to 95% for cod. These results were similar to those obtained when no clean-up was applied but obtaining a clean clear extract. This can be explained by the composition of the sorbent, which seemed to retain only the interferences, and by the simplified procedure, which enables the extract to be handled less than when other procedures are used. LipiFiltr was therefore selected for the clean-up.

Once we had selected LipiFiltr® as the clean-up procedure, we evaluated the matrix effect to compare the efficiency of the clean- up itself, not only the full method recovery. To do so, we extracted spiked fish samples using the method with and without LipiFiltr® clean-up. Finally, both extracts (with and without clean-up) were spiked at a concentration of 2.5 mg kg^{-1} (d.w.) and reconstituted to 2 mL. The extracts were then injected into the GC–MS system. The matrix effect was calculated using the equation described by Trabalón et al. [44]. Our results are given in Fig. 3, which shows that the matrix effects for both high and low lipid content species were below 10%, which indicates that the matrix effects were negligible.

3.3. Method performance parameters

To evaluate the analytical performance of the method under optimal conditions, we determined apparent recoveries (%), method detection limits (LOD), method quantification limits (LOQ), linear ranges, repeatability (intra-day), and reproducibility (inter-day). Due to the negligible matrix effects, external calibration using directly injected solvent standards with deuterated tributylphosphate as internal standard was preferred. Quantification was therefore performed using the instrumental calibration curves and applying the apparent recoveries depending on the lipid content. LOQ and LOD were estimated for each compound taking in consideration their respective instrumental detection limit (calculated as S/N = 3 using direct injection) and instrumental quantification limit (expressed as the first point of the instrumental linear range) and applying the recovery values as well as the extraction procedure calculations. Therefore, LODs ranged from 0.05 to 2.00 ng g^{-1} dw and LOQs ranged from 0.25 to 5.00 ng g^{-1} dw. Repeatability and

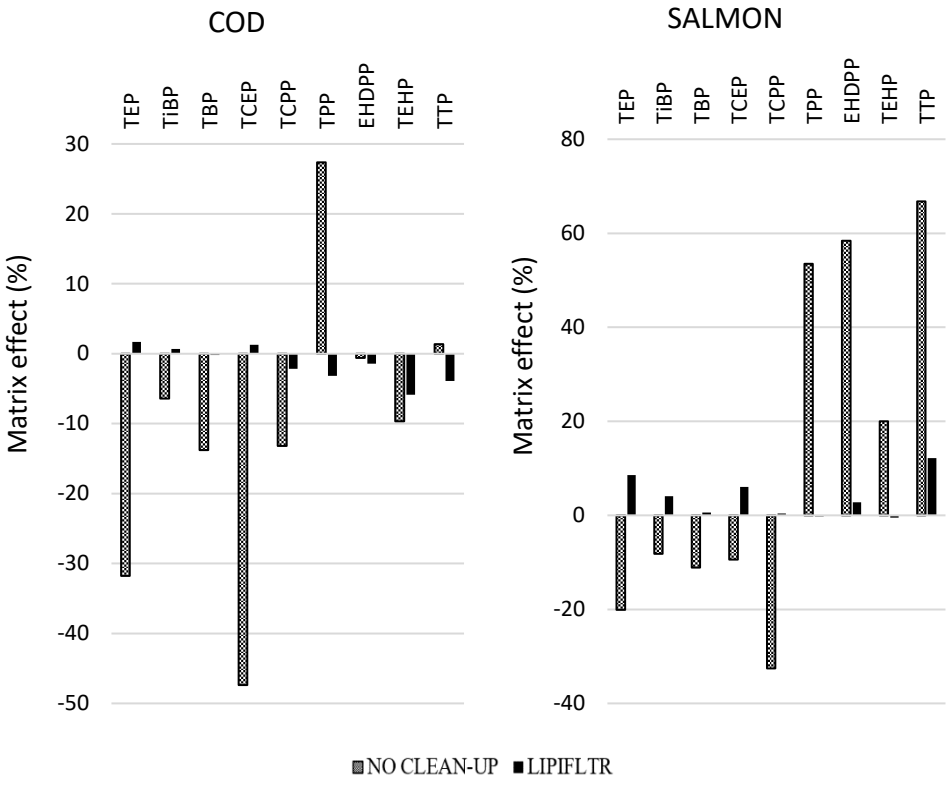

Fig. 3. Matrix effects (%) obtained without and with LipiFiltr clean-up.

reproducibility, expressed as relative standard deviation (%RSD) (n = 3, 10 ng g^{-1} dw.), were below 14 and 19%, respectively, for all compounds. The method quality parameters are summarized in Table 3.

3.4. Application to commercial fish samples

The QuEChERS LipiFiltr GC-QqQ-MS/MS method was applied to samples of four fish species to determine organophosphate esters. Triplicates of mackerel (*Scomber vincialis*), salmon (*Salmo salar*), cod (*Gadus morhua*) and hake (*Merluccius merluccius*) were analysed using the method described above.

The presence of target compounds in the samples was confirmed by comparing retention time (min), presence of qualifier product ions, and quantifier/qualifier transition ratios

with a standard. The acceptance criteria were applied in accordance with European Directive 2002/657/EC [45]. Quantification procedure was differentiated between low and high lipid content species. Therefore, low lipid content species (hake and cod) were quantified using the apparent recoveries obtained for cod and high lipid content species (salmon and mackerel) were quantified using the apparent recoveries obtained for salmon.

To evaluate our results and compare them to others in the literature, we calculated the concentrations found in fish samples in wet weight (w.w.) as the product of the d.w. concentration and the moisture content percentage of each species. The results are shown in Table 4.

Only TiBP, TBP, TCEP, TCPP, EHDPP and TEHP could be quantified in at least one fish species, while TEP and TCEP were also present at non-quantifiable levels. All compounds found in the samples were at similar levels of concentrations, which ranged from 0.45 to 3.35 ng g^{-1} (w.w.). Chlorinated compounds such as TCPP appeared at higher concentrations than the alkyl/aryl esters because they are more resistant to degradation in water than other compounds, which are more likely to be hydrolysed and removed in wastewater plants [3,31,46]. Although TCEP was expected to ap- pear in higher concentrations than the alkyl OPEs, it could not be quantified since it had the highest quantification limit (5 ng g^{-1} dw.).

As several studies have reported, the concentration patterns of OPEs in fish can differ significantly between species and even within the same species due to differences in fish feeding habits, body size, developmental stage or metabolic capacity [3,26,31]. The concentration patterns of OPE compounds may differ between lo- cations, which makes it difficult to compare data, though levels are usually found at low ng g^{-1} (w.w.) except in heavily contaminated spots. The median concentration of ΣOPEs found in this study was 5.31 ng g^{-1} (w.w.) for all species, which is comparable to those found in regions such as Southeast Queensland, Australia (1.8 ng g^{-1} (w.w), [47]); Albany, United States (7.11 ng g^{-1} (w.w), [48]); and Sweden (3.50 ng g^{-1} (w.w), [49]).

3.5. Dietary intake and risk assessment of OPEs in fish

Dietary exposure to OPEs was based on lower-, middle- and upper-bound scenario concentrations for six subgroups depending on age and gender: boys/girls, adult men/adult women and senior men/senior women. As reported by the ENCAT 2003 survey, the senior population is the most active

Table 3. Method quality parameters.

Compound	% R_{app}[a] *Low lipid*	% R_{app}[a] *High Lipid*	Instrumental linear range (µg L^{-1})	r^2	LOD[b] (ng g^{-1} dw.)	LOQ[c] (ng g^{-1} dw.)
TEP	67	78	0.1–5	0.9998	0.25	0.50
TiBP	82	82	0.05–5	0.9995	0.05	0.25
TBP	85	81	0.05–5	0.9985	0.05	0.25
TCEP	86	90	1–100	0.9999	2.00	5.00
TCPP	85	86	0.5–100	0.9991	0.05	2.00
TPP	95	93	1–100	0.9981	2.00	5.00
EHDPP	95	107	1–50	0.9991	0.50	5.00
TEHP	88	98	0.1–5	0.9939	0.25	0.50
TTP	90	116	1–50	0.9996	2.00	5.00

[a] Apparent recovery.
[b] LOD: method detection limit.
[c] LOQ: method quantification limit.

Table 4. Concentrations found in 4 different fish species (w.w.) using the developed method.

Compound	Mackerel (Scomber vincialis) ng g^{-1}	Cod (Gadus morhua) ng g^{-1}	Salmon (Salmo salar) ng g^{-1}	Hake (Merluccius merluccius) ng g^{-1}
TEP	<MQL	n.d.	n.d.	n.d.
TiBP	1.04	0.59	1.48	0.62
TBP	0.89	0.45	0.73	0.68
TCEP	<MQL	<MQL	<MQL	<MQL
TCPP	2.78	1.85	2.23	1.52
TPP	n.d.	n.d.	n.d.	n.d.
EHDPP	3.03	<MQL	<MQL	<MQL
TEHP	<MQL	<MQL	3.35	<MQL
TTP	n.d.	n.d.	n.d.	n.d.

*n.d.: not detected; <MQL: below method quantification limit.

fish-consuming group. Figure S1 shows the total exposure of these Tarragona-population subgroups in the three above scenarios. The results reflect exposure associated with the consumption of cod, mackerel, hake and salmon – species which are commonly consumed by this population. The exposure values of ΣOPEs ranged from 1.19 to 1.86 ng kg^{-1} bw day^{-1} for LB; from 1.83 to 3.34 ng kg^{-1} bw day^{-1} for MB; and from 2.53 to 4.72 ng kg^{-1} bw day^{-1} for UB. The same exposure pattern was observed in all three scenarios. Senior men was the most exposed subgroup, followed by senior women, adult women, adult men, boys and, finally, girls. With regard to compound-based exposition, in the LB scenario TCPP represented roughly 40% of total exposure followed by roughly 20% for TCEP, roughly 16% for TEHP, roughly 15% for TiBP, and below 10% for the remaining compounds. Chlorinated organophosphate esters are therefore the main contributors to OPE exposure. Differences in contribution patterns were observed in the other two scenarios. In the MB scenario, for example, TCPP decreased to roughly 25% and TCEP decreased to roughly 15%, while EHDPP (at roughly 23%) was the largest contributor. In the UB scenario the figures were roughly 23% for TCEP and roughly 23% for EHDPP, followed by roughly 18% for TCPP. This shows that chlorinated OPEs prevailed at higher concentrations (MB and UB), while EHDPP was the compound with the highest exposure. Figures summarising all compound-based distributions for each subgroup and scenario are provided in the supplementary data (S2).

To sum up, increased exposure associated with the intake of OPEs through fish consumption was observed as age increased. This was due to a higher rate of fish consumption. No significant differences were observed between genders, though the values for boys and senior men were slightly higher than those for their female counterparts. Chlorinated compounds contributed most to human exposure.

Risk assessment was calculated using the equations described earlier for toxic and carcinogenic compounds. Due to their carcinogenicity, the calculations for TCEP and TBP were made using the margin of exposure (MOE) model. The risk factors (Rt) and margins of exposure (MOE) were therefore calculated for the studied compounds in each scenario and subgroup. The values obtained for the toxic and carcinogenic compounds are shown in Figs. 4 and 5, respectively. The risk factors (Rt) for the non-toxic compounds ranged from $9.06 \bullet 10^{-5}$ (girls in the lower-bound scenario) to $1.55 \bullet 10^{-3}$ (senior men in the upper-bound scenario), which are extremely low and may be considered of low concern. On the other hand, MOE values

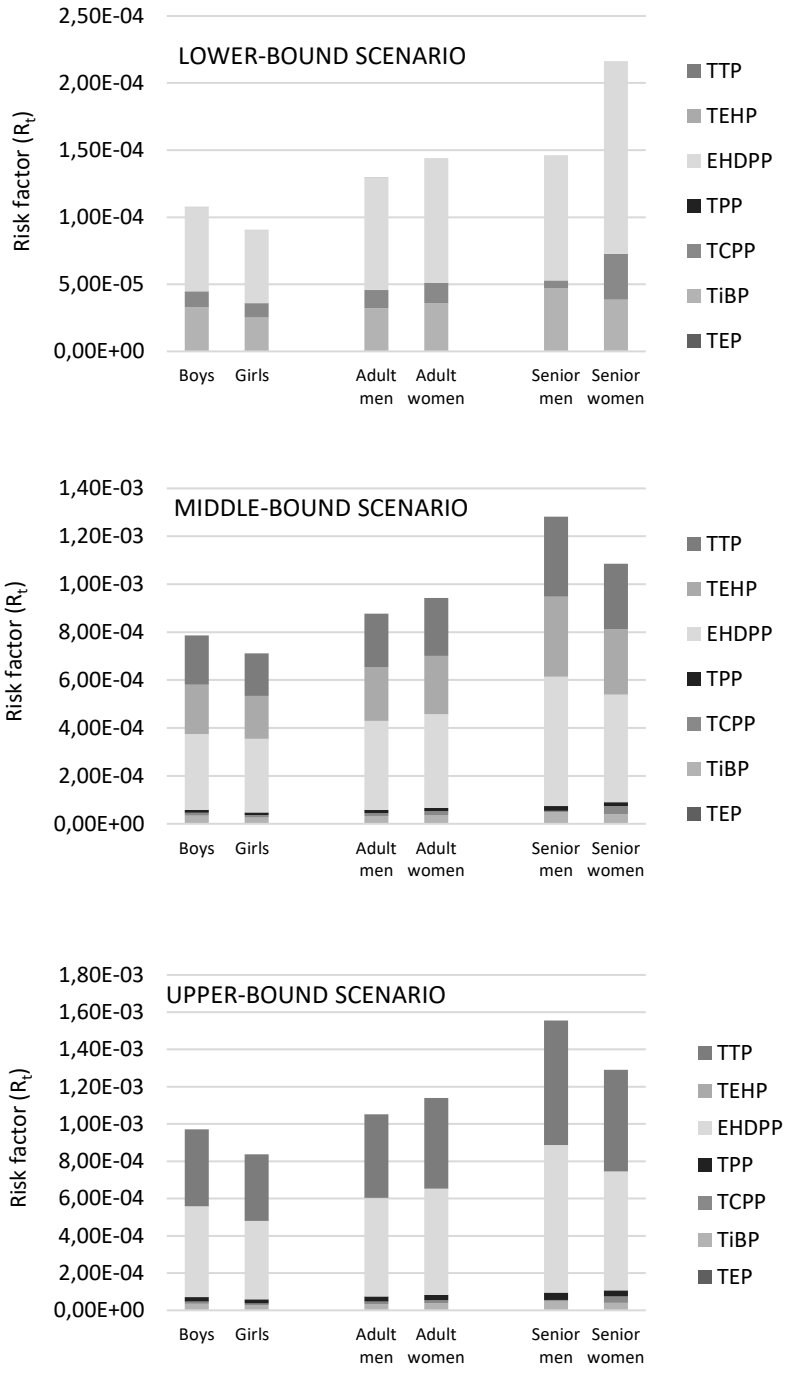

Fig. 4. Risk assessment associated to toxic OPEs exposure through fish consumption of the population of Tarragona. Estimation for low, middle and upper-bound scenarios

ranged from $4.79x10^7$ (senior men in the upper- bound scenario) to $11.0x10^7$ (girls at the lower-bound scenario), which are 5000 to 11,000 times higher than the $1x10^4$ value set by the EFSA Scientific Committee as the limit for low concern due to carcinogenic effects [50]. Therefore, for the population of Tarragona, Spain, no health risk is associated with the intake of OPEs though fish consumption.

4. Conclusions

We have successfully developed a new, cheaper and easy to handle method for determining nine organophosphate esters in fish samples. QuEChERs extraction followed by use of novel Lipi- Filtr® clean up cartridges and GC-QqQ-MS/MS is shown to be effective in determining these compounds in both low- and high- lipid fish species. We analysed four fish species using this method and obtained concentrations of TiBP, TBP, TCPP, EHDPP and TEHP ranging from 0.45 to 3.35 ng g^{-1} (w.w.). The first three of these compounds were present in all four species. Dietary exposure of OPEs via consumption of these species was estimated under three scenarios for six population subgroups in Tarragona, Spain. The results showed that the elderly populations (senior men and women) were the most exposed subgroups due to their higher consumption of fish in their daily diet. Finally, health risk calculations showed there is no current risk to human health associated with the in- take of OPEs via consumption of these fish species. All in all, our method obtained reliable data on OPE concentrations for carrying out risk calculations in fish samples from various species. However, further studies with other fish species based on the population's global diet should be conducted to provide more extensive health risk data.

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Supplementary Material

Determination of organophosphate ester flame retardants and plasticisers in fish samples by QuEChERS followed by gas chromatography-tandem mass spectrometry. Exposure and risk assessment through fish consumption.

CONTENTS

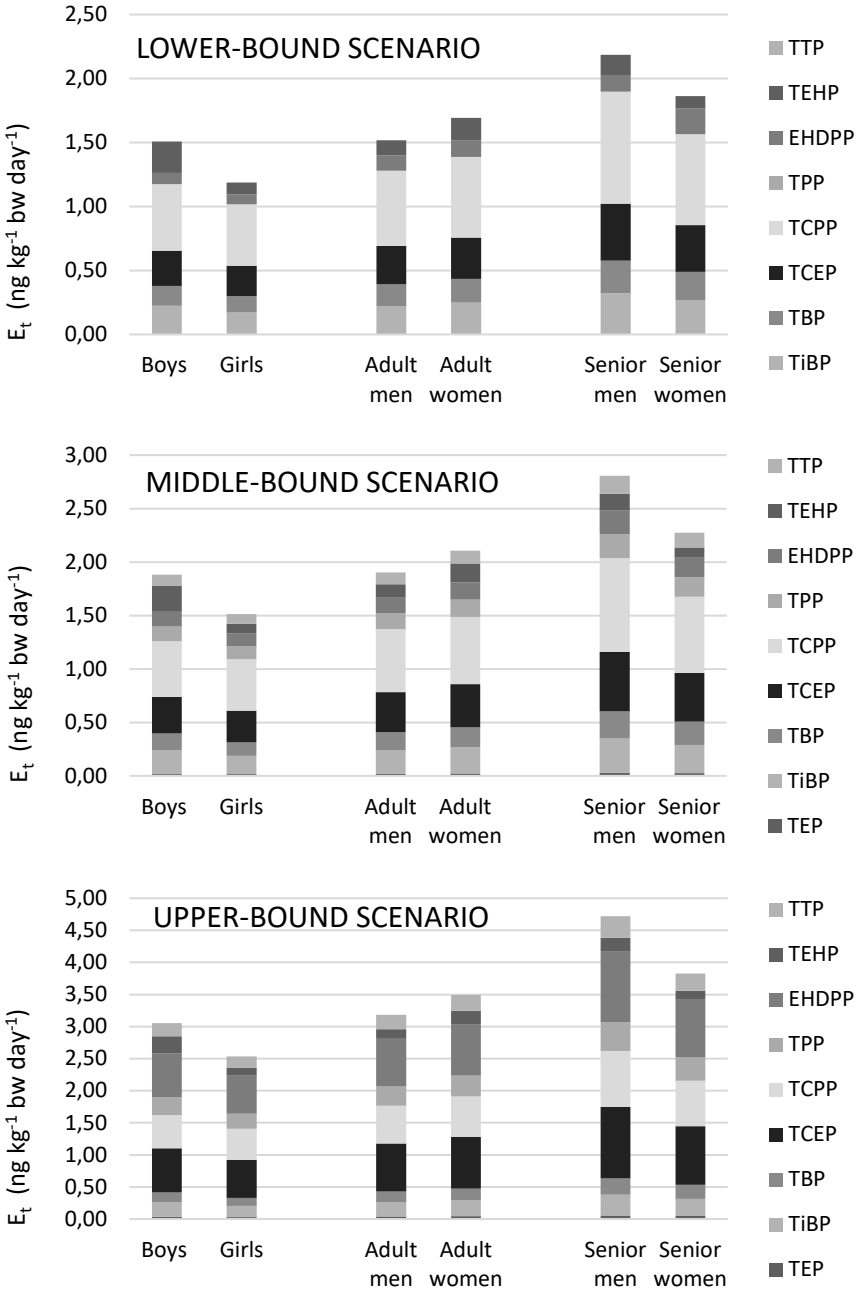

Figure S1. Human exposure to OPEs through fish consumption of the population of Tarragona. Estimation for low, middle and upper-bound scenarios.

3.1.3. Determination of benzothiazoles, benzotriazoles and benzenesulfonamides in seafood using QuEChERS followed by gas chromatography - tandem mass spectrometry: method development and risk assessment

DETERMINATION OF BENZOTHIAZOLES, BENZOTRIAZOLES AND BENZENESULFONAMIDES IN SEAFOOD USING QUECHERS FOLLOWED BY GAS CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY: METHOD DEVELOPMENT AND RISK ASSESSMENT

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Abstract

The common use of benzothiazoles, benzotriazoles and benzenesulfonamides has led to widespread ubiquity in several environmental fates. Their occurrence in edible fish could represents an additional exposure route for population. The present study aims to develop a method for the simultaneous determination of these three compound families in seafood samples. Based on QuEChERS extraction, different salt combinations and clean-up strategies have been evaluated to achieve the highest recoveries while reducing the matrix effect in low and high lipidic content species. The best results were obtained with the original method salts and the lipid-selective push-through clean-up, which combined with gas chromatography-tandem mass spectrometry led to recoveries between 50-112% with negligible matrix effects and method detection limits between 0.15 – 9.50 ng g-1 d.w. The application of the method to commercially available samples confirmed the presence of BTs as well as BSAs, with the latter being determined in seafood for the first time. Exposure and risk assessment calculations indicated a minor risk for the population when consuming fish.

Highlights

- A QuEChERS-GC-MS method enabled the determination of BTs, BTRs and BSAs in seafood.
- LipiFiltr appeared as the best clean-up option for lipid removal.
- BSA and Et-p-TSA have been determined in seafood for the first time.
- Risk linked to BTs, BTRs and BSAs intake via seafood ingestion is of low concern.

1. Introduction

The population has adapted to a way of life where daily commodities are fundamental. From furniture to electronics, all the surrounding objects have been produced and contain many chemical compounds. Some of them are produced in massive amounts every year, leading to a widespread ubiquity of chemicals that nowadays have been indicated as potential contaminants. It is by these negative effects that organisations such as the OECD (Organisation for Economic Co-operation and Development) and the EPA (Environmental Protection Agency) have listed some of them as High Production Volume chemicals (HPVs) [1]. This list is intended to prioritize the research on compounds with huge production over the year to collect enough data to evaluate the consumer, occupational and environmental exposure. The list contains compounds from different families including benzothiazoles (BHTs), benzotriazoles (BTRs), and benzenesulfonamides (BSAs). These compounds are usually found on their own, i.e., 1-H-benzothiazole, 1-H-benzotriazole, and benzenesulfonamide, or as derivatives of the previously stated (usually methylated and halogenated variants). Both benzothiazoles and benzotriazoles share a heterocyclic structure with a 1,3-thiazole or a 3 nitrogen five-membered respectively [2,3]. As for benzene-sulfonamides, these are formed by a benzene or toluene ring with a sulfonamide moiety where the derivates are formed. As high production volume chemicals, these are used in industrial as well as consumer products with applications including vulcanization acceleration, antifreeze formulations, UV light stabilizers, or dye synthesis precursors, among others [4]. Thus, their extensive use in many everyday commodities has become a major source of these reaching the environment and becoming a potential threat. The presence of these benzocompounds in environmental fates such as air [5–7], water, soil, and even biota [8,9] has been described by several authors. The pathway followed by these compounds to reach the aquatic environment starts upon their release from household and industrial runoffs. Their removal in wastewater treatment plants (WWTP) is mostly ineffective, especially for some benzenesulfonamides, which concen-trations become 4-6 times higher in the effluents due to biodegradation / bio-conversion processes [10]. Because of their high mobility, their path reaches the aquatic environment, where benzothiazoles, benzotriazoles and benzensulfonamides become available to aquatic organisms [4]. Their possible accumulation on seafood becomes a problem as these could then become accessible to population through dietary intake, thus turning seafood

consumption in an alternative exposure route.

With seafood species being considered high complexity samples due to the huge number of interferences and coextracted compounds found when analysing them, it represents an analytical challenge to determine compounds. Regarding the methodologies used for the determination of these compounds in seafood samples, scarce information is found in the literature. Whereas benzotriazoles have been determined using different extraction techniques with good results such as ultrasonic assisted extraction (USE) [11,12], QuEChERS [9,13,14] or pressurized liquid extraction (PLE) [15], methods involving the determination of benzothiazoles in seafood samples are scarce in literature (use of USE [11] and PLE [8]) and no methods exists for the determination of benzenesulfonamides. After the extraction, the use of an efficient clean-up is mandatory for this type of samples. On this purpose, authors have used strategies such as solid-liquid extraction (SPE) or dispersive solid-liquid extraction (d-SPE) using sorbent like florisil, PSA or C18 [9,11,13,14,16]. Regarding the determination techniques used for the analysis of these samples, chromatography, especially liquid chromatography, is the go to technique, mostly coupled with tandem mass spectrometry to reach the desirable levels [8,13,17].

To the best of our knowledge, this is the first method for the simultaneous determination of benzotriazoles, benzothiazoles and benzenesulfonamides in fish samples. Moreover, the study aims to find an effective clean-up to diminish the matrix effect to a level where it can be disregarded, thus enabling the use of a single external calibration in the analysis of several fish and seafood species. The application of the following method to commercially available fish specimens is intended to provide reliable data for the calculation of exposure and risk assessment related with the ingestion of these compounds via dietary intake.

2. Materials and methods

2.1. Chemicals and reagents

The present study focuses on the determination of three different compound families: benzothiazoles, benzotriazoles, and benzenesulfonamides. The studied benzothiazoles included 1-H-benzothiazole (BT), 2-aminobenzothiazole (NH2BT), chlorobenzothiazole (ClBT), 2-hydroxybenzothiazole (OHBT), and 2-(methylthio)-benzothiazole (MeSBT). Benzotriazoles comprised 1-H-benzotriazole (BTR), 4-methyl-1-H-ben-

zotriazole (4TTR), 5-methyl-1-H-benzotriazole (5TTR), and 5,6-dimethyl-1-H-benzotriazole (XTR). Finally, benzenesulfonamides included benzenesulfonamide (BSA), para-toluenesulfonamide (p-TSA), N-methyl-para-toluenesulfonamide (Me-p-TSA), N-ethyl-para-toluenesulfonamide (Et-p-TSA), and ortho-toluenesulfonamide (o-TSA). All compounds were purchased at Sigma-Aldrich (Darmstadt, Germany). Deuterated compounds supplied by LGC Standards (Teddington, UK) were used as internal standards: d4-benzothiazole (d4-BT) for benzothiazoles, d4-benzotriazole (d4-BTR) for benzotriazoles, and d4-p-toluenesulfonamide (d4-p-TSA) for benzenesulfonamides. Individual stock solutions of 1000 mg L-1 were prepared using ethyl acetate as solvent. Working mixture solutions of 100 mg L-1 of each of the compound families (benzothiazoles, benzotriazoles, and benzenesulfonamides) were prepared also with ethyl acetate and stored at -20ªC until further dilution. Solvents used throughout the study were all GC grade with purity higher than 99%. Ethyl acetate, acetone, and acetonitrile were purchased from Scharlab (Barcelona, Spain), hexane from JT Baker (Deventer, The Netherlands). Ultrapure water was obtained from a Synergy water purification system from Millipore (Massachusetts, USA). Helium and nitrogen used for the chromatographic and mass spectrometer systems were both of a 99.999% purity and were supplied by Carburos Metálicos (Tarragona, Spain). Materials such as QuEChERS salt packets (Original, AOAC, and EN15662), disposable syringes, and PTFE filters were purchased from Scharlab (Barcelona Spain) while LipiFiltr cartridges were supplied by Carlo Erba (Barcelona, Spain).

2.2. Sample preparation

Fish samples used for the method optimization process as well as for the application on real samples were purchased at different local stores. Specimens of cod (*Gadus morhua*), mackerel (*Scomber vincialis*), squid (*Loligo vulgaris*) and tuna (*Thunnus thynnus*) were purchased and divided into two groups depending on the lipid content: low lipid content (1% for cod and 6% for squid) and high lipid content (17% for mackerel and 16% for tuna). Samples were covered with aluminium foil once purchased to prevent cross-contamination and kept at -24ºC until further treatment. Due to the study being focused on the ability to obtain data for its posterior use on ingestion risk, only the edible parts of the specimens were selected. Therefore, muscle fillets were dissected from each specimen and then freeze-dried using a miVac Duo freeze-drying system from Genevac (Ipswich, United Kingdom). Moisture content (%) of the species was calculated as the weight difference

before and after lyophilization. Once completely dried, samples were ground and homogenised. Finally, samples were sieved through a 500 µm mesh to obtain a homogenised particle size and kept in a glass container until their analysis. As for spiked samples, these were prepared by adding the compound mixture at the desired concentration to the freeze-dried fish samples previously weighted and covered in GC grade acetone. Samples were mixed so as to propitiate the contact between the matrix and the compounds and then left to dry overnight.

2.3. QuEChERS extraction method

The followed procedure for the determination of benzothiazole, benzotriazole, and benzenesulfonamide in fish samples was adapted from a previous study for which organophosphate esters were determined in the same matrix [18]. The procedure was carried out using 100 mg of lyophilized sample weighted in a 50 mL centrifuge tube. Next, 10 mL of ultrapure water and 10 mL of acetonitrile were added to the tube, and it was vortex mixed for 1 minute. A packet containing the salts corresponding to the original method for QuEChERS extraction (4g of magnesium sulfate and 1 g of anhydrous sodium acetate) was added to the tube and it was subsequently vortex mixed for 3 minutes. Tubes were then centrifuged at 4000 rpm for 5 min using a Hettich Universal 32R centrifuge (Tuttlingen, Germany) and the supernatant (acetonitrile layer) was collected and passed through a LipiFiltr push through cartridge. Extracts were then evaporated to circa 0.5 mL under a gentle nitrogen stream. The extracts were filtrated with a 0.22 µm PTFE syringe filter and spiked with the internal standard at 50 µg L^{-1}. Finally, these were reconstituted to 2 mL using ethyl acetate.

2.4. Gas chromatography-tandem mass spectrometry

The extracts obtained by the extraction process were analysed using an Agilent 8890 GC system coupled to an Agilent 7000D triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Samples were automatically injected by a PAL RSI 120 automatic injector from CTC Analytics (Zwingen, Switzerland). The chromatographic system worked with two HP-50 capillary columns (15 m x 0.25 mm i.d. and 0.25 µm film thickness) from Agilent connected by a pneumatic switching device (PSD) which permitted the use of backflush mode. Target analytes were separated using an oven temperature program as follows: the temperature was initially set at 75ºC (held for 2.87 minutes) and then raised at a rate of 15ºC/min to 300ºC (held for 5 minutes) with a total run time of 22.87

minutes and a 6-minute solvent delay. The carrier gas used was helium at a constant rate of 1.2 mL/min in the first column and 1.4 mL/min in the second column. A volume of 25 µL was injected using an Agilent Multi-Mode Inlet (MMI) in solvent vent mode with an initial temperature of 75ºC held for 0.37 min and subsequently raised at 600ºC/min rate to 325 ºC (held for 5 min). Solvent vent mode operated at a vent flow of 120 mL/min and 5 psi for 0.37 min and purge to split vent was set at 60 mL/min at 2.87 min. The triple quadrupole mass spectrometer system operated in electron ionisation mode at 70 eV with temperatures of 230ºC, 150ºC, and 150ºC for ion source, quadrupole 1 and quadrupole 2, respectively. For the quantification, multiple reaction monitoring mode was used with one quantifier transition (Q) and two qualifier transitions (q) to identify the target compounds. Data analysis was performed using Agilent MassHunter Workstation (Qualitative and Quantitative Analysis) version 10.0. Table 1 gathers the information regarding the parameters used for the GC-MS/MS(QqQ) system.

2.5. Exposure assessment and risk characterization

Values of exposure associated with the dietary intake of benzothiazoles, benzotriazoles, and benzene-sulfonamides by the population of Tarragona were assessed by using a methodology that combines the concentrations found of the target compounds in the fish samples along with the fish consumption data. Exposure values (E_t) were obtained as the result of applying equation 1 to the obtained dataset [19]. Thus, the global human exposure for contaminant *t* appears as the sum of the contribution of each fish species, considering the mean consumption of the individual species *f* (C_f, g kg bw^{-1} day^{-1}) previously normalised by dividing by the mean body weight for each gender/age population subgroup, and the concentration of each of the analysed contaminants t for the species *f* ($X_{t,f}$, ng g^{-1}) expressed in wet weight basis (w.w.).

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

Based on the method detection limit (LOD) and method quantification limit (LOQ), three scenarios were estimated for below-LOQ and non-detected compounds. An upper-bound scenario (UB) was assessed assuming LOQ and LOD concentration values for below-LOQ and non-detected compounds. The middle-bound scenario was estimated as half of the concentration of the LOQ and LOD for below-LOQ and non-detected compounds respectively. Finally, for the lower-bound scenario (LB), compounds under the LOQ were estimated as the LOD value and non-

Table 1. GC-QqQ-MS/MS parameters.

	Rt (min)	MRM Q[a]	CE[b] (eV)	MRM q[c]	CE[b] (eV)
Benzothiazoles					
BT	9.28	135 → 108	51	135 → 82 135 → 69	33 49
ClBT	10.16	169 → 108	21	169 → 82 169 → 69	37 53
NH_2BT	13.27	150 → 96	27	150 → 70 150 → 123	43 15
MeSBT	12.77	181 → 148	15	181 → 135 181 → 108	19 41
OHBT	13.79	151 → 70	41	151 → 96 151 → 123	25 13
BT-d4	9.26	139 → 70	51	139 → 84 139 → 112	35 21
Benzotriazoles					
BTR	11.98	119 → 63	37	119 → 52 119 → 91	33 7
4TTR	12.42	104 → 77	15	104 → 51 133 → 104	35 13
5TTR	12.78	104 → 77	15	104 → 51 133 → 104	35 13
XTR	13.27	118 → 65	27	118 → 91 147 → 91	15 31
BTR-d4	11.96	123 → 95	7	123 → 66 123 → 53	39 35
Benzenesulfonamides					
BSA	13.07	157 → 93	5	157 → 77 77 → 51	25 19
o-TSA	13.58	90 → 63	33	91 → 65 106 → 79	13 15
p-TSA	13.88	171 → 65	45	91 → 65 155 → 91	15 9
Me-p-TSA	13.77	185 → 91	29	155 → 91 91 → 65	9 15
Et-p-TSA	13.89	155 → 91	11	155 → 65 184 → 91	33 19
p-TSA-d4	13.86	95 → 68	11	159 → 95	9

a Multiple Reaction Monitoring Quantification transitions
b Collision Cell energy
c Multiple Reaction Monitoring Qualifier transitions

detected compounds concentrations were reduced to zero. Calculations regarding the risk assessment were performed using the methodology suggested by the European Safety Authority (EFSA) and the Environmental Protection Agency (EPA) [20,21]. Non-genotoxic and non-carcinogenic compounds were evaluated using NOAEL (non-observed-adverse-effect - level) values. These compound-dependant values were used to calculate the acceptable daily intake (ADI_t), which can be easily obtained by dividing the oral NOAEL value by an uncertainty factor. The risk associated with the intake of the compound t can be then calculated using equation 2 where ADI_t, E_t, and R_t are the acceptable daily intake, the global dietary exposure, and the risk factor, respectively.

$$R_t = (E_t / ADI_t) *100 \qquad \text{Eq. 2}$$

2.6. Study of population and data collection

The data concerning the consumption of seafood by people living in Tarragona was gathered from a survey conducted in Catalonia over the year comprised between 2002 and 2003 (ENCAT, 2003) [22]. The data is segregated by population groups depending on age and gender. A total of 2160 individuals with ages ranging between 10-80 (1164 women and 996 men) participated in the study which consisted of a food frequency questionnaire and two 24-hour recalls along with anthropometric measurements (height, body weight, circumferences, etc.) and a socioeconomic general questionnaire. Moreover, participants also administered data about food habits, chronic diseases, physical activity, smoking, and nutrition knowledge. All the data obtained was collected by trained dietitians via personal interviews at the volunteers' homes. The mean consumption of the species analysed in the present study by the population of Tarragona is summarized in Table 2.

3. Results and discussion

3.1. GC-MS conditions

Benzotriazoles, benzothiazoles, and benzenesulfonamides are polar compounds not chromatographically easy to determine, especially when using GC. Their low signal response along with its usually tailed peaks turns this group of compounds, benzotriazoles specifically, into an analytical challenge. Tailing peaks can be associated with instrumental-related issues, however, for this group of benzocompounds, specifically benzo-triazoles, tailing can be linked to the higher polarity of the compounds, which may cause secondary interactions with

Table 2. Mean consumption (g day^{-1}) by the population of Tarragona of the determined fish species.

	FoodEx2 code	Boys (10-19)	Adult men (20-65)	Senior men (>65)	Girls (10-19)	Adult women (20-65)	Senior women (>65)
Mackerel	A02CV	0.36	1.13	0.50	0.32	1.27	2.86
Squid	A02JJ	1.88	3.17	3.18	5.18	3.17	0.77
Tuna	A02DX	0.71	1.62	1.07	0	1.45	0.52
Cod	A02BV	2.13	4.18	8.08	0.60	4.61	8.15

active sites present in the liner and column. The low signal response can be typically addressed with strategies such as sample treatment enrichment, higher injection volumes and/or sample weights, or even derivatization. However, one of the disadvantages of using QuEChERS is its low enrichment factors, especially since a minimum weight of sample is used to prevent higher matrix effects. Thus, experiments regarding injection volume and different sample weights were performed. In this case, the use of derivatization agents was avoided to simplify the procedure. The use of the Multi-Mode Inlet (MMI) present in the instrument allowed the injection of 25 µL of the sample extract using the Solvent vent mode. Parameters such as oven initial temperature, injector initial temperature, vent time, vent pressure, vent flow and purge time and flow were optimised using the Solvent Elimination Wizard present in the Agilent MassHunter software. As regards the increase of sample weight, further explanation of its optimisation can be found in the following section regarding the extraction optimisation. Backflush conditions were also optimised using the backflush wizard integrated with the Agilent MassHunter Software. The optimisation of the solvent vent mode and the application of higher injection volumes lead to the obtention of peaks of higher area at lower concentrations, thus leading to lower instrumental limits (low µg L^{-1}) and counteracting the dilution of the extraction process.

3.2. Extraction procedure optimisation

When working with fish or seafood samples, techniques such as solid-liquid (SLE) or ultrasonic-assisted extraction (USE) may require large solvent volumes and long extraction times to achieve effective extractions. Additionally, using techniques such as pressurized liquid extraction (PLE) reduces the time and solvent needed, but highly rises the amount of matrix components present in the extract. For these reasons,

QuEChERS are believed to be a good alternative. This extraction technique, as its acronym states, enables quick, easy, cheap, effective, rugged, and safe determinations. Its' use has widely expanded from its initial purpose. Since its first application for the determination of pesticides, QuEChERS are nowadays being used for the extraction of many other compound families [23–25].

For the present work, QuEChERS extraction efficiency of the target compounds in fish samples was studied. The extraction procedure was optimised using two different fish species with different lipid contents. Thus, cod and mackerel were used to perform the optimisation procedure representing low and high lipid content species, respectively. The differentiation of lipid content was based on the analytical differences that may be found when analysing fish species with a higher lipid content, as fat present in some of the species may interfere in the determination process concerning extraction efficiency and enhanced or suppressed signal (matrix effect).

The optimisation process for the present method relied on the selection of the salts used for the extraction as well as the selection of the most convenient clean-up strategy in terms of apparent recovery and matrix effect reduction for the studied compounds.

Previous method optimisation procedures started with the selection of the QuEChERS extraction salts, comparing the apparent recoveries of raw extracts directly injected after the salt addition step. However, in the present study, the injection of raw extracts (no clean-up) resulted in the apparition of high levels of interferences, thus hindering the determination of some of the compounds and making it impossible to obtain useful data. The appearance of these interferences may be a direct cause of the lipid content present in the samples combined with the use of a higher injection volume. Therefore, it was decided to start the optimisation process by selecting the most efficient clean-up procedure to reduce the interferences found in the raw extracts and enable the obtention of useful data.

3.2.1. Clean-up selection

Fish samples are considered of high complexity due to the elevated content of compounds present in the matrix. Among them, lipids stand out as one of the biggest challenges to overcome. Fat content may act as a compound retainer, thus hindering the extraction process and reducing its efficiency and it could also affect the instrumental signal, enhancing or suppressing it. It is, therefore, necessary to assess convenient strategies to minimize its effect when extracting fish samples.

Following QuEChERS extraction, several clean-up methodologies have been described in the literature amongst which dispersive solid-phase extraction (d-SPE) is one of the most common due to its simplicity. Other strategies include liquid-liquid extraction, solid-phase extraction, or the use of novel devices such as LipiFiltr. Dispersive-solid phase extraction is based on the use of sorbents to absorb interferences that might be present in the QuEChERS extract once the extraction is performed. The obtained extract is transferred to a tube containing the sorbent and is then thoroughly mixed. The variety of sorbents susceptible to being used for d-SPE is large and can be easily accessed commercially.

Nevertheless, as the present study focuses on the reduction of lipid content from the extract, only sorbents susceptible to achieve that aim were tested. Thus, sorbents such as primary secondary amine (PSA) and a combination of the same sorbent with C18 were tested. On the other hand, liquid-liquid extraction using hexane and LipiFiltr cartridges were also tested as alternatives to d-SPE. The use of hexane as a cleaning solvent has been described by several authors to extract lipids from matrices with high fat content, therefore, the use of hexane LLE after QuEChERS extraction may be an easy to handle alternative to d-SPE. Moreover, devices such as LipiFiltr have recently appeared as new alternatives to the previously mentioned. In this case, LipiFiltr is intended to selectively subtract lipids from QuEChERS extracts using a sorbent contained in a push-through cartridge.

Experiments comparing the previously stated strategies (d-SPE with PSA and PSA+C18, hexane LLE and LipiFiltr) were conducted using spiked samples of fish species from both lipid contents (cod and mackerel). Therefore, 0.1 g of fish were weighed in a glass tube and subsequently spiked with the target compounds following the protocol for spiked samples described in the previous section. QuEChERS extraction step was performed with the original method salts packet for all the experiments. Once the extracts were obtained, those were treated differently for each of the procedures. (a) For d-SPE clean-up, the extracts were transferred to a tub containing 200 mg PSA or 200 mg PSA + C18 (1:1) and vortexed for 3 min. The extracts were then centrifuged at 4000 rpm for 5 min and the supernatant (liquid layer) was transferred to a 20 mL glass vial. (b) For LLE, the extracts were transferred to a separatory funnel and 10 mL of hexane were added. The funnel was thoroughly mixed for 3 min and then left for 5 min to stabilize. The bottom layer (ACN) was collected in a 20 mL glass vial. (c) For LipiFiltr clean-up, the extracts were passed through a LipiFiltr push-trough

cartridge using a 5 mL syringe and then collected in a 20 mL glass vial. All the extracts obtained from the different clean-up strategies were then evaporated under a nitrogen stream until circa 0.5 mL and were subsequently filtrated with a 0.22 µm PTFE syringe filter and reconstituted with EtAc to 2 mL after including the I.S. mixture. The same procedure was performed for blank samples to subtract its signal from the spiked ones. The different clean-up strategies were evaluated in terms of apparent recoveries (%R, sample spiked prior extraction) and matrix effect (%M.E., extract spiked prior reconstitution). Figures 1 and 2 gather the results obtained for the studied clean-ups.

Observing the results, benzenesulfonamides signal is mostly enhanced for all the studied congeners while, on the other hand, benzothiazoles tend to suppress the signal when in presence of the matrix. As for benzotriazoles, mild signal enhancement and suppression is observed, being 5TTR the most affected compound. All in all, matrix effect values were comprised between -45 and 20% for the low lipid content species and between -43% and 30% for the high lipid content species. The effect of lipid content is notable in special for benzenesulfonamides, where a higher tendency to enhance the matrix is observed. The use of LipiFiltr clearly diminishes the matrix effect for all the target compounds, thus being the best option to use as clean-up. Regarding the apparent recoveries when including the clean-up step, better recoveries are obtained when using LipiFiltr. This could be explained due to the reduction of the matrix effects along with an easier to handle procedure, as no additional transfers are needed when using a push-trough cartridge, whereas d-SPE and LLE require an additional transfer and phase separation step. For these reasons, LipiFiltr was selected as the optimal clean-up strategy.

3.2.2. QuEChERS salts packet optimisation

Once the clean-up strategy was selected, the content of the salts packet used for the QuEChERS extraction was evaluated. Three different mixtures were tested corresponding to three different official methods described for QuEChERS extraction: the original method (OR), the Association of Official Agricultural Chemists method (AOAC), and the European Committee for Standardization EN 15662 method (EN). The methods mainly differ on the type and number of salts present in the mixture. Thus, the OR method firstly described by Anastassiades et al. [26] contains 4 g of magnesium sulfate and 1 g of sodium chloride. On the other side, the AOAC and the EN methods also contains 6 g of magnesium sulfate and

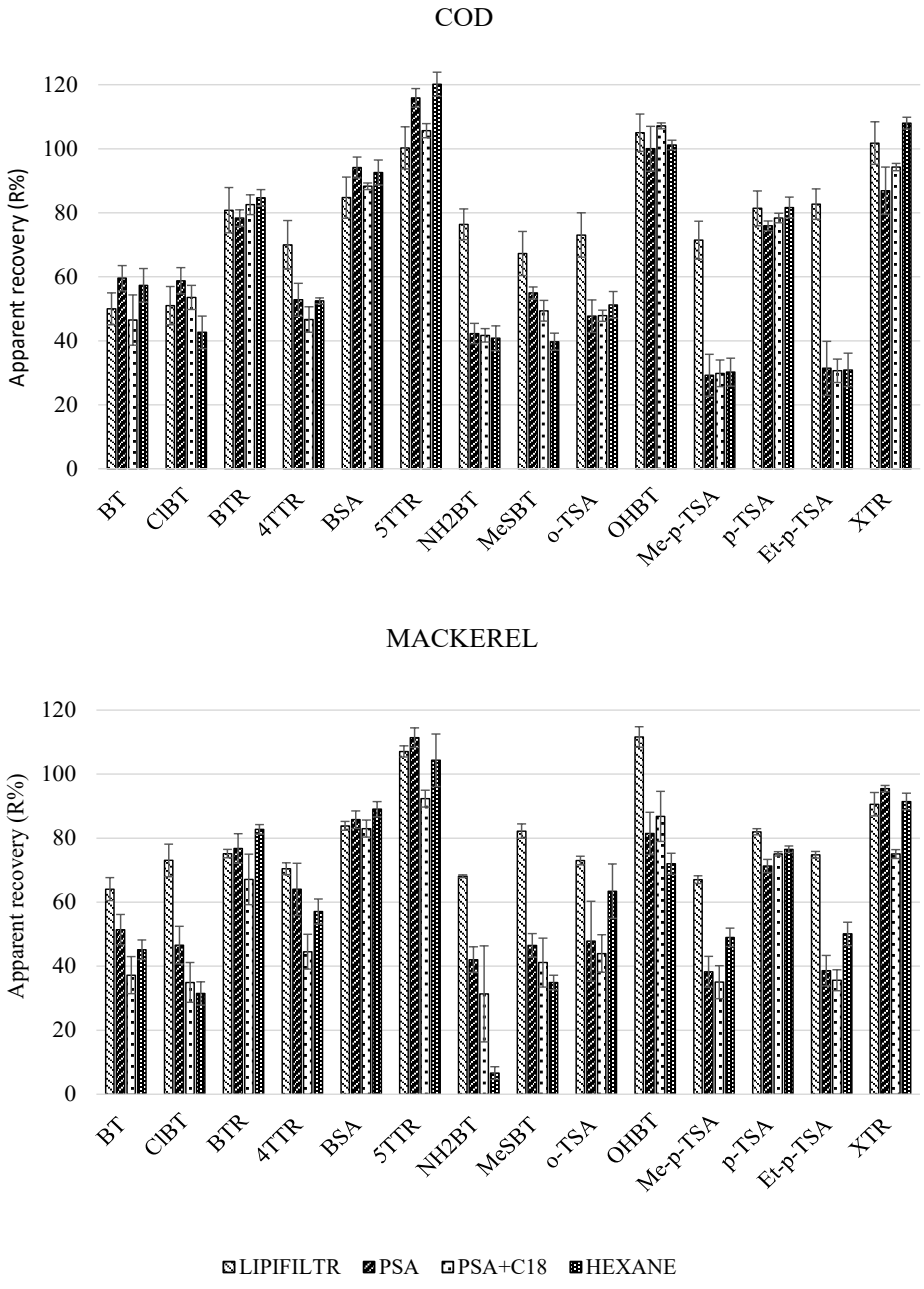

Figure 1. Apparent recoveries (R%) (50 ng g^{-1} d.w. n=3, %RSD < 19%) obtained for the different clean-up strategies: LipiFiltr, PSA, PSA+C18 and hexane.

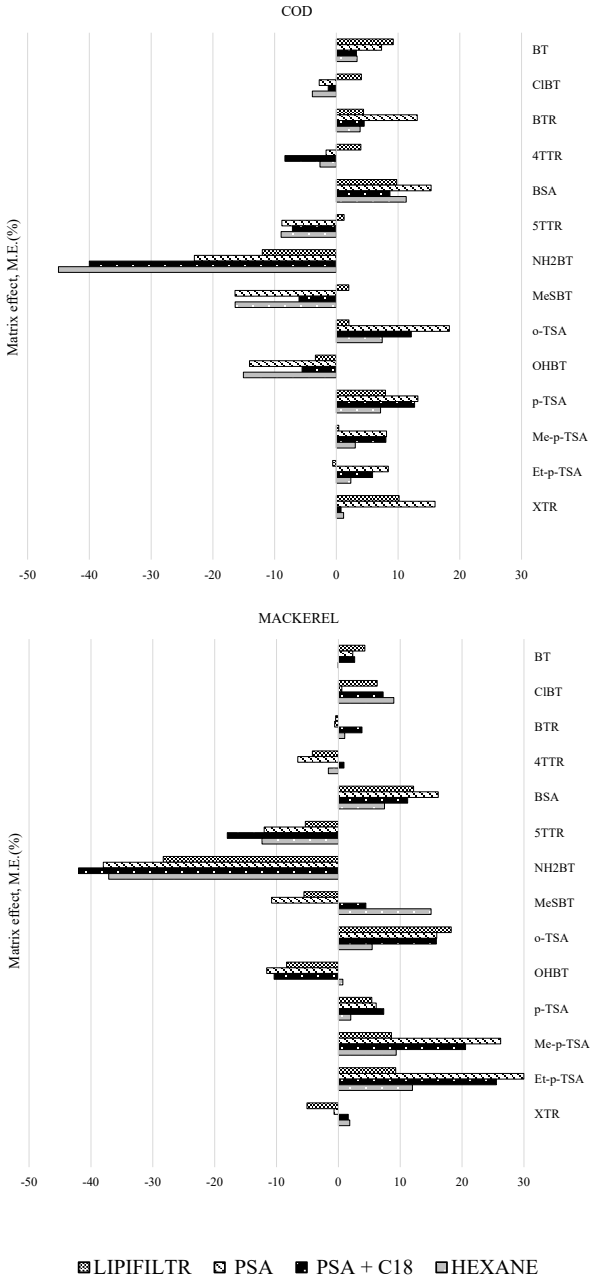

Figure 2. Matrix effects (M.E. %) (50 ng g^{-1} d.w. n=3, %RSD < 22%) obtained for the different clean-up strategies: LipiFiltr, PSA, PSA+C18 and hexane.

include a buffer to regulate pH, in the case of AOAC method, 1.5 g of anhydrous sodium acetate. As for the EN method, the mixture is composed of 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium hydrogen sesquihydrate, and 1 g of sodium citrate. Extraction of cod and mackerel samples were performed per triplicate for each of the salt combinations and the apparent recoveries of the method were evaluated. Results obtained can be observed in Figure 3. Differences were mostly negligible for most of the compounds in both fish species, being 5TTR and p-TSA the compounds with the most notable recovery differences. The use of the original salts resulted in a higher recovery value for these compounds in both low and high lipid content species. ClBT also depicted better recoveries when working with the original method in presence of the low lipid matrix. All in all, no significant differences were observed for these three method alternatives. Thus, the original method was selected due to its slightly better results for some of the compounds.

3.2.3. Sample weight

Once the clean-up and the salts were selected, some experiments were performed to observe the extraction and clean-up potential of the developed method. Thus, extractions of different sample weights were carried out to analyse the effect of a higher sample weight on the apparent recoveries and the method efficiency. The increase of sample weight could lead to a higher response, thus reducing the method limits of detection. Sample weights of 0.1, 0.25, and 0.5 g were used to perform the analysis with the same conditions previously described. Figure 4 comprises the apparent recoveries found when the sample weight was increased. As observed in Figure 4, most of the compounds were affected by the variation of sample amount for both species, reducing its apparent recovery as the quantity of sample to be extracted increased. This can be related to the sample/solvent ratio. Increasing the amount of sample without raising the volume of extraction solvent could cause the compounds not to be extracted enough from the matrix, as the contact between the sample and the solvent is reduced. Moreover, the matrix effects were also evaluated in this case, and no significant differences were observed when compared with the matrix effects previously achieved, thus reinforcing the theory of the extraction sample/solvent ratio not being correct. Even though a higher volume of extraction solvent could have been tested to enhance efficiency when extracting higher sample volumes, the experiments were eluded as a higher acetonitrile volume would drive to longer evaporation times and could also affect the efficiency of the LipiFiltr.

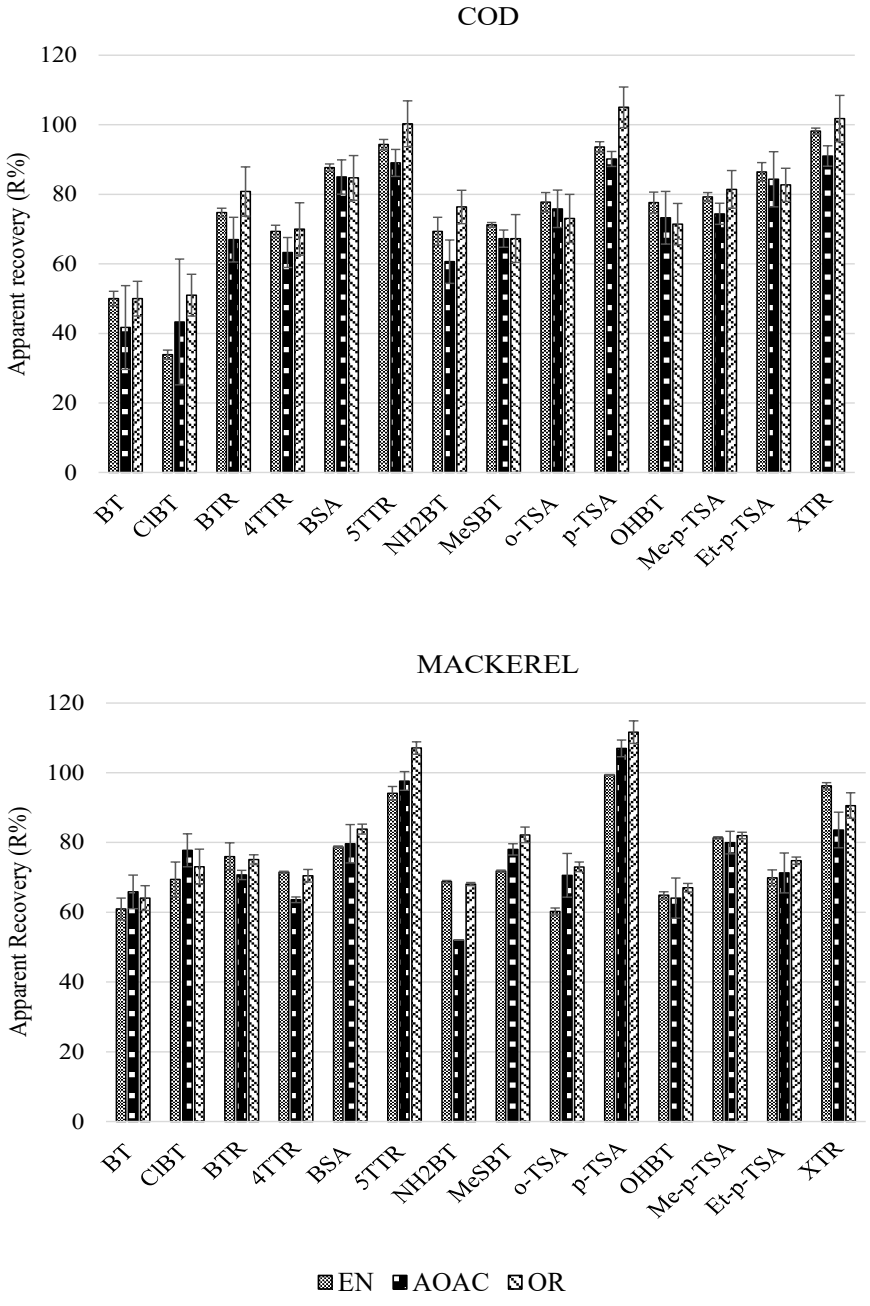

Figure 3. Apparent recoveries (R_{app}% 50 ng g^{-1} d.w. n=3, %RSD < 16%) when using different extraction salts packets corresponding to three different official methods.

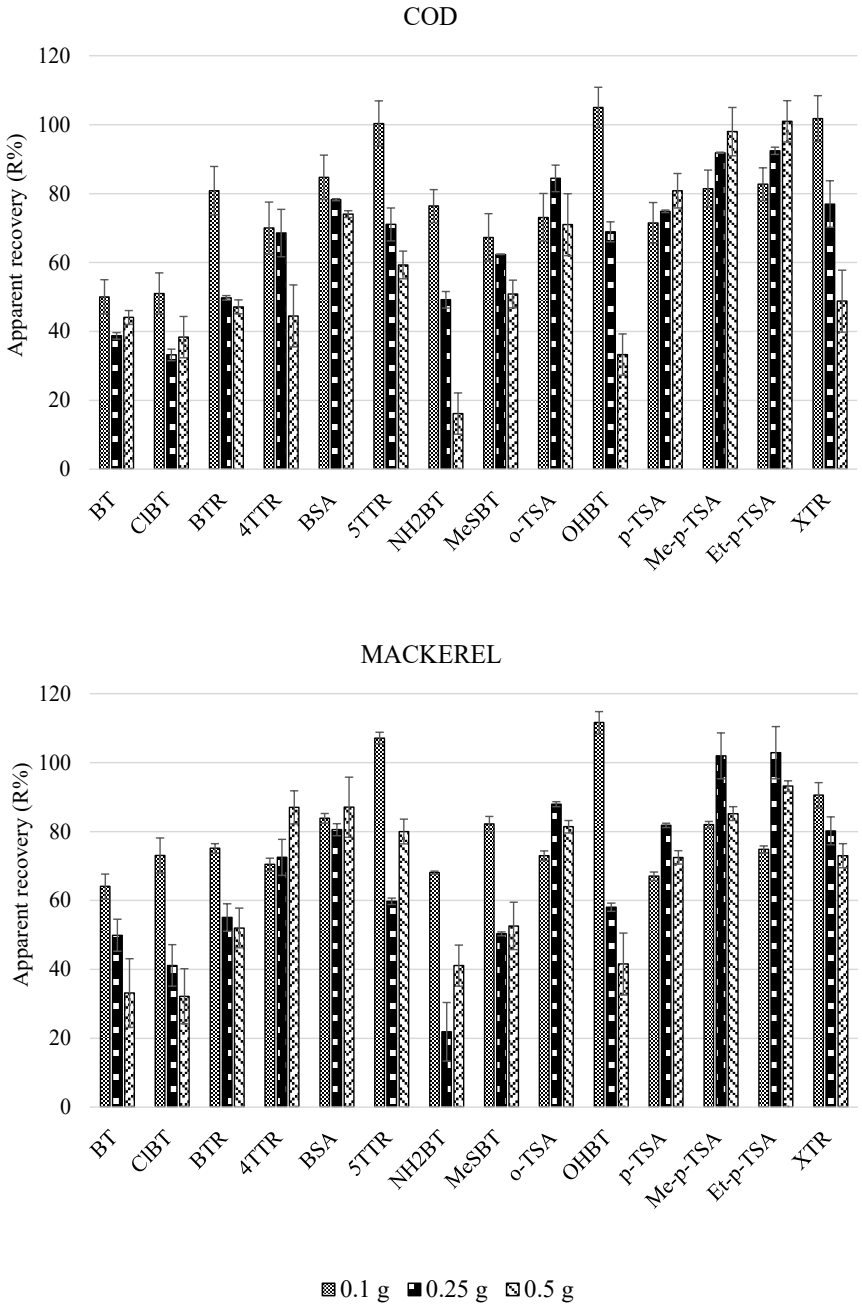

Figure 4. Effect on apparent recoveries (R_{app} % 50 ng g^{-1} d.w. n=3, %RSD < 21%) of the amount of fish extracted.

3.3. Method performance parameters

To test the efficiency of the developed method, parameters such as apparent recoveries (%Rapp), linearity, method detection limits (LOD), method quantification limits (LOQ), repeatability (intra-day), and reproducibility (inter-day) were evaluated under the optimal conditions. Cod and mackerel samples, being species with low and high lipid content, respectively, were used to perform the evaluation.

Matrix effects previously calculated were found between -10 and 10% for most of the compounds in both species, indicating that the matrix effect was negligible. Thus, the quantification of the analytes was performed with external calibration using solvent standards directly injected to the system along with the apparent recoveries depending on the lipid content of the analysed species.

Method limits of detection (MDL) and quantification (MQL) were estima-

Table 3. Method quality parameters.

Compound	Instrumental linear range ($\mu g\ L^{-1}$)	r^2	Low lipid content			High lipid content		
			R_{app} (%)	MDL ($ng\ g^{-1}$)	MQL ($ng\ g^{-1}$)	R_{app} (%)	MDL ($ng\ g^{-1}$)	MQL ($ng\ g^{-1}$)
Benzothiazoles								
BT	0.025 - 75	0.9995	50	0.40	1.00	64	0.30	0.75
ClBT	0.01 - 75	0.9996	51	0.20	0.40	73	0.15	0.25
NH_2BT	0.025 - 50	0.9958	76	0.20	0.70	68	0.20	0.75
MeSBT	0.01 - 75	0.9997	67	0.15	0.30	82	0.10	0.25
OHBT	0.05 - 75	0.9967	105	0.15	0.95	112	0.15	0.90
Benzotriazoles								
BTR	0.25 - 50	0.9990	81	0.60	6.20	75	0.65	6.65
4TTR	0.25 - 50	0.9997	70	2.15	7.15	70	2.15	7.15
5TTR	0.25 - 75	0.9992	100	1.50	5.00	107	1.40	4.65
XTR	1 - 100	0.9986	102	9.50	19.50	91	11.00	22.00
Benzenesulfonamides								
BSA	0.025 - 75	0.9999	85	0.15	0.60	84	0.15	0.60
o-TSA	0.5 - 100	0.9999	73	6.85	13.70	73	6.85	13.70
p-TSA	0.025 - 75	0.9992	81	0.15	0.65	82	0.15	0.60
Me-p-TSA	0.025 - 75	0.9994	71	0.15	0.70	67	0.15	0.75
Et-p-TSA	0.025 - 75	0.9999	83	0.15	0.60	75	0.15	0.70

[a.] Apparent recovery.
[b] MDL: method detection limit.
[c] MQL: method quantification limit.

ted for each of the target compounds based on their instrumental detection (direct injection, S/N = 3) and quantification limits (first concentration on the instrumental linear range), the application of the apparent recovery values (depending on its lipid content) and the dilution calculations from the extraction procedure. For compounds present in the matrix, MDL were estimated as the blanks' average signal plus three times the standard deviation of the blank. Thus, MDLs were comprised between 0.15 – 0.40 ng g^{-1} d.w. for BTs, 0.60 – 9.50 ng g^{-1} d.w. for BTRs and 0.15 – 6.85 ng g^{-1} d.w. for BSAs. Regarding the MQLs, those were set between 0.30 – 1.00 ng g^{-1} for BTs, 5.00 – 19.50 ng g^{-1} for BTRs and 0.60 – 13.70 ng g^{-1} for BSAs. Repeatability and reproducibility were tested at 10 and 50 ng g^{-1} d.w. with values of %RSD below 14 and 12% (repeatability) and below 19 and 15% (reproducibility). The quality parameters of the method can be found at Table 3.

3.4. Application to commercial fish samples

The developed method was applied to samples of four different fish species bought in local markets from Tarragona, Spain. Thus, triplicates of cod (*Gadus morhua*), mackerel (*Scomber vincialis*), squid (*Loligo vulgaris*) and tuna (*Thunnus thynnus*) were analysed using the QuEChERS LipiFiltr GC-QqQ-MS/MS method. To confirm the presence of the target compounds in the samples, parameters such as retention time (min) and the presence of the qualifier transitions as well as their ratios were compared with the data obtained from the analytical standard. Table 4 comprises the target compounds found in at least one of the samples. Concentrations are expressed in wet weight basis so as to enable an easier comparison between values found in the literature and the present study as well as to perform the exposure and risk calculations.

Four benzothiazoles and two benzenesulfonamides were quantified in the analysed samples, whereas no BTRs were found. ΣBTs highest values were found for squid (49.8 ng g^{-1} w.w.) followed by cod (25.9 ng g^{-1} w.w.), tuna (11.7 ng g^{-1} w.w.) and finally mackerel (6.4 ng g^{-1} w.w.). BT appeared in all the analysed samples with the highest concentrations ranging between 42.7 ng g^{-1} w.w. (squid) and 1.4 ng g^{-1} w.w. (tuna). Benzothiazole concentrations were followed by NH2BT (2 – 5,9 ng g^{-1} w.w.), OHBT (1.6 – 3.1 ng g^{-1} w.w.) and MeSBT (1.1 – 1.9 ng g^{-1} w.w.). Regarding the distribution of the compounds based on the lipid content, species with higher lipidic content such as tuna and mackerel appear to have lower levels of benzothiazoles. For ΣBSAs, cod presented the highest values (43.8 ng g^{-1} w.w.), followed by tuna (9.4 ng g^{-1}

Table 4. Concentrations (ng g^{-1} wet weight) of target compounds in samples of different seafood samples.

Compound	Cod (77%) *Gadus morhua*	Mackerel (71%) *Scomber vincialis*	Tuna (70%) *Thunnus thynnus*	Squid (77%) *Loligo vulgaris*
BT	24.3	5.3	1.4	42.7
MeSBT	n.d.	1.1	n.d.	1.9
BSA	15.0	n.d.	9.4	n.d.
OHBT	1.6	n.d.	4.4	3.1
NH_2BT	<MQL	n.d.	5.9	2.0
Et-p-TSA	28.8	n.d.	<MQL	3.3
Σ BTs	25.9	6.4	11.7	49.8
Σ BSAs	43.8	n.d.	9.4	3.3

*n.d.: not detected; <MQL; under the method quantification limit; % Moisture content in brackets

w.w.) and squid (3.3 ng g^{-1} w.w.). Samples of cod and tuna presented BSA concentrations of 15 and 9.4 ng g^{-1} w.w., whereas Et-p-TSA was detected in cod and squid at 28.8 and 3.3 ng g^{-1} w.w., respectively. Opposite to benzo-thiazoles, no tendency is observed for their distribution in different lipid content species.

To date, few studies have proven the presence of benzothiazoles in seafood samples. Trabalón et al. [8] and Jia et al. [11] reported the presence of BT, NH_2BT, and MeSBT in samples of fish (species from local markets of Tarragona, Spain) and molluscs (from the Bohai Sea, China), respectively. Both studies agreed on BT as the benzothiazole with the highest detection frequency as well as with the highest concentrations. Regarding the concentrations, substantial differences were found in both studies, as per the fish from Tarragona, BT concentrations ranged between 13-82 ng g^{-1} d.w., whereas molluscs from the Bohai Sea reached concentrations up to 13400 ng g^{-1} d.w. (132 – 13400 ng g^{-1}, geometric mean: 595 ng g^{-1}). Squid appeared as the species with the highest concentrations in both the Trabalón et al study and the present study. NH_2BT and MeSBT concentrations were comprised between 11-70/ 0.165 ng g^{-1} d.w. and 11-24/ 14.2 ng g^{-1} d.w. for both studies, respectively. Other benzo-thiazoles were also detected in some of the studies, Trabalón et al. reported the presence of ClBT at 6-38 ng g^{-1} d.w., whereas Jia et al. along with another study conducted by Chen et al. [27] on the presence of benzothiazoles in fish specimens from Chung-li City (Taiwan) reported the presence of OHBT at 20.1 ng g^{-1} d.w. and 15.4 – 26.1 ng g^{-1} d.w., respectively. Related with the literature,

the concentrations found in the analysed samples are similar to the ones found in fish samples from the other studies. It is necessary to point out that the concentrations obtained from the other studies are given in a dry weight basis, thus being slightly higher than the ones from the present study. The detected congeners (BT, NH_2BT, MeSBT and OHBT), their distribution (BT with the highest concentrations) and their concentrations all convey with what the literature has reported to date about the presence of benzothiazoles in fish samples.

Regarding benzenesulfonamides, no data is available on their presence in seafood as this is the first method developed for their determination in this matrix. The presence of these compounds in water is also scarcely found in the literature. Hence, some studies have reported their presence in superficial waters and wastewater influent and effluent samples. Richter et al. [28] studies in Berlin's WWTP showed the presence of BSA in both influent and effluent water samples with values of 0.05 µg L^{-1} and 0.35 µg L^{-1}, respectively. Further studies by the same authors also reported higher levels of BSA in effluent samples than in influent samples, thus reporting a possible biodegradation /bioconversion of higher molecular weight sulfonamides to BSA during the WWTP process. A high removal efficiency of p-TSA at WWTP (~90%) was also denoted by the authors [10]. Regarding other compounds such as Et-p-TSA, Jover et al. [29] and Herrero et al. [30] reported concentrations of 0.086 and 36 -70 µg L^{-1} for influent and 0.122-0.831 and 0.026-0.074 µg L^{-1} for effluent, respectively. A similar correlation as with BSA appears for Et-p-TSA, with higher concentrations also found in effluent waters, suggesting again a possible bioconversion. BSA and Et-p-TSA have also been detected in superficial waters. Samples from Spanish rivers such as Besós, Ebre, Llobregat or Turia presented concentrations between 0.006 – 0.011 ng L^{-1} for BSA and 0.002 – 0.155 ng L^{-1} for Et-p-TSA [29,30]. On account of the previously reported data, the migration of these compounds towards aquatic environments and further incorporation in seafood seems a possible alternative. The concentrations found in the present study are in agreement with the said theory, as the major detected benzenesulfonamides in the analysed samples, BSA and Et-p-TSA, coincide with the benzenesulfonamides with the higher release from WWTP. Moreover, the lack of p-TSA detection could also be linked to the high removal efficiency.

3.5. Dietary intake and risk assessment

The dietary exposure of BTs, BSAs and BTRs was calculated for six subgroups (boys / girls, adult

Table 5. Exposure assessment (ng kg^{-1} bw day^{-1}) of Tarragona's population to the intake of HPVs through seafood ingestion.

	Species	Boys (10-19)			Adult men (20-65)			Senior men (>65)			Girls (10-19)			Adult women (20-65)			Senior women (>65)		
		LB	MB	UB	LB	MB	UB	LB	MB	UB	LB	MB	UB	LB	MB	UB	LB	MB	UB
ΣBTs	Cod	2.25	2.26	2.28	1.57	1.58	1.59	3.04	3.06	3.08	0.63	0.64	0.64	1.74	1.75	1.76	3.07	3.09	3.11
	Mackerel	0.09	0.10	0.10	0.10	0.11	0.11	0.05	0.05	0.05	0.08	0.09	0.09	0.12	0.12	0.13	0.26	0.27	0.28
	Tuna	0.33	0.34	0.34	0.27	0.27	0.28	0.18	0.18	0.18	0.00	0.00	0.00	0.24	0.24	0.25	0.09	0.09	0.09
	Squid	3.74	3.74	3.75	2.25	2.26	2.26	2.26	2.26	2.27	10.30	10.32	10.34	2.25	2.26	2.26	0.55	0.55	0.55
	Total	**6.41**	**6.44**	**6.47**	**4.20**	**4.22**	**4.24**	**5.53**	**5.55**	**5.58**	**11.01**	**11.04**	**11.07**	**4.35**	**4.37**	**4.39**	**3.96**	**4.00**	**4.03**
ΣBSAs	Cod	3.73	4.04	4.34	2.62	2.83	3.04	5.06	5.47	5.88	1.05	1.14	1.22	2.88	3.12	3.36	5.10	5.52	5.93
	Mackerel	0.00	0.05	0.11	0.00	0.06	0.12	0.00	0.03	0.05	0.00	0.05	0.10	0.00	0.07	0.14	0.00	0.15	0.30
	Tuna	0.28	0.38	0.48	0.23	0.31	0.39	0.15	0.21	0.26	0.00	0.00	0.00	0.21	0.28	0.35	0.07	0.10	0.13
	Squid	0.25	0.52	0.80	0.15	0.31	0.48	0.15	0.32	0.48	0.68	1.44	2.20	0.15	0.31	0.48	0.04	0.08	0.12
	Total	**4.26**	**5.00**	**5.73**	**2.99**	**3.52**	**4.04**	**5.36**	**6.02**	**6.68**	**1.73**	**2.62**	**3.51**	**3.24**	**3.78**	**4.32**	**5.21**	**5.84**	**6.48**
ΣBTRs	Cod	0.00	0.59	1.17	0.00	0.41	0.82	0.00	0.79	1.59	0.00	0.17	0.33	0.00	0.45	0.91	0.00	0.80	1.60
	Mackerel	0.00	0.11	0.22	0.00	0.12	0.25	0.00	0.05	0.11	0.00	0.10	0.19	0.00	0.14	0.28	0.00	0.31	0.62
	Tuna	0.00	0.22	0.43	0.00	0.18	0.35	0.00	0.12	0.23	0.00	0.00	0.00	0.00	0.16	0.31	0.00	0.06	0.11
	Squid	0.00	0.52	1.03	0.00	0.31	0.62	0.00	0.31	0.62	0.00	1.42	2.85	0.00	0.31	0.62	0.00	0.08	0.15
	Total	**0.00**	**1.43**	**2.86**	**0.00**	**1.02**	**2.04**	**0.00**	**1.28**	**2.55**	**0.00**	**1.69**	**3.37**	**0.00**	**1.06**	**2.12**	**0.00**	**1.24**	**2.49**

men/women and senior men/women) based on lower-, middle- and upper-bound scenarios. Exposure values ranged between 3.96 – 11.07 ng kg^{-1} bw day^{-1} for ΣBTs, 1.73 – 6.68 ng kg^{-1} bw day-1 for ΣBSAs and 1.06 – 3.37 ng kg^{-1} bw day^{-1} for ΣBTRs. The highest exposure values for ΣBTs and ΣBTRs were observed for the girls' subgroup in all the studied scenarios, whereas ΣBSAs exposure reached the highest level for senior men. On the other side, adult men and senior women are the subgroups with the lowest exposure values. Table 5 compiles the obtained values for all the studied subgroups and scenarios. Benzothiazole is the only compound of the present study with an associated NOAEL value corresponding to an oral repeated dose. Thus, a pTDI was estimated from the oral NOAEL of 5.1 mg kg^{-1} bw day^{-1} [8] by dividing the present value by an uncertainty value of 100 to compensate the species difference, the human variation and the use of a sub chronic study for the obtention of the value. As BT appeared in all the analysed samples, only one scenario was evaluated. Calculated dietary risk values (Rt) were comprised

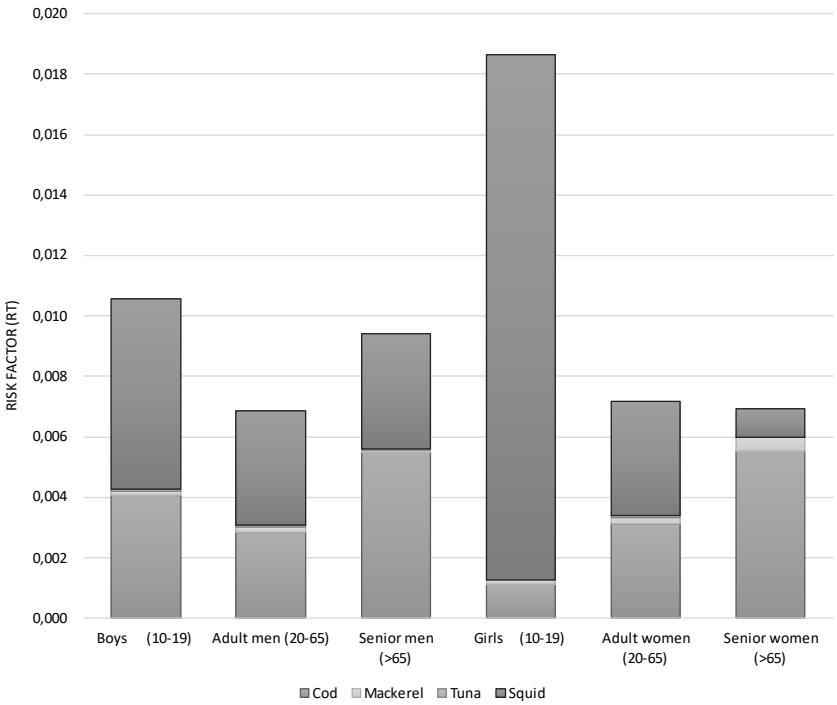

Figure 5. Risk factor for benzothiazole intake through seafood ingestion.

between $6.9x10^{-3}$ (senior women) and $1.86x10^{-2}$ (girls), being the later the group with the higher value. Regarding the contribution of each of the species, squid and cod are the responsible of up to 94-99% of the risk of each of the subgroups. Even though no threshold value to compare has been established for BT to date, the obtained values ($<1.86x10^{-2}$) suggest that minor risk is expected from the ingestion of the studied HPVs via dietary intake.

4. Conclusions

The use of QuEChERS extraction combined with a novel clean-up device such as LipiFiltr followed by GC-QqQ-MS/MS has been successfully developed for the determination of benzothiazoles, benzotriazoles and benzene-sulfonamides in seafood samples. Problematic analytical issues like matrix effect caused by a high complexity matrix such as seafood or low instrumental signal have been solved using a simple dilution of the extract combined with an effective clean-up step whereas instrumental signal was effectively enhanced thanks to large volume injection. All in all, the proposed method enables the analysis of a wide range of seafood species while avoiding the necessity of using matrix matched calibrations due to the negligible matrix effects. Its easy procedure and reliable quality parameters turn this method into a useful tool to generate data from both the aquatic environment contamination and the populations' exposure and risk associated with seafood intake. On this behalf, the method has been successfully applied to different local seafood samples, obtaining data on its benzothiazole, benzotriazole and benzene-sulfonamides contamination. The analysed samples reported the presence of low levels (<42.7 ng g^{-1} w.w.) of some benzothiazoles (BT, MeSBT, NH_2BT and OHBT) along with benzenesulfonamide and ethyl-p-toluenesulfoamide, which, to the best of our knowledge, have been determined in seafood samples for the first time. Studies on the exposure and risk associated with the dietary intake of the analysed samples by the population of Tarragona suggested values between 3.96 – 11.07 ng kg^{-1} bw day^{-1} for ΣBTs, 1.73 – 6.68 ng kg^{-1} bw day^{-1} for ΣBSAs and 1.06 – 3.37 ng kg^{-1} bw day^{-1} for ΣBTRs, being girls the population subgroup with the highest exposure. Regarding the risk assessment, BT showed a risk factor of $1.86x10^{-2}$ which indicates a minor risk of developing chronic adverse effects due to the ingestion of this compound through the diet. Future research should be focused on the development of novel methodologies including more target compounds of these high production volume chemical families. Moreover, assays aiming to elucidate NOAEL values of the compounds of the present study

are urgent, as to date, only benzothiazole has an assigned value.

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3.1.4. Discussion of results

The following section aims to summarize the most relevant findings of the previously presented studies while discussing some topics of interest.

Synthetic musk fragrances are easily found in many environmental fates, and as previously observed, seafood is not an exception. The use of SPME Arrow enabled the determination of these compounds in seafood samples with an instrumental signal 10 times higher than when using conventional SPME. Parameters such as coating appear to be critical, especially depending on the properties of the target compounds. In this case, PDMS and PDMS/DVB, which are usually used to retain low-mid polarity compounds with high volatilities, showed the best results according to what was expected. Extraction time and temperature were also evaluated reaching the equilibrium at 45 min and an optimal temperature of 100ºC. Other parameters such as the addition of water and desorption conditions did not show improvement when changed, thus being avoided (no water was added) or set at the previously tested conditions (desorption at 250ºC for 3 min). The reported method successfully determined the presence of HHCB and AHTN in all the analysed samples at concentrations between 6.5 – 17.5 ng g^{-1} d.w. and 2.9 – 5.1 ng g^{-1} d.w., respectively, thus demonstrating the consequences of the excessive use of these compounds in some personal care products. Other congeners such as DPMI, ATII and HHCB-lactone were detected under the limit of quantification. Regarding the experience of optimising a novel device for which no extensive data is found in the literature, the procedure was indeed really similar to the optimisation of a conventional SPME fiber. However, the major drawback was the system modifications. SPME Arrow requires a series of modifications in the chromatographic system (wider injection port) and in the autosampler (specific SPME Arrow holder), which, by the time the group was performing the modifications, were not easy to accomplish. The device was recently being tested, and these modifications were not easy to access, at least in a commercial way (major vendors did not have parts of the GC system that could fit the specific requirements, e.g. the wider injection port). Moreover, the addition of a new module required the reprogram of the autosampler for it to learn the new positions of the modules and perform the correct sequence throughout the extraction.

Since its appearance, the use of SPME Arrow as a substitute for conventional SPME has been increasing over the years. Literature compiles methods using this technique for fish samples determination of terpenes [1], polycyclic aromatic hydrocarbons [2] and even untargeted microbial metabolites [3] among others. Its use is also extended to other compounds and matrices e.g. volatile organic compounds in grapes [4], pesticides in water [5], furans in food [6] or fatty acid methyl esters in aqueous samples [7]. The broad and increasing use of this technique is linked to its good results, but also its compliance with the principles of green chemistry, as it can be fully automated and solventless. Moreover, vendors such as Agilent Technologies, Thermo Fisher or Gerstel have already created toolkits containing the necessary modules and have adapted their systems to fit with SPME Arrow, making it easier to incorporate this technique into laboratories. In my opinion, working with SPME Arrow overtakes conventional SPME results with a more robust device. The enlarged sorbent phase volume works nicely for compounds with low polarity and high volatility, but maybe gets more complicated with compounds with more polar characteristics due to them being, possibly, more difficult to desorb from an enlarged phase volume. This could be easily solved with the selection of a different phase. Another aspect that draws my attention is the use of SPME Arrow in immersion mode. Higher phase volume clearly equates to better sensitivity, but it should be noted that it could also mean that other compounds from the matrix can also enhance their presence, which could lead to a higher matrix effect. Further studies on the applicability of this technique are needed for compounds and matrices covering a wider range of characteristics, however, it would not be something unexpected to see SPME Arrow replace conventional SPME in a near future.

Results from the SPME Arrow method were promising, however, as previously explained, the acquisition of a new GC-MS/MS system with a triple quadrupole analyser made us put SPME Arrow aside until its future implementation in the new system. Hence, QuEChERS was selected as an easy and reliable technique to perform the extractions based on the previous experience of the group on the determination of synthetic musk fragrances in seafood samples. The use of QuEChERS was initially planned to focus on OPEs, as BTs, BTRs and BSAs were intended to be determined using liquid-chromatography. Moreover, previous

attempts to simultaneously determine BTs, BTRs and BSAs in seafood using PLE followed by SPME-GC-MS/MS(IT) were not fruitful, as only BTs exhibited good results. Halfway through the development of the OPEs-QuEChERS method, it was thought that maybe it was interesting to try QuEChERS for the determination of BTs, BTRs and BSAs followed by GC-MS/MS(QqQ). Hence, another method was developed with the intention of them being combined in the future if possible.

Regarding the methods developed for the determination of organophosphate esters and benzothiazoles, benzotriazoles and benzenesulfonamides, both methods were based on a previously reported method by Trabalón et al. [8] for which synthetic musk fragrances were determined in fish samples using QuEChERS. The election of QuEChERS as an extraction technique comes from its easy and quick procedure, as described by its name, but also because of its ability to extract a wide range of compounds with different physicochemical properties. As previously explained in section 1.2.1., QuEChERS combine useful characteristics such as wide selectivity and a minimal requirement of sample weight to extract, which is something greatly useful for seafood-focused methodologies. QuEChERS procedure includes an initial salting out step, where compounds extracted from the solid sample to the aqueous phase, are then forced into the organic layer. The combination of salts used differs from the preferred method. As observed in the results, lower molecular weight organophosphates (TEP, TiBP and TBP) were better extracted when using the EN 15662 method (4 g of magnesium sulfate + 1 g of sodium chloride + 0.5 g of sodium hydrogen citrate sesquihydrate + 1 g of sodium citrate) whereas the Original method (4 g of magnesium sulfate + 1 g of sodium chloride) seemed to work better for most of the benzothiazoles, benzotriazoles and benzenesulfonamides. Even though QuEChERS reduces the amount of matrix extracted, a convenient clean-up is also needed. Methods previously described in the literature for the determination of organophosphate esters in fish samples required exhaustive clean-ups to get rid of the matrix before its analysis, usually with techniques such as gel permeation chromatography [9,10] or multiple solid phase extraction cartridges [11,12]. Our goal was to develop a simple and effective method while reducing the number of steps needed. Initially thought to be solved with a dispersive solid phase extraction combining different sorbents, the apparition of LipiFiltr easily solved the problem. The evaluation of different

strategies resulted in LipiFiltr being the best option in terms of apparent recoveries and matrix effect reduction. This could be explained due to the selective lipid removal of the cartridge but also the reduction of the number of steps to perform. While liquid-liquid extraction and dispersive solid phase extraction require the separation of phases and/or centrifugation, LipiFiltr consists of a push-through cartridge. For most of the compounds of both methods, matrix effects were reduced to values between -10 – 10%. The reduction of matrix effects was also accomplished due to a dilution of the extract. For both methods, a dilution of the initial concentration present in the solid seafood sample was needed going from 0.5 g to 2 mL (4 times dilution) for organophosphate esters and from 0.1 g to 2 mL (20 times dilution) for benzothiazoles, benzotriazoles and benzenesulfonamides. Nevertheless, the dilution of the extracts combined with the low GC-MS signal of some compounds, especially benzothiazoles, benzotriazoles and benzenesulfonamides, requires the use of large-volume injection.

Large volume injection using the Multi-mode Inlet (MMI) of the GC-MS system has been key to accomplishing the required limits of detection. Using solvent vent mode, the signal of compounds such as TCEP, TCPP or TTP has been greatly enhanced when injecting 5 µL of the extract. Hence, method detection limits for organophosphate esters reached values between 0.05 – 2.00 ng g^{-1} d.w.. Benzothiazoles, benzotriazoles and benzenesulfonamides became a challenge as their GC-MS signal is notably poor. This combined with the 20 times dilution needed to diminish the matrix effect, led to the injection of 25 µL of the extract in the system. Hence, method detection limits for these compounds in seafood were comprised of between 0.15 – 11.00 ng g^{-1} d.w. being benzotriazoles the group with the highest detection limits.

If we were to sum up the highlights of the studies regarding methodology, the reduction of time achieved by the use of a single disposable LipiFiltr cartridge should be mentioned. The percolation of a QuEChERS extract through a push-through cartridge, which can be done in seconds, counteracts the time-consuming use of multiple d-SPE, SPE or GPC steps. Moreover, the use of LipiFiltr was not previously reported by other authors for the determination of any of these compounds given the novelty of the device. Nevertheless, a proper clean-

up is not enough to achieve these results. The possibility of injecting up to 25 µL should also be underlined. The combination of many other things such as a suitable sample weight or a proper dilution also influence the overall outcome, but the difference was clearly marked by the application of these two strategies. Even so, some drawbacks should be mentioned. LipiFiltr push-through cartridges are not reusable and especially not cheap, a fact that increases the overall method cost as well as increases the waste produced per analysed sample. However, the price could be on the level of the use of other non-reusable SPE cartridges or the cost of a GPC system. On the other hand, the injection of up to 25 µL requires a higher volume syringe. In our case, given the PAL autosampler and the software used, the system requires a syringe with double the volume intended to be injected, which means the syringe needs to reach up to 50 µL. Prices of typical 5-10 µL syringes are, as expected, much lower than a 50 µL one. Finally, the injector used was an MMI in solvent vent mode. This means the injection of high volumes is restricted to the possession of an injector supporting large-volume injection.

As for the occurrence of the target compounds in commercial seafood, at least one of the target OPEs appeared in all the samples, being TiBP, TBP, and TCPP the most detected compounds in concentrations ranging from 0.62 – 1.48 ng g^{-1} w.w. , 0.45 – 0.89 ng g^{-1} w.w. and 1.52 – 2.78 ng g^{-1} w.w., respectively. The concentrations and profiles follow the trends found in the literature as previously stated in section 1.2.1, for which OPEs tend to appear at low ng g^{-1} being chlorinated compounds the most detected at the highest concentrations. Regarding BTs, BTRs and BSAs, both BTs and BSAs were detected in all the samples, whereas no BTRs were found, probably due to this being not present or at lower concentrations than the detection limit. BT has a 100% detection frequency with values between 1.4 – 42.7 n ng g^{-1} w.w., being squid the species with the highest concentration. BSA and Et-p-TSA were detected for the first time in seafood species at concentrations from 9.4 – 15.0 ng g^{-1} w.w. and 3.3. – 28.8 ng g^{-1} w.w., respectively.

Exposure and risk assessment calculations were performed for the last two studies to exemplify the potential of the data obtained through the developed methods. Elder men/women and girls were the population subgroups with the

highest exposures towards OPEs and BTs+BSAs, respectively. The exposure was highly linked to the species that these subgroups mostly consume, and the concentrations found in the study. Even though being exposed to these chemicals, risk calculations indicated that a low level of concern risk was posed to the intake of HPVs via seafood consumption. Despite the results showing a low concern, it should be noted that most of the compounds included in the BTs, BTRs and BSAs do not have an associated NOAEL, which blocks the calculation of the risk associated with the ingestion of these compounds. The data needed for these calculations can be considered the bottleneck of risk assessment. Compounds with a relatively novel significance are mostly understudied, which is the case of BTs, BTRs, and BSAs. At the moment, only benzothiazole (BT) has an associated NOAEL value, meaning this is the only compound that can be further assessed in terms of risk. Dose-response studies are fundamental, and further research on this topic is needed to elucidate whether population health is put at stake or not.

All in all, the proposed methodologies show great potential to be used as tools to obtain reliable data on concentrations of HPVs in seafood samples, thus enabling calculations of population exposure and risk assessment of HPVs intake via seafood consumption.

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3.2. Occurrence and risk assessment of high production volume chemicals in commercial seafood

Seafood takes an important role in healthy and balanced diets. The benefits associated with its consumption cover a wide range, going from the prevention of cardiovascular disease to better cognitive function [1]. Spain, being influenced by the Mediterranean diet, relies on seafood consumption as part of its everyday diet. For instance, the 2021 annual consumption report of the Spanish Ministry of Agriculture, Fisheries and Foods reported an annual consumption of 22.72 kg of seafood per capita [2].

High production volume chemicals occurrence in seafood not only assures the presence of these compounds in the aquatic environment but also points out the possible ingestion of these compounds by the population via seafood consumption. In the previous section, different chromatographic methods were developed to be used as tools to evaluate the contamination status of seafood and obtain useful data to assess exposure and risk towards HPVs.

The present section aims to determine HPVs in commercially available seafood specimens. In this case, synthetic fragrances were dismissed as a previous study of our research group already evaluated their presence in seafood [3]. A method combining both QuEChERS methodologies previously discussed was developed, for which phthalate esters were also determined along with OPEs, BTs, BSAs and BTRs.

The first goal was to evaluate the occurrence of HPVs in samples from Tarragona, Spain. Seafood specimens of the ten most consumed species from Catalonia [4] were bought every four months from February 2019 to February 2020. Samples were bought in three different acquisition channels, these being the supermarket, the local fishmonger, and the central city market. The purpose of the wide variety of acquisition channels was to cover the most usual places where the population tends to buy seafood. The presence of HPVs can differ within species and even specimens especially because of their origin. Even though literature compiles articles reporting the different concentration profiles for compounds such as OPEs [5,6] and PAEs [7,8] from different origins, data regarding the same information for BTs or BSAs is still scarce. Edible parts of the seafood were dissected from the specimens and subsequently analysed using the developed method. The obtained results were studied in terms of concentration in each of the species, detection frequency and differentiation between low and

high lipid content species. Data on HPVs concentrations was further used to calculate the exposure and risk for the population living in Tarragona to the dietary intake of HPVs via seafood consumption. It is important to note that the origin of the specimens was not evaluated in this study, as the focus was on the exposure of the population who buys these seafood specimens independently of their origin.

The second goal was to compare the exposure and risk of the population of two locations with different seafood consumption habits. In this sense, Catalonia and Canary Islands, both being regions within Spain, where evaluated. Specimens of the most consumed seafood species from both locations were evaluated. However, Catalonia's most consumed species may be imported, whereas for the Canary Islands, these tend to be autochthonous. Seafood samples were analysed using the same method as the one used for the previous study to determine HPVs. This preliminary study evaluated both the HPVs profiles of the species and the exposure and risk associated. The study was conducted in collaboration with the Institute for Environmental Studies and Natural Resources (i-UNAT) from the University of Las Palmas de Gran Canaria (ULPGC).

Results found in the following projects have been already submitted for publication in Food and Chemical Toxicology, being the first already accepted.

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3.2.1. High production volume chemicals in the most consumed seafood species in Tarragona area (Spain): occurrence, exposure, and risk assessment

HIGH PRODUCTION VOLUME CHEMICALS IN THE MOST CONSUMED SEAFOOD SPECIES IN TARRAGONA AREA (SPAIN): OCCURRENCE, EXPOSURE, AND RISK ASSESSMENT

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Abstract

Seafood consumption has become a potential exposure route towards high production volume chemicals due to the pathway of these compounds reaching the aquatic environment via industrial and domestic discharges. The present study focuses on the determination of phthalate esters (PAEs), organophosphate esters (OPEs), benzothiazoles (BTs), benzotriazoles (BTRs) and benzenesulfonamides (BSAs) in the ten most consumed fish species in Catalonia. A total of 120 commercially available seafood specimens were purchased throughout February 2019-February 2020 in three different stores (supermarket, local market, and local fishmonger) of the city of Tarragona, Spain, to cover the most typical places where seafood can be obtained. ΣOPEs, ΣBTs, ΣBSAs and ΣPAEs concentrations ranged between 5.99 – 139.45 ng g^{-1} w.w., 8.41 – 54.08 ng g^{-1} w.w., 8.38 – 304.47 ng g^{-1} w.w and 2.86 – 323.80 ng g^{-1} w.w., respectively. PAEs and BSAs had similar contributions which combined represented nearly the 70% of detected compounds and sardine resulted as the species with the higher HPVs mean concentration. No considerable threat was posed due to the individual intake of these compounds via seafood consumption.

Highlights

- HPVs were determined in the ten most consumed seafood species from Catalonia.
- HPVs mean concentrations trended as PAEs > BSAs > BTs > OPEs.
- Sardine appeared as the most contaminated species.
- No differences were found between low and high lipid content species.
- Low risk concern is associated to the intake of HPVs present in seafood.

1. Introduction

The massive production of chemical compounds to cover the present day necessities has been the result of a rapid economic and industrial development of our society over the last century. Organisations such as the Organisation for Economic Co-operation and Development (OECD) [1] or the US Environmental Protection Agency (EPA) have listed the chemical compounds produced in more than 1000 or 500 tonnes per year, respectively, with the intention of generating useful data on these substances to provide screening information datasets (SIDS). The chemicals comprised in the list receive the name of High Production Volume chemicals (HPVs), and their prioritised study will be useful for the assessment of the exposure and risk towards both the environment and the population. HPVs constitute a diverse group of compound families including well-known compounds such as phthalate esters (PAEs), currently on the spotlight ones like organophosphate esters (OPEs) or even families yet to be further studied as benzothiazoles (BTs), benzotriazoles (BTRs) and benzenesulfonamides (BSAs). Most of these compounds are used in industrial as well as daily commodities, thus becoming generally present in our everyday life. Compounds such as OPEs or PAEs are used as plasticisers or flame retardants, mostly in food packaging, furniture, electronic devices, textiles and even toys [2,3]. On its part, BTs, BTRs and BSAs are usually found as corrosion inhibitors in antifreeze formulations or dishwasher detergents, as well as vulcanization accelerators in rubber, dye-synthesis precursors and even disinfectants [4,5]. Abrasion, dissolution and even volatilization are believed as the most common environment release mechanisms of OPEs and PAEs due to the lack of chemical bonding with the material [6]. BTs, BTRs and BSAs fate mainly initiates with their release as domestic and industrial effluents with non-efficient removal at wastewater treatment plants (WWTP). All in all, HPVs release pathways coincide in their arrival to the aquatic environment, becoming a threat not only for the biodiversity and wildlife inhabiting, but also for the population ingesting seafood with possibly bioaccumulated HPVs. The fact that some of these compounds are believed to be mutagenic, carcinogenic, endocrine disruptors and even respiratory irritant and dermal sensitizers at high concentrations has led to the urge of screening and quantifying their presence in many environmental fates as well as commodities, including foodstuffs [7-9]. In this case, seafood can be used for both elucidating the environmental status and potential threat that these compounds represent for the environment, while evaluating the

exposure and risk for population associated to their consumption via dietary intake. Seafood includes all edible fish species from marine and freshwater environments as well as molluscs, crustaceans, and aquatic plants. Seafood consumption, as part of the Mediterranean diet, is relevant for the Spanish population. As an example, in 2021 the mean seafood consumption per capita was 22.72 kg, which represented a 13.12% of the average family budget destinated for food and beverages acquisition [10]. It is therefore necessary to evaluate the possible bioaccumulation/biomagnification of HPVs in seafood to assess the risk of consumers.

In this study, a total of 120 samples of the ten most consumed seafood species of Tarragona, Spain, were purchased throughout February 2019 - February 2020 in three different stores to represent the different acquisition channels to determine nine organophosphate esters, six phthalate esters, five benzothiazoles, five benzenesulfonamides and four benzotriazoles. Based on the concentrations found in the samples, the study aims to calculate the exposure and risk for the population associated to the dietary intake of these HPVs via seafood consumption. To the best of our knowledge, this is the first monitorisation study including compounds of emerging concern such as benzothiazole and benzenesulfonamides. We believe the results obtained in the study may depict the contamination status of the seafood people is consuming along with an overview of the risk associated to different population subgroups, which may lead to future regulation of the use of some of these compounds.

2. Materials and methods

2.1. Standards and reagents

The present method compiles the determination of nine organophosphate esters (OPEs): triethyl phosphate (TEP), tributyl phosphate (TBP), tri-isobutyl phosphate (TiBP), tris (2- ethylhexyl) phosphate (TEHP), 2-ethylhexyl-diphenyl phosphate (EHDPP), triphenyl phosphate (TPP), tris(2-chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TCPP), tritolyl phosphate (TTP); five benzothiazoles (BTs): 1-H-benzothiazole (BTH), 2-chlorobenzothiazole (CIBT), 2-hydroxibenzothiazole (OHBT), 2-amino-1-H-benzothiazole (NH2BT), 2-(methylthio)-benzothiazole (MeSBT); four benzotriazoles (BTRs): 1-H-benzotriazole (BTR), 4-methyl-1-H-benzotriazole (4TTR), 5-methyl-1-H-benzotriazole (5TTR), 5,6-dimethyl-1-H-benzotriazole (XTR); five benzenesulfonamides (BSAs): benzenesulfonamide (BSA), para-toluenesulfonamide (p-TSA), ortho-toluene-

sulfonamide (o-TSA), N-methyl-para-toluenesulfonamide (Me-p- TSA), N-ethyl-para-toluenesulfonamide (Et-p-TSA); and six phthalate esters (PAEs): diethylphthalate (DEP), dimethyl-phthalate (DMP), diethylhexyladipate (DEHA), di-iso-butylphthalate (DiBP), diethylhexylphthalate (DEHP), di-n-octylphthalate (DnOP). The previously listed standards were analytical grade with purity > 98% and were purchased from Sigma Aldrich (St. Louis, USA). Five deuterated compounds acquired from LGC Standards (Teddington, UK) were used as internal standards: d4-benzothiazole (d4-BT), d4-benzotriazole (d4-BTR), d4-p-TSA (d4-p-TSA), d27-tributylphosphate (d27-TBP) and d4-diethylhexyl-phthalate (d4-DEHP).

The acetonitrile and ethyl acetate used during the extraction procedure were GC grade with purity >99.9% from Scharlab (Barcelona, Spain), whereas ultrapure water was obtained from a Synergy water purification system from Millipore (Massachusetts, USA). Helium and nitrogen gas (>99.999% purity) were supplied by Carburos Metálicos (Tarragona, Spain). Salt packets of the original method to perform QuEChERS extraction, disposable syringes and PTFE filters were purchased from Scharlab. Lipifiltr push-trough cartridges were obtained from Carlo Erba (Barcelona, Spain).

2.2. Sample collection

Samples of 10 different seafood species were purchased in the supermarket, the local market and local fishmonger from Tarragona, Spain, to provide variability to cover the usual stores where local population acquire seafood. The samples were purchased every four months from February 2019 until February 2020. Seafood species included the ten most consumed seafood species from Catalonia, Spain based on the ENCAT 2003 survey [11] and included: codfish (Gadus morhua), shrimp (Aristeus antennatus), hake (Merluccius merluccius), sole (Solea solea), squid (Loligo vulgaris), mussel (Mytilus galloprovincialis), sardine (Sardina pilchardus), tuna (Thunnus thynnus), mackerel (Scomber vincialis) and salmon (Salmo salar). Lateral fillets of the fish samples were dissected and only the soft parts of the shrimp and mussel samples were kept. All samples were freeze-dried using a miVac Duo freeze-drying system from Genevac (Ipswich, UK) and then ground and homogenised. As the aim of the article was to cover the total dietary intake, samples of the different locations were mixed per species and sampling date, thus obtaining a total of 40 samples to analyse. Seafood species were also segregated upon their lipidic content. Thus, species were divided as low lipid content (cod, hake, shrimp, sole and squid) or high lipid content (sardine,

tuna, mackerel, salmon, and mussel). The differentiation of lipid content was useful not only for the analysis of the samples, as the lipids may act as interferents during the extraction, but also to check the possible lipophilic character of some of the target HPVs. The moisture content was calculated as the difference between the weight prior and after lyophilisation. Once all the samples were lyophilised, composites of each of the species and each of the months were prepared (n=40) and stored in the same conditions. All the samples were kept in glass containers and stored at -24°C until their analysis.

2.3. Sample extraction

The samples were extracted using an adaptation of a previously described method for the determination of organophosphate esters from seafood samples [12]. Briefly, 0.1g of the lyophilised sample was weighted in a 50 mL glass centrifuge tube with 10 mL of ultrapure water and 10 mL of acetonitrile. The mixture was vortex mixed for 1 min and subsequently, a QuEChERS extraction salt packet of the original method (1g of anhydrous sodium acetate + 4g of magnesium sulfate) was added to the tube and further mixed for 3 min. The tubes were later centrifugated at 4000 rpm for 5 min using a Hettich Universal 32R centrifugation system (Tuttlingen, Germany) and the acetonitrile layer (supernatant) was collected. The extract was further cleaned using a LipiFiltr push-through cartridge and evaporated to ~0.5 mL under a gentle nitrogen stream. Finally, the extracts were reconstituted with ethyl acetate to 2 mL after an internal standard spike of 50 µg L^{-1}. The extracts were then filtrated using a 0.22 µm PTFE syringe filter and analysed using GC-QqQ.

2.4. Instrumental analysis

An Agilent 8890 GC system coupled to an Agilent 7000D triple quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA, USA) was used for the analysis of the obtained extracts. The extracts were automatically injected by a PAL RSI 120 automatic injector from CTC Analytics (Zwingen, Switzerland). A total of 25 µL were injected in the system using an Agilent Mulit-Mode Inlet (MMI) in solvent vent mode. The initial temperature for the injector was 75°C (held 0.37 min) and ramped to 325°C (held for 5 min) at 600°C/min. The optimised parameters for the solvent vent mode were as follows: vent flow of 120 mL/min and 5 psi for 0.37 min and purge to split vent at 60 mL/min at 2.87 min. The separation was performed using a ZB-50 capillary column (30 m x 25 mm i.d. and 0.25 µm film thickness) from Phenomenex with an oven temperature program starting at 75°C (held for 2.87 min) and raising to 300°C

(held for 5 min) at 15°C/min. The total run time of the analysis was of 22.87 min with a solvent delay of 6 min. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. The triple quadrupole system operated in electron ionisation mode at 70 eV with ion source, quadrupole 1 and quadrupole 2 temperatures set at 230 °C, 150°C and 150°C, respectively. Agilent MassHunter Workstation (Quantitative and Qualitative Analysis) version 10.0 was used to perform the data analysis. Table S1 compiles the list of target compounds along with their retention times, quantitative and qualitative transitions, and collision energies.

2.5. Quality assurance and quality control (QA/QC)

Phthalates and organophosphates may be present in the ambient as well as in the material used for the extraction, especially if the material is made of plastic. Therefore, usage of plastic was avoided when possible and glass flacon tubes were used for the QuEChERS extraction. Even though minimizing the use of plastic, some of the compounds were still present in procedural blank extractions. Thus, two procedural blanks were included in every batch of analysed samples (10 samples per batch) to subtract the blank signal from the obtained sample signal. To ensure the quality of the determination, quality controls were included every 5 samples along with system blanks to check the suitability of the system and prevent carry-over. Concentrations in the samples were quantified depending on the lipidic content of the species using external calibration along with internal standard to correct injection variations and applying the convenient apparent recovery (low or high lipidic content). For low lipid content species, recoveries ranged between 72 – 92 % for OPEs, 52 – 104% for PAEs, 50 – 105% for BTs, 70 - 102% for BTRs and 71 – 85% for BSAs, whereas the values for high lipid content species were 52 – 119 % for OPEs, 62 – 132% for PAEs, 64 – 112% for BTs, 70 - 107% for BTRs and 67 – 84% for BSAs. Further quality parameters can be found in the supplementary material (Table S2).

2.6. Exposure and risk assessment

The presence of HPVs in seafood turns dietary intake into an alternate exposure route for these compounds to reach our organisms. Thus, exposure and risk assessment calculations are used as an effective tool to estimate the possibility of this compounds causing adverse health effects. Human exposure values (Et) were calculated using the mean wet weight concentrations found in the study along with the mean fish consumption (g day^{-1}) obtained from the ENCAT 2003 survey conducted in Catalonia, which data is segregated between fish species and gender/age

(boys 10-19, girls 10-19, adult men 20-65, adult women 20-65, senior men >65, senior women >65) (Table S3). Equation 1 was used on this purpose, where Et is the human exposure, Cf the mean consumption of the individual species f and xt,f the concentration of the individual compound t, for the species f. It was assumed that 100% of the HPVs present in the seafood were absorbed when ingested.

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

For the risk assessment, non-genotoxic and non-carcinogenic compounds were assessed using the NOAEL (non-observed-adverse-effect-level) approach, for which equation 2 is used. E_t, ADI_t and R_t are the dietary exposure, acceptable daily intake and risk factor for compound t, respectively. Acceptable daily intake values were the result of dividing the oral NOAEL values by an uncertainty factor of 100.

$$Rt = (E_t / ADI_t) * 100 \qquad \text{Eq. 2}$$

As per carcinogenic compounds such as TBP and TCEP, the margin of exposure (MOE_t) was calculated using the equation 3, where MOE_t is the margin of exposure for the compound t, E_t is the dietary exposure to compound t and BMD is the benchmark dose for which a measurable response of 5-10% range above the control is caused.

$$MOE_t = BMD_t / E_t \qquad \text{Eq.3}$$

Calculations were performed for three different scenarios assuming method limit of detection and quantification values for non-detected and below-LOQ compounds: (a) Lower-bound scenario. Non-detected compounds concentra-tions are assumed as 0, while below-LOQ compounds are estimated as the LOD. (b) Middle-bound scenario. Non-detected and below-LOQ compound are estimated as half the LOD and half the LOQ, respectively. (c) Upper-bound scenario. Non-detected compounds are assumed as the LOD and below-LOQ compounds are estimated as the LOQ.

2.7. Statistical analysis

Principal components analysis (PCA) was used as multivariate statistical analysis to examine the possible patterns in HPVs associated to the lipidic content (low or high lipidic content). The statistical analyses were all performed using R (RStudio, 2022.02.3) with FactoMineR and factoextra packages.

3. Results and discussion

3.1. Occurrence of HPVs in commercial seafood

The target HPVs were divided in their respective families: OPEs (TEP, TBP, TiBP, TCPP, TCEP, TEHP, EHDPP,

TPP and TTP), PAEs (DMP, DEP, DiBP, DEHA, DEHP, DnOP), BTs (BT, ClBT, MeSBT, OHBT, NH2BT), BTRs (BTR, 4TTR, 5TTR, XTR) and BSAs (BSA, o-TSA, p-TSA, Me-p-TSA, Et-`p-TSA). One kind of each of the target HPVs (OPEs, PAEs, BTs and BSAs) was detected in all the analysed samples at the minimum, except for BTRs, which were not detected in any of the samples. Regarding the individual compounds' detection frequencies, these ranged between 8% for DEHP and 73% for BT. Among the analysed seafood species, the concentrations of ΣOPEs, ΣBTs, ΣBSAs and ΣPAEs ranged between 5.99 – 139.45 ng g^{-1} w.w. (GM: 31.99 ng g^{-1} w.w), 8.41 – 54.08 ng g^{-1} w.w (GM: 30.23 ng g^{-1} w.w), 8.38 – 304.47 ng g^{-1} w.w (GM: 56.75 ng g^{-1} w.w) and 2.86 – 323.80 ng g^{-1} w.w. (GM: 59.56 ng g^{-1} w.w), respectively. Thus, phthalate esters (PAEs) appeared as the group of compounds with the highest mean concentration as well as the group with the highest concentration of a single compound. Table 1 compiles the results obtained from each of the species, indicating the geometric mean, the minimum and maximum concentrations, and the detection frequency (DF%). Regarding the distribution of the detected compounds in the same group in terms of concentration, OPEs showed a trend of TTP < TiBP < TBP < EHDPP < TTP < TCPP < TCEP, with a difference of nearly an order of magnitude between the mean concentrations of TTP (1.74 ng g^{-1} w.w) and TCEP (18.04 ng g^{-1} w.w.). The presence of chlorinated organophosphate esters has been commonly reported in the literature by other authors, mainly by their lower degradation and higher persistence [13]. Studies on market basket total OPEs conducted in Australia [14] and the US [15] showed the presence of TCEP and TCPP at high detection frequencies and con-centrations between 0.10 – 0.13, 0.40 – 1.39 ng g^{-1} w.w. and 0.06 – 2.16, 0.06 – 25.6 ng g^{-1} w.w., respectively. The same studies also reported the presence of other compounds such as TBP, TiBP, TPP and EHDPP at levels of low ng g^{-1} w.w.. For BTs, the trend follows as MeSBT < NH2BT < OHBT < BT, with the highest mean concentration being five times higher than the lowest one (3.37 ng g^{-1} w.w. for MeSBT and 15.40 ng g^{-1} w.w. for BT). Although their presence in the literature is scarce, some authors have documented the presence of benzothiazoles in seafood. Trabalón et al. [4] reported concentrations between 6 – 82 ng g^{-1} d.w. of four benzotriazoles in seafood of common consumption, being BT the congener with the highest concentrations (up to 82 ng g^{-1} d.w.), followed by NH2BT, ClBT and MeSBT. Another study involving the determination of benzothiazoles and benzotriazoles in mollusc samples from the Bohai Sea also reported the presence of BT with a mean concentration of 595 ng g^{-1} d.w., OHBT

with 20.1 ng g^{-1} d.w., MeSBT with 14.2 ng g^{-1} d.w. and NH2BT with 0.165 ng g^{-1} d.w. [16]. As it can be observed, the benzothiazoles determined in the present study are similar to the ones reported by other authors in terms of concentrations and detection frequency. Regarding the concentrations it can be observed that except for BT, which values are slightly higher, values for the other found congeners are similar. It should also be noted that the presented values are given in wet weight basis, which tends to be lower than the literature values given in dry weight. In the case of BSAs, only two compounds were detected, Et-p-TSA and BSA, with mean concentrations of 27.5 and 29.2 ng g^{-1} w.w. To date, there is no available data on the presence of these compounds in seafood species. However, literature on the presence of BSAs in water indicates the potential of waste-water treatment plants of causing bioconversion/biodegradation of sulfonamides of higher molecular weight into BSA and Et-p-TSA. Studies such as the ones carried out by Jover et al. [17] and Herrero et al. [18] found higher concentrations of these compounds in effluent waters when compared with the influent. This bioconversion and concentration enhancement could explain the apparition of these compounds in seafood species. Opposite to the presence of BSA and Et-p-TSA, high removal efficiency of p-TSA at WWTP explains the inexistence of this compound in the analysed samples [19]. Lastly, PAEs followed a trend of DiBP < DEHP < DEP < DEHA, with DEHA mean concentration (45.8 ng g^{-1} w.w.) being 15 times higher than DiBP (2.94 ng g^{-1} w.w.). Even though literature depicts DEHP as the congener with the most detection frequency and with the highest concentrations [20-22], this fact disagrees with the PAEs distribution found in the present study. This could be directly linked to the apparition of an interference during the determination process disabling the determination of DEHP for high lipid content species. It should be noted that the punctual apparition of DEHA at high concentrations turns this compound as the one with the highest concentration, however, DEP appears as the phthalate ester with the highest detection frequency (43%). Concentration profiles for PAEs in fish are rather despair. Authors such as Gu et al. [23] reported total concentrations of Σ5PAEs, in which DEHP was included, of 5 – 46.3 ng g^{-1} w.w. for fish, 3.3. – 219.3 ng g^{-1} w.w. for mollusc and 5.0 – 57. 3 ng g^{-1} w.w. for shrimp. These values share similarities with the ones found in the present study in terms of concentrations. Distribution of the total HPVs concentrations in the analysed seafood samples is summarized in Figure 1A.

In order to check possible correlations between the HPVs

Table 1. Mean concentrations, range and detection frequencies (%DF) of the determined HPVs in seafood (ng g^{-1} wet weight, w.w.) classified by species.

		TBP	TiBP	TCPP	TCEP	EHDPP	TPP	TTP	BT	MeSBT	OHBT
Cod	mean	3.33	1.36	14.05	5.82	1.63	n.d.	8.09	34.1	n.d.	10.73
Gadus morhua	range	0.23 - 6.73	n.d. - 1.75	n.d. - 14.05	n.d. - 7.36	n.d. - 1.63	n.d.	n.d. - 9.55	n.d. - 72.08	n.d.	n.d. - 16.38
	DF %	100	75	25	50	25	0	50	75	0	75
Hake	mean	0.51	0.97	2.95	1.31	n.d.	n.d.	0.25	13.56	n.d.	3.52
Merluccius merluccius	range	n.d. - 0.89	n.d. - 0.97	n.d. - 2.95	n.d. - 1.51	n.d.	n.d.	n.d. - 0.25	n.d. - 35.90	n.d.	n.d. - 4.90
	DF %	50	25	25	50	0	0	25	75	0	50
Shrimp	mean	2.07	1.02	6.31	3.24	n.d.	0.6	3.4	27.91	n.d.	5
Aristeus antennatus	range	n.d. - 3.33	n.d. - 1.02	n.d. - 9.61	n.d. - 4.69	n.d.	n.d. - 0.60	n.d. - 3.40	n.d. - 31.46	n.d.	n.d. - 12.01
	DF %	50	25	50	50	0	25	25	50	0	75
Sole	mean	2.48	1.95	12.35	2.73	1.04	1.25	n.d.	1.64	1.02	38.18
Solea solea	range	n.d. - 4.29	n.d. - 3.64	n.d. - 16.41	n.d. - 4.46	n.d. - 1.04	n.d. - 1.76	n.d.	n.d. - 2.75	n.d. - 1.02	n.d. - 96.08
	DF %	50	50	50	50	25	50	0	50	25	75
Squid	mean	0.55	1.2	10.41	2.46	n.d.	n.d.	n.d.	30.41	6.8	3.14
Loligo vulgaris	range	n.d. - 1.18	n.d. - 1.53	n.d. - 18.99	n.d. - 4.71	n.d.	n.d.	n.d.	n.d. - 43.28	n.d. - 11.66	n.d. - 3.14
	DF %	75	50	50	75	0	0	0	75	50	25
Sardine	mean	1.31	4.37	10.2	n.d.	2.33	n.d.	9.46	4.9	0.87	5.17
Sardina pilchardus	range	n.d. - 2.4	n.d. - 4.37	n.d. - 10.2	n.d.	n.d. - 3.5	n.d.	n.d.- 9.46	1.10 - 7.00	n.d. - 1.37	n.d. - 5.17
	DF %	50	25	25	0	50	0	25	100	50	25
Tuna	mean	n.d.	1.84	n.d.	130.8	3.44	3.37	n.d.	2.65	1.74	4.86
Thunnus thynnus	range	n.d.	n.d. - 3.6	n.d.	n.d. - 259.4	n.d. - 4.1	n.d. - 3.37	n.d.	n.d. - 5.1	n.d. - 1.74	n.d. - 5.3
	DF %	0	50	0	50	50	25	0	75	50	50
Mackerel	mean	2.53	n.d.	2.73	5.59	2.37	n.d.	n.d.	31.59	4.62	3.74
Scomber vincialis	range	n.d. - 3.4	n.d.	n.d. - 2.73	n.d.- 5.59	n.d. - 2.40	n.d.	n.d.	n.d. - 84.9	n.d. - 11.2	n.d. - 3.74
	DF %	50		25	25	50		0	75	75	25
Salmon	mean	6.14	5.27	n.d.	7.59	7.94	n.d.	11.1	5.09	2.21	6.17
Salmo salar	range	n.d. - 7.0	n.d. - 6.80	n.d.	n.d. - 11.20	n.d. - 11.3	n.d.	n.d. - 11.10	n.d. - 5.30	n.d. - 2.6	n.d. - 6.30
	DF %	50	50	0	50	75	0	25	75	50	50
Mussel	mean	1.03	1.62	n.d.	2.82	2.75	n.d.	n.d.	2.11	6.3	n.d.
Mytilus galloprovincialis	range	n.d. - 1.5	n.d. - 2.5	n.d.	n.d. - 2.82	n.d. - 4.1	n.d.	n.d.	n.d. - 3.5	0.4 - 19.3	n.d.
	DF %	75	50	0	50	75	0	0	75	100	0
All	mean	2.22	2.18	8.43	18.04	3.07	1.74	6.46	15.40	3.37	8.95
	range	n.d. - 7.0	n.d. - 6.80	n.d. - 18.99	n.d. - 259.4	n.d. - 11.3	n.d. - 3.37	n.d. - 11.10	n.d. - 84.90	n.d. - 19.3	n.d. - 96.08
	DF %	55	44	25	45	35	11	15	73	40	45

Table 1. Mean concentrations, range and detection frequencies (%DF) of the determined HPVs in seafood (ng g^{-1} wet weight, w.w.) classified by species.

		NH_2BT	BSA	Et-p-TSA	DEP	DiBP	DEHA	DEHP	ΣOPEs	ΣBTs	ΣBSAs	ΣPAEs
Cod	mean	9.25	9.13	17.69	11.43	n.d.	1.22	n.d.	34.28	54.08	26.82	12.65
Gadus morhua	range	n.d. - 18.03	n.d. - 14.97	n.d. - 28.78	n.d. - 11.43	n.d.	n.d. - 1.22	n.d.				
	DF %	50	75	50	25	0	25	0				
Hake	mean	2.3	4.77	3.61	9.03	1.31	n.d.	n.d.	5.99	19.38	8.38	10.34
Merluccius merluccius	range	n.d. - 3.00	n.d. - 8.16	0.20 - 9.81	n.d. - 9.03	n.d. - 1.51	n.d.	n.d.				
	DF %	50	50	100	25	50	0	0				
Shrimp	mean	1.39	11.19	6.03	36.92	n.d.	1.52	10.92	16.64	34.3	17.22	49.36
Aristeus antennatus	range	n.d. - 1.39	n.d. - 11.19	n.d. - 11.34	n.d. - 36.92	n.d.	n.d. - 1.52	n.d. - 10.92				
	DF %	25	25	50	25	0	25	25				
Sole	mean	2.38	19.21	6.98	15.03	0.95	3.26	8.26	21.8	43.22	26.19	27.5
Solea solea	range	n.d. - 3.40	n.d. - 33.07	n.d. - 13.72	n.d. - 32.35	n.d. - 0.95	n.d. - 3.26	n.d. - 8.26				
	DF %	50	50	50	75	25	25	25				
Squid	mean	5.21	4.82	7.05	n.d.	n.d.	1.03	13.75	14.62	45.56	11.87	14.78
Loligo vulgaris	range	n.d. - 8.44	n.d. - 4.86	n.d. - 10.80	n.d.	n.d.	n.d. - 1.79	n.d. - 13.75				
	DF %	50	50	50	0	0	75	25				
Sardine	mean	4.15	11.57	7.58	102.07	n.d.	221.73	n.d.	27.67	15.09	19.15	323.8
Sardina pilchardus	range	n.d. - 4.15	n.d. - 11.57	n.d. - 7.58	n.d. - 117.5	n.d.	n.d. - 221.73	n.d.				
	DF %	25	25	25	75	0	25	0				
Tuna	mean	6.88	47.85	74.33	19.43	n.d.	n.d.	n.d.	139.45	16.13	122.18	19.43
Thunnus thynnus	range	n.d. - 7.9	n.d. - 86.2	n.d. - 148.20	n.d. - 19.50	n.d.	n.d.	n.d.				
	DF %	50	50	50	50	0	0	0				
Mackerel	mean	5.82	165.71	138.76	2.86	n.d.	n.d.	n.d.	13.22	45.77	304.47	2.86
Scomber vincialis	range	n.d. - 5.82	n.d. - 165.71	n.d. - 138.76	n.d. - 2.86	n.d.	n.d.	n.d.				
	DF %	25	25	25	25	0	0	0				
Salmon	mean	6.85	13.69	6.59	60.99	n.d.	n.d.	n.d.	38.04	20.32	20.28	60.99
Salmo salar	range	n.d. - 9.90	n.d. - 15.70	n.d. - 6.59	n.d. - 72.5	n.d.	n.d.	n.d.				
	DF %	75	75	25	75	0	0	0				
Mussel	mean	n.d.	4.4	6.5	67.28	6.57	n.d.	n.d.	8.22	8.41	10.9	73.85
Mytilus galloprovincialis	range	n.d.	n.d. - 7.00	n.d. - 10.6	n.d. - 127.5	n.d. - 6.57	n.d.	n.d.				
	DF %	0	75	50	50	25	0	0				
All	mean	4.91	29.23	27.51	36.12	2.94	45.75	10.98	31.99	30.23	56.75	59.56
	range	n.d. - 9.90	n.d. - 165.71	n.d. - 148.20	n.d. - 117.5	n.d. - 6.57	n.d. - 221.73	n.d. - 13.75				
	DF %	40	50	48	43	10	18	8				

concentrations, a Pearson correlation test was performed. Figure 1B shows the results of the correlations. As observed in the figure, most of the compounds show weak or non-significant correlation values. Positive correlations appear between TBP and TiBP, EHDPP and TTP, as well as between TiBP with EHDPP and TPP or TCEP with TPP. As observed, most of the compounds with positive correlation values belong to the same family. This correlation agrees with previous studies suggesting the diffusive spread of OPEs, which leads to similar profiles in different fates. A strong positive correlation is also found between BSA and Et-p-TSA, which may be explained due to a possible bioconversion of Et-p-TSA to BSA or a common higher molecular weight benzenesulfonamides to Et-p-TSA/BSA.

3.2. Comparison among seafood species

HPVs total concentrations and contributions were determined in the ten most consumed seafood species from Catalonia, Spain. The total contribution of the different compound families included in the target HPVs was evaluated among the ten seafood species. As observed in Figure 2B, ΣPAEs and ΣBSAs represented the 65% of the total concentrations, with 34% and 31%, respectively. ΣBTs and ΣOPEs accounted the 19% and 16%. Regarding the levels of HPVs concentrations found in each of the species, the trend was hake < squid < mussel < shrimp < sole < cod < salmon < tuna < mackerel < sardine (Figure 2C). Sardine presents the highest value with a total HPVs mean concentration of 386 ng g^{-1}, this value being nearly ten times higher than the species with the lowest concentration (hake, 44 ng g^{-1}). If compared between compound families, differences are found regarding the distribution of the HPVs among the studied species. Figure 2A compiles the contributions of each of the HPVs families to the total concentrations of each of the species. It can be observed that species such as cod, hake, sole and squid have similar profiles, being benzenesulfonamides the group with the higher contribution, followed by OPEs/BTs with a similar contribution and finally PAEs. For mussel, salmon and sardine, phthalate esters represent from nearly a 50% of the contribution at the minimum (salmon and shrimp) to 70-80% (mussel and sardine). Most of these species are included in the higher lipid content subgroup except for shrimp. This trend could be explained by the known lipophilicity of phthalate esters, which would cause higher lipid content species such as sardine or salmon to bioaccumulate these compounds in greater concentrations. Shrimp higher phthalate esters concentration levels could be possible explained by a contaminated source on its habitat or the presence of higher

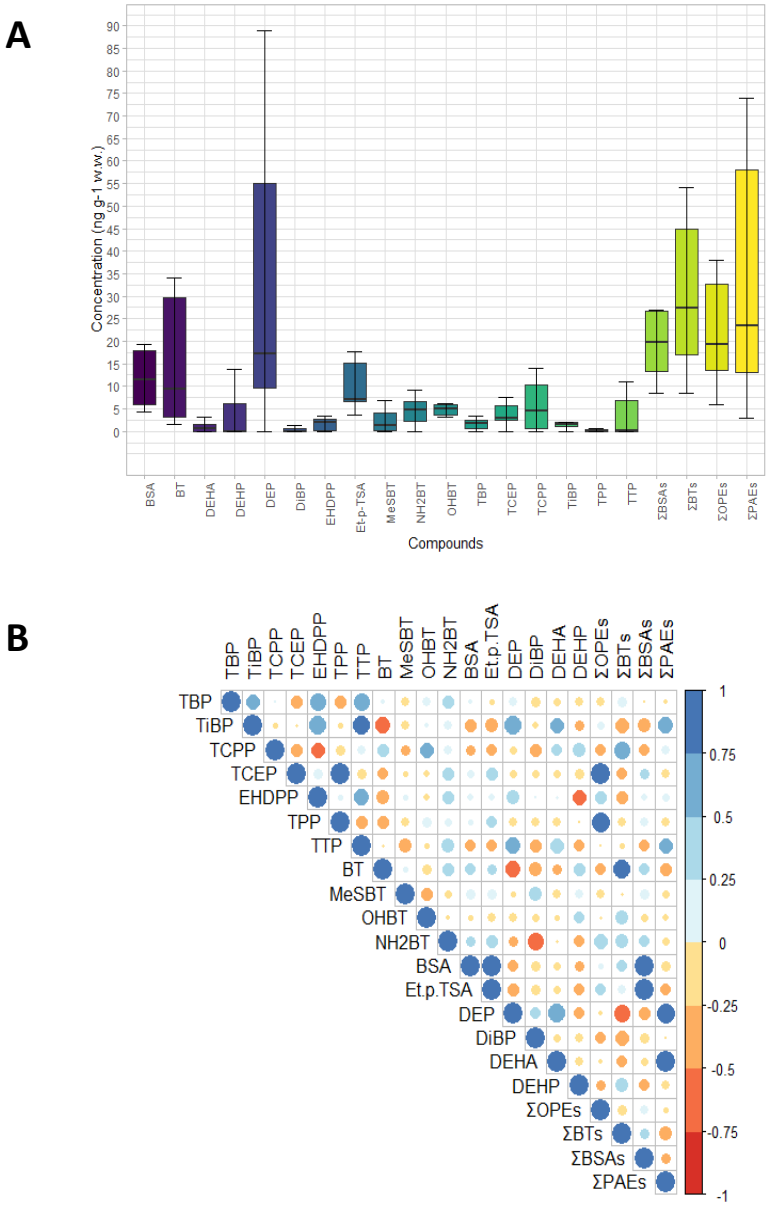

Figure 1. A) Total concentrations of HPVs in the analysed samples. The box plot shows the 25th and 75th percentile concentrations (bottom and top edges of the box), the maximum and minimum concentrations (bottom and top whiskers) and the median concentration (line within the box). B) Pearson correlation among the concentrations of HPVs in seafood samples.

phthalate esters concentration in the waters were these were captured. Cross contamination coming from any of the manufacturing processes involved throughout the whole selling process should not be discarded, as these compounds can be found in packaging and other plastic made materials. Contrary to shrimp, tuna levels of PAEs are lower than expected for this high lipid content species. Despite most of the species having similar profiles, mackerel, as tuna and sardine, differs in its distribution. A larger percentage of BSAs, for both detected compounds BSA and Et-p-TSA is observed. As no data is found in the literature referred to the possible bioaccumulation or presence of these compounds in seafood or other aquatic organisms, no explanation can be given to this phenomenon. If focused on single HPVs family's contamination, hake appears as the species with the least concentration for OPEs (5.99 ng g^{-1} w.w) and BSAs (8.38 ng g^{-1} w.w.), whereas mussel and mackerel present the lowest concentrations of BTs (8.41 ng g^{-1} w.w.) and PAEs (2.86 ng g^{-1} w.w.), respectively. On the other hand, tuna, cod, mackerel, and sardine show the highest mean concentrations for OPEs (139.45 ng g^{-1} w.w.), BTs (54.08 ng g^{-1} w.w.), BSAs (304.47 ng g^{-1} w.w.) and PAEs (323.8 ng g^{-1} w.w.), respectively.

A principal component analysis was performed for all the analysed samples to check the possible correlation between the HPVs families' contributions and the species lipidic contents. Thus, samples were represented in biplots including PC1 (16.4%) vs PC2 (12.3%) and PC2 vs PC3 (10.7 %) (Figures 3A and 3B, respectively). Convex hulks for low lipid content species (yellow) and high lipid content species (blue) were mostly overlapped in both cases, thus indicating that no differentiation could be possibly made between low/high lipid content species when comparing their HPVs concentrations. All in all, the target HPVs comprised in the present study compile compounds with different characteristics, in which lipophilicity can be included. Compounds such as PAEs are highly fat dependant, whereas OPEs are believed to be non-lipophilic. As seen in figure 3C and 3D, contribution of PAEs to the PC1 and PC2 is minimal, except for DEP. Most of the compounds with the highest contributions are OPEs such as EHDPP and TiBP, which are believed to be non-lipophilic. Apart from OPEs, compounds such as MeSBT, BT, Et-p-TSA, NH2BT or BSA have higher contributions than PAEs. To date, there is not sufficient data regarding the lipophilicity of these compounds, however, given the obtained data, it seems that these may not be fat-dependant. Further experiments should be carried out in this topic to elucidate the relationship between BTs and BSAs and the lipid content.

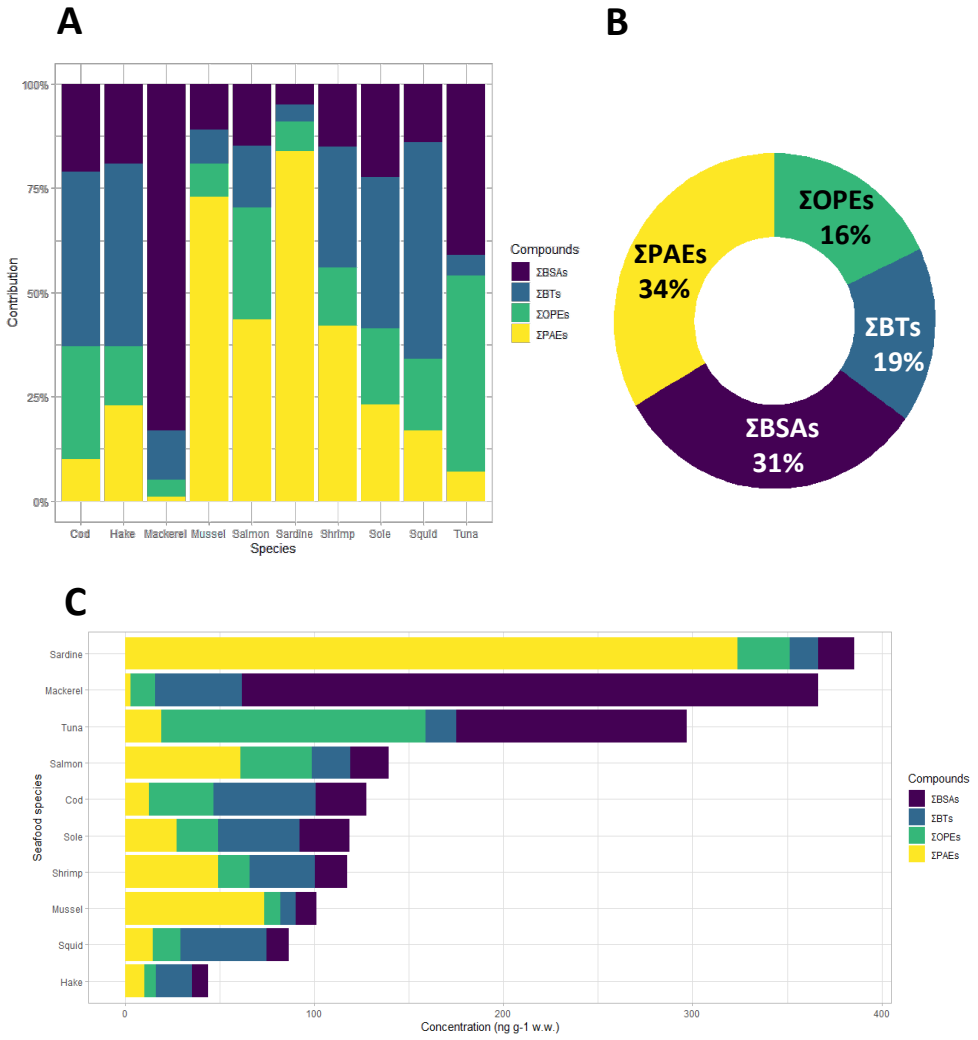

Figure 2. A) Composition profiles of HPV families in the different seafood species. B) Total contribution of the different HPV families to the total analysed samples. C) Total concentrations of HPVs in the analysed seafood species.

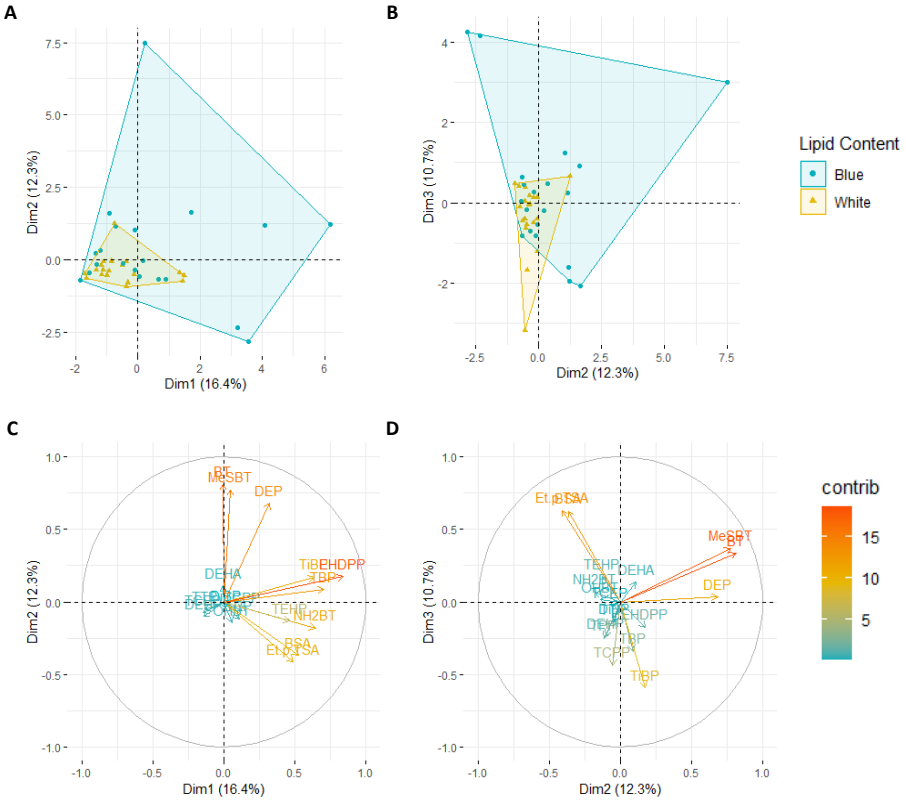

Figure 3. Principal component analysis of HPVs in seafood. Convex hulls for low (white) and high (blue) lipidic content species delimited in different colours for PC1 vs PC2 (A) and PC2 vs PC3 (B). Subplots C and D show the congeners loadings and contributions for PC1 vs PC2 (C) and PC2 vs PC3 (D).

3.3. Dietary intake of HPVs and risk characterization

The values regarding HPVs exposure via seafood consumption are gathered in Table 2. The table shows the results of the sum of the exposures of all the analysed species for each of the target HPV families. As no compounds were found below the method limit of quantification, the values for the lower-bound scenario correspond to the real exposure based on the concentrations determined in the present study. The exposure to the contaminants trends as follows: OPEs < BSAs < BTs < PAEs, which agree with the trend followed for the concentrations. Regarding the most

affected groups, boys appear as the subgroup with the highest exposure values for 3 out of the 4 families, OPEs, BSAs and PAEs, with values of 32.8, 38.4 and 50.9 ng kg^{-1} bw day^{-1}, respectively. On the other hand, girls' subgroup tops on BTs exposure with 68.6 ng kg^{-1} bw day^{-1}. Compounds responsible of the higher contributions for each group were DEP (36.0 ng kg^{-1} bw day^{-1}), BT and OHBT (25.7 and 27.4 ng kg-1 bw day^{-1}), BSA (20.9 ng kg^{-1} bw day^{-1}) and TCEP (11.6 ng kg^{-1} bw day^{-1}). Sole appeared as the seafood species with the highest exposure contribution for BTs, BSAs and OPEs, whereas PAEs were more present in sardine. As seen in Table 2, values regarding the middle and higher-bound scenarios mainly differ in the incorporation of the BTRs exposure along with other non-detected compounds with no significant differences in the obtained values. Extended data on single compounds exposure values for each of the species can be found in Table S2. Several studies have reported exposure values for some HPVs through seafood consumption. Trabalón et al. [4] studies on the presence of benzothiazoles in seafood of common consumption reported values up to 48 ng kg^{-1} bw day^{-1} for Σ5BTs, being MeSBT and BT the major contributors (22 and 11 ng kg^{-1} bw day^{-1}). Jia et al. [16] exposure values for Σ6BTs ranged between 76.1 and 121 ng kg^{-1} bw day^{-1}, with BT being responsible of approximately the 80% of the total exposure. OPEs exposure values from different seafood species from Nansha Islands (China), the Great Lakes (Canada/USA) and the western Mediterranean Sea (Spain) have been reported with values between 4.74 6.5 ng kg^{-1} bw day^{-1} (Σ9OPEs) [24], 16.9 ng kg^{-1} bw day^{-1} (Σ13OPEs) [25] and 2.56 – 31 ng kg^{-1} bw day^{-1} (Σ12OPEs) [26], respectively. Regarding PAEs, most of the studies present in the literature focus on the exposure to DEHP, reporting values between 50 – 1390 ng kg^{-1} bw day^{-1}. As previously stated, no data on benzenesulfonamides is available to date. None of the obtained exposure values were higher than the acceptable daily intake (ADI), also known as reference dose (RfD), which ranged between 5.1x104 – 106 ng kg^{-1} bw day^{-1} result of dividing the NOAEL values provided by the EPA by a safety factor of 100. Results gathered in Table 2 are several orders of magnitude lower than the ADIs, thus suggesting a low concern regarding the exposure to HPVs through seafood consumption.

The risk associated to the obtained exposure values was calculated for both non-carcinogenic and carcinogenic compounds using the equations previously described. Risk factors (Rt) values for the lower-bound scenario ranged between 1.38x10-5 – 1.12x10-3 for OPEs (TPP < TTP < TiBP < TCPP < EHDPP), 3.19x10-2 – 9.32x10-2 for BT, and 4.09x10-6 – 1.14x10-3 for PAEs

Table 2. Exposure values (ng kg^{-1} bw day^{-1}) of the different subgroups of population for three different scenarios

		ΣOPEs	ΣBTs	ΣBSAs	ΣPAEs	ΣBTRs
BOYS	LB[a]	32.8	61.3	38.4	50.9	0.0
	MB[b]	33.0	61.4	42.3	51.2	7.9
	UB[c]	33.3	61.5	46.3	51.6	15.9
GIRLS	LB	19.1	68.6	24.1	48.4	0.0
	MB	19.4	68.8	27.7	48.8	7.3
	UB	19.6	68.9	31.4	49.2	14.6
ADULT MEN	LB	16.7	29.6	22.2	26.6	0.0
	MB	16.9	29.7	21.1	26.8	4.0
	UB	17.0	29.8	26.1	27.0	8.0
ADULT WOMEN	LB	17.5	31.6	23.4	28.9	0.0
	MB	17.7	31.7	25.5	29.1	4.3
	UB	17.8	31.8	27.6	29.3	8.5
SENIOR MEN	LB	17.2	37.4	21.2	28.0	0.0
	MB	17.4	37.5	23.7	28.3	5.0
	UB	17.6	37.6	26.2	28.5	10.0
SENIOR WOMEN	LB	14.5	34.8	28.0	33.2	0.0
	MB	14.6	34.8	30.0	33.4	4.2
	UB	14.8	34.9	32.1	33.6	8.3

[a] Lower-bound scenario
[b]Middle-bound scenario
[c]Upper-bound scenario

(DEHP < DiBP < DEP < DEHA). As for TBP and TCEP, the margin of exposure values ranged between 4.12x108 – 1.60x109 and 2.33x108 – 6.80x108, respectively. As risk factor values are expressed as the percentage of the ADIs, the closer the obtained values are to 100, the higher is the risk. In this case, all the obtained values are found lower than 0.1%, thus posing a low risk of negative health effects related to the consumption of seafood. For TBP and TCEP, values of margin exposure are higher than the 10,000-limit set by the EFSA for genotoxic and carcinogenic compounds [27], thus meaning their presence in fish and their associated risk is of low concern. Figure S4 compiles the risk factor values divided by compound families and scenarios.

4. Conclusions

The presence of several HPVs in the most consumed seafood species in Tarragona has been confirmed, being phthalate esters the group with the highest mean concentration (59.56 ng g^{-1} w.w.), followed by BSAs (56.75 ng g^{-1} w.w.), BTs (30.23 ng g^{-1} w.w.), and OPEs (31.99 ng g^{-1} w.w.). Sardine appeared as the seafood species with the highest

HPVs mean concentration (385.7 ng g^{-1} w.w.), mainly to a higher presence of PAEs, specially DEHA. To the best of our knowledge this is the first study focused on the monitoring of compounds such as BSAs and BTs. As observed in the results, their presence in seafood is higher than for compounds such as organophosphate esters, thus suggesting that their presence in aquatic environments could be even higher. No differentiation could be made between low and high lipid content species for their HPVs concentrations, thus indicating a potential lack of lipophilicity for most of the compounds. Exposure and risk related to the intake of HPVs through seafood consumption was assessed, concluding that the threat to population's health was of low concern. It must be noted that the obtained results were based on the diet of people from Tarragona consuming all the studied seafood species, which could differ from the average diet. We believe the present study enlightens the necessity of including novel or less studied HPVs families to the current field and hopefully helps to impulse new regulations on the use of some of these compounds in daily commodities.

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Supplementary Material

High production volume chemicals in the most consumed seafood species in Tarragona area (Spain): occurrence, exposure, and risk assessment

CONTENTS

Table S1. GC-MS/MS-(QqQ) conditions

Compound	Rt (min)	MRM Q[a]	CE (eV)	MRM q[b]	CE (eV)
Benzothiazoles					
BT	9.28	135 → 108	51	135 → 82	33
				135 → 69	49
ClBT	10.16	169 → 108	21	169 → 82	37
				169 → 69	53
NH_2BT	13.30	150 → 96	27	150 → 70	43
				150 → 123	15
MeSBT	12.77	181 → 148	15	181 → 135	19
				181 → 108	41
OHBT	13.30	151 → 70	41	151 → 96	25
				151 → 123	13
BT-d4	9.26	139 → 70	51	139 → 84	35
				139 → 112	21
Benzotriazoles					
BTR	11.98	119 → 63	37	119 → 52	33
				119 → 91	7
4TTR	12.42	104 → 77	15	104 → 51	35
				133 → 104	13
5TTR	12.78	104 → 77	15	104 → 51	35
				133 → 104	13
XTR	13.27	118 → 65	27	118 → 91	15
				147 → 91	31
BTR-d4	11.96	123 → 95	7	123 → 66	39
				123 → 53	35
Benzenesulfonamides					
BSA	13.12	157 → 93	5	157 → 77	25
				77 → 51	19
o-TSA	13.58	90 → 63	33	91 → 65	13
				106 → 79	15
p-TSA	13.92	171 → 65	45	91 → 65	15
				155 → 91	9
Me-p-TSA	13.77	185 → 91	29	155 → 91	9
				91 → 65	15
Et-p-TSA	13.89	155 → 91	11	155 → 65	33
				184 → 91	19
p-TSA-d4	13.86	95 → 68	11	159 → 95	9
				175 → 95	23

Compound	Rt (min)	MRM Q[a]	CE (eV)	MRM q[b]	CE (eV)
Organophosphate esters					
TEP	7.61	155 → 99	5	99 → 63	40
				99 → 81	25
TBP	10.64	99 → 81	20	99 → 63	45
				99 → 47	50
TiBP	11.90	99 → 81	25	99 → 63	25
				99 → 47	45
TCPP	13.66	125 → 99	10	124 → 81	25
				99 → 81	20
TCEP	13.91	249 → 125	10	249 → 63	25
				249 → 187	5
TEHP	16.45	99 → 81	25	99 → 81	45
				113 → 57	10
EHDPP	17.53	251 → 77	20	251 → 152	20
				251 → 175	20
TPP	18.15	326 → 233	15	326 → 170	20
				77 → 51	15
TTP	19.81	368 → 165	30	368 → 91	35
				368 → 243	30
TBP-d27	12.48	103 → 83	25	103 → 63	45
				147 → 62	20
Phthalate esters					
DMP	11.34	163 → 77	27	163 → 51	53
				163 → 92	33
DEP	12.27	149 → 65	25	149 → 121	15
				149 → 93	15
DiBP	13.88	149 → 65	27	149 → 93	19
				149 → 121	15
DEHA	16.19	129 → 55	17	129 → 101	5
				129 → 111	5
DEHP	17.53	149 → 65	29	149 → 121	15
				149 → 93	15
DnOP	18.59	149 → 65	29	149 → 121	15
				149 → 93	15
DEHP-d4	17.53	153 → 69	25	153 → 125	25
				153 → 97	25

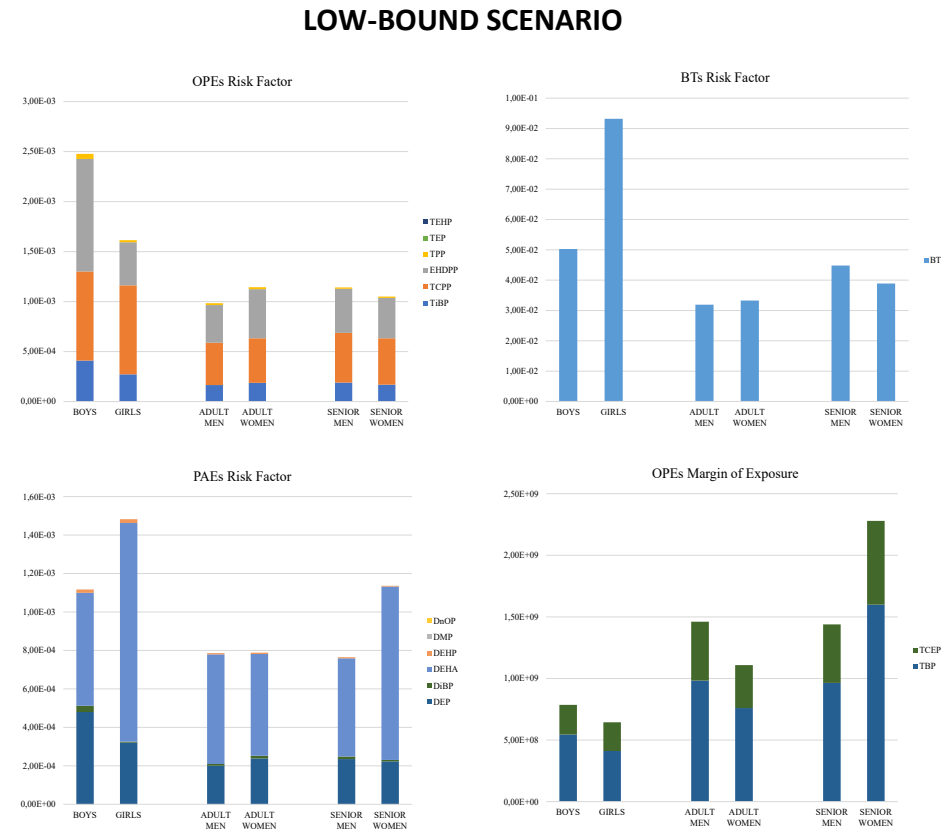
Table S2. Method quality parameters

Compound	Instrumental linear range ($\mu g\ L^{-1}$)	r^2	Low lipid content			High lipid content		
			R_{app} (%)	MDL ($ng\ g^{-1}$ d.w.)	MQL ($ng\ g^{-1}$ d.w.)	R_{app} (%)	MDL ($ng\ g^{-1}$ d.w.)	MQL ($ng\ g^{-1}$ d.w.)
Organophosphate esters								
TEP	0.025 - 75	0.9995	91	0.10	0.55	119	0.10	0.40
TBP	0.025 - 75	0.9992	82	0.10	0.60	85	0.10	0.60
TiBP	0.025 - 75	0.9991	82	0.10	0.60	85	0.10	0.60
TCPP	0.025 - 75	0.9997	78	0.15	0.65	79	0.15	0.65
TCEP	0.01 - 10	0.9996	92	0.10	0.20	96	0.10	0.20
TEHP	0.01 - 250	0.9997	63	0.15	0.30	52	0.20	0.40
EHDPP	0.01 - 100	0.9999	77	0.15	0.25	71	0.15	0.30
TPP	0.025 - 75	0.9997	72	0.15	0.70	68	0.15	0.75
TTP	0.025 - 100	0.9999	81	0.10	0.60	82	0.10	0.60
Phthalate esters								
DMP	0.01 - 50	0.9994	80	0.15	0.25	113	0.10	0.20
DEP	0.01 - 250	0.9991	75	0.15	0.30	132	0.10	0.15
DiBP	0.01 - 75	0.9995	52	0.20	0.40	98	0.10	0.20
DEHA	0.01 - 75	0.9998	59	0.20	0.35	62	0.20	0.30
DEHP	0.01 - 75	0.9984	104	0.10	0.20	104	0.10	0.20
DnOP	0.025 - 75	0.9959	82	0.25	0.60	82	0.25	0.60
Benzothiazoles								
BT	0.025 - 75	0.9995	50	0.40	1.00	64	0.30	0.75
ClBT	0.01 - 75	0.9996	51	0.20	0.40	73	0.15	0.25
NH_2BT	0.025 - 50	0.9958	76	0.20	0.70	68	0.20	0.75
MeSBT	0.01 - 75	0.9997	67	0.15	0.30	82	0.10	0.25
OHBT	0.05 - 75	0.9967	105	0.15	0.95	112	0.15	0.90
Benzotriazoles								
BTR	0.25 - 50	0.9990	81	0.60	6.20	75	0.70	6.65
4TTR	0.25 - 50	0.9997	70	2.15	7.15	70	2.10	7.15
5TTR	0.25 - 75	0.9992	100	1.50	5.00	107	1.40	4.70
XTR	1 - 100	0.9986	102	9.50	19.50	91	11.00	22.00
Benzenesulfonamides								
BSA	0.025 - 75	0.9999	85	0.15	0.60	84	0.15	0.60
o-TSA	0.5 - 100	0.9999	73	6.85	13.70	73	6.85	13.70
p-TSA	0.025 - 75	0.9992	81	0.15	0.65	82	0.15	0.60
Me-p-TSA	0.025 - 75	0.9994	71	0.15	0.70	67	0.15	0.75
Et-p-TSA	0.025 - 75	0.9999	83	0.15	0.60	75	0.15	0.70

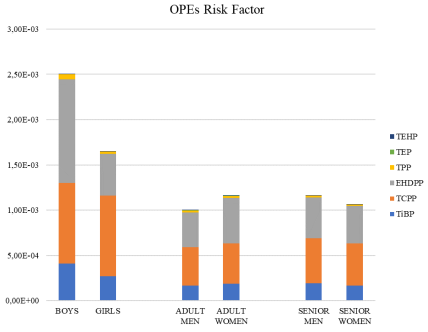
Table S3. ENCAT 2003 data on seafood dietary intake of Catalonia population

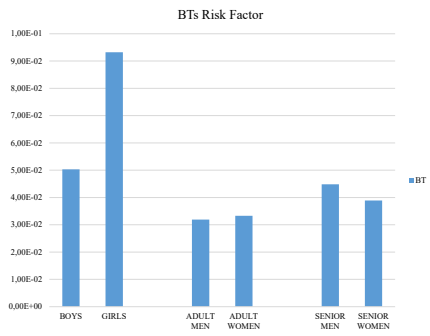
	Boys	Adult men	Senior men	Girls	Adult women	Senior women
Hake (*Merluccius merluccius*)	7,82	15,03	23,02	10,84	14,49	14,56
Sardine *(Sardina pilchardus)*	0,99	2,92	2,60	2,08	2,69	4,7
Tuna *(Thunnus thynnus)*	0,71	1,62	1,07	0,00	1,45	0,52
Sole *(Solea solea)*	6,22	4,84	3,65	2,44	5,28	5,17
Cod *(Gadus morhua)*	2,13	4,18	8,08	0,60	4,61	8,15
Mackerel *(Scomber vincialis)*	0,36	1,13	0,50	0,32	1,27	2,86
Salmon *(Salmo salar)*	3,30	1,80	2,23	1,00	3,00	1,14
Mussel *(Mytilus galloprovincialis)*	1,26	0,97	2,06	0,00	1,84	0,67
Squid *(Loligo vulgaris)*	1,88	3,17	3,18	5,18	3,17	0,77
Shrimp *(Aristeus antennatus)*	2,71	2,83	2,42	2,94	3,44	1,68

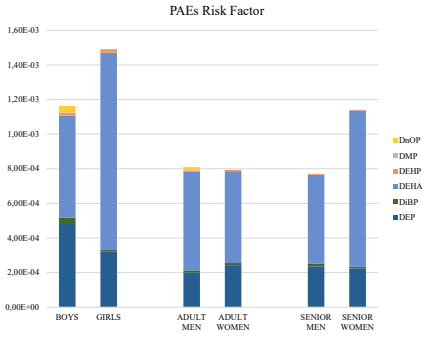
Figure S4. Risk assessment of HPVs for all the subgroups in different exposure scenarios

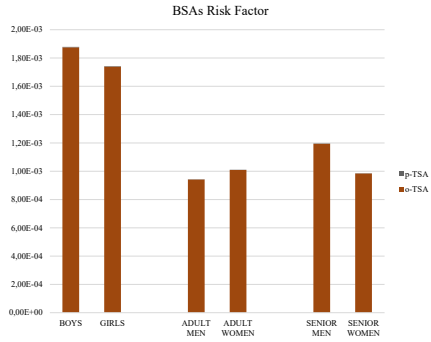


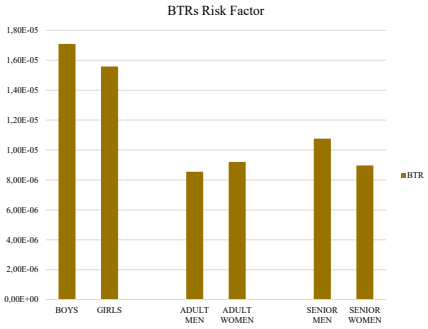
MIDDLE-BOUND SCENARIO

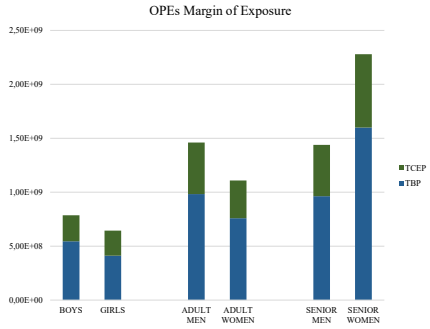




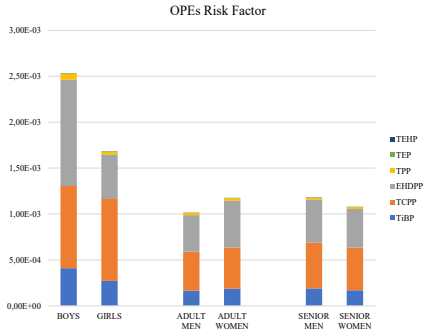


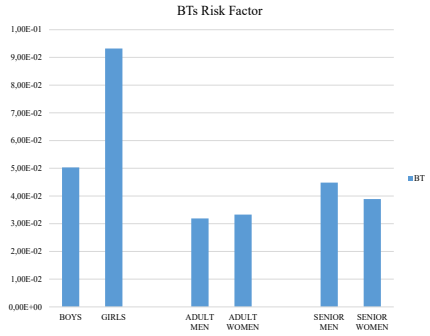


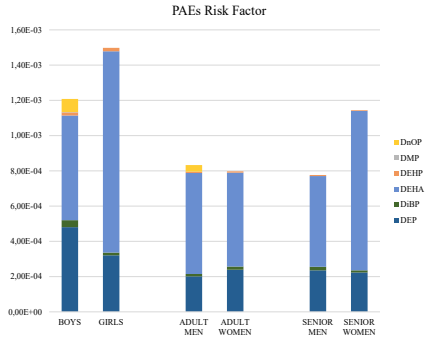


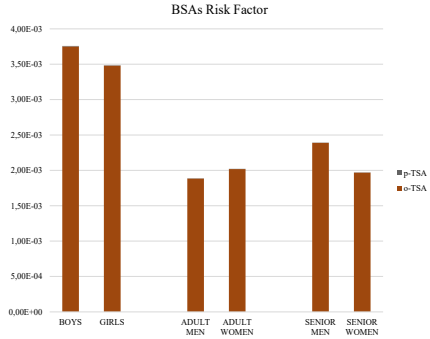


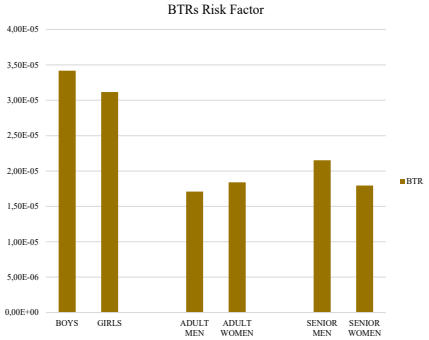
UPPER-BOUND SCENARIO

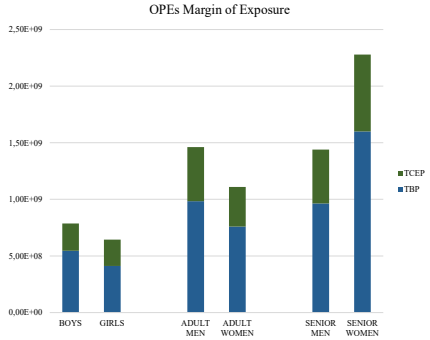












3.2.2. Seafood consumption as a source of high production volume chemicals exposure: a comparison between Catalonia and the Canary Islands

SEAFOOD CONSUMPTION AS A SOURCE OF HIGH PRODUCTION VOLUME CHEMICALS EXPOSURE: A COMPARISON BETWEEN CATALONIA AND THE CANARY ISLANDS

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Abstract

Seafood takes an important role in population's diet due to the health benefits usually tied to its consumption. The presence of chemical compounds such as high production volume chemicals could lead to a potential risk towards people due to the intake of these compounds via seafood consumption. On this purpose, specimens of the most consumed seafood species in Catalonia and in the Canary Islands were collected to assess the occurrence of HPVs and further estimate the exposure and risk associated to their ingestion. Results showed higher levels of HPVs in samples from Catalonia with a prevail of phthalate esters and benzenesulfonamides over the rest of the target compounds for samples from both locations. Multivariate analysis showed spatial differences between HPVs mean concentration profiles for Catalonia and the Canary Islands samples. Exposure values were higher for Catalonia samples, however, none of the locations posed a high level of concern for the intake of HPVs via seafood consumption.

Highlights

- HPVs were detected in all the analysed samples from both locations.
- HPV profiles enable the classification of seafood species from different locations.
- Catalonia and Canary Islands based diets pose no risk towards population.

1. Introduction

High production volume chemicals (HPVs) list appeared as a form of prioritising chemicals to gather information to produce screening information datasets (SIDS) relative to environmental, occupational and consumer exposure. Initially started by the OECD countries in the nineties, compounds with productions over 1,000 tonnes per year were susceptible to being included in the list [1]. In the same direction, the US environmental protection agency (EPA) also started to list compounds manufactured over 500 tonnes per year to produce information on hazard assessment. Compound families in the present list include many contaminants belonging to various compound classification groups such as legacy contaminants, e.g., some polycyclic aromatic hydrocarbons, as well as emerging contaminants (organophosphate flame retardants, synthetic phenolic antioxidants, benzo-triazole ultraviolet stabilizers, etc.) [2,3]. The present study focuses on the occurrence of four of the families present in the HPVs group: phthalate esters (PAEs), benzothiazoles (BTs), benzenesulfonamides (BSAs) and organophosphate esters (OPEs). These groups have a wide range of physicochemical properties; hence their use is intended in different applications. PAEs and some OPEs are used as plasticisers to soften materials made of plastic, whereas other OPEs are mainly used as an alternative to poly-brominated diphenyl ethers (PBDEs) to prevent or delay fire ignition [4,5]. These two groups are therefore found in many daily commodities such as electronics, furniture, toys and even textiles [6,7].

On the other hand, benzothiazoles and benzenesulfonamides have been widely used as rubber vulcanization accelerators or anticorrosive agents in antifreeze formulations for the first, and disinfectants or fungicides for the later [8]. Regarding their toxicological properties, some of these compounds are classified as endocrine disruptors (PAEs) [9], neurotoxic (OPEs) [10] and even carcinogenic (e.g. TBP, TCEP) [11]. BTs have been linked with dermal sensibilisation and respiratory irritation [12], whereas for BSAs, compounds such as p-TSA are considered moderately toxic [8]. Due to these compounds not being chemically bonded to the materials, their release towards the environment can be easily achieved by abrasion, volatilization, and domestic/ industrial discharges [13]. Their release towards the environment has resulted on a widespread occurrence in many environmental fates including air [13,14], dust [15], water [16–18], biota [19–21], and even biological samples [22,23]. As water bodies receive effluents flooded with remaining of these compounds which may not have been removed at waste-water

treatment plants (WWTP) [8], HPVs reach seas and oceans, where these have also been determined by other authors [24–26]. Once they reach the aquatic environment, these become bioavailable to the species inhabiting, e.g. seafood. Several studies have reported the presence of HPVs in seafood samples around the world, especially for PAEs and OPEs [27]. The extensive presence of these compounds in seafood samples along with the concern on how the negative health effects associated to them can affect population has led to the necessity of further research on this topic. Studies involved in the determination of HPVs in seafood samples mainly convey in the necessity of providing tools to evaluate the exposure and risk associated to the dietary intake of HPVs via seafood consumption.

The suspected toxicity of some of these compounds has led to the development of studies of dose-response relationship so as to assess No-Observed-Adverse-Effect Level (NOAEL) values for their oral ingestion. These values can then be used to evaluate the exposure and risk towards population. As part of risk analysis, exposure assessment is fundamental. The determination of HPVs in fish species commercially available along with data concerning fish consumption enables the calculation of the exposure via dietary intake of HPVs towards the population. It is also necessary to point out that these assessment calculations take into consideration the amount of fish consumed as well as the concentrations found for a certain compound. Hence, differences are found depending on the population's diet. Spanish gastronomy is rich and varied inside the country itself, which means not all regions have a certain diet in common. Most consumed seafood species of each location mainly differ on what is commercially available in that place. Catalonia, being a Mediterranean coast region, usually offers proximity species from the Mediterranean Sea in its markets, but mostly relies on imported fish specimens from species not usually found in the Mediterranean Sea. On a counterpart, the consumption of autochthonous seafood species has a bigger role in the Canary Islands, these being an archipelago in the Atlantic Sea. Differences in the consumed species could lead to a differentiation on the levels of HPVs found in the fishes, thus leading to diverse exposure values between diets containing on or another species.

The present study aims to compare the concentrations of HPVs in the most consumed fish species from Catalonia and the Canary Islands. Once the HPVs are determined, calculations on the exposure and risk associated to the intake of these compounds via dietary intake will be performed.

2. Materials and methods

2.1. Standards and reagents

Compounds from four different HPV families were included in the present study: organophosphate esters (OPEs), benzothiazoles (BTs), benzene-sulfonamides (BSAs) and phthalate esters (PAEs). Organophosphate esters included tri-isobutyl phosphate (TiBP), tributyl phosphate (TBP), triethyl phosphate (TEP), 2-ethylhexyl-diphenyl phosphate (EHDPP), tris (2- ethylhexyl) phosphate (TEHP), tritolyl phosphate (TTP), tris(2-chloroethyl) phosphate (TCEP), triphenyl phosphate (TPP), tris(2-chloroisopropyl) phosphate (TCPP); benzothiazoles (BTs) included 2-amino-1-H-benzothiazole (NH2BT), 2-hydroxibenzothiazole (OHBT), 1-H-benzothiazole (BTH), 2-(methylthio)-benzothiazole (MeSBT), 2-chlorobenzothiazole (ClBT); pththalate esters (PAEs) included dimethyl phthalate (DMP), diethylhexyl phthalate (DEHP), diethyl phthalate (DEP), di-iso-butyl phthalate (DiBP), diethylhexyl adipate (DEHA) and di-n-octylphthalate (DnOP); benzenesulfonamides (BSAs) included benzenesulfonamide (BSA), ortho-toluenesulfonamide (o-TSA), para-toluenesulfonamide (p-TSA), N-ethyl-para-toluenesulfonamide (Et-p-TSA) and N-methyl-para-toluene-sulfonamide (Me-p- TSA). All the analytical standards previously listed were purchased from Sigma Aldrich (St. Louis, USA) with a purity grade >98%. Deuterated compounds used as internal standards were acquired from LGC Standards (Teddington, UK): d4-benzothiazole (d4-BT), d27-tributyl phosphate (d27-TBP), d4-diethylhexyl-phthalate (d4-DEHP) and d4-p-TSA (d4-p-TSA). Each of the internal standards were applied for the group of compounds of the same family as the deuterated compound.

Helium and nitrogen gas were supplied by Carburos Metálicos (Tarragona, Spain) with >99.999% purity. Acetonitrile and ethyl acetate used for the extraction were GC grade from Scharlab (Barcelona, Spain) with purity >99.9% and ultrapure water was obtained from a Millipore (Massachusetts, USA) Synergy water purification system. Original method salt packets for QuEChERS extraction and disposable PTFE 0.22 µm filters and syringes were purchased at Scharlab, while LipiFiltr push-through cartridges for the clean-up were obtained from Carlo Erba (Barcelona, Spain).

2.2. Sample collection and treatment

Samples of four different seafood species, mainly fish, were bought in two different locations. Samples of the most consumed species from Catalonia were bought in local markets from Tarragona, Spain and included: sole (*Solea solea*), cod (*Gadus morhua*), mackerel

(*Scomber vincialis*) and yellowfin tuna (*Thunnus thynnus*) specimens. As regards the most consumed species from the Canary Islands, these were bought in local markets from Las Palmas de Gran Canaria (Canary Islands), Spain. Local species specimens of mackerel (Scomber colias), comber (Serranus cabrilla), common pandora (Pagellus belloti) and skipjack tuna (Katsuwonus pelamis) were bought. Each of the species were bought per triplicate and subsequently mixed to create a composite sample. Once the samples were obtained, lateral fillets were dissected and stored at -20ºC until its lyophilisation. Samples were then freeze-dried using a miVac Duo freeze-drying system from Genevac (Ipswich, UK). Once lyophilised, samples were ground and sifted through a 300 µm mesh to obtain powder. The powders obtained for each of the species were kept in sealed glass containers until their analysis.

2.3. QuEChERS extraction

Samples were extracted using a previously developed method. Concisely, 0.1g of dry weight fish sample were weighted in a 50 mL glass centrifuge tube. Subsequently, 10 mL of ultrapure water and 10 mL of acetonitrile were added to the tube and the mixture was vortex mixed for 1 min. A packet of QuEChERS extraction salts containing 1g of anhydrous sodium acetate and 4g of magnesium sulfate was added to the tube and mixed for 3 min. The tubes were then centrifuged for 5 min at 4000 rpm using a Hettich Universal 32R centrifuge (Tuttlingen, Germany). The upper layer (acetonitrile) was then collected and further cleaned using a LipiFiltr push-through cartridge. The extract was finally evaporated to circa 0.5 mL under a gentle nitrogen stream and reconstituted to 2 mL with ethyl acetate after incorporating the internal standard mixture at 50 µg L-1. The obtained extracts were filtrated with a 0.22 µm PTFE syringe filter and kept at -20ºC until their analysis with GC-QqQ.

2.4. Instrumental analysis

The extracts obtained from the QuEChERS extraction were analysed using an Agilent 8890 GC system coupled to an Agilent 7000D QqQ mass spectrometer from Agilent Technologies (Palo Alto, USA). The instrument included a PAL RSI 120 system from CTC Analytics (Zwingen, Switzerland) for the automatic injection of the extracts. A sample volume of 25 µL was injected into the Agilent Multi-Mode Inlet (MMI) using solvent vent mode. Solvent vent mode parameters were as follows: purge to split vent set at 60 mL/min at 2.87 min and vent flow of 120 mL/min and 5 psi for 0.37 min. The inlet ramped from 75ºC (held for 0.37 min) to 325 ºC (held for 5 min) at 600ºC/min. The

separation was achieved using a ZB-50 capillary column (30 m x 25 mm i.d. and 0.25 µm film thickness) from Phenomenex with the following temperature program: initial temperature of 75ºC (held 2.87 min) raised to 300ºC (held 5 min) at 15ºC/min. The overall runtime was of 22.87 min using a constant flow of 1.2 mL/min of helium as carrier gas. The mass spectrometry system operated in electron ionisation mode (70 eV) at 230ºC, 150ºC and 150ºC for ion source, quadrupole 1 and quadrupole 2, respectively. Multiple reaction monitoring (MRM) mode was selected for the quantification using one quantifier transition (Q) and two qualifier transitions (q) for each of the target compounds. For qualitative and quantitative analysis, Agilent Mass-Hunter Workstation was used in its 10.0 version.

2.5. Quality assurance and quality control (QA/QC)

Quantification of the samples was carried out using internal standard calibration. Apparent recoveries were applied varying on the lipid content of the species, thus being low lipid content species: sole (Solea solea), cod (Gadus morhua), comber (Serranus cabrilla) and common pandora (Pagellus belloti), for which apparent recoveries ranged between 50 – 104 % and high lipid content species: mackerel (Scomber vincialis), yellowfin tuna (Thunnus thynnus), mackerel (Scomber colias), and skipjack tuna (Katsuwonus pelamis) for which apparent recoveries ranged between 52 – 132%. Additional method quality parameters are found in Table S1. Regarding quality assurance, it is important to note that phthalate esters and some organophosphate esters may be present in plastic materials as well as in the ambient, which could lead to background signal, thus leading to non-reliable determinations. In order to reduce this effect, plastic material was avoided when possible and glass alternatives were used. Two procedural blank extractions were included in every batch of samples (10 samples per batch) to subtract the signal. Quality controls were also included every 5 samples along with system blanks to check the system and prevent carry-over.

2.6. Exposure and risk assessment

Human exposure values (E_t) were computed for three different scenarios. For the lower-bound scenario non-detected compounds and below-LOQ compounds were estimated as 0 and the LOD, respectively. Middle-bound scenario included half the LOD for non-detected compounds and half the LOQ for below-LOQ compounds. Finally, upper bound scenario was assessed with values of the LOD and the LOQ for non-detected and below-LOQ, respectively. The exposure values were

calculated according to the following equation (Eq. 1):

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

Where E_t was the human exposure (ng kg^{-1} b.w. day^{-1}), C_f the mean fish consumption of the individual fish species *f* and $X_{t,f}$, the concentration found in the present study for the individual compounds *t* for the *f* species (ng g^{-1} w.w.). Data regarding fish consumption was obtained from the 2021 annual Spanish food consumption report conducted by the Spanish Ministry of Agriculture, Fisheries and Food [28]. Consumption per capita of the most common fish species was segregated between total Spanish population, Catalonia population and Canary Islands population. As no available data exists for the consumption of the autochthonous fish species from the Canary Islands specifically, the consumption of comber, common pandora and skipjack tuna were assumed as the same as other species with the same lipid content from the Catalan species. Hence, common pandora and comber were assumed as cod and sole, respectively, while skipjack tuna was assumed as white tuna. Table 1 compiles the mean consumption values in g day^{-1} for the selected species.

All the calculations were performed assuming that ingested HPVs were 100% absorbed. Risk was assessed for the obtained exposure values in the three scenarios. For non-genotoxic and non-carcinogenic compounds, the NOAEL (no-observed-adverse-effect-level) approach was followed, for which the risk was calculated as the result of the division between the dietary exposure (E_t) previously obtained by the acceptable daily intake (ADI_t) multiplied by 100. At the same time, the acceptable daily intake was obtained by

Table 1. Consumption data (g day^{-1}) of the selected fish species for each of the studied regions. Data extracted from the 2021 annual Spanish food consumption report (Ministerio de Agricultura Pesca y Alimentación, 2022).

Species	**FOODX2 code**	**Location**	
		Catalonia	**Canary Islands**
Sole (*Solea solea)*	A02CB	0.63	0.78
Cod (*Gadus morhua*)	A02BX	3.51	1.82
Mackerel *(Scomber colias)*	A02DX	0.70	1.01
Tuna (*Thunnus thynnus)*	A02DX	6.15	8.97

dividing the NOAEL value by an uncertainty factor of 100. For carcinogenic and genotoxic compounds such as TBP and TCEP, benchmark dose (BMD) was used to calculate the margin of exposure (MOE_t). In this case, the BMD for which a response of 5-10% above the control is caused was divided by the dietary exposure.

2.7. Statistical data analysis

PLS Toolbox 9.0 (Eigenvector Inc, Manson, WA, USA) for Matlab 2022a (Mathworks Inc, Natick, MA, USA) was used for data analysis. Principal component analysis (PCA) was used for exploratory data analysis. Classification of the different seafood species was carried using the soft independent modelling of class analogies (SIMCA) and the k-nearest neighbours (knn) techniques. Prior to any model calculation, data were autoscaled to take into account differences in concentration scales of the different compounds.

The criterion to select the number of PCs for the SIMCA model corresponding to each class was to select the minimum number of PCs that explained at least 95% of the variance for each class. In the SIMCA classification, samples were assigned to the class with the highest probability. In the knn classification, k, the number of nearest neighbours, was selected to 1 because of the low number of available samples in each class. Sensitivity and specificity were the parameters used to assess the goodness of the classification [29].

3. Results and discussion

3.1. Occurrence of HPVs in Catalonia and the Canary Islands samples

OPEs, BSAs, PAEs and BTs were detected in all the analysed samples from both locations. For Catalonia, BSAs were detected in 100% of the analysed samples followed by BTs with 81%, OPEs with 53% and PAEs with 44%. In the case of the Canary Islands, OPEs leads in detection frequency (66%) closely followed by BSAs (63%) and further BTs (44%) and PAEs (31%). Differences are observed between locations in terms of detection frequencies. While BSAs maintain a high detection frequency in both, OPEs leads in Canary Islands samples. BTs also appear to half their rate in Catalonia samples than in the Canary Islands ones. As for PAEs, their detection frequency appears as the lowest in both locations, being slightly higher for samples from Catalonia. Table 2 gathers the concentrations determined for each of the species of the two locations, Catalonia and the Canary Islands, their mean concentrations, range and detection frequency. If focused on the concentrations, samples from Catalonia showed the highest mean concentration

for BSAs with 94.2 ng g^{-1} w.w. (ranging from <MQL to 166 ng g^{-1} w.w.), then PAEs with 83.6 ng g^{-1} w.w. (range between n.d. – 290 ng g^{-1} w.w.), OPEs with 20.5 ng g^{-1} w.w. (range between n.d. – 16.4 ng g^{-1} w.w.) and finally BTs with 15.4 ng g^{-1} w.w.(range between n.d. – 24.3 ng g^{-1} w.w.). For the Canary Islands samples, trend follows as BSAs with 113 ng g^{-1} w.w. (ranging from n.d. to 242 ng g^{-1} w.w.), then PAEs with 82.1 ng g^{-1} w.w. (range between n.d. – 122 ng g^{-1} w.w.), OPEs with 16.5 ng g^{-1} w.w. (range between n.d. – 28.0 ng g^{-1} w.w.) and finally BTs with 5.3 ng g^{-1} w.w.(range between n.d. – 24.3 ng g^{-1} w.w.). Regarding differences between concentrations of each of the target HPV families, no significant differences were found for any of the families except for benzothiazoles, for which levels in Catalonia samples (15.4 ng g^{-1} w.w.) were significantly higher than those in the Canary Islands (5.3 ng g^{-1} w.w.) ($p<0.05$). Catalonia's cod appeared as the species with the highest OPEs and BTs mean concentration with 4.8 and 6.5 ng g^{-1} w.w., respectively. As for BSAs and PAEs, Catalonia's mackerel appears as the predominant with 152 and 73 ng g^{-1} w.w., respectively. Catalonia contributes with the species with the highest mean concentrations for all the studied HPV families. Nevertheless, skipjack tuna and common pandora, both species from the Canary Islands, appear as the second species with the highest mean concentration for PAEs and BSAs, respectively.

Focusing on congeners, Figure 1 displays the boxplot compound such as NH2BT, OHBT, DEHA and DEHP were only found in samples from Catalonia whereas TEHP was only found in the Canary Islands. Regarding the rest of compounds, MeSBT, TPP, TTP, BSA, DiBP, Et-p-TSA and DEP were found at higher concentrations in the Canary Islands than in Catalonia, whereas, as a counterpart, BT, EHDPP, TBP, TCEP, TCPP and TiBP were higher in the later. It can be observed that benzothiazoles are more keen to appear in the samples from Catalonia, thus being the cause of the significative differences between the mean concentrations of BTs between both locations.

Presence of these compounds in seafood samples has been reported by other authors. Studies such as the ones conducted by Poma et al. [30] or Sala et al. [31] reported the occurence of OPEs congeners like TBP, TCEP, TCPP, TEHP, EHDPP in samples of seafood from either the Belgian market or different locations of the Mediterranean Sea, respectively. Both studies agree on the occurrence of aryl and chlorinated OPEs in most of the analysed samples at concentration levels between 5.3 – 174 and 1.39 – 73.4 ng g^{-1} w.w., respectively. Phthalate esters ocurrence in fish covers a wide range of concentration levels. As

Table 2. Mean concentrations, range and detection frequencies (%DF) of the determined HPVs in seafood (ng g-1 wet weight, w.w.) classified by species.

	Canary Islands							
	Common pandora	Mackerel	Skipjack tuna	Comber	TOTAL	Mean	Range	DF%
Benzothiazoles								
BT	3.1	5.4	8.3	1.5	18.3	4.6	1.5 - 8.3	100
OHBT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
NH2BT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
MeSBT	2.2	0.5	n.d.	<MQL	2.7	1.4	n.d. - 2.2	75
ΣBTs	5.3	5.9	8.3	1.5	21.0	5.3	1.5 - 8.3	44
Organophosphate esters								
TBP	3.9	n.d.	2.1	4.6	10.6	3.5	n.d. - 4.6	75
TiBP	0.8	n.d.	n.d.	1.7	2.5	1.2	n.d. - 1.7	50
TCPP	1.4	n.d.	n.d.	n.d.	1.4	1.4	n.d. - 1.4	25
TCEP	2.2	2.3	2.1	0.4	6.9	1.7	0.4 - 2.3	100
TEHP	0.9	2.4	n.d.	<MQL	3.4	1.7	n.d. - 2.4	75
EHDPP	3.1	0.3	n.d.	<MQL	3.4	1.7	n.d. - 3.1	75
TPP	n.d.	3.3	4.1	n.d.	7.4	3.7	n.d. - 4.1	50
TTP	1.4	n.d.	28.0	1.1	30.6	10.2	n.d. - 28.0	75
ΣOPEs	13.6	8.3	36.2	7.9	66.0	16.5	7.9 - 36.2	66
Benzenesulfonamides								
BSA	242	<MQL	14	56.4	312	104	<MQL - 242	75
Et-p-TSA	n.d.	n.d.	140	<MQL	140	140	n.d. - 140	50
ΣBSAs	242	n.d.	154	56.4	452	113	n.d. - 242	63
Phthalate esters								
DEP	n.d.	n.d.	184	n.d.	184	184	n.d. - 184	25
DiBP	122	20.9	1.8	n.d.	145	48.3	n.d. - 122	75
DEHA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
DEHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
ΣPAEs	122	20.9	185	n.d.	328	82.1	n.d. - 185	31

Table 2. (Continued)

	Catalonia							
	Sole	Cod	Mackerel	Yelowfin tuna	TOTAL	Mean	Range	DF%
Benzothiazoles								
BT	<MQL	24.3	4.5	1.4	30.3	10.1	<MQL - 24.3	100
OHBT	6.8	1.6	3.7	4.4	16.5	4.1	1.6 - 6.8	100
NH2BT	3.4	<MQL	5.8	2.9	12.1	4.0	<MQL - 5.8	100
MeSBT	1.0	n.d.	1.5	n.d.	2.6	1.3	n.d. - 1.5	50
ΣBTs	11.3	25.9	15.6	8.7	61.5	15.4	8.7 - 25.9	81
Organophosphate esters								
TBP	4.3	6.7	3.4	n.d.	14.4	4.8	n.d. - 6.7	75
TiBP	3.6	1.7	n.d.	n.d.	5.3	2.7	n.d. - 3.6	50
TCPP	16.4	14.0	2.7	n.d.	33.2	11.1	n.d. - 16.4	75
TCEP	1.0	7.4	n.d.	n.d.	8.36	4.18	n.d. - 7.4	50
TEHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
EHDPP	1.0	1.6	2.4	4.1	9.2	2.3	1.0 - 4.1	100
TPP	1.8	n.d.	n.d.	3.4	5.1	2.6	n.d. - 3.4	50
TTP	n.d.	6.6	n.d.	n.d.	6.6	6.6	n.d. - 6.6	25
ΣOPEs	28.1	38.0	8.5	7.5	82.2	20.5	7.5 - 38.0	53
Benzenesulfonamides								
BSA	5.4	15.0	166	9.5	195	48.9	5.4 - 166	100
Et-p-TSA	13.7	28.8	139	<MQL	181	60.4	<MQL - 139	100
ΣBSAs	19.1	43.8	304	9.5	377	94.2	19.1 - 304	100
Phthalate esters								
DEP	11.3	n.d.	n.d.	19.5	30.8	15.4	n.d. - 19.5	50
DiBP	1.0	n.d.	n.d.	n.d.	1.0	1.0	n.d. - 1.0	25
DEHA	3.3	1.2	n.d.	n.d.	4.5	2.2	n.d. - 3.3	50
DEHP	8.3	n.d.	290	n.d.	298	149	n.d. - 290	50
ΣPAEs	23.7	1.2	290	19.5	334	83.6	1.2 - 290	44

its sources include different routes such as incorporation from the aquatic envionment, ingestion of microplastics and even migration from food packaging [32,33], the reported concentrations for these compounds are generally dispair, with congeners such as DEHP and DBP being usually found [34]. In the present study, phthalate esters were found to a lesser extent in terms of detection frequency (up to 31 and 44% in the Canary Islands and Catalonia, respectively), at concentrations ranging between 1.2 – 290 ng g^{-1} w.w.. DEHP appears as the compound with the highest concentration (290 ng g^{-1} w.w. in Catalonia), but other compounds such as DiBP or DEP are also found at concentrations above 100 ng g^{-1} w.w. Benzothiazoles occurrence is not as widley found in literature. Trabalón et al. [35] and Jia et al. [36] reported the presence of BT, MeSBT, NH2BT and OHBT among other congeners in fish samples from Tarragona and mollusc samples from the Bohai Sea of China, respectively. Levels of concentration ranged from low ng g-1 (8-88 ng g^{-1} w.w.) in fish samples to values up to 13800 ng g^{-1} w.w. in the most contaminated mollusc samples from China. Results of the present study for the presence of BTs agree with the reported data, with levels found surrounding the low ng g^{-1} as expected. No data on the presence of BSAs in seafood has been reported to date. However, data on the presence of BSA and Et-p-TSA in WWTP influent and effluent waters suggest a possible biomagnification of these compounds as results of the procedures conducted at the wastewater treatment plants [8]. Hence, these compounds are expected to be present in the aquatic environment and thus, seafood. Thus, their presence in samples from both locations suggest a widespread ubiquity of these compounds.

Multivariate statistical analysis was conducted using principal component analysis (PCA) to check possible spatial related patterns for HPVs present in seafood species from both locations (Catalonia and Canary Islands). Results of the PCA were displayed using PC1 (47.88%) vs. PC2 (39.38%) vs. PC3 (10.35%) scores. In this case, variables were reduced to the sum of each of the target HPV families (ΣOPEs, ΣBTs, ΣPAEs and ΣBSAs) mean concentrations. Figure 2A shows the biplot (where the scores and the loadings of the PCA analysis are shown in the same plot) of the above-mentioned PCA analysis, for which it can be observed that a separation pattern exists for samples of Catalonia (Cat) and the Canary Islands (Can) along positive values of PC2 and PC3. For the sake of clarity Figures 2B and 2C show the scores and loading plots to help the reader to better understand the position of the scores and loadings in the 3D space, and their relationships. The relative position of the scores in

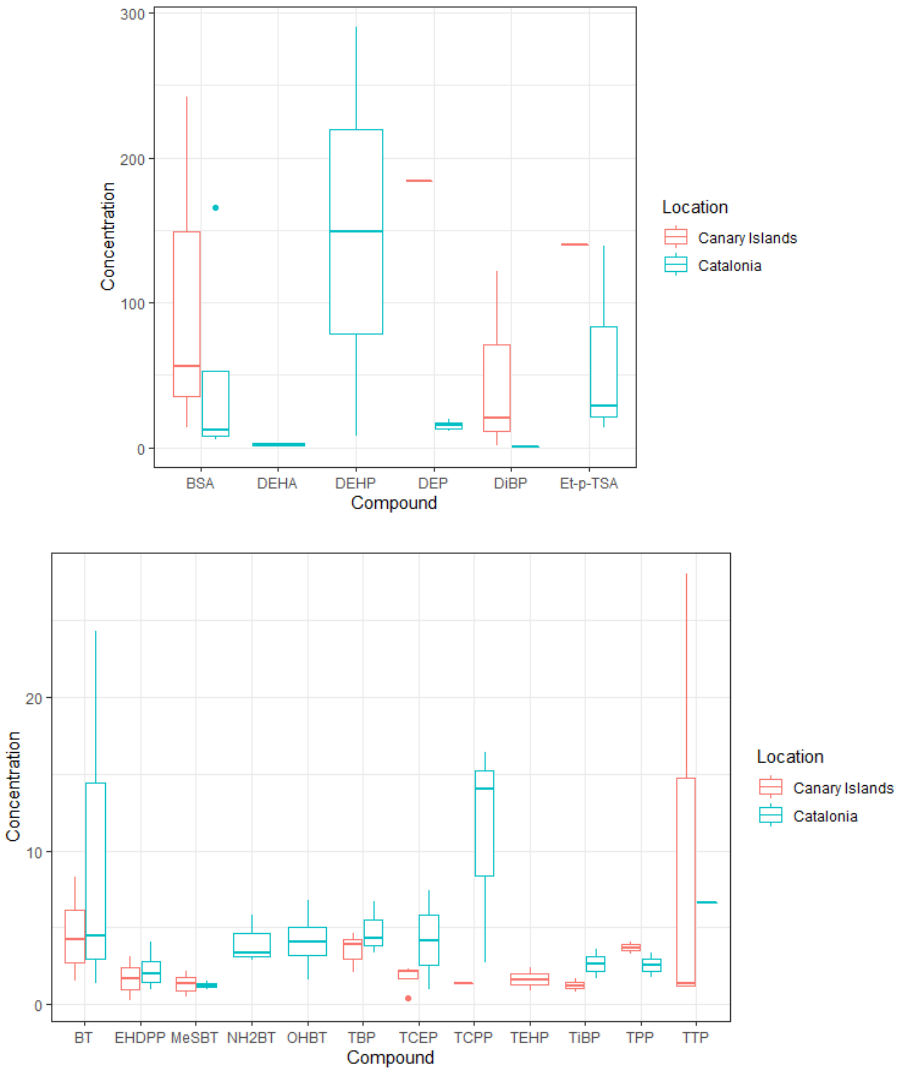

Figure 1. Total concentrations of HPVs in the analysed samples for the Canary Islands (blue) and Catalonia (red) samples. The box plot shows the 25th and 75th percentile concentrations (bottom and top edges of the box), the maximum and minimum concentrations (bottom and top whiskers) and the median concentration (line within the box).

Figure 2 means that samples in Catalonia tend to higher concentrations of ΣBTs and ΣOPEs and samples in Canarias tend to higher concentrations of ΣPAEs and ΣBSAs, what can be seen along PC1. In this way, PC1 may discriminate between HPV families that tend to give more peaks of concentration (ΣPAEs and ΣBSAs) from HPV families with more homogeneous values (ΣBTs and ΣOPEs). Samples of the Canary Islands appear with a lesser dispersion when compared with Catalonia samples, thus indicating a higher similarity between mean HPVs concentrations for samples from the Canary Islands than for the Catalonia ones. In consideration of the previously stated, spatial differences could be considered. These differences could be also linked to the fact that most of these species come from a common origin (Atlantic Sea surrounding the Canary Islands), which differs from the different origins of the usually consumed species from Catalonia. To check this difference, two classification techniques (SIMCA and knn) were used to classify the samples from the two locations. Due to the small number of total samples (four samples for each class), only results for the classification of the samples used to make the models (training set) are presented. More samples needed to be included in future studies to perform a more adequate validation including an external set of samples (test set) or internal validation using cross-validation. Considering these premises, all the specificities and sensitivities for the two classes (Catalonia and Canary Islands) were 1 using both SIMCA and knn, concluding that the study of HPVs is a good option to differentiate seafood samples from Catalonia and Canary Islands.

3.2. Exposure and risk assessment

The seafood samples analysed in the present study comprise species which are commonly consumed by the population of both regions. Hence, data regarding the concentrations of HPVs found in the samples can be used to estimate the exposure and risk of people of both locations towards HPVs present in seafood via dietary intake. Calculations performed in the present study consider a 100% ingestion of the compounds present in the fish. Human exposure values (E_t) for the different compound families for the three studied scenarios are summarized in Table 3. If the upper-bound scenario values are compared, the highest contributors of each of the compound's families for Catalonia and the Canary Islands were TCPP (1.71 ng kg^{-1} bw day^{-1}) and TTP (0.82 ng kg^{-1} bw day^{-1}) for OPEs; BT (1.60 and 0.49 ng kg^{-1} bw day^{-1}) for BTs; BSA (10.02 and 8.31 ng kg^{-1} bw day^{-1}) for BSAs; and DEHP (15.28 ng kg^{-1} bw day^{-1}) and DEP (4.89 ng kg^{-1} bw day^{-1}) for PAEs, respectively. Regarding the species with the most contribution for each of the

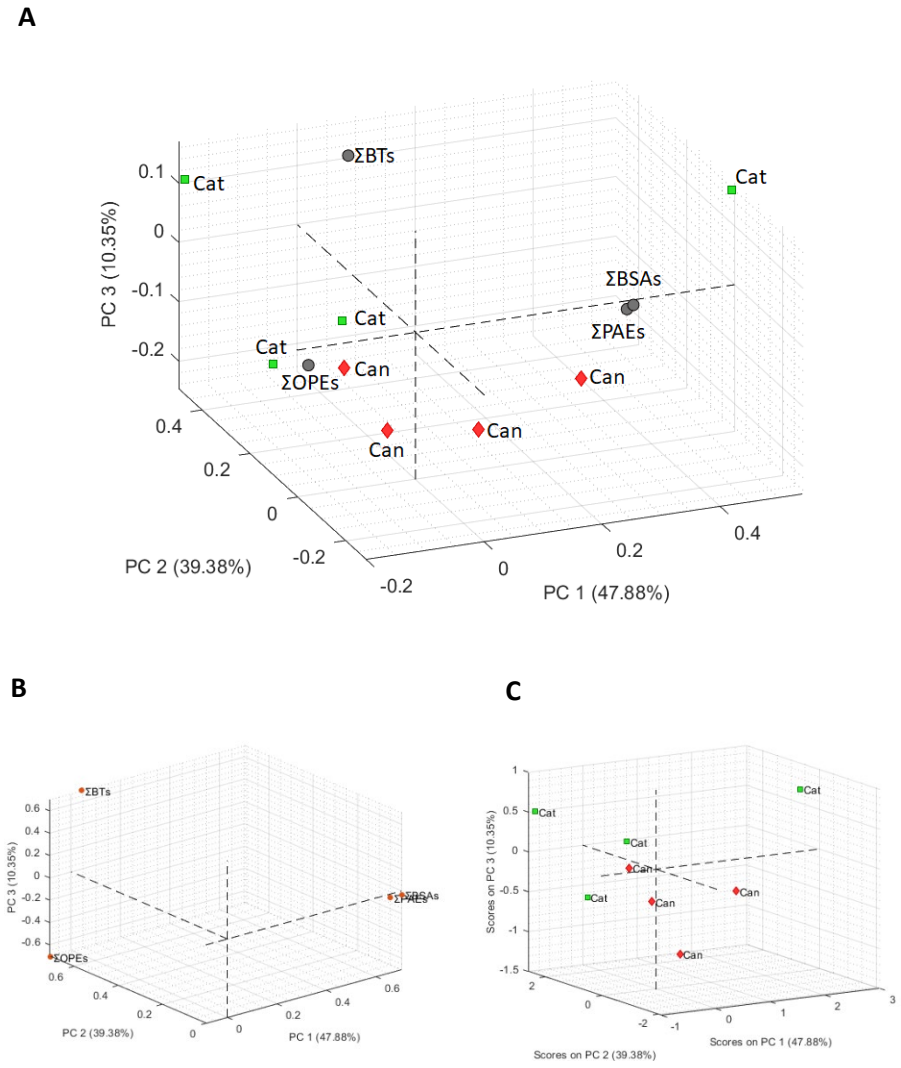

Figure 2. A) Biplot showing the scores and loadings of the principal component analysis for the samples from Catalonia and the Canary Islands. B) Scores C) Loadings

groups: in Catalonia, cod appeared as the species with the highest contributions to OPEs and BTs with values of 1.97 ng kg^{-1} bw day^{-1} and 1.38 ng kg^{-1} bw day^{-1}, respectively, whereas mackerel was responsible of the highest contribution for BSAs and PAEs with values of 15.97 and 14.89 ng kg^{-1} bw day^{-1}, respectively. On the other hand, in Canary Islands skipjack tuna reported the highest contributions for OPEs, PAEs and BTs (0.98, 4.94 and 0.24 ng kg^{-1} bw day-^{1}) and common pandora for BSAs (6.62 ng kg^{-1} bw day^{-1}). For both regions, BSAs represented the highest exposure levels (20.81 and 12.83 ng kg^{-1} bw day^{-1}), followed by PAEs (17.26 and 8.83 ng kg^{-1} bw day^{-1}), OPEs (4.33 and 1.82 ng kg^{-1} bw day^{-1}) and finally BTs (3.28 and 0.62 ng kg^{-1} bw day^{-1}). It must be noted that these exposure values are the sum of the target compounds included in each of the families, thus being a different number of congeners for each of the groups. All in all, exposure values for all compound families are lower for Canary Islands diet than Catalonia diet, thus meaning people from Catalonia are more exposed to HPVs than people from the Canary Islands. However, none of the values obtained for neither location surpassed the reference doses established by US EPA [37] for compounds with an available value. These values range from 600– 100,000 ng kg^{-1} bw day^{-1} for OPEs (EHDPP and TEHP, respectively) and from 20,000 – 800,000 ng kg^{-1} bw day^{-1} for PAEs (DEHP and DEP, respectively). No reference doses are available either for benzothiazoles nor benzenesulfonamides.

Risk factors for each of the compounds determined in the samples were calculated for the exposures of both regions. For Catalonia, risk factor values were comprised between $3.59x10^{-7}$ % (TEHP) – $2.26x10^{-4}$ % (EHDPP) for OPEs; between $1.78x10^{-6}$ % (DMP) – $1.18x10^{-4}$ % (DnOP); $3.14x10^{-3}$ % (BT) for BTs and between $1.24x10^{-6}$ % (p-TSA) – $7.02x10^{-4}$ % (o-TSA) for BSAs. For Canary Islands, the values ranged between $5.31x10^{-7}$ % (TEP) – $8.15x10^{-5}$ % (TTP) for OPEs; between $5.31x10^{-8}$ % (DEHP) – $2.18x10^{-4}$ % (DiBP); $9.52x10^{-4}$ % (BT) for BTs and between $6.43x10^{-7}$ % (p-TSA) – $3.64x10^{-4}$ % (o-TSA) for BSAs. Figure 3 compiles the risk factors for each of the HPV families for both locations. It can be observed that Catalan overall risk doubles the values found for samples from the Canary Islands. As risk factors are expressed as a percentage of the acceptable daily intake, values close to 100% are believed to be of extreme concern for population health, whereas as the percentage decreases, so does the risk. As the values found for the present study, these are several orders of magnitude below, the concern upon the risk caused by the ingestion of these compounds via seafood consumption is low. For carcinogenic compounds with

an available BMDL value, TCEP and TBP, margin of exposure (MOE) estimated values were $1.08x10^7$ and $1.57x10^7$ for Catalonia and $2.83x10^7$ and $3.77x10^7$ for Canary Islands, respectively. These values lay two orders of magnitude above the value set by EFSA for which MOE values equal or higher than 10,000 are considered of low concern from a public health point of view [38]. Therefore, risk associated to the intake of TBP and TCEP via consumption of seafood from both locations is of low concern.

All in all, no risk is associated for neither non-carcinogenic nor carcinogenic compounds intake via seafood consumption from any of the studied diets.

4. Conclusions

The presence of several HPV chemicals has been successfully proved in seafood samples from both Catalonia and the Canary Islands. Benzenesulfonamides appeared as the group of compounds with the highest detection frequency (100%) and mean concentration in both locations (113 ng g^{-1} w.w. for Canary Islands and 94.2 ng g^{-1} w.w. for Catalonia), whereas PAEs, even though presenting low detection frequencies, were found as the second HPV family with the highest mean concentration (82.1 and 83.6 ng g^{-1} w.w., respectively). Samples from both Catalonia and the Canary Islands showed HPV profiles with BSAs and PAEs being the top contributors, while BTs

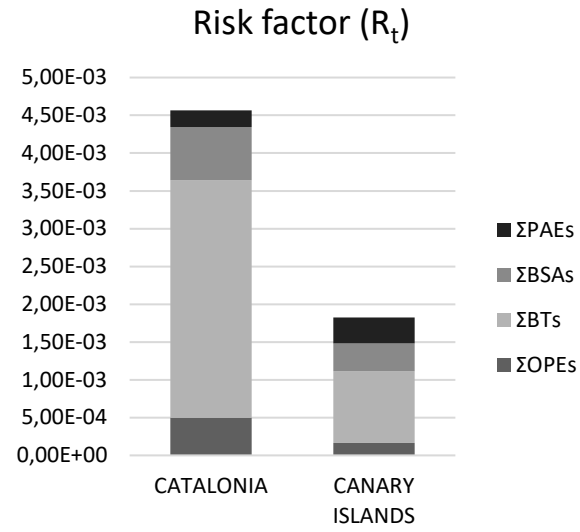

Figure 3. Compiled risk factors for all the compounds with available risk values from both locations.

and OPEs appeared as the least for the Canary Islands and Catalonia samples, respectively. Catalonia species (cod and mackerel) appeared as the species with the highest total concentrations for all the studied families. No significant differences were found between individual mean concentration of the target families between both locations except for BTs, for which significant differences were found with concentrations being higher for Catalonia samples than for Canary Islands ones. Using multivariate analysis and classification techniques, samples from both locations were successfully differentiated upon their HPVs concentration profiles. Regarding exposure and risk, neither Catalonia nor Canary Islands samples posed a high level of concern for the intake of HPVs via seafood consumption.

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3.2.3. Discussion of results

A brief discussion of the most relevant findings of the previously described studies is summarized in the following section.

Multitarget methods are required to easily evaluate the presence of a wide number of analytes simultaneously. In this sense, we intended to merge both QuEChERS methods described in the previous section to have a unique method for organophosphate esters, benzothiazoles, benzotriazoles and benzenesulfonamides. We decided to incorporate phthalate esters due to their widespread ubiquity in seafood as demonstrated by other authors [1-3]. Thus, a method based on QuEChERS extraction followed by gas chromatography-tandem mass spectrometry was developed for the determination of a wide range of relevant high production volume chemicals. As benzothiazoles, benzotriazoles and benzenesulfonamides required a higher injection volume to reach desirable detection limits, the method conditions regarding the instrumental part (GC-MS/MS, injector specifically) for these compounds were selected. As a result of the application of these conditions, organophosphate and phthalate esters were evaluated in terms of apparent recovery, limits of detection, limits of quantification, repeatability and reproducibility.

Comparing the method quality parameters of the updated method for organophosphate esters, these reached similar and even lower limits of detection for certain compounds, especially TCEP, TPP and TTP. It is important to note that while the injection volume was 5 times higher than in the original method, the amount of sample was 5 times less, a fact that explains why some of the compounds obtained similar detection limits whereas others became better. The improvement of the detection limits could be linked to a better penetration of the solvent in the sample, as a minor sample weight was extracted with the same amount of solvent, thus probably improving the analyte transfer between the sample and the solvent. Moreover, a reduction of the sample weight also leads to a minimisation of the extracted interferences. Phthalate esters also accounted for method detection limits between 0.10 - 0.25 ng g^{-1} d.w. and apparent recoveries between 59 -132%. All compounds showed repeatability and reproducibility values under 19 and 22% (n=5, 5/100 ng g^{-1}), respectively. The addition of phthalate esters to the method also brought the incorporation of additional quality control and assurance procedures to the method. Due to these

compounds being present everywhere, especially plastic materials, the use of plastic material used for the extraction was avoided when possible. Hence, extractions were performed in glass centrifuge tubes and glass Pasteur pipettes were used when collecting phases. Tube caps and other plastic parts were covered in aluminium foil if in contact with the extraction solvent. Even though sample treatment was conducted under controlled plastic usage, the presence of the compounds in the air and the system, required the addition of several procedure blanks as well as system blanks to control the background presence of these compounds.

Regarding the results found in the monitoring, these are mainly described in the article itself. Briefly, the presence of HPVs was demonstrated in all the analysed samples. Phthalate esters and benzenesulfonamides appeared as the compound families with the highest relevance in terms of mean concentration and contribution to the total. No benzotriazoles were found in any of the analysed samples, which led us to believe that either these could be at concentrations lower than the method limit of detection or that these were truly not present in the samples. Literature reported concentrations up to nearly 100 ng g^{-1} w.w. of some BTRs congeners in molluscs from the Bohai Sea, China [4] and fish specimens from different locations of the Pearl and Yangtze rivers from China (mean concentrations between 2.48 – 4.38 ng g^{-1} w.w. with detection frequencies below 47%), but was not detected in any sample from specimens collected from European cities such as Portugal, Spain, Italy, Netherlands or Norway as stated by Álvarez-Muñoz et al.[5]. This led us to believe that the presence of BTRs in seafood samples could be linked to specimens from countries with higher use of these compounds, as could be the case in China.

In respect of phthalate esters, even though their mean concentrations appeared as the highest, it was expected to be even higher, as an interference disabled the quantification of DEHP for most of the samples of high lipid content. This explains the lower detection frequency of DEHP in the described project, as the literature has DEHP as one of the most detected phthalate esters [1,6]. As commented in the article, profiles and concentrations for organophosphate esters and benzothiazoles agree with the values found in the literature. As for benzenesulfonamides, this is the first time that BSA and Et-p-TSA are determined

in seafood samples. As previously explained, the ubiquity of these compounds in effluent waters has been reported by some authors, suggesting a possible bioconversion of higher molecular weight benzenesulfonamides in the wastewater treatment plants, which concurs with the possibility of finding these compounds in organisms inhabiting the outfalls of these WWTP discharges, in this case, seafood. Regarding the species with the higher HPVs level, sardine topped the chart with a mean concentration of 385.7 ng g^{-1} w.w., whereas hake appeared as the least contaminated species, with 44.1 ng g^{-1} w.w.. Focusing on the differentiation of species based on their lipid content, no conclusive results were found for the grouping of species in low and high lipid content depending on their HPVs concentrations.

Multivariate analysis using PCA did not show a specific separation based on this premise. Hence, HPVs profiles do not seem to be closely linked to the lipid content of the species. As stated in the article, this could be associated with the lipophilicity of each of the compound families itself. While phthalate esters are believed to be highly lipophilic, other compounds such as organophosphate esters seem to act the opposite way. Scarce to no information on the lipophilic character of BTs, BSAs and BTRs appear in the literature, thus complicating the interpretation of the results. However, as no separation was observed between low and high lipid content species, it is kind to believe that these compounds' lipophilicity is low. Finally, the calculations for the exposure and risk assessment indicated that the population with a diet based on the consumption of these ten species at rates established by the ENCAT 2003 survey would have a low level of concern about their health having a negative impact because of the intake of HPVs via seafood consumption. However, as stated in the previous section, further calculations should be performed once more risk data on BTs, BTRs and BSAs is available.

Concerning the second study, seafood samples from two locations in Spain were analysed. As it was intended to evaluate the differences in exposure and risk associated with the intake of HPVs via seafood consumption, species typically consumed by both locations were analysed. On one hand, insular diet-based species from the Canary Islands were selected from highly consumed and autochthonous species, these being common pandora, skipjack tuna, mackerel

and comber. On the other hand, the selection of species from Catalonia was performed upon the most consumed, trying to match them with the samples from the Canary Islands in terms of the lipid content of the species. Thus, mackerel, yellowfin tuna, cod and sole were selected. In this case, the study aimed to characterize the presence of HPVs in samples to possibly find some differentiation between locations. In this sense, no differences were found between mean concentrations found for OPEs, PAEs, or BSAs, however, a significative difference was found for BTs. This could lead to the interpretation of BTs being the most variable group within the target HPVs in terms of the spatial occurrence of these compounds.

A second and equally important goal of the study was to evaluate the exposure and risk associated with the concentrations found in the analysed samples for specimens from both locations. While exposure values resulted higher for peninsular diets (Catalonia) than, for insular diets (Canary Islands), both the exposure and the risk values were several orders of magnitude below the threshold value set to indicate an elevated risk. Hence, the risk from both locations could be taken as of low concern. The results of the study should be taken as preliminary due to the limited number of samples available, however, we hope this study opens the door to a more exhaustive project focused on the determination of these HPVs in seafood samples from many other locations.

All in all, both studies successfully reported the presence of HPVs in seafood samples. Compounds of high interest like organophosphate esters and phthalate esters were detected in several specimens, whereas compounds such as benzothiazoles, with a lesser spotlight, have also been detected in most of the analysed samples. We would like to emphasize the presence of benzenesulfonamides, these being determined for the first time in seafood samples. HPVs occurrence in seafood has been confirmed and, thus, the current elevated contamination levels of the aquatic environment. This is the result of the massive chemical production and industrial/consumer use in our current society. We hope studies like the previously described contribute to the development and implementation of guides and regulations on the use and production of these chemicals to reduce their presence in the environment, seafood and consequently our organisms.

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3.3. Bioaccessibility of relevant high production volume chemicals

To this point, all the previously described studies assumed a total absorption of the compounds present in the analysed specimens to perform the exposure and risk assessments. However, this approach may be not 100% accurate, as the amount of ingested compound, either being a nutrient or a contaminant, may not reflect the real amount which is further available for our organism to absorb. Hence, it is necessary to investigate how the compound behaves once it enters our digestive system, and to which extent it may be absorbed, or, as it is commonly called, how much of it is bioaccessible.

The bioaccessibility of chemical compounds has not been extensively studied. To date, some studies have reported the bioaccessibility of essential and toxic elements such as mercury or cadmium [1,2] as well as organic contaminants like perfluorinated compounds, brominated flame retardants or personal care products, among others [3,4]. Results conclude that the chemical properties of the contaminants along with the type of matrix for which these are assessed heavily influence bioaccessibility. Hence, several bioaccessibility experiments regarding different kinds of matrices and chemical compounds are needed to obtain useful data to perform more accurate risk assessment calculations. To do so, in-vitro models have been described for which human digestion steps are simulated [3].

With bioaccessibility studies being heavily influenced by the matrix, another parameter susceptible to being tested is the cooking process. In the case of seafood, it can be ingested raw or cooked, so the evaluation of how the compounds' bioaccessibility is affected is something that deserves to be tested. Moreover, culinary treatments not only affect bioaccessibility, as the concentrations of the compounds may be also altered during the cooking process, either decreasing or increasing. Several studies have reported the different effects that culinary treatments like steaming, grilling, or frying have on the global concentration of a compound, mostly reporting differences when compared with the initial values [1,3,5,6].

The following section includes a preliminary study of the bioaccessibility of tri-n-butyl phosphate, tris(2-chloroethyl) phosphate and benzothiazole in raw and cooked seafood samples. Specimens of hake and tuna were selected to represent both low and high lipid content species and were further analysed for the

determination of the aforementioned compounds in raw and cooked samples. Steaming and grilling were selected as common culinary treatments aimed to evaluate the behaviour of these compounds when cooked. Once the culinary treatment was performed, an in-vitro digestion of each of the raw and cooked samples as well as for each of the compounds was conducted to check the bioaccessibility.

The presented study was performed in collaboration with the Portuguese Institute for Sea and Atmosphere (IPMA) in Lisbon, Portugal as part of the PhD stay in the Division of Aquaculture, Upgrading and Bioprospection (DivAV). The results of the study are currently in preparation for publication in Food and Chemical Toxicology.

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3.3.1. Preliminary assessment of tri-n-butyl phosphate, tris(2-chloroethyl) phosphate and benzothiazole bioaccessibility in raw and cooked fish

PRELIMINARY ASSESSMENT OF TRI-N-BUTYL PHOSPHATE, TRIS(2-CHLOROETHYL) PHOSPHATE AND BENZOTHIAZOLE BIOACCESSIBILITY IN RAW AND COOKED FISH

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Abstract

Seafood consumption takes an important role in diets, providing compounds linked with health-beneficial effects. Other chemicals present in the environment can bioaccumulate in these species, posing a threat to the population due to their ingestion. The occurrence of high-production volume chemicals with negative health effects like tri-n-butyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP) and benzothiazole (BT) has been reported in many seafood species. Culinary treatments can cause differences in the fish contents of these compounds. Moreover, only a fraction is accessible to our organism (bioaccessible fraction). The present study evaluated the effects of steaming and grilling on the BT, TBP and TCEP content in hake and tuna samples. Results showed a TBP loss of nearly 50% for steamed and grilled specimens, whereas a 12-20% loss was observed for BT. TCEP showed differences between culinary treatments as well as between species, reducing its content in a range between 10 – 50%. Regarding their bioaccessibility (in-vitro study), BT and TCEP showed bioaccessibility of nearly 100%, whereas TBP exhibited a reduced bioaccessibility (32 -65%), mainly linked to the compounds' physicochemical properties (higher Kow values led to lower bioaccesibilities).

Highlights

- BT, TBP and TCEP content is reduced when steaming and grilling fish.
- Overall losses account a 50% of TBP, 10-20% of BT and 10-50% of TCEP.
- Bioaccessibility of BT and TCEP was nearly 100%, while for TBP was 32-65%.
- TCEP showed statistical differences between species and culinary treatments.

1. Introduction

Balanced and healthy diets are usually linked to seafood consumption [1]. The excellent and essential nutrients present in fish pose several health benefits i.a. prevention of hypertension and cardiovascular diseases and improvement of fetal and infant development [2,3]. These benefits are related to the presence of numerous vitamins, minerals, high biological value proteins, amino acids and omega-3 fatty acids [2]. Nevertheless, chemical contami-nants may also accumulate in fish specimens, thus counteracting the benefits and turning seafood consumption into an issue of concern. From toxic elements such as mercury or cadmium to organic contaminants such as poly-chlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) or organophosphate esters (OPEs), the presence of these compounds in seafood has become an issue for which the benefits and risks of seafood consumption are constantly being evaluated.

Among these compounds, tri-n-butyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP) and benzothiazole (BT), considered High Production Volume chemicals (HPVs) due to their production /manufacture being over 1,000 tonnes per year in the European Economic Area [4], have been reported in seafood species by several authors. Studies on the occurrence of TBP and TCEP among other OPEs have been undergone worldwide. For instance, Sala et al. [5] reported the presence of TCEP and TBP in sardine and anchovy specimens from the Mediterranean Sea with mean concentrations and detection frequencies of 2.38 ng g^{-1}, 80% (TCEP) and 2.09 ng g^{-1}, 60% (TBP) for sardine and 0.16 ng g^{-1}, 21% (TCEP) and 0.17 ng g^{-1}, 64% (TBP) for anchovy. Wang et al. [6] reported concentrations between 0.04 – 4.98 and 0.06 – 7.96 ng g^{-1} w.w. in seafood samples from New York, US, for TBP and TCEP, respectively. More recently, Zhang et al. [7] evaluated seafood specimens from the Beibu Gulf in China, reporting con-centrations of 3.13 – 25.5 and 0.47 – 2.36 ng g^{-1} w.w. for TCEP and TBP, with both having a 100% detection frequency. Benzo-thiazoles, although being less studied, have also been reported by Trabalón et al. [8] in the most consumed seafood species from Catalonia, Spain, with values ranging between 13 – 82 ng g^{-1} d.w., and squid showing the highest concentration. Molluscs from the Bohai Sea, China were also analysed by Jia et al. [9] with values of BT up to 13800 ng g^{-1} d.w.

The widespread ubiquity of these compounds is the result of their extended production and application as flame retardants (TCEP and TBP) [10] or anti-corrosion agents (BT) [11] in many industrial and consumer products.

Despite their extended use, TCEP and TBP have been classified as possible carcinogens [12] while TCEP has also been confirmed to be toxic for reproduction [13]. On its part, BT has been linked to endocrine-disrupting effects regarding the thyroid hormone as well as cytotoxicity in human gastric and lung carcinoma cells [14,15]. Moreover, it can also irritate sensory organs if exposed [11]. On account of the negative health effects associated with these compounds, their presence in seafood and its possible dietary intake, several analytical metho-dologies have been developed for their occurrence control and further risk assessment [16–19].

Despite most of the risk assessment studies regarding the intake of these compounds through seafood consumption taking into account the reported concentrations in fish, not all the amount present in seafood is then released during the digestive process. This means only a certain part is susceptible to being available for absorption in the intestines and further entering the bloodstream [20]. The amount of contaminant reaching the blood is known as the bioaccessible fraction. The evaluation of the percentage of bioaccessible fraction from a certain compound enables more accurate calculations of exposure and risk assessments. To date, literature regarding the bioaccessibility of organic compounds present in seafood is scarce, especially those taking the cooking process into account. Some studies have reported the bioaccessibility of essential and toxic elements like cadmium or mercury [3,20,21] as well as some perfluorinated compounds, brominated flame retardants and personal care products [22,23], but to date, no data is available for organo-phosphate esters or benzothiazoles.

The present study aimed to preliminary assess the bio-accessibility of three high production volume chemicals compounds such as TBP, TCEP and benzothiazole BT in raw and cooked fish. The effects on their concentrations in fish after common culinary treatments like steaming and grilling were evaluated for two fish species from different lipid content: hake (Merluccius merluccius) and tuna (Thunnus thynnus). Further evaluation of the bioaccessible fraction of TBP, TCEP and BT was performed for raw and cooked fish samples from both species using an in vitro digestion protocol.

2. Material and methods

2.1. Standards and reagents

Analytical standards of tri-n-butyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP) and benzothiazole (BT) were purchased at Sigma Aldrich (St. Louis, USA) with a purity >98%.

Deuterated benzothiazole (d_4-benzothiazole) and tri-n-butyl phosphate (d_{27}-TBP) were acquired from LGC Standards (Teddington, UK). Stock solutions of 2500 mg L^{-1} were prepared in ethyl acetate for TBP, TCEP and BT for the cooking effect and bioaccessibility expe-riments. Ethyl acetate and acetonitrile were GC grade with purity >99.00% supplied by JT Baker (Deventer, The Netherlands). Ultrapure water for the digestion was obtained from a Synergy purification system from Millipore (Massachusetts, USA). Helium and nitrogen for the GC-MS system were of 99.999% purity and supplied by Carburos Metálicos (Tarragona, Spain).

The *in-vitro* digestion procedure included the use of four digestive fluids: saliva, gastric fluid, duodenal fluid, and bile. Fluids were prepared as a combination of inorganic, organic and bioactive reagents. Inorganic components included: $CaCl_2{\cdot}2H_2O$ (Sigma, C3881), HCl (Merck, 37% w/w), KCl (Merck, 99.5%), KH_2PO_4 (Merck, 90%), KSCN (Sigma, P2713), $MgCl_2$ (Riedel-de Haen, 99.5%), NaH_2PO_4 (Merck, 99.5%), Na_2SO_4 (Merck, 90%), NaCl (Merck, 99.5%), $NaHCO_3$ (Merck, 99.5%) and NH_4Cl (Riedel-de Haen, 99.5%). Organic components included: D-(+) glucosamine hydrochloride (Sigma, G4875), glucose (Sigma, G5400), glucuronic acid (Sigma, G5269), urea (Sigma, U5128) and uric acid (Sigma, U2625). Finally, bioactive components included: albumin from bovine serum (Sigma, A7906), bile porcine extract (Sigma, B8631), lipase from porcine pancreas, type II (Sigma, L3126), mucin from porcine stomach (Sigma, M2378), pancreatin from porcine pancreas (Sigma, P8096), pepsin from porcine stomach mucosa (Sigma, P7125), trypsin from porcine pancreas (Sigma, T6567), α-amylase from Aspergillus oryzae (Sigma, 86250) and α-chymotrypsin from bovine pancreas (Sigma, C4129).

2.2. Sample collection and culinary treatments

Specimens of hake (Merluccius merluccius) and tuna (Thunnus thynnus) were bought in stores from Tarragona, Spain. The collected specimens had uniform sizes and weights. Fish species from two different lipid content were purchased to evaluate the effect of the lipid content on the cooking and bioaccessibility procedures. These two species were selected among the most consumed seafood species from Catalonia, Spain [24]. Thus, hake was selected as representative of the low lipid content (1%) and tuna of high lipid content (16%). Lateral fillets of the hake specimens were dissected and portions for each of the species were made to perform triplicates of each of the culinary treatments. In the same sense, tuna portions were bought and these were also divided into smaller portions with the same purpose.

Grilling and steaming were selected as cooking treatments due to them being the most common cooking processes for fish. Cooking conditions were adapted from previously reported methods for the study of bioaccessibility in different seafood species ([3,20,25,26]. Thus, the steaming procedure was carried out in an electric oven at 110ºC for 25 min with fish wrapped in aluminium foil while for grilling, fish was cooked over an electric grilling device for 10 min at 175ºC.

Given the expected low concentrations of these compounds in the specimens, an addition of the compounds was performed to better observe the results. Hence, each of the portions previously prepared was weighted and further added with each of the compounds using a needle syringe with the proper weight dependant calculated volume. In order to accurately introduce the compounds, the addition volume was divided into four and injected into different sections of the portion. Added portions were left overnight at 4ºC to promote analyte-sample interaction prior to their cooking process. The addition of the compounds was performed individually (one compound per portion), meaning triplicates of each of the culinary treatments were performed for each of the three compounds for both tuna and hake.

All raw and cooked samples were later homogenised using a grinder with a stainless-steel cup until the complete visual disruption of the tissues. A portion of each of the obtained homogenates was kept for the bioaccessibility procedure and the rest was lyophilised using a miVac Duo freeze-drying system from Genevac (Ipswich, United Kingdom). Moisture content (%) was evaluated for the freeze-drying procedure as well as for the cooking procedure to quantitatively check the water loss. To do so, samples were weighted prior and after the culinary treatment as well as prior and after the freeze-drying step.

2.3. In vitro human digestion model

2.3.1. In vitro digestion protocol

The model followed for the in-vitro digestion of the samples was adapted from Alves et al. [26]. Raw and cooked samples of each of the selected fish species were digested per triplicate as follows: 2g of homogenized sample were weighed in a glass centrifuge tube and 4 mL of saliva fluid were added. The mixture was then incubated (Select 400W) at 37 °C using a Rotary Tube Mixer with Disc at 25 rpm (LSCI, Portugal) for 5 min at pH 7.0 ±0.2. Once finished, 8 mL of gastric fluid were incorporated, and the tube was subsequently incubated for 2 h at pH 2.0

±0.2 under the same conditions as before. Finally, 8 mL of duodenal fluid and 4 mL of bile were added and incubated for 2 h at pH 7.0 ±0.2 at 37 °C. NaOH or HCl (1M each) were used to adjust the pH in each of the digestion steps. Finally, the digestion process was stopped by placing the tubes in ice to stop the reactions. The tubes were then centrifuged at 2750xg at 10 °C for 10 min to split the liquid bioaccessible part (BIO) from the solid residues non-bioaccessible part (NBIO). Triplicates of the digestion were performed for each of the fish species and compounds. Negative controls were included, performing the digestion with only the fluids.

2.3.2. Digestion efficiency

Digestion efficiency was evaluated following the procedure described by [22]. Briefly, protein levels in raw and cooked wet weight fish samples before digestion (BD) and in the bio and non-bioaccessible fractions (BIO and NBIO) were measured using a FP-528 DSP LECO nitrogen analyser (LECO, St. Joseph, USA). The quantification was performed with a calibration standard curve using EDTA following the methodology described by Saint-denis and Goupy et al. [27]. Protein recovery (%) and bioaccessible protein (%) were then calculated using equation 1(Eq. 1) and equation 2 (Eq.2), respectively, for which BIO + NBIO are the sum of the levels of protein determined in the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions and BD the determined protein before digestion.

$$\text{Protein recovery (\%)} = ((BIO+NBIO) \times 100)/(BD) \quad \text{Eq. 1}$$

$$\text{Bioaccessible protein (\%)} = (BIO \times 100)/(BD) \quad \text{Eq. 2}$$

2.4. Sample analysis methods

Samples generated throughout the study were analysed using two different methods. Hence, solid samples of raw and cooked fish were extracted following a method using QuEChERS, whereas liquid samples result of the digestion were extracted using supported liquid extraction (SLE). Both extracts were further analysed using gas chromatography-mass spectrometry (GC-MS).

2.4.1. QuEChERS extraction

Solid samples were extracted using QuEChERS. Solid fish samples of both raw and cooked samples were extracted following the method described by Castro et al. [19]. A portion of 0.1 g of dry weight fish was introduced in a 50 mL glass centrifuge tube. 10 mL of ACN and 10 mL of ultrapure water were then added to the tube and were vortex mixed for 1 min. A complete packet of QuEChERS salts from the original

method including 1g of anhydrous sodium acetate and 4g of magnesium sulfate were introduced in the tube and it was then vortex mixed for 3 more minutes. Tubes were later centrifugated using a Hettich Universal 32R centrifugation system (Tuttlingen, Germany) at 4000 rpm for 5 min. The organic layer (upper layer) was collected and passed through a LipiFiltr push-through cartridge to retain the lipids present in the extracts. Finally, these were evaporated using a nitrogen stream to ~0.5 mL and reconstituted with ethyl acetate to 2 mL after an internal standard spike of 1 mg L^{-1}. The extracts were filtrated with a 0.22 µm PTFE syringe filter and stored at -20 °C until GC-MS analysis.

2.4.2. Supported liquid extraction

Liquid samples derived from the in-vitro digestion protocol were extracted using supported liquid extraction (SLE). Bioaccessible fractions obtained for each of the species and compounds as well as blanks were extracted as follows: 3 mL of liquid bioaccessible fraction were introduced in a Strata DE SLE cartridge from Phenomenex (Torrance, CA, USA) and left for 10 min until complete absorption in the cartridge sorbent. Then, 10 mL of DCM were added to the tube and further collected in a glass vial after its gravity percolation. Extracts were then evaporated to circa ~0.5 mL and finally reconstituted to 1 mL with DCM while including the internal standards at 1 mg L^{-1}. Extracts were stored at -20 °C until their GC-MS analysis.

2.4.3. Gas chromatography -tandem mass spectrometry

Extracts from both solid and liquid samples were analysed using an Agilent 8890 GC system coupled to a 7000D triple quadrupole tandem mass spectrometer from Agilent Technologies (Palo Alto, USA). A PAL RSI 120 automatic injector from CTC Analytics (Zwingen, Switzerland) was used to automatically inject the extracts into the system. An Agilent Multi-Mode Inlet (MMI) in solvent vent mode was used for the injection of 25 µL of the extract. Injector's temperature was set at 75 °C (held for 0.37 min) and ramped at 600 °C /min to 325 °C (held for 5 min). A ZB-50 capillary column (30 m x 25 mm i.d. and 0.25 µm film thickness) from Phenomenex was used to perform the separation. The oven temperature program started at 75 °C (held for 2.87 min) and was raised at 15 °C/min to 300 °C (held for 5 min), which resulted in a total run time of 22.87 min. The carrier gas was Helium at a constant flow rate of 1.2 mL/min. The triple quadrupole system operated in electron ionisation mode (70 eV) with the quadrupoles and ion source set at 150 and 230 °C, respectively. Retention times and MRM transitions and collision energies used

for the quantification were as follows: BT, 9.28 min, 135 → 108, 51 eV (quantifier), 135 → 82, 33 eV and 135 → 69, 49 eV (qualifiers); TBP, 1.64 min, 99 → 81, 20 eV (quantifier), 99 → 63, 45 eV and 99 → 47, 50 eV (qualifiers); TCEP, 13.91, 249 → 125, 10 eV (quantifier), 249 →63, 25 eV and 249 → 187, 5 eV (qualifiers); d4-BT, 9.26, 139 → 70, 51 eV (quantifier), 139 → 84, 35 eV and 139 → 112, 21 eV (qualifiers); d27-TBP, 12.48, 103 → 83 25 eV (quantifier), 103 → 63, 45 eV and 147 → 62, 20 eV (qualifiers).

2.4.4. Quality control assurance

Procedural (only the used solvents) and sample (non-spiked sample) blanks were included in each of the analysed batches to check the presence of any of the target compounds in the whole procedure or the blank samples (two procedural blanks every ten samples and three sample blanks for each of the culinary treatments). Quality controls along with system blanks were included in every 5 samples to check the suitability of the system and prevent carry-over. Quantification was performed using calibration with internal standard for which standards containing the target compounds as well as internal standards (d_4-benzothiazole and d_{27}-TBP) were injected directly into the chromatographic system. A good linearity of the calibration curves was obtained with values of $R^2 > 0.9989$. Recoveries for the QuEChERS method were tested for low (hake) and high (tuna) lipid content species with values in hake of 64, 69 and 74% for BT, TBP and TCEP, respectively, and values in tuna of 64, 85 and 96% for BT, TBP and TCEP, respectively. In the case of the liquid samples, recoveries regarding the supported liquid extraction were 73, 120 and 100% for BT, TBP and TCEP, respectively. Method detection limits and method quantification limits ranged between 0.1 – 0.4 ng g^{-1} d.w. and 0.20 – 1 ng g^{-1} d.w., respectively, for QuEChERS and between 0.001 – 0.005 µg L^{-1} and 0.003 – 0.01 µg L^{-1}, respectively, for SLE. Correct identification of the target compounds was achieved by following the following criteria: (1) retention time shift could not exceed ± 0.1 min when compared with the standard; (2) quantifier/qualifier ratios could not differ in more than a 15% with the ratios obtained for the standard.

2.4.5. Statistical analysis

Differences in BT, TBP and TCEP's concentration/ bioaccessibility were analysed for each of the culinary treatments (steamed or grilled) using a one-way ANOVA with a significance level set at 5%.

3. Results and discussion

As previously explained, concentrations of these compounds present in seafood samples are generally low,

which combined with the fact that the extraction method for the solid samples required a 20-fold dilution to obtain a reliable and free of matrix effect value, led to the spike of the samples used for the study at 20 µg g^{-1} w.w. in order to better observe the possible loses. Non-spiked samples were analysed, reporting concentrations in tuna of 1.4, n.d. and 2.2 ng g^{-1} w.w. for BT, TBP and TCEP, respectively; and in hake of 0.44, 0.12 and 1.10 ng g^{-1} w.w. for BT, TBP and TCEP, respectively. These values confirmed the necessity of spiking the samples prior to the culinary treatments, in this case at 20.0 µg g^{-1} w.w. The spiking of the pre-treated fish portions followed for the study did not assure the total incorporation of the compounds in the fish prior its cooking treatment. Thus, all raw portions were previously analysed and calculations were performed taking into account the water lost during the lyophilisation process. Thus, the average concentrations (n=3) found for each of the species were: for hake, BT was at 17.0 µg g^{-1} w.w. (RSD = 1%), TBP was at 14.0 µg g^{-1} w.w. (RSD = 55%) and TCEP was at 8.1 µg g^{-1} w.w. (RSD = 17%); for tuna, BT was at 6.4 µg g^{-1} w.w. (RSD = 1%), TBP was at 18.8 µg g^{-1} w.w. (RSD = 55%) and TCEP was at 14.3 µg g^{-1} w.w. (RSD = 41%). It can be observed that all the compounds showed lower concentrations compared with what was theoretically spiked and that the spiking procedure entails high %RSD values. This could be associated with the high complexity of the procedure and the capacity of the compounds to synthetically incorporate into the sample. Hence, calculations on the differences caused by the culinary treatments as well as for bioaccessibility were performed taking into account the real concentration found in the sample.

3.1. Effect of cooking on HPVs concentration

Most of the seafood consumed by the population is usually cooked. With chemicals being susceptible to volatilisation, leaching or degradation, it is important to evaluate the possible losses or increments of these. Steaming and grilling are common culinary treatments for seafood, thus, these were selected for the study. Once all the fish portions were cooked, a portion of these was kept to perform the bioaccessibility while the other was lyophilised to analyse the target chemicals content. As not the same amount of each of the portions was lyophilised, results on the content were divided by the lyophilised amount to normalise the results. This was done for all the analysed samples (raw, steamed and grilled) and the results are compiled in Figure 1.

As observed in Figure 1, TBP and TCEP concentrations mostly decrease with the culinary treatments, whereas

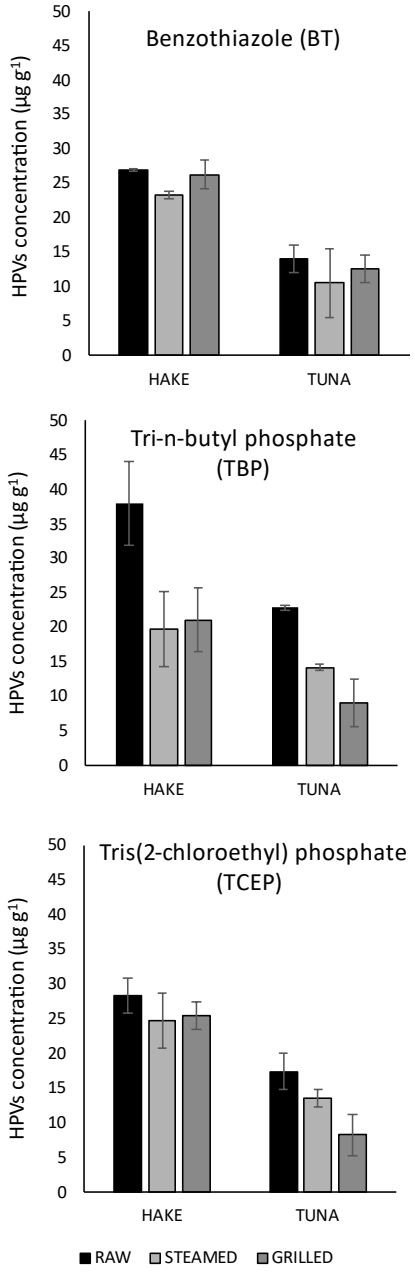

Figure 1. Variation of the concentration of the compounds for each of the culinary treatments.

BT, even also decreasing for some treatments, shows a lower tendency towards analyte loss. TBP lost the most on the grilling of tuna (61% loss), followed by steamed hake (49% loss), grilled hake (46% loss) and finally steamed tuna (38% loss). It is observed that, while no statistical differences are observed between the two culinary treatments for hake, the opposite occurs for tuna. Statistical differences between both cooking processes ($p = 0.0458$) were found for tuna's TBP content, being grilled, the culinary treatment that affects the most. In the case of TCEP, the highest loss is observed for grilled tuna (50% loss), followed by steamed tuna (22% loss), steamed hake (13% loss) and grilled hake (10% loss). Similar to TBP, TCEP's content in tuna was also statistically different between both culinary treatments ($p = 0.0357$), being also grilled the treatment with the greatest reduction. Finally, BT lost at most 25% for steamed tuna, followed by steamed hake (14% loss), grilled hake (12% loss) and grilled tuna (11%). For BT, no differences were found between both culinary treatments for either of the species.

Regarding the differences between species, no differences were found for the culinary treatments for BT and TBP. On the opposite, for TCEP's content in grilled hake and tuna as well as for steamed hake and tuna, statistical differences between the two species

were found with p = 0.0143 and p = 0.0465, respectively. Given that no statistical differences were found between treatments for BT and TBP, an average of the losses caused by each of the treatments for both species combined was calculated, resulting in a 12% and 20% loss for grilled and steamed BT, respectively, and 53% and 44% for grilled and steamed TBP, respectively. Based on the previously reported results, it can be concluded that TBP is the most affected compound by the culinary treatments, reducing its content by approximately half (44% steamed and 53% grilled), while BT only reduced its content a 12% when grilled and a 20% when steamed. On its part, TCEP experimented mixed values, with the highest being a 50% loss for grilled tuna and the lowest being a 10% for grilled hake.

3.2. Bioaccessibility of BT, TBP and TCEP in raw and cooked fish

Digestion efficiency was evaluated analysing the total protein levels prior and after digestion. For the present study, protein digestibility was always above 85%, thus indicating a successful performance of the in-vitro procedure.

In general, all the studied compounds are bioaccessible to a certain level, meaning these could be incorporated into our organism at least for a small fraction (Figure2). Benzothiazole appears as the compound with the highest bioaccessibility for all the culinary treatments (raw, steamed and grilled). All the BT bioaccessibility values are comprised between 95 - 129%. Values higher than 100% can be linked to experimental variability, which also aligns with the fact that the highest variance between the values is found for the ones with the highest mean bioaccessibility values (raw and steamed hake and tuna). TCEP follows a different trend, mostly linked to culinary treatment. For hake, a high bioaccessibility value was obtained for raw and grilled, while steaming dropped the bioaccessibility to nearly half the other values (57%). On the other hand, tuna's bioaccessibility seems to increase with its cooking, with raw bioaccessibility being the lowest (48%) and steaming and grilling offering complete bioaccessibility of the compound (106 and 123%, respectively). Finally, TBP bioaccessibility was the lowest, with its highest value being 65% for raw hake and the lowest being 32% for steamed tuna. In this case, culinary treatments caused bioaccessibility reduction for both species.

Statistical differences were found only for TCEP, in particular between raw and steamed hake (p = 0.0009) and between steamed and grilled hake (p = 0.0014). Also, between raw and steamed tuna (p = 0.0435) and between raw and grilled tuna (p = 0.0289). Thus, TCEP was

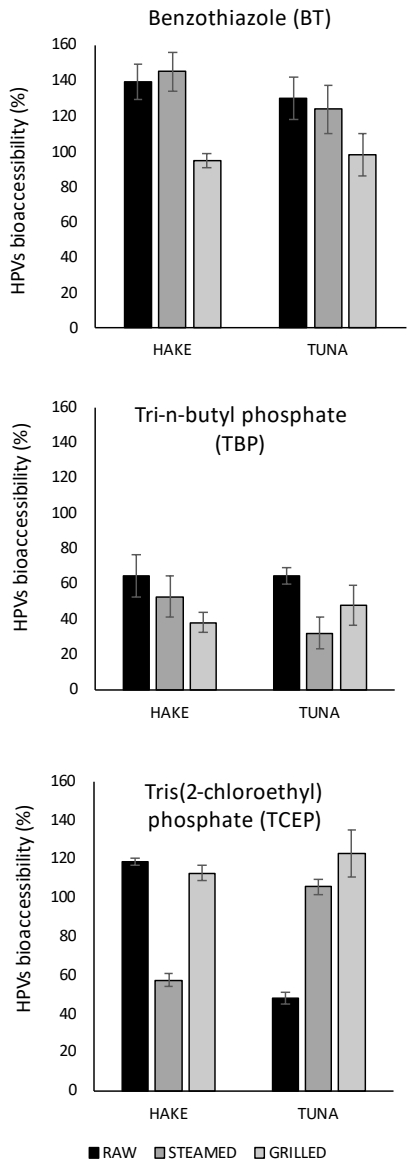

Figure 2. Bioaccessibility of the studied compound for each of the raw and cooked samples.

the only compound for which bioaccessibility differences were found not only between raw and cooked samples but also between culinary treatments (hake). Regarding the differences between species, these were also found only for TCEP, between raw hake and tuna ($p = 0.0013$) and between steamed hake and tuna ($p = 0.0048$).

Differences between bioaccessibility values can be linked to parameters such as food matrix, the composition of digestion fluids or physicochemical characteristics of the studied compounds. For instance, octanol/water partition coefficients (K_{ow}) are closely linked to chemicals' behaviour in bioaccessibility studies. Alves et al. [22] preliminary assessed the bioaccessibility of contaminants from different families in seafood samples, including perfluorinated compounds (PFCs), brominated flame retardants (BFRs), pharmaceuticals and personal care products (PCPs). The authors reported the correlation between higher bioaccessibility values and their respective Kow values for 2,2',4,4'-tetrabromodiphenyl ether (BDE47) and 2,2',4,4',6-pentabromodiphenyl ether (BDE47) in mussel and mackerel (BIO: 18 – 37%) and for hexabromocyclodecane (HBCD) in plaice and mackerel (BIO: 63 – 90%). In this case, PBDEs have a value between 6.02 – 7.39, whereas HBCD has a value of 5.63. Authors associated the lower hydrophobic character of the

compounds (lower K_{ow}) with an easier transition to the aqueous phase, which is bioaccessible. Even higher bioaccessibility values were found for venlafaxine (85 – 95%) and methylparaben (85%), with K_{ow} values of 3.28 and 1.96, respectively, thus reinforcing the theory. For the present study, the same trend is observed for BT, TBP and TCEP, being their K_{ow} values 2, 4 and 1.78, respectively. As observed, a higher hydrophobic character (higher Kow) leads to a reduction of bioaccessibility.

4. Conclusions

The effect on the content of three relevant high production volume chemicals in raw and cooked fish has been preliminary studied. Results show that TBP was the most affected compound, with a reduction of approximately 50% when either steamed or grilled. On the opposite, BT exhibited a lower reduction of its content, mostly being a 10-20% loss. No statistical differences were found between species for these two compounds, whereas TCEP accounted for differences between species (tuna and hake) and also between culinary treatments. In this case, TCEP content was greatly reduced when grilled for tuna (50% loss), while the same treatment only caused a 10% reduction for hake. Regarding steaming, nearly twice the reduction was found for tuna than for hake (22% vs. 13%). It is difficult to draw conclusions on this topic, as several parameters such as the ability of the compound to thermally degrade, or even the possible leaching with the cooking fluid or the compounds' volatilisation due to higher temperatures could lead to the loss of compound. However, it can be seen that BT and TBP content reduction due to cooking is not closely matched with the matrix, in this case with different lipid content. TCEP, on its part, showed a counter effect, being reduced differently based on the matrix it was present and also behaving differently depending on the culinary treatment.

Bioaccessibility is extremely linked to matrix composition, the effect of the cooking process on the matrix composition or even the physico-chemical properties of the compounds. The present study showed the higher tendency of compounds with a lower Kow value like BT and TCEP of being bioaccessible (nearly a 100% BIO fraction), whereas an increase in the same value led to a reduction of the bioaccessibility, as observed for TBP (32 – 65% BIO fraction). As for content reduction, TCEP was the only compound exhibiting differences between raw and cooked bioaccessibility as well as between species. A BIO fraction of 57% was found for steamed hake, which was opposite to the nearly 100% BIO fraction

for raw and grilled hake, as well as for steamed and grilled tuna.

All in all, the values obtained were the result of a complex study that included critical steps such as spiking, which led to moderate %RSD values. A higher number of repetitions and the application for non-spiked samples containing the target compounds would lead be of great interest to reduce the possible variability. Moreover, the obtention of data regarding the reduction of the content as well as the bioaccessibility enables more accurate exposure and risk assessments. Hence, more experiments based on these and other high production volume chemicals should be performed to further advance in the characterization of risk towards population based on seafood consumption.

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3.3.2. Discussion of results

The bioaccessibility of three relevant HPVs such as BT, TBP and TCEP was preliminary studied along with the possible effects product of the cooking of the specimens. To the best of our knowledge, this is the first time that the modification of the content of these compounds in fish by a culinary treatment as well as their bioaccessibility have been studied.

Results showed a general loss of the target compounds, being TBP the most affected compound. Its concentration was reduced by approximately 50% in both species (tuna and hake) and for both culinary treatments (44% for steam and 53% for grill). BT resulted in the compound with an overall lower loss, with only a 12 and a 20% loss when grilled and steamed, respectively. On its part, TCEP showed differences between both the species and the treatments. While a 50% loss was achieved by the grilling of tuna, only 10% occurred for hake. Moreover, steamed tuna reduced its content by approximately double the same procedure in hake (22 % vs. 13%). The %RSD achieved for the different cooking procedures was mostly high, probably a result of the complexity of the study. Several portions of the same fish are needed for just one culinary treatment, if we take into account that the study was performed with triplicates, the number of portions increases. Even though most of the specimens bought were similar in size, their compositions may vary, which could later induce differences in how the analytes of the spiking procedure interact with the matrix, or how the cooking affects them. Moreover, another critical factor is the necessity of spiking the portions. Despite trying to closely weigh the portion and spike the correct amount of analyte for each of the weights to perfectly have the same concentration, several steps are required, a fact that increases the uncertainty of the process and leads to further variability.

For bioaccessibility, a protocol described by Alves et al. [1] was followed for the in-vitro digestion. Protein digestibility indicated a good performance of the followed protocol. Regarding the results, high accessibility values were found for most of the compounds and culinary treatments. A correlation between the hydrophobicity/Kow partition coefficient and the compound's bioaccessibility was found similar to what was previously stated by Alves et al. [1]. Chemicals with a lower K_{ow} value (lower hydrophobicity), are keener to partition towards the aqueous phase, e.g., the bioaccessible fraction, thus conducting higher

bioaccessibility values. In this case, differences were observed between BT/TCEP and TBP, for which K_{ow} values are 2, 1.78 and 4, respectively. Given no differences between species or between culinary treatments were found for BT and TBP, the differences could then be explained by these physicochemical properties. In the case of TCEP, differences were found between species and between culinary treatments. For hake, a substantial loss was observed when steaming (~50%), whereas for tuna the lowest bioaccessibility was found for the raw. Theories regarding the loss when steaming could be explained by the protein denaturation result of the high temperatures, which could lead to lower digestive enzyme activity as previously stated by other studies [2,3]. However, if linked to the higher temperatures, it should also be expected for grilling, which was not the case.

Other studies have also reported the effects of cooking and the bioaccessibility of other high production volume chemicals. For instance, Trabalón et al. [4] followed the same in-vitro digestion protocol to assess the bioaccessibility of HHCB in raw fish, which yielded values of 100%, meaning all the ingested HHCB was accessible. On the other hand, HHCB content was decreased by 50% and 70% in cod and mackerel when steamed and grilled, respectively.

All in all, studies such as the present are of high complexity, given the extensive procedures and steps required to obtain the results. Several repetitions should be performed to minimize the variability produced by the extended procedure. It should also be noted that, given the low concentrations of these compounds in the samples, it is challenging to observe how these are lost in non-spiked samples. Prior analysed samples with known concentrations, preferably high, should be used for the development of the study. This would drastically increase the required time and would also be difficult to replicate. However, the data obtained would be of high relevance to the field.

If we take into account the results obtained in the study, we could retrospectively approach how the exposure to the population of these compounds would change. Levels of BT, TBP and TCEP exposure would decrease up to 50% when people consuming the seafood species from the previous studies cook their meals. Hence, only half of the exposure would occur, which combined for example with the lower bioaccessibility of TBP, would reduce the amount for

which the population is exposed to this compound. On the other hand, authors like Bhavsar et al. [5] or Alves et al. [1] have reported the increase of some compounds once cooked, which would then lead to higher exposure values. This gives us an overview of the importance of performing these studies, as exposure and risk assessments are necessary to prevent any negative health effects on the population. Thus, the application of correction factors on the losses/gains of some compounds as well as their bioaccessibility would end in more accurate and reliable assessments.

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CHAPTER 4. CONCLUSIONS

The main conclusions drawn from the results obtained of the studies presented in this Doctoral Thesis are summarized below:

1. A method involving the novel SPME Arrow was successfully applied for the first time in the determination of synthetic musk fragrances in fish samples, being a greener alternative due to its fully automatic and solventless procedure. PDMS SPME Arrow emerged as the sorbent option with the highest efficiency for the extraction, achieving a 10-fold higher instrumental signal than conventional SPME fibers. The method yielded limits of detection reaching the low ng g^{-1} d.w., which were comparable with other reported methods involving QuEChERS or PLE. The application of the SPME Arrrow-GC-IT-MS/MS method to commercially available seafood species reported the occurrence of HHCB and AHTN with a 100% detection frequency.

2. Two QuEChERS followed by GC-MS/MS(QqQ) methods were successfully developed for the determination of OPEs and BTs/BTRs/BSAs in seafood samples, reaching low ng g^{-1} levels. The novel LipiFiltr push through cartridge appears as an excellent clean-up strategy to conveniently reduce the matrix effect in seafood samples. The MMI injector enables the injection of volumes up to 25 µL, thus making it possible to reach low detection limits for compounds with limited GC-MS instrumental signal such as BTs, BTRs and BSAs.

3. HPVs occurrence in the ten most consumed seafood species from Catalonia has been demonstrated, showing a mean concentration trend as follows: PAEs > BSAs > BTs > OPEs. Sardine appeared as the species with the highest HPVs concentration and no differences were found between low and high lipid content species. Exposure and risk assessment calculations performed for the HPVs intake via consumption of these samples resulted in a low level of concern.

4. HPVs were found in all the analysed samples from two different Spanish regions: Catalonia and the Canary Islands. The HPVs profiles enabled the classification of seafood species from both locations. Regarding the risk associated to HPVs intake, a low level of concern was assessed for both regions.

5. Cooking treatments such as steaming and grilling resulted in a reduction of BT, TBP and TCEP content in seafood with overall losses of 10-20% of BT, 50% of TBP and 10-50% of TCEP. No statistical differences were found between culinary treatments or seafood species for the reduction of BT and TBP content. On the other part, TCEP showed differences between grilling and steaming for tuna, being the later, the treatment which accounted a higher reduction (50%). Further studies on the effectivity of the reduction should be performed for other compounds and seafood species.

6. Bioaccesibility of BT and TCEP accounted mostly a 100% of the ingested amount. For TBP, values of 32-65% were found, mostly given the higher hydrophobicity when compared with the other studied compounds. Similar with the cooking treatments, TCEP presented statistical differences between its raw and cooked bioaccesibility, being steamed hake and raw tuna, the processes with lower bioaccessibilities.

Appendix

Appendix I. List of abbreviations used in the present Doctoral Thesis

4TTR	4-methyl-1-H-benzotriazole
5TTR	5-methyl-1-H-benzotriazole
ACN	acetonitrile
ADBI	celestolide
ADI	acceptable daily intake
AHMI	phantolide
AHTN	tonalide
AOAC	Association of Official Agricultural Chemists
APC	aminopropyl-silica
ATII	traseolide
BBP	benzyl butyl phthalate
BFRs	Brominated flame retardants
BMD	benchmark dose
BMDL	benchmark dose lower-confidence limit
BSA	benzenesulfonamide
BSAs	Benzenesulfonamides
BT	benzothiazole
BTR	1-H-benzotriazole
BTRs	Benzotriazoles
BTs	Benzothiazoles
BUVs	Benzotriazole UV-light stabilizers
C18	silica C18
CE	capillary electrophoresis/collision energy
CEPA	Canadian Environment Protection Act
ClBT	2-chlorobenzothiazole
CoCAP	Chemicals Assessment Programme
DCM	dichloromethane
DEHA	bis(2-ethylhexyl) adipate
DEHP	bis(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DiBP	di-iso-butyl phthalate
DiNP	di-isononyl phthalate
DMP	dimetyl phthalate

DnOP	di-n-octyl phthalate
DPMI	cashmeran
d-SPE	dispersive solid phase extraction
DVB	divinylbenzene
d.w.	dry weight
ECHA	European Chemicals Agency
EDI	estimated daily intake
EFSA	European Food Safety Authority
EHDPP	2-ehtylhexyl diphenyl phosphate
EI	electron ionisation
EOCs	Emerging Organic Compounds
EPA	United Estates Environmental Protection Agency
ESI	electrospray ionisation
EtAc	ethyl acetate
EtOH	ethanol
Et-p-TSA	N-ethyl-p-toluenesulfonamide
EU	European Union
FAO	Food and Agricultural Organisation of the United Nations
FPD	flame photometric detector
FUSLE	focused-ultrasound solid liquid extraction
GC	gas chromatography
GCB	graphitised carbon black
GCxGC	multidimensional gas chromatography
GM	geometric mean
GPC	gel permeation chromatography
H_2O	water
HBCD	hexabromocyclodecane
Hex	hexane
HHCB	galaxolide
HLB	hydrophilic-lipophilic balanced polymer
HPVs	High Production Volume chemicals
HQ	hazard quocient
HRMS	high resolution mass spectrometry
HS	headspace
ICCA	International Council of Chemical Associations

ICSC	International Chemical Safety Cards
IT	ion trap detector
LB	lower bound
LC	liquid chromatography
LLE	liquid-liquid extraction
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LVI	large volume injection
l.w.	lipid weight
MAE	microwave assisted extraction
MB	middle bound
MDL	method detection limit
MeOH	methanol
Me-p-TSA	N-methyl-p-toluenesulfonamide
MeSBT	2-(methyltio)-benzothiazole
MK	musk ketone
MMI	multimode Inlet
MOE	margin of exposure
MQL	method quantification limit
MRM	multiple reaction monitoring
MS	mass-spectrometry detector
MS/MS	tandem mass spectrometry
MSPD	matrix solid-phase dispersion
MX	musk xylene
NaCl	sodium chloride
NH_2BT	2-aminobenzothiazole
NOAEL	no-observed-adverse-effect-level
NPD	nitrogen-phosphorous detector
OECD	Organisation for Economic Cooperation and Development
OHBT	2-hydroxybenzothiazole
OPEs	Organophosphate esters
o-TSA	orto-toluenesulfonamide
PA	polyacrylate

PAEs	Phthalate esters
PBDEs	Polybrominated diphenyl ethers
PCA	principal component analysis
PCPs	Personal care products
PDMS	polydimethylsiloxane
PLE	pressurized liquid extraction
POD	point of departure
PSA	primary-secondary amine
PSD	pneumatic switching device
PTFE	polytetrafluoroethylene
p-TSA	para-toluenesulfonamide
PVC	polyvinyl chloride
QqQ	triple quadrupole mass spectrometer
QTOF	quadrupole-time of flight mass spectrometer
QTRAP	quadrupole-ion trap mass spectrometer
QuEChERS	quick, easy, cheap, effective, rugged and safe extraction
REACH	Registration, Evaluation and Authorization of Chemicals
R_{app}	apparent recovery
RfD	reference dose
RSD	relative standard deviation
SHVC	Substances of Very High Concern
SIDS	Screening Information Data Set
SLE	solid-liquid extraction
SPAs	Synthetic phenolic antioxidants
SPE	solid phase extraction
SPME	solid-phase microextraction
STP	sewage treatment plant
TBP	tributyl phosphate
TCEP	tris(2-chloroethyl) phosphate
TCPP	tris(2-chloroisopropyl) phosphate
TDCP	tris[2-chloro-1-(chloromethyl) ethyl] phosphate
TDI	tolerable daily intake
TEHP	tris(2-ethylhexyl) phosphate
TEP	triethyl phosphate
TiBP	tri-iso-butylphosphate

TOF	time of flight mass spectrometer
TPP	triphenyl phosphate
TSCA	US Toxic Substances Control Act
TTP	tritolyl phosphate
UB	upper bound
UF	uncertainty factor
USE	ultrasonic extraction
UV	ultraviolet detector
WHO	World Health Organisation
WWTP	waste-water treatment plant
XIC	extracted ion chromatogram
XTR	5,6-dimethyl-1-H-benzotriazole

Appendix II. List of publications

Publications derived of the present doctoral thesis:

- Ó. Castro, L. Trabalón, B. Schilling, F. Borrull, E. Pocurull, Solid phase microextraction Arrow for the determination of synthetic musk fragrances in fish samples, J. Chromatogr. A (2019) 1591, 55–61. doi:10.1016/j.chroma.2019.01.032

- Ó. Castro, E. Pocurull, F. Borrull, Determination of organophosphate ester flame retardants and plasticisers in fish samples by QuEChERs followed by gas chromatography-tandem mass spectrometry. Exposure and risk assessment through fish consumption. J. Chromatogr. A (2020) 1626, 461356. doi:10.1016/j.chroma.2020.461356

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