DEVELOPMENT AND VALIDATION
OF A LC-MS/MS METHOD FOR
QUANTITATIVE ANALYSIS OF BISPHEMOL A
AND TETRABROMOBISPHENOL A IN
SEAFOOD AND SEAWEED

Cátia Adriana Almeida Oliveira

Master in Quality Control
Faculty of Pharmacy of University of Porto

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Desenvolvimento e validação de um método de LC-MS/MS para a análise quantitativa de bisfenol A e tetrabromobisfenol A em amostras de peixe e algas

Cátia Adriana Almeida Oliveira

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO DO INTERESSADO, QUE A TAL SE COMPROMETE.
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ABSTRACT

In last years a significant increase in public concerns about the adverse effects of substances with endocrine disrupting properties, like bisphenol A (BPA) and its analogous tetrabromobisphenol A (TBBPA) has been observed. BPA is highly used in the production of polycarbonate plastics and epoxy resins used in several food containers, while TBBPA is a known brominated flame retardant (BFR) present in epoxy, polycarbonate and phenolic resins. When conditions that trigger it occur, these chemicals may leach into the ambient and accumulate, with harmful effects for both human and wildlife.

The aim of this work was the optimization of an analytical procedure based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of BPA and TBBPA on forty-six seafood samples and fourteen mussels samples collected in different European regions and on thirty-eight seaweed samples, both included in the ECsafeSEAFOOD project. Initially, three extraction techniques described in literature were compared. Best results were achieved with the use of QuEChERS (quick, easy, cheap, rugged, safety) procedure followed by liquid-liquid extraction, with recoveries above 70%. The optimized technique was validated concerning to linearity, precision (intraday and interday precision) and recovery.

In a total of forty-six seafood samples analysed, BPA was detected in eleven (approximately 24%) while five (approximately 11%) presented TBBPA. In seafood, the results obtained where highly variable and disperse, being different even on samples from the same specie but collected in different time or region. In mussels, both BPA and TBBPA were detected and quantified. Seaweeds did not presented any traces of these contaminants.

Despite BPA and TBBPA levels found in seafood and mussels were generally low and within the regulated and safety limits, the contamination of the endocrine disruptors (EDs) should not be ignored since there are researchers who consider that the toxicity of these compounds takes place at levels far below these established as limits. The absence of both EDs in the seaweeds subjected to study may indicate that the contaminants are not absorbed or phytoremediated by these. More studies should be developed concerning these contaminants in foodstuff. Optimized extraction techniques, particularly regarding fat removal from the sample prior to analysis should be developed in order to improve precision and recovery.
RESUMO

Nos últimos anos tem sido observado um aumento significativo da preocupação do público sobre os efeitos adversos das substâncias com propriedades de desregulação do sistema endócrino, como o bisfenol A (BPA, do inglês) e o seu análogo tetrabromobisfenol A (TBBPA, do inglês). O BPA é um composto sintético muito utilizado na produção de plásticos de policarbonato e de resinas epóxidas utilizadas em várias embalagens de alimentos, enquanto o TBBPA é conhecido como um retardador de chama bromado (BFR, do inglês) presente em resinas epóxidas, policarbonatadas e fenólicas. Em condições propícias, estes compostos podem ser libertados no ambiente e acumular-se, com efeitos nocivos para homens e animais.

O objetivo deste trabalho foi a otimização de um procedimento analítico baseado em cromatografia líquida com espectrometria de massa tandem (LC-MS/MS, do inglês) para a determinação simultânea de BPA e TBBPA em quarenta e seis amostras de peixes e catorze amostras de mexilhões recolhidas em diferentes regiões europeias e em trinta e oito amostras de algas, trabalho incluído no âmbito do projeto europeu ECsafeSEAFOOD. Inicialmente, compararam-se três técnicas de extração descritas na literatura, tendo os melhores resultados sido obtidos com o uso do procedimento QuEChERS, seguido de um clean up líquido-líquido, com recuperações acima de 70%. A técnica optimizada foi validada relativamente à linearidade, precisão (precisão intra-dia e inter-dias) e recuperação.

Num total de quarenta e seis amostras de peixes analisados, onze (cerca de 24%) apresentam níveis de BPA enquanto cinco (cerca de 11%) apresentam níveis de TBBPA. Nos peixes, os resultados obtidos foram altamente variáveis e dispersos, mesmo para amostras da mesma espécie recolhidas em tempo ou região diferente. Em mexilhões, ambos o BPA e o TBBPA foram detetados e quantificados. As algas não apresentaram quaisquer vestígios destes contaminantes.

Apesar dos níveis de BPA e TBBPA encontradas em peixes e mexilhões terem sido baixos e dentro dos limites regulamentados, a contaminação com desreguladores endócrinos não deve ser ignorada, até porque existem investigadores que consideram que a toxicidade destes compostos tem lugar mesmo em níveis muito inferiores aos limites em vigor. A ausência de BPA e TBBPA nas algas em estudo pode indicar que estes contaminantes não são absorvidos nem metabolizados por estas. Mais estudos devem ser desenvolvidos a respeito destes contaminantes. Técnicas de extracção optimizadas, especialmente no que respeita à remoção de gordura das amostras, devem ser desenvolvidas a fim de se obter melhores precisões e recuperações.
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ABBREVIATIONS AND SYMBOLS

AA Anhydride acetic
ABS Acrylonitrile Butadiene Resins
AP Alkaline phosphatase
APCI Atmosphere-pressure chemical ionization
BADGE Bisphenol A dyglicyl ether
BA-ELISA Biotin-streptavidin amplified ELISA
BAN Bromoacetonitrile
BFDGE Bisphenol F dyglycidyl ether
BFR Brominated flame retardant
BPA Bisphenol A
BPA-G Bisphenol A monoglucuronide
BPA-LC Bisphenol A leaching velocity
BPB Bisphenol B
BSA Bovine serum albumine
BSTFA N-O-bis(trimethylsilyl) trifluoroacetamide
bw body weight
C18 Octadecyl bonded endcapped silica
C8 Octyl bonded endcapped silica
DCM Dichloromethane
DDT Dichlorodiphenyltrichloroethane
DLLME Dispersive liquid-liquid microextraction
dw dry weight
ECNI Electron chemical negative ionization
ED Endocrine disruptor
EFSA European Food Safety Authority
ELISA Enzyme-Linked Immunosorbent Assay
EPA Environmental Protection Agency
ESI Electrospray ionization
EU European Union
FDA Food and Drug Administration
FMS Fluid Management Systems
GC Gas chromatography
GC-MS Gas chromatography-mass spectrometry
GC-MS/MS Gas chromatography tandem mass spectrometry
GPC Gel permeation chromatography
HBB Hexabromobenzene
HBCDD Hexabromocyclododecane
IPMA Instituto Português do Mar e da Atmosfera
LC Liquid chromatography
LC-MS Liquid chromatography-mass spectrometry
LC-MS/MS Liquid chromatography tandem mass spectrometry
LD50 Median lethal dose
LC50 Median lethal concentration
LLE Liquid-liquid extraction
LOD Limit of detection
<table>
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<tr>
<td>Log Pow</td>
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</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
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<td>MCF</td>
<td>Methylchloroformate</td>
</tr>
<tr>
<td>MDL</td>
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<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
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<td>MeOH</td>
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<td>MIPs</td>
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</tr>
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<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
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<tr>
<td>MSPD</td>
<td>Matrix solid-phase dispersion</td>
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<tr>
<td>MTBE</td>
<td>tert-butylmethyl ether</td>
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<td>NP</td>
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<tr>
<td>NTP</td>
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</tr>
<tr>
<td>OP</td>
<td>4-tertoctylphenol</td>
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<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonates</td>
</tr>
<tr>
<td>PCB</td>
<td>Perchlorinated biphenyls</td>
</tr>
<tr>
<td>PCP</td>
<td>Polycarbonate plastic</td>
</tr>
<tr>
<td>PFC</td>
<td>Perfluorinated compounds</td>
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<td>PLE</td>
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<tr>
<td>QMS</td>
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<td>Tolerable dose intake</td>
</tr>
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<td>Tetrahydrofuran</td>
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<td>Trimethylchlorosilane</td>
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<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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I. Theoretical Part
1. World fisheries and seafood consumption

According to European Regulation No. 853/2004 “seafood comprises fishery products and live bivalve molluscs, and no distinction is made between products coming from the sea and other sources, nor between wild catch and aquaculture species”. It states that fishery products include “all seawater or freshwater animals (except for live bivalve molluscs, live echinoderms, live tunicates and live marine gastropods, and all mammals, reptiles and frogs) whether wild or farmed, and including all edible forms, parts and products of such animals” (ECR, 2004).

Fish production has been growing in the last five decades, as can be seen in Figure 1, in aquaculture as well as the capture of rivers and oceans. Global capture fishery production of 93.7 million tonnes in 2011 was the second highest ever (93.8 million tonnes in 1996) (FAO, 2014). In 2009, the Northwest Pacific area was the largest contributor to global fish supply (25%), followed by Southeast Pacific (16%) and Western Central Pacific (14%), while the sixteen remaining marine fishing areas comprise 45% of average catches (FAO, 2011). In 2014, the Northwest and Western Central Pacific are the areas with highest and still-growing catches (FAO, 2014).

Figure 1 – World capture fisheries and aquaculture production. Adapted from: FAO World Review of Fisheries and Aquaculture. Rome, 2014
In 2011, the highest caught groups at the global level were: herrings, sardines, anchovies; tunas, bonitos, billfishes, and cods, hakes, haddocks (FAO, 2013b). These species are the more commonly consumed by human population and in 2011, 85% of production derived for human consumption while the other 15% was destined to non-food purposes like reduction to fishmeal and fish oil, utilization as fish for ornamental purposes, for culture (fingerlings, fry, etc.), for bait, for pharmaceutical uses as well as raw material for direct feeding in aquaculture, for livestock and for animals (FAO, 2012). However, a considerable portion of the fish consumed in developed countries is not due to their own production but from imports, being expected an increasing tendency in coming years, mostly owing to steady demand and declining of domestic fishery catch (FAO, 2012).

Table 3 - Fish production in 2000 and 2004 and expectations for future years. Adapted from: FAO Review of the state of world marine fishery resources. Rome, 2005.

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<tr>
<td>Capture (MT)</td>
<td>95.6</td>
<td>95.0</td>
<td>93</td>
<td>105</td>
<td>93</td>
<td>116</td>
<td>93</td>
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<tr>
<td>Aquaculture (MT)</td>
<td>35.5</td>
<td>45.5</td>
<td>53</td>
<td>74</td>
<td>70</td>
<td>54</td>
<td>83</td>
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<tr>
<td>Total production (MT)</td>
<td>131.1</td>
<td>140.5</td>
<td>146</td>
<td>179</td>
<td>163</td>
<td>170</td>
<td>176</td>
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<tr>
<td>Percentage used for food fish</td>
<td>74%</td>
<td>75%</td>
<td>82%</td>
<td>85%</td>
<td>77%</td>
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In Europe, seafood consumption varies widely across countries. Consumption is concentrated in the South, especially Spain and Portugal, where each consumer eats more than 40kg of seafood per year. In the South, consumers eat a wide variety of seafood including squid, shrimp, tropical fish and locally captured fish. In the Netherlands and Western Europe, each consumer eats 15-25 kg of seafood per year. The variety of fish is smaller in Western Europe than in the South. Eastern European consumers eat 5-10 kg of seafood per year and tend to eat locally produced carp or imported fish such as pangasius and tuna (FAOSTAT, 2014).
1.1. **Nutritional value of seafood**

Researchers worldwide are in agreement that eating fish regularly - one or two servings weekly - may be very effective on the promotion of a healthy life. Being a high-protein, low-fat food and an excellent source of omega 3 fatty acids, fish consumption are known to reduce the risk of various diseases and disorders like cancer, cardiovascular, dementia, depression, diabetes, among others (Domingo, 2014) as well as may prolong life after a heart attack, lowers blood triglycerides (fats), may improve heart, can lower blood pressure and may decrease symptoms of inflammatory diseases, arthritis and psoriasis.

A portion of 150 g of fish can provide about 50–60 percent of an adult’s daily protein requirements. In 2010, fish accounted for 16.7 percent of the global population’s intake of animal protein and 6.5 percent of all protein consumed (FAO, 2014). The cholesterol content of most fish is similar to red meat and poultry, however some fish are relatively high in fat such as salmon, mackerel and catfish but most of the fat it has is poly-unsaturated. Omega 3 fatty acids help lower blood pressure and triglyceride levels. Fish is also a good source of B Vitamins B-6, B-12, biotin and niacin. Vitamin A is found mainly in fish liver oils, but some high fat fish are good sources of this vitamin. Fish is also a good source of several minerals, especially iron, phosphorus, potassium and zinc. Canned fish with edible bones, such as salmon or sardines, are also rich in calcium (Domingo, 2014).

1.2. **Contaminants and Seafood Accumulation**

Marine ecosystems are the end point of several chemicals that are present in rivers by discharges or atmospheric deposition. These environmental problem is not only a potential ecological hazard but also a public health hazard since these pollutants can be present in marine food items through accumulation in marine biota (Vandemeersch et al., 2015). Contamination of seafood during production, processing and storage can also occur.

Fish and shellfish have been identified as the food items typically showing the highest concentrations of a number of harmful environmental contaminants (Llobet et al., 2003, Bocio et al., 2005) like perchlorinated biphenyls (PCBs), dioxins, residues of pesticides, toxic elements, and new emerging contaminants (Domingo, 2007).

Nowadays, emerging organic pollutants can vary from pharmaceuticals and hormones to pesticides, surfactants and plasticizers. Simultaneously to the decrease of many legacy persistent organic contaminants (POCs), such as dichlorodiphenyltrichloroethane (DDT)
and PCBs, in Arctic marine biota, new contaminants are emerging, like perfluorinated compounds (PFCs) (Cruz et al., 2015).

According with the European Union (EU) Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC; ECD, 2008), “Priority Contaminants” embraces all harmful contaminants in seafood that might constitute a risk for human health and for which there are scarce scientific knowledge. It includes substances for which no maximum levels have been laid down yet (in EU legislation or international standards), as well as substances for which maximum levels have been provided but require revision.

In 2010, the MSFD compiled the regulatory levels for some substances, including heavy metals (lead, cadmium and mercury), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, dioxin-like PCBs, and radionuclides, establishing seven classes of compounds as chief Priority Contaminants, ordered according to priority as: 1) Non-dioxin-like PCBs (congeners #28, 52, 101, 138, 153 and 180); 2) BFRs; 3) PFCs; 4) Arsenic (total and inorganic); 5) Organotin compounds (tributyltin, triphenyltin, dibutyltin); 6) Organochlorine pesticides (chlordane, dichlorodiphenyltrichloroethane, dicofol, endosulfan, heptachlor, aldrin, dieldrin, endrin, hexachlorocyclohexane, toxaphene, hexachloro-benzene), and 7) Phthalates (benzylbutylphthalate, dibutyl phthalate, di-2-ethylhexyl phthalate, diisodecyl phthalate, diisononyl phthalate, diisobutyl phthalate) (Swartenbroux et al., 2010).

Meanwhile, several governmental and health authorities became highly concerned with seafood quality and safety, increasing regulation for specific contaminants and supporting the development of specific actions regarding major sea-related challenges.

The information currently available on the levels of several emerging environmental contaminants in seafood is rather fragmented and is not harmonized which may hinder the elaboration of seafood risk assessment. Therefore, a unique European database (www.ecsafeseafooddbase.eu), based on information collected from scientific literature, reports and monitoring programs concerning emerging contaminants levels in seafood, was developed within the ECsafeSEAFOOD project (www.ecsafeseafood.eu).
2. Seaweeds

Seaweeds are macroscopic, multicellular, marine algae attached to rock or other hard substrata that have been used over the centuries in many applications: food, filtration, fertilizer, gelling agents and others (Fleurence, 1999).

According to CEVA, the French study center for algal development, world production of seaweed has been increasing constantly since 1950, reaching 14.7 million tonnes in 2009 (Figure 2). Almost all of this production comes from Asia with Europe representing just 0.01%. An estimated 800 tonnes per year of seaweed is harvested in France with 23 different varieties being authorized for food use (CEVA, 2009).

![Figure 2 – World seaweed production. CEVA](image)

This is leading to an innovative product range including spreadable, salads, marinates as well as salted and dehydrated presentations. It has gained more acceptances in regions like California and Hawaii, where communities of Japanese are larger. On the east coast of United States of America and Canada, some companies have begun cultivating seaweeds for human consumption, and their markets are growing. With the current trend for consumers to embrace organically grown foods and "natural" foods from clean environments, seaweeds should receive an increasing acceptance (FAO, 2003).

These algae are consumed by coastal people, particularly in East Asia, e.g. Japan, China, Korea, Taiwan, Singapore, Thailand, Cambodia, and Vietnam and also in South Africa, Indonesia, Malaysia, Belize, Peru, and Chile, where they are normally...
eaten fresh seaweeds as salad components. Since people from these countries have migrated to other parts of the world, nowadays there are more countries consuming seaweeds in a normal daily basis. Although still a niche product in Europe, seaweed is also becoming better known and is considered a natural food and ingredient (CEVA, 2009).

2.1. **Nutritional value of seaweeds**

There are three types of seaweeds that can be distinguished based on their nutrient and chemical composition: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta), and green algae (phylum Chlorophyta, classes Bryopsidophyceae, Chlorophyceae, Dasycladophyceae, Prasinophyceae, and Ulvophyceae). Red and brown algae are mainly used as human food sources (Ratana-arporn and Chirapart, 2006).

Compared to terrestrial animals and plants, seaweeds are rich in dietary fibre, omega 3 fatty acids, essential amino acids, and vitamins A, B, C and E. Despite having a simpler appearance, these seaweeds are high in nutrients and pack a variety of health benefits. These include digestive health, cholesterol-lowering effects and weight loss (Rajapakse and Kim, 2011).

In addition to their use as food, macroalgae have been much in demand for environmental technology.

2.2. **Contaminants and Seaweed Phytoremediation**

The introduction of organic pollutants and heavy metals in the aquatic systems through industrial discharges, agricultural uses, or waste disposal can cause serious problems. The persistence of these chemicals in the environment can lead to its destruction as well as damage to organisms, affecting negatively the stability of many aquatic ecosystems and can also cause adverse effects on human health (Perelo, 2010).

Recently, there has been increasing interest on a green technology that uses certain plant species, as algae, which accumulate, translocate and concentrate high amounts of certain toxic elements in their aboveground/harvestable parts. This happens via
mechanisms involving photosynthesis, transpiration, metabolism and mineral nutrition to remove degrade or render harmless pollutants in aquatic systems. It is called phytoremediation (Jatav and Singh, 2015) and has several different process associated with it (Figure 3).

Natural contamination of freshwater by heavy metals (Cd, Pb, Se, As…) has become a crucial water quality problem in many parts of the world. For example, one of the main causes of the widespread arsenic poisoning is the growing current trend around the world of drinking water from polluted underground and surface water, naturally and anthropogenic sources.

Macroalgae are capable of accumulating metals within their tissues by their immobilization in the cell wall or by their compartmentalization in vacuoles. This ability led to their widespread use as biomonitors of metals availability in marine systems. *Chlorophyta* and *Cyanophyta* are hyper-absorbents and hyper-accumulators for arsenic and boron, absorbing and accumulating these elements from their environment into their bodies (Chekroun and Baghour, 2013). Another mechanism of remotion of heavy metals from the aquatic environment results from the strong affinity of these metals to some cell wall
components in macroalgae. It is the example of the brown algae (*Phaeophyta*) which accumulate metals due the high levels of sulfate polysaccharides and alginates within their cell walls (Chekrour and Baghour, 2013). Other metals have been eliminated from the environment like lead, nickel, copper, cadmium, strontium, chromium, mercury U(VI) by marine micro and macroalgae (eg. *Chlorella*, *Ulva*, *Sargassum*, *Fucus* and *Ascophyllum*) (Cheney *et al.*, 2014).

There have been very few studies, however, that have examined the ability of macroalgae to accumulate persistent organic pollutants from marine waters or sediments. The earliest report of a macroalgae taking up and concentrating PCB from sediment is that of Larsson (1987) who described the ability of a freshwater green macroalga, *Cladophora glomerata*, to take up and concentrate PCBs to a level of 3.6 µg/g dry weight (dw) from an artificial fresh water pool containing sediment with a PCB concentration of 2.7 µg/g dw after two months (Larsson, 1987). Recently, Wang and Zhao (2007) reported that *Laminaria japonica*, in laboratory conditions, have great ability to take up and metabolize phenanthrene and pyrene. At a PAH concentration level of 0.1 mg/L, the seaweed tolerated and survived well for up two weeks and 90% phenanthrene and pyrene were removed and subsequently degraded (Wang and Zhao, 2008).

Some microalgae species have shown to biotransform organic pollutants such as chlorophenol, bisphenol A and tetrabromobisphenol A, which can be an important first step for its subsequent degradation in the environment (Hirooka *et al.*, 2005, Sun *et al.*, 2007). Hirooka *et al.* in 2005, studied the removal of BPA by the green alga *Chlorella fusca*. The investigators considered that *C. fusca* can be considered a useful organism to remove BPA from landfill leachates since this seaweed was able to remove BPA from the media with a capacity depending on the light/dark conditions (Hirooka *et al.*, 2005). The accumulation of TBBPA and its consequent biological responses were examined in coontail (*Ceratophyllum demersum L.*) in the study of Sun *et al.* (2007). Most of the TBBPA was accumulated after 4 day exposure and TBBPA concentration in plant increased with decreasing TBBPA concentration in growth solution. The researchers found that the TBBPA exposure increased total free radicals generation in the plants as well as lipid peroxidation and decreasing on the chlorophyll content. These results suggested that *C. demersum* is able to accumulate TBBPA removing it from the environment, which induces oxidative stress (Sun *et al.*, 2007).
3. Importance of studying BPA and TBBPA in seafood and seaweed

Synthetic chemicals like TBBPA and BPA are included in the group of priority environmental contaminants in seafood. Because of the persistence, bioactivity and bioaccumulation potential, concern is increasing about the possible harmful effects on ecosystems and human health.

Despite having a soil half-life of only 1–10 days, BPA’s ubiquity makes it an important pollutant. In 2010, the U.S. Environmental Protection Agency reported that over one million pounds of BPA are released into the environment annually (Erler and Novak, 2010). BPA can enter the environment either directly or indirectly. Directly in the leaching from chemical, plastics coat and staining manufacturers, from paper or material recycling companies, or indirectly in the leaching from plastic, paper and metal waste in landfills (EPA, 2011) or ocean-borne plastic trash (Barry, 2009). This could lead to aquatic environment contamination and consequently to the wild life and plants in that habitat. Even though a study conducted in the US in 2005 had found that up to 91% of BPA may be removed from water during treatment at municipal water treatment plants (Drewes et al., 2005), a 2009 analysis of BPA in the water system showed that this ED is present in the surface water and sediment in the US and Europe (Klečka et al., 2009).

Resulting from the bromination of BPA, TBBPA is its tetrabrominated form, TBBPA released into wastewater would likely be transported to a treatment facility. Most TBBPA entering a treatment plant will sequester into sludge, which can be applied to soil; however, small amounts (Kuch and Ballschmiter, 2001) have also been measured in final effluents discharged into receiving waters. Materials in landfills are subject to weathering, releasing TBBPA particulates or polymer-associated TBBPA, primarily into soil and, to a lesser extent, water and air. Currently, there have been no experiments conducted on the leachability of TBBPA from polymers in landfills; however, leaching over extended time periods is a possibility given that TBBPA has some solubility in water (EU RAR 2008). Uncontrolled burns and accidental fires may release TBBPA into air, and ash from both controlled and uncontrolled incineration may contain TBBPA and other potentially hazardous degradation products (EU RAR 2008).
Aware that seafood is a major dietary route for human exposure to these widespread contaminants, one of the main purposes of ECSafeSEAFOOD was the monitorization of the priority environmental contaminants in seafood and the assessment of the effects of industrial and home preparation on contaminant content. In this work, we tried to access the levels of BPA and TBBPA in several seafood from rivers, seas, oceans, and aquaculture in their raw form as well as cooked. This evaluation was also made in seaweeds commonly used in feeding a large part of world population. Regarding this, is fundamental to introduce these two chemicals with a resume of their most important properties, sources of exposure, metabolism routes and associated legislation, further enhancing various aspects of possibilities for their detection and quantification in food products.
Chapter I

Theoretical Part

4. Bisphenol A

4.1. Properties of BPA

Bisphenol A, also known as BPA or 4-[2-(4-hydroxyphenyl)propan-2-yl]phenol, is one of the highest-volume chemicals produced worldwide (Schug et al., 2012). It results from the condensation of 2 mol of phenol with 1 mol of acetone in the presence of an acid catalyst (Figure 4). It has the chemical formula C\textsubscript{15}H\textsubscript{16}O\textsubscript{2}, with a molecular weight (MW) of 228.29 g/mol belonging to the group of diphenylmethane derivatives and bisphenols. BPA is a moderately water-soluble compound (300 mg/L at room temperature) and it dissociates in an alkaline environment (pK\textsubscript{a} 9.9–11.3).

![Figure 4 – Synthesis of bisphenol A from the reaction of phenol with acetone in the presence of an acid catalyst. Adapted from: http://commons.wikimedia.org/wiki/File:Synthesis_Bisphenol_A.svg](http://commons.wikimedia.org/wiki/File:Synthesis_Bisphenol_A.svg)

In 2011, the global BPA production reached approximately 4.4 million tonnes. In 2012, it grew by just over 372,000 tonnes if compared to the previous year and surpassed the 4.7 million tonnes mark. Asia is the dominant BPA manufacturer with nearly 53% share of the overall production volume followed by Europe and North America, holding 25% and 18% market shares, respectively. The USA, Taiwan, China, South Korea and Japan are the top five BPA producers worldwide (World BPA Production Grew by Over 372,000 Tonnes in 2012. Available from: http://mcgroup.co.uk/news/20131108/bpa-production-grew-372000-tonnes.html).
4.2. **Sources of exposure to BPA**

4.2.1. **Packages**

BPA is used in the production of Polycarbonates (PC). These are a group of thermoplastic polymers produced by the condensation/polymerisation reaction of BPA and carbonyl chloride (Figure 5) or by melt-transesterification reaction between BPA and diphenylcarbonate used in a wide variety of common products including baby bottles, water bottles, digital media (e.g. CDs, DVDs), electronic equipment, automobiles and medical devices.

![Figure 5 – Production of polycarbonate by the condensation polymerisation reaction of BPA and carbonyl chloride. Adapted from: http://en.wikipedia.org/wiki/Polycarbonate](image)

Leakage of BPA from PC into liquid foods can occur in two different manners: i) diffusion of residual BPA present in PC after the manufacturing process and hydrolysis of the polymer, metal ion or acid base catalysed when in contact with aqueous food and stimulants (Mercea, 2009); ii) release of BPA from PC containers into food dependent on the contact time, temperature, and type and pH of the food stimulant (Hoekstra and Simoneau, 2013, Aschberger et al., 2010, Kitahara et al., 2010). High temperatures as well as acidic and alkali solutions cause polymer degradation via hydrolysis, resulting in increased BPA migration. After incubation for 8, 72, and 240 h in food-simulating solvents (10% ethanol at 70°C and corn oil at 100°C), mean BPA migration increased with incubation time (Wong et al., 2005). After a sequence of washing and rinsing, Le et al. (2008) found that new PC bottles leached 1.0 ± 0.3 µg/mL BPA (mean ± standard deviation (STD)) into the bottle content after incubation at room temperature for 7 days (Le et al., 2008). Sajiki and Yonekubo have observed that BPA leaching velocity (BPA-LV) from a polycarbonate plastic (PCP) to a solution of 50 mM glycine at pH 6 or 7 was twice that to control water, and leaching was enhanced above pH 8. At pH 11, BPA-LV was significantly higher in 50 mM glycine and methionine solutions than in 50 mM NaOH. These results indicate that
basic pH and amino acids contained in water could accelerate BPA leaching (Sajiki and Yonekubo, 2004).

As an answer to consumer worries, companies have been producing “BPA-free” plastic water bottles, alternatives to PC plastic bottles. However, epoxy derivatives of BPA are still the most common monomer substrates used to form the “epoxy resin” polymer (Cooper et al., 2011).

Epoxy resins represent the second largest use for BPA (Figure 6). With good mechanical properties (high temperature and chemical resistance), they are used as thermosetting polymers with an extensive range of applications. This polymer is present in nearly all soda and beer cans and also in the most diverse canned foods such as fish, vegetables, fruit and other foods with this type of conservation (WUR, 2012). Despite of epoxy resins as inner coatings protect several metallic food cans from rusting and corrosion, the sterilization process can be responsible for the leach of BPA from the can into the food (Múngia-López et al., 2005, Sajiki et al., 2007). Factors such as coating types, amount of coating, manufacturing and processing conditions appear to be highly important factors influencing the amount of migrated BPA into the food (Goodson et al., 2004).

Several studies report the presence of BPA in canned foodstuff worldwide. For example, recently the study of Geens et al. in 2010, where BPA concentrations in canned beverages ranged from <0.02 µg/l to 8.10 µg/l (Geens et al., 2010), and the study of Cunha et al. (2011) that reported the presence of BPA and bisphenol B (BPB) in canned beverages.
and powdered infant formula in the Portuguese market. In this study, BPA was detected in twenty-one of thirty canned beverages (ranging from 0.03 to 4.70 µg/l) and in two of seven powdered infant formula samples (0.23 and 0.40 µg/l) (Cunha et al., 2011). The potential exposure is dependent on contact surface, time and temperature and is more likely to happen with the high temperature processing conditions and the long shelf-life of canned foods (Rathee et al., 2012).

Another application of BPA is on thermal paper, which is used as point-of-sale receipts, labels, tickets, and print-outs from recording for example. This is a special fine paper that is coated with a chemical that changes color when exposed to heat (Figure 7). This coating is made from a leuco dye and a phenol developer such as BPA. On printing, a thermal head causes the coating components to melt and react with each other, causing the dye to become dark (Mendum and Stoler, 2011, Bierdermann et al., 2010).

Exposure can occur through oral and dermal exposure, although there is no consensus about absorption of BPA through the skin (Environmental Working Group, 2010). Biedermann et al. (2010) discovered that the total mass of BPA on a receipt is 250–1000 times higher than the amount of BPA typically found in a can of food or baby formula, as well as the amount that leaches from a BPA based plastic baby bottle (Biedermann et al., 2010). The hypothesis of dermal absorption of BPA after contact with thermal paper becomes more probable since Zalko et al. (2011) observed that viable skin efficiently absorbs BPA in short-term cultures and also an extensive metabolism of BPA into BPA-monogluturonide (BPA-G) and BPA-monosulfate into the skin (Zalko et al., 2011). This suspicion was also raised by Braun et al. (2011) who found significantly higher urinary BPA
concentrations in cashiers, who had frequent contact with thermal paper, compared to women with other occupations (Braun et al., 2011).

4.2.2. Ambient

The atmosphere is a geochemical reservoir of various organic compounds, interacting with the oceans, land, and living organisms including human beings. One important environmental issue is the origin, transport and fate of organic pollutants in atmospheric aerosols and their health effects (Fu and Kawamura, 2010).

BPA is a pseudo-persistent chemical, ubiquitous in the environment because of continuous release which can occur during chemical manufacture, transport, and processing. Post-consumer releases are primarily via effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, and the natural breakdown of plastics in the environment (US Environmental Protection Agency, 2011).

Characterized with a moderate hydrophobicity, BPA has a modest capacity for bioaccumulation. Based on these various characteristics, it is estimated that the largest environmental compartments of BPA are abiotic and are associated with water and suspended solids (~53%), soil (~25%), or sediments (~22%) (Flint et al., 2012).

BPA has a moderate affinity for soil organic matter, being improbable its mobility or bioavailability in soils (Fent et al., 2013). However, mobility can be affected by soil chemistry and texture. Reports of increased BPA sorption in the presence of iron, cadmium, and lead are consistent but results conflict with regards to the influence of soil pH (Li et al., 2007).

4.2.3. Wildlife

BPA is rapidly degraded in the environment through both microbial biodegradation and photodegradation and has a low potential to bioaccumulate in animals. Still, aquatic organisms that are in proximity of point source outputs of BPA are at the greatest risk of harmful effects of BPA (Crain et al., 2007, Oehlmann et al., 2009), including bioaccumulation and biomagnification in the food chain.
4.3. Metabolism and toxicity of BPA

Rodents have been used in toxicokinetic studies of BPA. However, there is a significant difference in the distribution of BPA in humans and rodents (Figure 8).

Figure 8 – Schematic diagram depicting the glucuronidation of BPA in the liver and the route of elimination of unconjugated BPA from serum in rodents and primates after initial absorption from the gut and transport to the liver. Adapted from: Taylor, J. et al. (2011) *Similarity of Bisphenol A Pharmacokinetics in Rhesus Monkeys and Mice: Relevance for Human Exposure* Environmental Health Perspectives 119, 422-430

In humans, metabolism of BPA is dominated by Phase II conjugation reactions of detoxification where the metabolite monoglucuronide conjugate (BPA-G) derived by glucuronidation reaction catalyzed by the uridine-5'-diphospho(UDP)-glucuronosyltransferase UGT2B15 (enzyme localized in the endoplasmic reticulum responsible for the conversion of small lipophilic compounds, such as BPA, to charged, water-soluble glucuronides) (Hanioka et al., 2008, Mazur et al., 2010). In a lower extent, BPA sulfation can also occur and is mediated probably by the sulfotransferase isoform SULT1A1, the isoform with the highest $k_{cat}/K_{M}$ value for BPA conjugation (Nishiyama et al., 2002). Unlike the aglycone form of BPA, BPA-G does not bind to the estrogen receptor or show estrogen activities (Matthews et al., 2001). Hence, it is important to investigate the toxicokinetics of unchanged BPA, not metabolites, for prediction of the toxicity of BPA in humans. In rats BPA-G is excreted predominantly via the bile into feces and then undergoes enterohepatic recirculation while in humans and monkeys it is rapidly excreted into the urine.
with a faster clearance (Volkel et al., 2002) (less than 6h) than in rats (over 19h) (Mazur et al., 2010).

There are others limitations for BPA metabolism studies such as difficulties in identifying individuals that are completely unexposed to BPA from the environment. Further, all of the current metabolic studies are based on kinetics following a single, usually high dose, while current evidence indicates that humans are experiencing multiple exposures each day.

A threshold model is being used by toxicologists when assessing the effects of possible EDs. This model relies on the principle that “the dose makes the poison,” implying that higher doses were expected to cause greater harm, defining NOEL (no observable effect level) by assessing different doses of a chemical.

The European Food Safety Authority (EFSA, 2007) established a value of 50 ng/g day as the tolerable daily intake (TDI) and the US Environmental Protection Agency (EPA, 2009) established the same as the reference dose (RfD). These organizations conclude that current BPA levels present no risk to the general populations. However, non-linear relationships between dose and response have been observed for some EDs when it triggers observable effects at very high and low doses but almost no effect at moderate doses, for example (Lemos et al., 2009).

EDs can have detrimental effects during specific stages of development and no discernible effect during other life stages. Such chemicals may necessitate changes to toxicological study methods. It has been suggested by some toxicologists that the threshold model routinely used for risk assessment purposes by government agencies, including the EPA, should be rejected and replaced entirely (Matsumoto, 2002). Many of these studies examine only animals exposed during adulthood and thus lack information about progenies of animals treated during pregnancy. Conclusions reported by Lang et al. in 2008 suggest that follow-up longitudinal studies are crucial on infants, children, and adolescents, as well as pregnant women and fetuses. The fetus and infant are believed to be more susceptible to the estrogenic effects of BPA because of small body size and limited capacity to metabolize this substance (Lang et al., 2008).
4.4. **Legislation**

In last years, the National Institute of Environmental Health Sciences (NIEHS) and the Food and Drug Administration (FDA) have been working together to address potential health concerns about BPA. In September 2008, the National Toxicological Program (NTP) completed a review of available research on BPA and concluded that there was “some concern for effects on the brain, behavior, and prostate gland in fetuses, infants, and children at current human exposures.” (NTP-CERHR, 2008). In 2009, FDA provided updates to the previous assessment and expressed its agreement with the NTP’s perspective (FDA, 2013).

In order to improve risk assessment studies concerning this ubiquitous chemical, NIEHS launched in 2009 a multipronged research program designed to fill remaining gaps and resolve controversies about BPA toxicity studies (Figure 9) (Birnbaum et al., 2012).

![Figure 9 – Elements of the NIEHS BPA research program. NIOSH, National Institute for Occupational Safety and Health. Adapted from: Birnbaum, L; Bucher, J; Collman, G; Zeldin, D; Johnson, A; Schug, T; Heindel, J. (2012) “Consortium-Based Science: The NIEHS’s Multipronged, Collaborative Approach to Assessing the Health Effects of Bisphenol A.” Environmental Health Perspectives 120, 1640-44](image)

In Europe, after concluding full risk assessment of BPA in 2006, EFSA set a TDI of 50 µg/kg bw.day for this substance. Between 2008 and 2011, new risk evaluations were made however the results did not justified the alteration of the TDI stablised in 2006. New scientific studies request that EFSA carries out a full re-evaluation of the human risks...
associated with exposure to BPA through the diet, also taking into consideration the contribution of non-dietary sources to the global exposure to this chemical (EFSA, 2013).

In January 2014, EFSA presented the draft opinion in which concluded that for all population groups diet is the major source of exposure to BPA and exposure is lower than previously estimated and also identified likely adverse effects on the liver and kidney and effects on the mammary gland as being linked to exposure to the chemical. It therefore recommended that the current TDI be lowered from its current level of 50 µg/kg bw.day to 5 µg/kg bw.day. At the beginning of 2015, EFSA released a new report where it concluded that “BPA poses no risk to human health from foodstuffs because current levels of exposure are well below the temporary TDI of 4 µg/kg bw.day. This also applies to pregnant women and to the elderly. In addition, EFSA’s experts concluded that the health concern from the aggregated exposure to BPA from foodstuff, toys, dust, cosmetics and thermal paper is also below the temporary TDI of 4 ng/g bw.day. The uncertainty in the exposure estimate from toys, dust, cosmetics and thermal paper is considerable due to the very limited availability of data. The new TDI is dependent on the results of the NTP Program in 2016.
5. Tetrabromobisphenol A

5.1. Properties of TBBPA

According to EFSA, “Brominated flame retardants (BFRs) are anthropogenic chemicals that are added to a wide variety of consumer/commercial products in order to improve their fire resistance. There are 5 major classes of BFRs: brominated bisphenols, diphenyl ethers, cyclododecanes, phenols and phthalic acid derivatives.” (EFSA, 2011). TBBPA falls within the category of the brominated bisphenols.

TBBPA, from the IUPAC name 2,2′,6,6′-Tetrabromo-4,4′-isopropylidenediphenol, is a BFR derived from the bromination of BPA, consisting of two hydroxyphenyl rings linked by a carbon bridge, with bromine substitution at the 3, 3′, 5 and 5′-positions (Figure 10). TBBPA is produced by the bromination of BPA with various solvents such as halocarbon alone, hydrobromic acid, aqueous alkyl monoethers, acetic acid or methanol (MeOH). With the molecular formula C₁₅H₁₂Br₄O₂ and a MW of 543.9 g/mol, it has two pKₐ values (7.5 and 8.5) since it has two phenol groups (EFSA, 2012). TBBPA is characterized by a high lipid solubility (the protonated compound with a log Kow of 9.7), and low volatility (EFSA, 2011). At 25°C, it has low solubility in water (4.16 mg/l), in MeOH (920 g/l) and in acetone (2400 g/l) (http://www.inchem.org/documents/ehc/ehc/ehc172.htm#SubSectionNumber:1.1.2, accessed on March 2015).

![Figure 10 – Tetrabromobisphenol A. Adapted from: http://www.bsef.com/about-tbbpa/](http://www.inchem.org/documents/ehc/ehc/ehc172.htm#SubSectionNumber:1.1.2)
In 2004, TBBPA had an annual global production of more than 170 million tons, though only 20-30% of the total volume produced was used as an additive flame retardant on material subject to environmental leaching (ECB, 2006). Today, TBBPA is now the most heavily manufactured BFR in the world, with global production currently topping 200 million tons a year. It is produced in Israel, the United States, Jordan, Japan and China. The region with the highest demand for TBBPA is Asia, due to the high volume of printed wiring boards and electronics components manufactured in that region (Masten, 2002).

5.2. Sources of exposure to TBBPA

5.2.1. Epoxy resins and polymers

TBBPA is mainly used as a reactive flame retardant in epoxy, vinyl esters and PC resins. The main application of TBBPA in epoxy resins is in printed circuit boards where the bromine content may be 20% by weight (Figure 11). Its application can be additive or reactive. When used as a reactive component, TBBPA is covalently bounded to the polymer by the phenolic hydroxy groups, being incorporated into it. However, the polymer can also contain a portion of unreacted TBBPA in excess not bounded to the polymer which can easily leach out from the polymer matrix into the environment and subsequently result in exposure of animals and humans (EFSA, 2011). On the other hand, when TBBPA is used as an additive component, the molecules are not part of the structure of the polymer itself and can be released into the environment more readily (Birnbaum, 2004). Printed circuit boards are used in communication and electronics equipment, electronic appliances, transportation devices, sports and recreation equipment, lighting fixtures and signs (Covaci et al., 2009).

Figure 11 – TBBPA application in epoxy resins in printed circuit boards. Adapted from: https://www.olimex.com/PCB/
TBBPA was measured in the air near a printed circuit boards production site at a level of 1.8 μg TBBPA per m³ (Zweidinger et al., 1979). Studies in Japan have found TBBPA in soil and sediment at concentration ranges of 0.5–140 μg/kg dw and 2–150 μg/kg dw, respectively (Watanabe et al., 1983). Data are very limited regarding the presence of TBBPA in biota, which may reflect its relatively short half-life in air, water, and sediment. Human TBBPA serum levels were measured by Jakobsson et al. (2002), who found TBBPA in 8 of 10 samples from computer technicians, at levels ranging from 1 to 3.4 pmol/g lipid (Jakobsson et al., 2002).

The second major application of TBBPA is when used also as a BFR in polymers such as acrylonitrile butadiene styrene (ABS) resins, high impact polystyrene (HIPS), phenolic resins, adhesives, paper, and textiles and others. Additive use accounts for approximately 10% of the total use of TBBPA (ECB, 2006).

More recently, TBBPA has been quantified in sewage sludge samples in Spain, along with other BFRs, being detected in concentration range of nd-472 ng/g (Gorga et al., 2013). Harrad and Abdallah (2011) determined TBBPA presence in dust from the four seats in five different cars, measuring usually higher levels in the front seats (Harrad and Abdallah, 2011). Ni and Zeng (2013) found considerable amounts of TBBPA in air conditioning filter dust, showing how inhalation might constitute a major pathway for human exposure to this BFR (Ni and Zeng, 2013).

TBBPA is also used in the manufacture of derivatives such as TBBPA bismethyl ether (TBBPA-bME), TBBPA bis(2,3-dibromopropyl)ether (TBBPA-bDiBPrE), TBBPA bisallyl ether (TBBPA-bAE), TBBPA bis(2-hydroxyethyl) ether (TBBPA-bOHEE), TBBPA brominated epoxy oligomer, and TBBPA carbonate oligomers. The main use of these derivatives is as flame retardants, usually in niche applications. (EFSA, 2011).

5.2.2. Ambient

Releases of TBBPA into the environment occur primarily through various waste streams generated during manufacture, processing and upon disposal of the substance and products containing the substance (i.e. dismantling, recycling, landfills, incineration,
accidental fires and sewage sludge applications for agricultural purposes), normally where it was introduced as an additive flame retardant (EU RAR, 2008).

Experimental microbial degradation studies have shown that in sediments under anaerobic reducing conditions TBBPA can be completely dehalogenated to BPA (Ronen and Abeliovich, 2000; Voordeckers et al., 2002; Gerecke et al., 2006). Ronen and Abeliovich (2000) also showed that BPA can be further degraded under aerobic conditions, indicating that a sequential anaerobic-aerobic process may possibly be used to completely degrade TBBPA present in contaminated soil.

5.3. Metabolism and toxicity of TBBPA

The studies concerning the metabolism of TBBPA are limited. In an early study, Brady (1979) concluded that TBBPA is poorly absorbed from the gastrointestinal tract after administered a single oral dose (6.5–7.5 mg/kg) of 14C-labeled TBBPA to rats. The researcher found more than 95% of the parent TBBPA dose in the feces and 1.1% in the urine in 72h following administration (Brady, 1979).

Later in 2001, other researchers detected peak concentrations of 14C-TBBPA 1h following intraperitoneal administration (250 or 1,000 mg/kg) in all tissues, with higher concentrations in the fat, followed by the liver, sciatic nerve, muscles, and adrenals (Szymanska et al., 2001). The analysis of the feces showed that 10% of the radiolabeled material in the feces was tribromobisphenol A, suggesting rapid elimination in the bile and possible debromination by gastrointestinal flora (Szymanska et al., 2001).

One human study examined the half-life of TBBPA in exposed Swedish computer technicians, observing that this BFR has an estimated half-life in blood serum of 2.2 days. (Hagmar et al., 2000).

Concerning the effects of TBBPA in fish, a few studies have been made more recently. In 2012, Chan and Chan have observed the effects of TBBPA in zebrafish. Based on their results, it can be perceived the induction of thyroid α-receptor, thyroid stimulating hormone, and transthyretin genes in zebrafish embryo–lарvae (Chan et al., 2012), since this chemical compete with the binding of T4 to plasma transport proteins (Meerts et al., 2000, Hamers et al., 2006). Regarding its endocrine disruption in fish, TBBPA has shown low but multiple hormonal activities in mosquitofish (Gambusia affinis) with significant up-regulation of vitellogenin and estrogen receptors mRNAs in the liver and testis, after exposure to 500 nM and 50 nM of TBBPA, respectively (Huang et al., 2013).
Liver enzymatic activity upon TBBPA chronic exposure has been evaluated in crucian carp, revealing irreversible damage for concentrations higher than 0.92–1.30 μm (S. Yang et al., 2013). Hepatic oxidative stress was observed in goldfish (*Carassius auratus*) after prolonged exposure to TBBPA (Feng et al., 2013).

About its toxicity, the higher median lethal dose (LD₅₀) and median lethal concentration (LC₅₀) values for mice, rats, guinea pigs, and rabbits indicate that the acute toxicity of TBBPA is low. For the mouse and guinea pig, LC₅₀ values were >500 mg/m³, while for the rat, it was >10,920 mg/m³. Oral LD₅₀ values for mice and rats were >2000 mg/kg, while an LD₅₀ >50,000 mg/kg was calculated for the rat via intubation. Dermal LD₅₀ values >1000 mg/kg were reported for rabbits and guinea pigs. The intraperitoneal LD₅₀ values were ≥3200 mg/kg for the mouse and rat (Toxicological Summary for Tetrabromobisphenol A [79-94-7] 06/2002).

### 5.4. Legislation

Among the available BFRs, TBBPA is by far the most deeply investigated from a health and environmental point of view as it has undergone an 8-year EU Risk Assessment for the environment and human health (TBBPA EU Risk Assessment report for Health & the Environment: http://ecb.jrc.it, accessed in January 2015). The conclusions of the EU Risk Assessment were published in the EU Official Journal on 18 June 2008 and also confirmed by the Scientific Committee on Health and Environmental Risks (SCHER). In May 2005, the human health part of the Risk Assessment report concluded that at current exposure levels TBBPA poses no risk to human health.

The EFSA report in December 2011 on the exposure of TBBPA and its derivatives in food concluded that “current dietary exposure to TBBPA in the European Union does not raise a health concern” (EFSA, 2011), in agreement to the conclusion of the SCHER committee.

TBBPA is classified as a “Persistent Bioaccumulative Toxic” chemical, being included in a chemical category which is subject to reporting under Emergency Planning and Community Right-to-Know Act, Section 313, 1986 (Environmental Protection Agency Toxics Release Inventory (TRI) Program. http://www2.epa.gov/toxics-release-inventory-tri-program/persistentbioaccumulative-toxic-pbt-chemicals-covered-tri, accessed on March 2015).
Nevertheless, TBBPA has been registered in REACH (Commission Regulation No. 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals). TBBPA as a substance is classified as H4105 (very toxic to aquatic species). However, this classification no longer applies when it is reacted into the epoxy resin, as TBBPA becomes one of the building blocks for a different substance (Directive 2002/95/EC of the European Parliament and of the Council - 27 January 2003 – on the restriction of the use of certain hazardous substances in electrical & electronic equipment).
6. Brief review of analytical methodologies

6.1. Sample preparation

After collecting the samples, the determination of BPA and TBBPA in complex matrices, like fresh or canned solid and liquid foodstuffs, requires extensive sample preparation preceding instrumental analysis. Special treatments are necessary depending on the matrix composition. As common in food residue analysis, solid samples are first fully homogenized while liquid ones are filtered and/or centrifuged. The removal of lipids from the extract is obligatory for samples of animal origin (e.g. fish, meat) since they can significantly reduce the analytical performance of the chromatographic techniques.

Solvent extraction and solid-phase extraction (SPE) are the most widely used techniques to isolate BPA from liquid and solid samples, respectively, mainly because of its simplicity and wide-range applicability. Other techniques might include microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), matrix solid-phase dispersion extraction (MSPD), etc. These procedures are not so typical but are known to improve the extraction of BPA in terms of reduced sample size, automation and solvent consumption. Often, different techniques are employed and methods become frequently matrix-dependent (Ballesteros-Gómez et al., 2009).

In case of TBBPA, the Soxhlet procedure is usually executed for extraction of solid material because its simplicity and high extraction efficiency (Morris et al., 2004). Other techniques include PLE (Webster et al., 2009, Zhou et al., 2010) and liquid-liquid extraction (LLE) and SPE are employed for liquids (milk, blood) (Cariou et al., 2005, Covaci et al., 2009). Since this BFR has pKa values of 7.5 and 8.5, meaning the pH should be carefully controlled in order not to have losses of TBBPA in the analytical procedure.
6.2. **Liquid-liquid extraction (LLE) and solid-phase extraction (SPE)**

LLE, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent.

The LLE of BPA in food matrix typically uses water as one of the solvents and another liquid that does not dissolve very well in water, such as diethyl ether (this is the most common type of ether, and it is often called simply "ether"), ethyl acetate, chloroform or dichloromethane (DCM) ([Ballesteros-Gómez et al., 2009](#)). Acetonitrile (MeCN) is usually preferred as a solvent for solid foods samples. However, this chemical is miscible in water not allowing good separation. This problem was overpassed with the development of QuEChERS extraction technique, in which a strong salting-out effect allows the separation of MeCN from water matrixes (a more detailed description is given in section 5.5). Although overall recoveries of LLE are usually well above 75%, low recoveries (<50%) due to matrix–analyte interactions were reported for a variety of foods ([Thomson and Grounds, 2005](#)).

The second most applied extraction technique for BPA is SPE. SPE is a sample preparation process by which compounds dissolved or suspended in a liquid mixture (mobile phase) are separated from other compounds in the mixture according to their physical and chemical properties. The compounds with affinity for a sorbent through which the sample is passed (stationary phase) are separated from the rest of the mixture.

For the retention of BPA in the column, non-selective or selective sorbents can be used. If the goal is a reversed phase extraction, a stationary phase of silica with carbon chains is commonly used. Octadecyl bonded endcapped silica (C18) or octyl bonded endcapped silica (C8) are usually the first choice sorbents. For a normal phase SPE, a stationary phase of C8 will retain organic analytes from polar solutions due to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. The absorbed compound can be further collected with a nonpolar solvent that disrupt the forces that bind the compound to the packing. Quaternary amine bonded silica with Cl- counterion (SAX) is typically used for strong anion exchange for BPA ([Sigma-Aldrich, 1998](#)). The commercial sorbent divinylbenzene/N-vinylpyrrolidone copolymer (OASIS HLB from Waters, 30–200 mg) has been the most used to date for BPA analysis. The hydrophilic N-vinylpyrrolidone polymer acts as a hydrogen acceptor, while the hydrophobic divinyl benzene polymer provides reversed-phase retention for BPA. It offers advantages over classical silica based sorbents, i.e. high specific area (800 m²/g), possibility to dry out during the extraction procedure without reducing its ability to retain BPA and like other polymeric resins, stability over the entire pH range ([Camel, 2003](#)). Further clean-up with Florisil
cartridge is sometimes also required, namely in the treatment of fish, fruit and vegetable samples (Ballesteros-Gomez et al., 2009). In particular for BPA, the addition of water reduces the viscosity of the sample, thus resulting in a better flow rate during SPE (Ballesteros-Gomez et al., 2009).

Comparatively to LLE, SPE has less organic solvents consumption and requires less quantity of sample; however, this method is costly because a new cartridge is required for each sample to be analysed (Nollet L., 2010).

The introduction of MIPs (Molecular Imprinted Polymers) as selective sorbents into SPE, a technique commonly referred to as MISPE, is emerging as a very popular tool. These synthetic polymers have molecular recognition ability for a target analyte. Currently a number of approaches have been used to prepare BPA imprinted polymers, which have been applied to the determination and removal of BPA and other phenolic estrogen pollutants in environmental waters (Ren et al., 2014). Architectures to develop BPA-MIPs, such as hybrid molecularly imprinted membranes have been described in the literature (Takeda and Kobayashi, 2006).

6.3. Solid Phase Microextraction (SPME)

SPME is a sample preparation technique that involves the use of a fibre coated with an extracting phase which extracts different kinds of analytes, normally volatile or semi-volatile, from liquid or gas phase media (Pawliszyn, 2012). Because no solvent is injected and the analytes are rapidly desorbed onto the column, low detection limits are allowed while resolution is maintained. For BPA extraction, a Carbowax fibre, with high polarity is normally used.
Chapter I

Theoretical Part

SPME followed by Gas Chromatography-Mass Spectrometry (GC–MS) has been applied to the determination of BPA in aqueous food simulants (Salafranca et al., 1999) and water from plastic containers and tableware (Lee et al., 2011). In the first study, detection limits ranged from 0.1 to 2.0 ng/g for BPA, with a linear range from the low ng/g to several µg/g range.

6.4. Matrix Solid Phase Dispersion (MSPD)

Barker et al. first reported MSPD in 1989 which can be applied for the extraction of solid, semi-solid or viscous food and biological matrices (Barker et al., 1989). In this technique, the sample is mixed with a sorbent such as C8 or C18 bonded silica, followed by packing the dispersant sorbent material into an empty SPE cartridge before elution.

MSPD is simple and versatile and offers the possibility of performing extraction and clean-up in one step. MSPD has several advantages over classical sample treatment procedures. This method is simpler and less time consuming, with no emulsion formation and low solvent consumption. The negative point is that the operation is tough and is not possible to automatize.

In 2007, Shao et al. applied a method based on MSPD using C18 as dispersant and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for the simultaneous determination of nonylphenol, octylphenol and BPA in eggs and milk. Recoveries of BPA were 79% and relative standard deviations were equal or lower than 15% for egg samples. In milk, recoveries ranged from 84 to 86% for BPA and relative standard deviations were equal to or lower than 8% (Shao et al., 2007).
6.5. QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)

QuEChERS is a recently developed extraction technique for food analysis, involving two steps. It is initialized with an extraction of the compounds of interest from the aqueous matrixes with MeCN, in the presence of considerable amounts of MgSO\textsubscript{4} and NaCl salts. When the salt concentration is increased, some of the water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the analyte. The procedure is followed by a dispersive SPE cleanup of an aliquot of the obtained extract. Non-polar solvents, such as \textit{n}-hexane, \textit{n}-heptane and trimethylpentane, have been used together with MeCN for the extraction of BPA from fatty samples (Fernández \textit{et al.}, 2007, Grumetto \textit{et al.}, 2008) because these solvents extract the lipidic material efficiently and therefore also extract BPA, taking into account its lipophilicity.

In addition to allowing the achievement of extracts with lower levels of interferents compared to a conventional extraction process, QuEChERS seems to have some advantages over processes that use packed stationary phases, and also reduces the use of organic solvents (Cunha, 2007). The method is simple, effective and saves solvent, being both flexible and selective at the same time. Its weakness lies in the low enrichment factor, which can be solved combining QuEChERS with the dispersive liquid-liquid microextraction (DLLME).

6.6. Dispersive Liquid-Liquid Microextraction (DLLME)

DLLME is a novel sample extraction procedure proposed by Assadi and co-workers in 2006, able to provide great enrichment factors and good yields in a simple and fast way (Razaee \textit{et al.}, 2006).

A high density extractant solvent together with a dispersive solvent with high miscibility in the extractant and water are rapidly added to an aqueous sample. This will result in a cloudy solution of extractant solvent dispersed through the aqueous phase. Hydrophobic solutes are rapidly and efficiently enriched in the extractant solvent and, after centrifugation, they can be determined in the phase settled at the bottom of the tube (Cunha \textit{et al.}, 2010).

The selection of an appropriate solvent extractor is the most important parameter in DLLME. The extracting solvent should be selected from those who have higher density than water, extraction capability of the compounds of interest and good chromatographic behavior. Usually for BPA, the most used solvents extractors are chlorinated solvents, among them stand out chlorobenzene, carbon tetrachloride and tetrachloroethylene (Razaee \textit{et al.}, 2006). To choose the dispersing solvent, the main feature to be considered is their
miscibility in the organic phase (extracting solvent) and in the aqueous one. The need for these characteristics generally limited to the use of MeOH, MeCN, acetone and tetrahydrofuran (THF).

A new simple and reliable method combining an MeCN partitioning extractive procedure followed by dispersive solid-phase cleanup (QuEChERS) with DLLME and further GC-MS analysis was developed by Cunha et al. (2012) for the simultaneous determination of BPA and BPB in canned seafood samples. The DLLME procedure involved the use of tetrachloroethylene as extractive solvent while the own MeCN extract obtained from QuEChERS was used as dispersive solvent, and anhydride acetic (AA) as derivatizing reagent. Besides the enrichment factor provided, the final DLLME extractive step allowed the simultaneous acetylation of the compounds required for their GC analysis. This process showed over 68% recovery, reproducibility with a relative standard deviation under 21% and high sensitivity for the target analytes (MDLs of 0.2 μg/kg for BPA and 0.4 μg/kg for BPB) (Cunha et al., 2012). Another work from the same researchers addressed to the determination of BPA in canned beverages showed limits of detection (LOD) of 5.0 ng/l and limits of quantification (LOQ) of 10.0 ng/l (Cunha et al., 2010).

6.7. Soxhlet extraction

TBBPA with a Log Pow (octanol-water partition coefficient) of 4.5-5.3, is apolar and hardly dissolves in aqueous solutions. This extraction technique is based in the greater solubility of a given compound in nonpolar solvents than in water. Often it uses a mixture of two apolar extraction solvents to improve the yield instead of just one solvent. Although DCM has been used as extraction solvent, soxhlet extractions normally involve the use of an hexane:acetone mixture varying from 1:1 to 3:1. Morris et al. in 2004 performed liquid solid extractions by Soxhlet or homogenization by Ultra Turrax using binary solvent mixtures (1:1 or 1:3 (v/v) acetone: n-hexane mixture) on sediments and biota. TBBPA extract was reduced to just dryness, reconstituted in MeOH, and stored at -20 °C prior to LC-MS analysis (Morris et al., 2004). Later in 2007, Granby and Cederberg also used this method to extract TBBPA from fish samples, proceeding to a Soxhlet extraction with a mixture of acetone:n-hexane (1:1, v/v) for seven hours. The extract was evaporated until a clear residue of lipid remains, which was dissolved in hexane and cleaned up with sulphuric acid. The cleaned hexane phase was evaporated using a gentle stream of nitrogen. The sample was then dissolved in 200 μl MeOH:water (4:1) and analysed in LC-MS/MS with over 79% recovery achieved (Granby and Cederberg, 2007).
Despite TBBPA could be fully extracted from the matrix, Soxhlet extraction is time and solvent consuming, making it undesirable for routine analysis (ten Dam et al., 2012).

Due to their advantages, such as minimum sample pre-treatment required, simplicity, and high recoveries (>80%) (Morris et al., 2004), binary solvent mixtures typically containing acetone:n-hexane (Boer et al., 2002) or DCM:n-hexane (Berger et al., 2004) have been preferred for Soxhlet-based extractions.

6.8. Pressurized Liquid Extraction (PLE)

PLE is similar to Soxhlet extraction, but in this technique the elevated temperature applied allows the sample to become more soluble and achieve a higher diffusion rate while the elevated pressure keeps the solvent below its boiling point.

Iso-hexane was the extraction solvent selected by Webster et al. (2009) for TBBPA determination in fish. After mixed with sodium sulfate and spiked with appropriate labelled internal standard (I.S.) (TBBPA\textsuperscript{13C_{12}}), samples were refrigerated overnight before being ground to a fine powder using a mortar and pestle. Samples were extracted by PLE using an ASE 300 (Dionex Ltd., Camberley, Surrey, UK) under elevated temperatures and pressures. Fish tissue samples were extracted using an oven temperature of 60 °C and a pressure of 1500 psi. Five minutes heating was followed by two static cycles of five minutes each. The cell flush was 50% total cell volume (i.e. 25% of the cell volume for each flush ¼ 25 ml per flush) with a 120 second purge (using nitrogen) at the end of each sample extraction. After extraction, TBBPA was analysed by LC-MS with over 75% recovery (Webster et al., 2009). In the study of Kolic et al. in 2009, a mixture of hexane and DCM solvents were used for extraction on the Fluid Management Systems (FMS) (Waltham, MA) automated PLE system. An acid silica column was initially employed for clean-up to remove bulk chemical interferences. Final extracts were brought to dryness via nitrogen evaporation. The individual residue was re-dissolved using IPA/toluene (9:1, v/v) containing hexabromobenzene (HBB\textsuperscript{13C_{6}}) as the instrumental internal standard (I.S.) prior to LC-APPI-MS/MS analysis (Kolic et al., 2009). The same technique was applied in 2010 by Zhou and its colleagues (Zhou et al., 2010).
6.9. **Clean-up**

Because of the limited selectivity of solvent-based extractions there is a need for extensive clean-up prior to instrumental analysis. The extracts containing BPA are commonly subject to extensive clean-up and in this respect SPE is preferred. In SPE, the non-selective sorbent OASIS HLB has been used also as a clean-up step for a variety of foods (fish, fruit and vegetables, and canned foods) after solvent extraction, removing hydrophilic and lipophilic interferences (Covaci et al., 2009). Further clean-up with Florisil - a highly selective adsorbent comprised of extremely white, hard-powdered synthetic magnesium-silica gel, with an extensive utility in preparative and analytical chromatography - cartridge is sometimes also required, namely in the treatment of fish, fruit and vegetable samples (Ballesteros-Gómez et al., 2009).

In order to isolate TBBPA from the co-extracted interfering compounds such as lipids and other matrix constituents, a following step of clean-up can be executed. Several methods, or combinations thereof, have been employed including gel permeation chromatography (GPC) (Morris et al., 2004; Webster et al., 2009), neutral or acidified silica (Harrad et al., 2009), Florisil (Klif, 2010) or sulphuric acid treatment (Fernandes et al., 2008).

GPC is based on the partition of the molecules by size allowing the separation of the desired analyte from the interfering ones. In the case of TBBPA, a GPC system with two crosslinked divinylbenzene gel columns in series has been already used aiming for lipid remotion (Morris et al., 2004). Large compounds elute earlier since they cannot enter the pores of the packing material. Clean-up by GPC might not result in complete separation of the lipid content and the compounds of interest and additional clean up procedures might be required (Frederiksen et al., 2007). When Frederiksen et al. discover that GPC is not sufficient to remove efficiently lipids from biotic samples, they used sulphuric acid; other research groups also used additional treatments like silica and florisil solid SPE after GPC (Budakowski and Tomy, 2003, Stapleton et al., 2006). The treatment with sulphuric acid is the most applied treatment, since it thoroughly removes the lipid content from extracts (Morris et al., 2004; Morris et al., 2006; Bethune et al., 2005).

A less exhaustive method and more easily automated is the acid silica digestion, although it is a less common technique (Zhou et al., 2010, Janak et al., 2005).
Another subsequent step in the extraction procedure is fractionation, which allows the separation of TBBPA from other pollutants (such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCDDs) and others), functioning also as a clean-up step. This is typically done by silica column fractionation (Covaci et al., 2009; Morris et al., 2004), although another methods have been used. Deactivated silica gel has been applied for the separation of TBBPA from PBDEs. *Iso*-octane was used for the elution of PBDEs, while a more polar solvent elute TBBPA (Morris et al., 2004).

Florisil has also been successfully used to isolate phenolic analytes like TBBPA from neutral organohalogen compounds. In this case, neutral compounds were first eluted with mixtures of DCM:*n*-hexane (1:3, v/v), while polar mixtures of acetone:*n*-hexane (15:85, v/v) and MeOH:DCM (12:88, v/v) elute TBBPA (Berger et al., 2004).

Another method can resort to the commercial sorbent Oasis HLB®, which allows the fast separation of TBBPA from HBCD diasteroisomers. The mixture of DCM:*n*-hexane (1:1, v/v) was used to elute HBCDs from the SPE cartridge, while TBBPA was afterward eluted with DCM (Cariou et al., 2005).

6.10. **Analytical techniques**

6.10.1. **Liquid Chromatography (LC)**

As BPA can be analysed by LC directly without the derivatization step in sample preparation, LC is the technique used most often for the determination of BPA in both food and biological samples. LC of BPA is usually carried out in reversed-phase C18 columns. Mobile phases vary according to the detector coupled to LC. Elution conditions highly depend on the analytes to be determined along with BPA and the food matrices under study. It is frequent to determine BPA with other phenols, EDs and migrants from food packaging and in this case gradient elution is always performed; 15 and 40 min are the range of run times, depending on the number of contaminants to be determined and the matrix composition. This separation technique is usually performed at room temperature.

Several detectors can be coupled to LC, like the UV detector or the electrochemical detector. However, the fluorescence detection is frequently the preferential non-MS-based method used for LC determination of BPA in both food and biological samples. The fluorophore in the BPA molecule is fairly strong, showing native fluorescence with excitation and emission wavelengths at 275 and 305 nm, respectively, which remain constant in the
solvents more frequently used in LC mobile phases, namely water, MeCN and MeOH (Ballesteros-Gómez, 2009). There is a possibility of interference from other fluorescent food migrants from can coatings, e.g. bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) or novolacs glycidyl ethers (NOGE), which may produce false-positives since they are very similar. Indeed, confirmation by MS is essential (Inoue et al., 2003, Schorningumer and Cichna-Markl, 2007).

6.10.2. Liquid Chromatography coupled to Mass Spectrometry (LC-MS) and tandem Mass Spectrometry (LC-MS/MS)

LC coupled to mass spectrometer (LC-MS) is the second most frequently used LC method after LC-fluorescence for the determination of BPA in both food and biological samples, providing much more confidence in peak identification based on the mass spectrum. This method can reduce sample treatment and enable the “extraction” of an analyte at the detection stage of a method by selection of specific ions or transitions.

After sample injection, the analysis of BPA is carried out using atmospheric pressure ionization interfaces, namely electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). In a simple explanation, ESI is a technique used in MS to produce ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol. ESI may produce multiply charged ions, effectively extending the mass range of the analyser to accommodate the kDa - MDa orders of magnitude but in a ‘soft ionization’ technique, since there is very little fragmentation. This can be advantageous in the sense that the molecular ion (or more accurately a pseudo molecular ion) is always observed, however very little structural information can be gained from the simple mass spectrum obtained. This disadvantage can be overcome by coupling ESI with tandem mass spectrometry (ESI-MS/MS) (Ho et al., 2003). APCI is characterized for an electrode discharge on a solvent spray that produces primary ions. It is mainly used with polar and relatively nonpolar compounds with a molecular weight of less than 1500 Da, generally giving monocharged ions (Bruins, 1991).

ESI is more frequently used for BPA than APCI (Dorival-García et al., 2012) because it generally provides better sensitivity (Ballesteros-Gómez, 2009) despite APCI being generally less susceptible to matrix effects (Iparraguirre et al., 2014). Normally these two ion sources are used in the negative mode (ESI(−) and API(−)), applied to acidic compounds [M-H]−, [M-nH]− and [M+I]− like BPA. Instrumental quantitation limits for BPA of 5 and 20.7
ng/ml have been reported using ESI(-) and APCI(-), respectively (Mendiola et al., 2007) and instrumental detection limits for BPA of 1 and 15 ng/ml in the same conditions (Iparraguirre et al., 2014).

After ionization, the product ions formed follow to the mass analyser. In studies for determination of BPA is ordinarily used the quadrupole mass analyzer (QMS), consisting of four cylindrical rods, set parallel to each other (Hoffmann and Stroobant, 2003). In a quadrupole mass spectrometer the quadrupole is the component of the instrument responsible for filtering sample ions, based on their mass-to-charge ratio (m/z). Ions are separated in a quadrupole based on the stability of their trajectories in the oscillating electric fields that are applied to the rods (Hoffmann and Stroobant, 2003). When we have a linear series of three quadrupoles, it is known as a triple quadrupole mass spectrometer. The first (Q₁) and third (Q₃) quadrupoles act as mass filters, and the middle (q₂) quadrupole is employed as a collision cell. This collision cell is an RF-only quadrupole (non-mass filtering) using Ar, He, or N₂ gas (~10⁻³ Torr, ~30 eV) for collision induced dissociation of selected parent ion(s) from Q₁. Subsequent fragments are passed through to Q₃ where they may be filtered or fully scanned. This process allows for the study of fragments that are useful in structural elucidation by MS/MS (Glish et al., 1982).

To overcome sample preparation losses and matrix effects, MS methods for BPA include the addition of an I.S, normally an isotopic analogue of BPA with a specific mass spectrum, being possible its identification. The most used I.S. have been 4-nonylphenol (when alkylphenols were also determined), deuterated BPAD₁₆ and isotope labelled ^13C₁₂BPA. Independently of the type of analyzer and ionization source, the most abundant ion in the BPA mass spectrum, and therefore used for quantitation purposes, is [M-H]⁻ m/z 227. In LC-MS/MS, [M-H-CH₃]⁻ m/z 212 was the most prominent product ion obtained being used for confirmation and/or quantitation of BPA. Other fragments were reported, like the ion [M−H−C₆H₅OH]⁻ m/z 133, resulting from the cleavage of the hydroxybenzyl group, and the ion [M−H−C₉H₁₀O]⁻ m/z 93, formed by the loss of hydroxyphenyl propyl.

TBBPA is also analysed mostly by LC-MS, since it needs no derivatization (Morris et al., 2004; Harrad et al., 2009; Covaci et al., 2009) whereas this step is necessary for its determination by GC. This is an advantage since derivatization has been reported to produce errors or analyte losses (Boer, 2006). Zhou et al. (2010) presented an LC method for the analysis of TBBPA together with several other BFRs. For TBBPA, both LC and Ultra Performance LC (UPLC) have been applied, using reversed phase columns (mostly C18) for the analysis of TBBPA (EFSA, 2011). Chu et al. found that the efficiency of the LC-MS
for TBBPA is dependent on the mobile phase used. They observe a 30% increase in response when replacing MeCN with MeOH in the mobile phase, which resulted in a lower LOQ (0.05 ng/g). The addition of 1mM ammonium acetate to the mobile phase, which may enhance the ionization, also increase the LC-MS response (Chu et al., 2005). According to Tollback et al., for TBBPA ionization ESI gave 30-40 times lower LODs compared to APCI, a feature also observed in the analysis of BPA. In addition, it permits monitoring of the intact TBBPA molecule through the soft ionization of ESI resulting in improved method selectivity and accuracy (Tollback et al., 2006). This finding agrees with results of Morris et al. (Morris et al., 2004).

Another advantage of the LC-MS/MS determination of TBBPA is that it enables the use of the $^{13}$C-labelled TBBPA as an I.S., which compensate any matrix-related effects or losses during extraction and clean-up that can affect analyte ion intensity.

The molecular ion $[M-H]^{-}$ $m/z$ 543 has an isotopic distribution in accordance with the presence of the four bromine atoms on the ion. Two daughter ions at $m/z$ 528 and $m/z$ 448 correspond to the loss of one methyl group $[M-\text{CH}_3]^{-}$, and the subsequent loss of one bromine $[M-\text{CH}_2-\text{Br}]^{-}$ (Figure 13).

![Figure 13](https://example.com/f13.png)

**Figure 13** – ESI fusi scan mass spectrum of tetrabromobisphenol acquired in negative ion mode. Adapted from: Saint-Louis, R. and Pelletier, E. (2004) "LC-ESI-MS-MS method for the analysis of tetrabromobisphenol A in sediment and sewage sludge" Analyst 129, 724-730
6.10.3. Gas Chromatography coupled to Mass Spectrometry (GC-MS)

GC-MS is frequently applied to quantify BPA in environmental samples. Determination of BPA by GC-MS requires derivatization since this chemical has insufficient volatility for direct analysis by GC and shows poor chromatographic properties. Therefore, time consuming sample preparation protocols using different extraction and preconcentration procedures are required (Dekant and Völkel, 2008). Despite the disadvantage of derivatization, GC-MS presents better separation than LC-MS, which is an advantage that can not fail to be considered.

The most common derivatization processes to BPA are cyanometilation where samples are dissolved in acetone, bromoacetonitrile (BAN) and K$_2$CO$_3$ and the solution is heated at 60°C for 60 min (Shin et al., 2001); trimethylsilylation using N-O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) (Cobellis et al., 2009) and heating of the samples mixed at 80°C for 30 min; or acetylation with AA or trifluoroacetic anhydride, not involving temperature or time. It was proved that this derivative of BPA was more sensitive than the corresponding trimethylsilyl derivative, which was a consequence of the higher molecular mass of the former (Ballesteros-Gómez et al., 2009). The base peak in the EI spectrum correspond to the fragment ion [M−15]$^+$ (m/z 405) formed from the ion molecular (m/z 420) by the loss of a methyl group (Figure 15).
The study of Cunha et al. (2012) determined BPA concentrations in canned seafood using GC-MS for the quantification and confirmation of this compound. The LOD was 0.02 ng/g. BPA was detected in 85% of the analysed samples at concentration ranging between 1 and 99 ng/g. In this study, AA was added to derivatize BPA in the samples (Cunha et al., 2012).

The column dimensions for BPA in GC-MS are typically 30 m length, 0.25-0.32 mm diameter and 0.25-0.5 μm film thickness (Li and Park, 2001; Mead and Seaton, 2011; Markham et al., 2011; Cunha et al., 2012).

A GC-MS method for TBBPA detection requiring derivatization with methylchloroformate (MCF) was developed by Berger et al. in 2004. After evaporation of the subsamples to dryness under a gentle stream of nitrogen, a 150 μL aliquot of the derivatization solvent MeCN/MeOH/water/pyridine (5:2:2:1; v/v/v/v) was added to the residues, and the mixture was placed into an ultrasonic bath for 10 min. The resulting suspension was filtered and a 10 μL aliquot of MCF was added, and the reaction mixture was allowed to stand for 5 min before it was diluted with 300 μL of water. However, this method suffered from a rather restricted linear range and low recoveries due to incomplete derivatization. Another derivatization method was employed with the use of diazomethane to obtain the TBBPA dimethyl ether derivative (Jakobsson et al., 2002). Although the chromatography of TBBPA on a GC column can be improved by derivatization of the hydroxyl groups (Covaci et al., 2009), GC analysis can also be performed without it (Korytár et al., 2005).

The column dimensions are typically 15-30 m length, 0.25 mm diameter and 0.1-0.25 μm film thickness (Gauthier et al., 2009; Shi et al., 2009; Cariou et al., 2005; Korytár et al., 2005) and with an apolar or slightly polar stationary phase. In GC-MS, electron chemical negative ionization (ECNI) can be used for ionization of TBBPA, monitoring the bromine

isotopes m/z 79 and 81. Comparing to EI ionization, this method is more sensitive, although less selective (Covaci et al., 2009).

6.10.4. Immunochemical Techniques

Other analytical method is the application of immunochemical techniques to the determination of BPA in food. As a small molecule, BPA is not able to initiate an immune response itself and needs to be conjugated with a protein to form a complete antigen. Recently, Enzyme-Linked Immunosorbent Assay (ELISA) methods based on monoclonal (Goda et al., 2000; Nishi et al., 2003) and polyclonal (Kim et al., 2007) mammalian antibodies and chicken immunoglobulins (De Meulenaer et al., 2002) were developed for the determination of BPA. The LOD ranged from 0.1 to 200 ng/mL, depending on the immunogen and the type of antibody.

More recently, Moreno et al. (2011) produce monoclonal antibodies to BPA, conjugating four synthetic compounds (BPA derivatives) to the protein bovine serum albumin (BSA) to avoid the loss of part of the structural characteristics of BPA and used as immunizing haptons in mice. The LOD of the most sensitive ELISA was 0.05 ng/mL. With regard to recovery, the analytical data obtained were also acceptable. The authors proved the potential of this immunoassay as a new tool for the rapid, sensitive and accurate determination of BPA in canned food (Moreno et al., 2011).

![Chemical structures of the haptons of BPA used to develop the immunoassay. BPAA: 2-[4-[(1-[4-hydroxyphenyl]-1-methylethyl) phenoxyl]acetic acid; BPAB: 4-[4-[(1-[4-hydroxyphenyl]-1-methylethyl) phenoxyl]butyric acid; BPAH: 6-[4-[(1-[4-hydroxyphenyl]-1-methylethyl) phenoxy]hexanoic acid; BPVA: 4,4'-Bis[4-hydroxyphenyl]-valeric acid. Adapted from: Moreno, M., D’Arienzo, P., Manclús, J. and Montoya, A. (2011) “Development of monoclonal antibody-based immunoassays for the analysis of bisphenol A in canned vegetables.” Journal of Environmental Science and Health 46, 509-517](image-url)
Cross-reactivity is one of the possible problems with the ELISA method, which cannot distinguish between free BPA and conjugated BPA, as both can generate responses with the kit. The results obtained must be compared with those obtained with well-established methods at different levels for accuracy. It is thus logical to predict that ELISA methods are unlikely to be applied widely for the determination of BPA in food and biological samples, even for qualitative screening purposes. ELISA can be a good fast screening method for BPA, but, again, only for samples with a simple matrix such as water (Kuruto-Niwa et al., 2007).

Up to now, the determinations have been focused on the analysis of liquid foods, mainly milk, water and food stimulants. LODs ranged from 0.05 ng/ml to 500 ng/ml (Ballesteros-Gómez et al., 2009).

TBBPA immunoassays have been developed and improved in the last years. The first ELISA method for TBBPA detection was developed by Xu et al. in 2012. In this study, they aim to create a new methodology for TBBPA detection in soil and sediments of an e-waste recycling area, comparing the results obtained with those obtained with LC-MS/MS. Cross-reactivity values of the ELISA with a set of important BFRs analogues to TBBPA were negligible (<0.05%). An antiserum was produced using the immunogen of which the hapten has a propanoic acid linker via an hydroxyl at the terminal position of TBBPA. A heterologous coating hapten having an acetic acid spacer attached to the same position resulted in the highest assay sensitivity. When compared, the average concentrations of TBBPA obtained by ELISA were slightly higher than those by LC–MS/MS, but not statistically significant according to a paired t-test (p > 0.05). The authors consider that this divergence may be due to the matrix effects or cross-reactivity of unknown compounds in the extracts by ELISA method (Xu et al., 2012).

In 2014, Bu et al. created a modified indirect competitive ELISA for TBBPA using a biotin–streptavidin amplification system. This system improve sensitivity because of the potential for amplification due to multiple site binding (Figure 17). Specific antibodies were produced and the proposed biotin–streptavidin-amplified ELISA (BA-ELISA) was sensitive and effective for the rapid detection of TBBPA in electronic waste samples. This proposed method also had negligible cross-reactions with structural TBBPA analogues (Bu et al., 2014).
In another recent study (Wang et al., 2015), a highly selective anti-TBBPA VHH T3-15 fused with alkaline phosphatase (AP) from *E. coli* showing both an integrated TBBPA-binding capacity and enzymatic activity was expressed (Figure 18). VHH is a variable domain of the heavy chain antibody naturally occurring in camelids and it approaches the lower size limit of functional antigen-binding entities. Wang et al. (2015) developed a one-step immunoassay for TBBPA based on the fusion protein T3-15-AP. Compared to the parental VHH T3-15, T3-15-AP was able to bind to a wider variety of coating antigens and the assay sensitivity was slightly improved. Cross-reactivity with a set of brominated analogues was negligible (<0.1%). The recoveries of TBBPA from urine samples via this immunoassay ranged from 96.7% to 109.9% and correlated well with a LC-MS/MS method.
7. **BPA and TBBPA occurrence in seafood**

The combination of population growth, rising incomes and increasing urbanization as well as economic development and advances in the availability and quality of food normally accompanied improvements in the supply chain of food that is, in production, processing and marketing. To protect the contents from the environment, avoiding the goods’ damage during transportation, for example, the first plastic packages appear at the end of World War II. Nowadays, packages are developed to promote food security from climatic conditions, such as temperature, humidity, precipitation and solar radiation.

However, there is a potential contamination risk when the product is in direct contact with the packaging, providing the conditions for migration of undesirable compounds. As already discussed above, both the plastics PC with BPA involving fish as TBBPA, a possible constituent of the epoxy resins that coat the inside of cans, can migrate into food contained therein. It is thus important to access the levels found in this seafood in order to evaluate if this event is contaminating the commercial food available for human consumption and as a consequence putting into risk human health.

7.1. **BPA**

As outlined above, BPA, as a main component of some polymeric plastics and as epoxy resin used as protective coating is one of the pollutants that could leach from the canned containers into food and beverages, when in conditions prone to such.

Table 2 shows data from several studies from 2010, 2011 and 2012 where the BPA levels in some canned seafood as well as seafood captures from estuaries and seas where measured. BPA was found in more than 56 % of the samples (total 228) with levels ranging from non-detected to 169.3 ng/g.
Table 2 – BPA levels (ng/g) found in canned seafood from different studies since 2010. *average; nd: non-detected

<table>
<thead>
<tr>
<th>Year</th>
<th>Sample</th>
<th>Country</th>
<th>Sample preparation</th>
<th>Method</th>
<th>Sampling, positive samples</th>
<th>BPA (ng/g) range; average</th>
</tr>
</thead>
</table>
| 2010 | Tuna   | US (Shecter et al., 2010) | • Extraction with MeCN in an ultrasonic bath  
• LLE (hexane)  
• Purification on ENVI-Carb column (hexane)  
• Derivatization with BSTFA  
• Purification on silica column | GC-MS | n=3, 3 BPA* | 1.66-4.16; 2.91 |
|      | Tuna in oil | Belgium (Geens et al., 2010) | • Extraction with MeCN by mixing and sonication  
• Wash of lipophilic impurities with hexane  
• Derivatization with pentafluorobenzoylchloride (PFBCl)  
• Purification on acidified silica | n=3, 3 BPA* | 169.3* |
|      | Tuna in water |  |  |  | 126.4* |
|      | Salmon | Canada (Cao et al., 2010) | • Extraction with MeCN  
• Dilution with pH 7.0 phosphate buffer solution  
• Purification through the C18 SPE cartridge (50% MeCN/water elution)  
• Derivatization with AA in a K₂CO₃ solution  
• Extraction with isooctane followed by methyl t-butyl ether (MTBE) | n=154, 55 BPA* | 3.4* |
|      | Fish |  |  |  | 106* |
| 2011 | Tuna   | US (Noonan et al., 2011) | • Extraction with MeCN  
• Dilution 1:2 with water | HPLC-MS/MS | n=4, 4 BPA* | 5.8-17; 11.4 |
|      | Tuna in oil |  |  |  | n=2, 2 BPA* | 4.5* |
|      | Mackerel |  |  |  | |  |
|      | Tuna   | Iran (Rastkari et al., 2011) | • Addition of an MeCN:H₂O (90:10, v/v) solution  
• Addition of KHCO₃, AA and NaCl  
• Extraction with SPME fused-silica fiber and exposure for 40min  
• In situ derivatization and extraction to the fiber | GC-MS | n=3, 3 BPA* | 4.5-17; 10.75 |
Theoretical Part

Chapter I

2012

<table>
<thead>
<tr>
<th>Product</th>
<th>BPA Detected</th>
<th>GC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuna</td>
<td>n=12, 12 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>32.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tuna in vegetable oil</td>
<td>n=13, 13 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>21.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tuna in olive oil</td>
<td>n=7, 7 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>5.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tuna in tomato sauce</td>
<td>n=3, 3 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>27.6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anchovy fillets in vegetable oil</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>Codfish in vegetable oil and garlic</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>Eels in escabeche sauce</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>9.9</td>
</tr>
<tr>
<td>Mackerel fillets in vegetable oil</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>40.4</td>
</tr>
<tr>
<td>Mackerel fillets in tomato</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>33.5</td>
</tr>
<tr>
<td>Mackerel fillets in vegetable oil</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1.4</td>
</tr>
<tr>
<td>Mussels in pickled sauce</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>49.2</td>
</tr>
<tr>
<td>Mussels in escabeche sauce</td>
<td>n=2, 2 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>30.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octopus in garlic</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>39.9</td>
</tr>
<tr>
<td>Octopus stew</td>
<td>n=5, 5 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.7&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sardines in vegetable oil</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.5</td>
</tr>
<tr>
<td>Sardines in spicy vegetable oil</td>
<td>n=3, 3 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>5.9&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Squid suffed</td>
<td>n=1, 1 BPA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>33.2</td>
</tr>
</tbody>
</table>

- Additive of n-heptane and deionized water
- Addition of MeCN, anhydrous MgSO4 and NaCl
- Purification of MeCN extract with MgSO4, C18 and GCB
- DLLME procedure:
  - Addition of 5% k2co3 solution until pH≥10 to MeCN extract
  - Addition of the extractive solvent tetrachloroethylene
  - Derivatization with aa

Portugal (Cunha et al., 2012)
When assessing commercial seafood samples contamination with BPA, the results are speckled. In the US, the values found for BPA are very low either in the study of Sherter et al. (2010) or in the study of Noonan et al. (2011), ranging from the minimum of 1.66 ng/g in a tuna sample in the first study to the maximum of 22 ng/g found in a commercial mackerel sample from US by the second research group. Noonan et al. found similar similar BPA levels in the tuna and the albacore samples with an average of 11.4 and 11.5 ng/g respectively and a minimum concentration in tuna in oil (4.5 ng BPA per gram of canned tuna in oil). Both studies results report very low levels of BPA in canned food found in US markets. There is no migratory limits in the USA at present.

Similar levels were detected by Rastkari et al (2011) in three samples of tuna from Iran ranging from 4.5 to 17 ng/g of BPA. At the moment, there is no specific legislation in Iran about migratory limits of BPA. However, the scientists regulate their evaluation by the European legislation. Since levels measured were below the EU migratory limits for BPA in food (600 ng/g), there is no restriction to the use of these foods which are considered safety. Contrarily, two tuna samples from Belgium (Geens et al., 2010) presented levels about ten-fold higher than the above cited, of 169.3 and 126.4 ng/g in tuna in oil and in water respectively, still below the EU migratory limits.

Cao et al. (2010) also found a similar level - 106 ng/g - in a fish sample from Canada markets. Health Canada conducted several risk assessments on BPA, and again in 2012 reconfirmed that consumer exposure to BPA is "very low" and that BPA is "not expected to pose a health risk to the general population." Nevertheless, the use of BPA in baby bottles is restricted in Canada since March 2010.

The study of Cunha et al. (2012) in Portugal allowed the determination of BPA levels in many types of canned fish and other sea products (octopus and clams) preserved with different sauces. Detected levels were generally low being the highest values found 49.2 ng/g in mussels, 39.9 ng/g in mackerel, 32.6 ng/g in tuna, and 33.2 ng/g in a squid sample. This study showed that the different sauces in which the fish may be involved in packaging did not significantly interfere with the migration of BPA. Levels found did not shown also a good correlation with the type of fish, e.g. two mussel samples showed levels of 49.2 and 1.4 ng/g.
7.2. **TBBPA**

Being a BFR, TBBPA can be leached to the environment as discussed before. Despite only 20-30% of the total volume produced was used as an additive flame retardant on material subject to environmental leaching (ECB, 2006), several studies were conducted to access the levels of this chemical in fishes from oceans and rivers around the world.

Since few studies have been made on this BFR, Table 3 aggregates studies since 2004 to 2013 where the TBBPA levels in some fishes samples were measured. TBBPA was found in more than 56% of the samples (total 214) with levels ranging from non-detected to 418 ng/g.
### Table 3 – TBBPA levels (ng/g) found in river and sea fish samples from different studies since 2004. *average; nd: non-detected

<table>
<thead>
<tr>
<th>Year</th>
<th>Sample</th>
<th>Country</th>
<th>Sample preparation</th>
<th>Method</th>
<th>Sampling, positive samples</th>
<th>TBBPA (ng/g) range; average</th>
</tr>
</thead>
</table>
| 2004 | Hermit crab           | UK (Morris et al., 2004)        | • SPE with Soxhlet or homogenization by Ultra Turrax using binary solvent mixtures  (1:1 or 1:3 (v/v) acetone n-hexane mixture)  
• Concentration with sulfuric acid to degrade lipid material  
• Gel permeation chromatography system  
• Elution with DCM  
• Reconstitution in isooctane  
• Fractionation with silica gel column chromatography  
• Evaporation to dryness | LC-MS     | n=9, - TBBPA+             | <1-35; 17                           |
|      | Whiting               |                                 |                                     |           | n=3, - TBBPA+              | <97-245; 136            |
|      | Cod                   |                                 |                                     |           | n=2, - TBBPA+              | <0.3-1.8; 1.05           |
|      | Hake                  |                                 |                                     |           |                            |                            |
|      | Eel                   |                                 |                                     |           |                            |                            |
|      | Cormorant             |                                 |                                     |           |                            |                            |
|      | Common tern           |                                 |                                     |           |                            |                            |
|      | Harbor seal           |                                 |                                     |           |                            |                            |
|      | Harbor porpoise       |                                 |                                     |           |                            |                            |
| 2008 | Bottlenose dolphin   | Florida (Johnson-Restrepo et al., 2008) | • Soxhlet extraction with DCM/hexane (3:1, v/v) for 16h  
• Concentration and purification by gel permeation chromatography column  
• Filtration through a nylon siringe filter 0.22 and evaporation to dryness | LC-MS/MS  | n=15, 15 TBBPA+            | 0.06-8.48; 3.27          |
|      | Bull shark            |                                 |                                     |           | n=13, 13 TBBPA+            | 0.04-35.6; 17.82         |
|      | Atlantic sharpnose    |                                 |                                     |           |                            |                            |
|      | shark                 |                                 |                                     |           |                            |                            |
| 2009 | Rainbow trout         | UK (Harrad et al., 2009)        | • Pressurized liquid extraction (PLE) hexane/DCM (1:9, v/v) at 90ºc and 1500psi  
• Heating during 5min, static time 4min, purge time 90s  
• Purification in SPE cartridges containing 8g of acidified silica (44% sulfuric acid with 2 g Cu powder)  
• Elution in hexane/DCM (1:1, v/v)  
• Evaporation to dryness | LC- MS/MS  | n=1, - TBBPA+             | nd          |
<p>|      | European perch        |                                 |                                     |           | n=6, 2 TBBPA+              | nd-1.3; 6.5             |
|      | Carassius             |                                 |                                     |           |                            |                            |
|      | Common roach          |                                 |                                     |           |                            |                            |
|      | Common carp           |                                 |                                     |           |                            |                            |
|      | Common rud            |                                 |                                     |           |                            |                            |
|      | Bream                 |                                 |                                     |           |                            |                            |
|      | Tench                 |                                 |                                     |           |                            |                            |
|      | Northern pike         |                                 |                                     |           |                            |                            |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Year</th>
<th>Country/Region</th>
<th>Extraction Method</th>
<th>Purification/Lipid Removal Method</th>
<th>Concentration Steps</th>
<th>Analysis Method</th>
<th>TBBPA+</th>
<th>nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common carp</td>
<td>2009</td>
<td>China (Shi et al., 2009)</td>
<td>Soxhlet extraction with n-hexane/acetone (1:1, v/v) for 48h</td>
<td>- Extracts concentration to 1-2mL</td>
<td>- Purification on a silica/aluminia column</td>
<td>GC-MS</td>
<td>n=1</td>
<td>- TBBPA+</td>
</tr>
<tr>
<td>Bighead carp</td>
<td></td>
<td></td>
<td></td>
<td>- Gel permeation chromatography to remove lipids</td>
<td></td>
<td>n=1, - TBBPA+</td>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>Tilapia</td>
<td></td>
<td></td>
<td></td>
<td>- Evaporation to 100µl</td>
<td></td>
<td>n=1, - TBBPA+</td>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>Species from Vltava</td>
<td>2013</td>
<td>Czech Republic</td>
<td>Extraction with water/MeCN (2:3, v/v)</td>
<td>- Addition of anhydrous MgSO4 and NaCl</td>
<td>- Centrifugation for 5min at 11000 rpm</td>
<td>HPLC-MS/MS</td>
<td>n=5</td>
<td>- TBBPA+</td>
</tr>
<tr>
<td>Species from Labe</td>
<td></td>
<td></td>
<td></td>
<td>- Addition of C18 and MgSO4 to the extract</td>
<td>- Centrifugation for 5min at 11000 rpm</td>
<td>n=1, 1 TBBPA+</td>
<td>0.20*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Concentration of the purified extract with evaporation at 40°C</td>
<td>n=5, 5 TBBPA+</td>
<td>0.03*</td>
<td></td>
</tr>
<tr>
<td>Species from Biilina</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Reconstituation in MeOH and filtration through 0.2 µm filter</td>
<td>n=2, 2 TBBPA+</td>
<td>2.16*</td>
<td></td>
</tr>
<tr>
<td>Species from Lusatian Neisse</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>n=3, 3 TBBPA+</td>
<td>0.11*</td>
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<tr>
<td>Species from Dyje</td>
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<td></td>
<td></td>
<td></td>
<td>n=2, - TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species from Morava</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=3, - TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud carp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=4, - TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=2, - TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plecostomus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=5, - TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species from Morava</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=2, 2 TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud carp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=1, 1 TBBPA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=9, 9 TBBPA+</td>
<td>6.5-66; 35.2</td>
<td></td>
</tr>
<tr>
<td>Plecostomus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=15, - TBBPA+</td>
<td>nd-51; 18.1</td>
<td></td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=10, - TBBPA+</td>
<td>nd-53.4; 21.2</td>
<td></td>
</tr>
</tbody>
</table>

* Denotes significant differences compared to controls.
When evaluating the levels of TBBPA found in fish from different sources, whether rivers or oceans, it is possible to observe levels below those found in the case of BPA, with the detected maximum level of 418 ng/g in harbor porpoise observed in Morris et al. study (Morris et al., 2004). In this study, fish samples were collected from different regions: the ermit crab, whiting, cod and harbor porpoise are from the North Sea; the hake from the Atlantic (South Ireland); one of the eels from Belgium and the other from rivers in Netherlands; the cormorant from England; the common tern from the Western Scheldt; and the harbor seal from the Western Wadden Sea (Morris et al., 2004). Despite the different origins, all the levels found were low, highlighting the harbor porpoise from the North Sea, with an average concentration of 209.05 ng/g. However, in the nine samples of harbor porpoise, the levels were quite disperse, ranging from 0.1 to 418 ng/g and a standard deviation of 187 ng/g (Morris et al., 2004).

Also in England, Harrad and his colleagues studied several fish samples from English lakes. In this study, only four of the thirty three samples reveal the presence of TBBPA although in minor quantities with a maximum observed of 1.7 ng TBBPA per g of carassius fish. Even in this species, seven of the eight individuals analysed did not show TBBPA in a quantifying level (Harrad et al., 2009).

In the USA, Johnson-Restrepo et al. found TBBPA in all of the thirty one fish samples captured from coastal and estuarine waters between 1991 and 2004 they have analysed. The levels of this BFR were generally low, with a maximum observed of 35.6 ng TBBPA per g of bull shark from the East coast of Florida (Johnson-Restrepo et al., 2009). However, the values found for this shark specie were quite disperse, since it ranged from 0.035 to 35.6 ng/g.

Despite the samples analysed in the study of Shi et al. have been collected from a e-waste area (extent of the country where are discarded electrical or electronic devices), the farmed fish samples analysed showed non detectable TBBPA traces. In this study, the authors also compared the presence of TBBPA with other BFRs in this species and between fishes and birds and conclude that “Plausible explanations for the different BFR pattern between bird and fish are the difference in their dietary habits or bioaccumulation tendencies.” (Shi et al., 2009).

TBBPA was determined in 23% of scanned samples and its concentrations were relatively low ranging from 0.14 to 4.43 ng/g. Labe River was acknowledged as the most contaminated locality by TBBPA with the mean concentration of 2.16 ng/g. In this area, the fishes were collected downstream from the chemical factory. In the Dyje River and the Morava River, mean concentrations of 0.98 and 0.79 ng/g were measured,
respectively. The locations bathed by these two rivers spill their sewage treatment plants waste to the river, where the fish samples were collected. In other monitored localities, only background concentrations of TBBPA in the range of non-detected to 0.39 ng/g were determined (Hlouskova et al., 2013).

The authors consider that the low amounts of TBBPA found in aquatic biota can be explained by the fact that “TBBPA emissions are probably low compared to other BFRs, since this chemical belongs to the group of reactive BFRs which are chemically bound into polymeric matrix” or because “TBBPA has a lower bioaccumulation potential compared to other BFRs as PBDEs” and also due to the elimination of TBBPA from the organisms (Hlouskova et al., 2013).

In the study of He et al. (2013), TBBPA was detected in thirty one out of thirty four fish samples. There are no significant differences in TBBPA level between the three fish species although the mud carp exhibits relatively higher mean value (35.2 ng/g) than the nile tilapia with 18.1 ng/g and the plecostomus with 21.2 ng/g. This levels were significant higher than those found in fish collected from the UK lakes where TBBPA was only detected in 13% of the samples at very low level (<2 ng/g) (Harrad et al., 2009). The TBBPA levels (<0.1 ng/g) in fish collected from Netherlands rivers were lower than the present study by 100 orders of magnitude (Morris et al., 2004). The TBBPA levels in this study were even higher than those in high trophic level organism such as marine predators (9.5 ng/g) from Florida (Johnson-Restrepo et al., 2008).
8. **BPA and TBBPA occurrence in seaweed**

To the best of our knowledge, there are only two studies reporting levels of BPA in algae. Gattullo *et al.* in 2012, studied the ability of the green algae *Monoraphidium braunii* to remove BPA in different concentrations from the environment where they were grown in the presence and absence of natural organic matter, since it may interfere with xenobiotics and modify their effects, modulate algal growth performances or produce a trade-off of both effects (Gattullo *et al.*, 2012). The determination of BPA in algal culture is synthetized in Figure 19.

![Flowchart](image)


At the highest concentration, BPA reduced the algal growth and photosynthetic efficiency. After 4-day growth, good removal efficiency was exerted by *M. braunii* at
concentrations of 2, 4 and 10 mg/l removing, respectively, 39%, 48% and 35% of the initial BPA. The natural organic matter added to the media hardly influenced this ED removal. In view of these results, the authors consider that *M. braunii* could be recommended for the phytoremediation of aquatic environments from BPA (Gattullo *et al*., 2012).

The study of Yang *et al.* in 2014 aims to understand the distribution and bioconcentration of EDCs in water, algae, and wild carp bile of the Pearl River Delta in South China. The method used for extraction, derivatization and chromatographic analysis of BPA in algae samples is synthetized in Figure 20.

**Figure 20** – Determination of BPA in algae samples collected from the Pearl River Delta, South China, and GC-MS detection. Yang, J., Li, H., Ran, Y. and Chan, K. (2014) "Distribution and bioconcentration of endocrine disrupting chemicals in surface water and fish bile of the Pearl River Delta, South China." *Chemosphere* 107, 439-446
In the algae studied, BPA was found in concentrations ranging from 16 to 94 ng/L. The presence of 4-tert octylphenol (OP), and 4-nonylphenol (NP) was also investigated. The authors considered that “phenolic EDCs can be accumulated by wild carp bile and algae in the investigated aquatic ecosystems, which is also affected by the degree of the eutrophication” (Yang et al., 2014).
II. Experimental Part
1. **Chemicals and reagents**

Bisphenol A (BPA; 99 % purity) and tetrabromobisphenol A (TBBPA; 99 % purity) were purchased from Sigma-Aldrich (West Chester, PA, USA). Tetrabromobisphenol A ring-\(^{13}\text{C}_{12}\) (TBBPA\(^{13}\text{C}_{12}\); 99 % purity) and d16-bisphenol A (BPAd\(_{16}\); 98 atom % D) used as I.S. were purchased from Sigma-Aldrich and Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA) respectively. Bisphenol B (BPB; >98 % purity) used as I.S. in alternative to BPAd\(_{16}\) for quantification of BPA in seaweeds was also purchased from Sigma-Aldrich.

QuEChERS solvents: acetonitrile (MeCN, gradient grade for HPLC; 78.6% purity) and anhydrous magnesium sulfate (anhydrous MgSO\(_4\); 99.5% purity) were purchased from Sigma-Aldrich; sodium chloride (NaCl; 99.5% purity) and ammonium acetate (97% purity) were purchased from AppliChem Panreac ITW Companies (Barcelona, Spain). To ensure efficient removal of phthalates and residual water, anhydrous MgSO\(_4\) was treated for 5 h at 500 °C in a muffle furnace. LLE solvents: \(n\)-hexane (gradient grade for HPLC), MTBE (pro-analysis) and benzene (pro-analysis) were purchased from MERCK (Darmstadt, Germany).

Ultra-pure Milli-Q water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA) and MeOH (MeOH, for HPLC LC-MS grade) was purchased from VWR (Radnor, PA, USA).

2. **Standards and quality control materials**

Individual stock solutions of BPA (200 mg/L) and TBBPA (200 mg/L) were prepared in MeOH. Standard working solutions containing both BPA and TBBPA in concentration of 1000 ng/mL were prepared in 10% ultra-pure MilliQ water with 5mM ammonium acetate and 90% MeOH. Individual working solutions of BPAd\(_{16}\) and TBBPA\(^{13}\text{C}_{12}\) (1000 µg/L) were also prepared in 10% ultra-pure MilliQ water with 5mM ammonium acetate and 90% MeOH. All the solutions were stored at −28 °C when not in use.

Matrix-matched calibration curves were achieved by analyzing blank samples (free of both BPA and TBBPA) spiked with known amounts of the analytes. Analytes concentration in the analyzed samples was obtained by the I.S. method.
3. Sampling

3.1. Seafood

The ECsafeSEAFOOD project consortium comprises 18 institutions from nine European member states (Belgium, Denmark, France, Ireland, Italy, the Netherlands, Portugal, Slovenia and Spain) and one associated country (Norway). Led by the Portuguese Institute of Sea and Atmosphere (IPMA) with the contribution of twelve partners, this multidisciplinary project intends to access environmental contamination and respective impact on public health by determination of some priority contaminants e.g., HBCDD, PBDEs, HBB, TBBPA, BPA, triclosan, methylparaben, nonylphenol, TBEP, inorganic arsenic, methyl mercury, microplastics, pharmaceuticals (like diclofenac, sulfamethoxazole, sotalol, diazepam, carbamazepine, and venlafaxine) PAHs, and UV-filters, in seafood collected in different European regions between 2014 and 2015 as well as the effects of industrial and home preparation on contaminant content.

The seafood samples were obtained in two periods of the year (Round I and Round II) and the target species and respective information are summarized in Table 4 (species from Round I) and Table 5 (species from Round II). The number of species was selected according to the chances of success for detection and identification of selected priority contaminants. The criteria used for selecting target species were the following: a) most common species consumed in the studied area; b) potential to accumulate high concentrations of chemicals; c) wide geographic distribution; d) easy identification; e) abundance; f) easy to capture; g) large enough to provide adequate tissue for analysis; h) from different geographical origins; i) from different habitats; j) from extra-EU origin or from EU production; and k) from wild or farmed origin. The tissues collected from mussels and macroalgae were all edible content, from seafood and shrimp the muscle was collected and from brown carb the brown meat.

Briefly, in each round for each species and location a minimum of twenty-five specimens (at least 800 g) were sampled. Each specimen, of each species, was divided in three portions (except for bivalves) namely raw, cooked and save (freeze). To obtain the cooked samples, the seafood samples were steamed for 15 min at 105ºC after adding salt (2% fillet weight) and then cooled in room temperature. Both the pooled samples performed of raw or cooked species were homogenized, placed in weighted recipients, freeze-dried and homogenised again, before being sent to the laboratories.
Table 4 - Seafood species from Round I (captured in April-June, 2014) for BPA and TBBPA analysis and their respective location.

<table>
<thead>
<tr>
<th>Code</th>
<th>Matrix</th>
<th>Location</th>
<th>Specie</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mackerel</td>
<td>Goro (IT)</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>2</td>
<td>Farmed seabream</td>
<td>Greece</td>
<td>Saprus aurata</td>
</tr>
<tr>
<td>3</td>
<td>Mussel</td>
<td>Goro (IT)</td>
<td>Mytilus galloprovincialis</td>
</tr>
<tr>
<td>4</td>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>5</td>
<td>Atlantic Cod</td>
<td>North Sea (DK)</td>
<td>Gadus morhua</td>
</tr>
<tr>
<td>6</td>
<td>Farmed salmon</td>
<td>Norway</td>
<td>Salmo solar</td>
</tr>
<tr>
<td>7</td>
<td>Mussel</td>
<td>Limfiord (DK)</td>
<td>Mytilus edulis</td>
</tr>
<tr>
<td>8</td>
<td>Canned mackerel</td>
<td>Portugal</td>
<td>Scomber sp.</td>
</tr>
<tr>
<td>9</td>
<td>Small monkfish</td>
<td>Atlantic coust (PT)</td>
<td>Lophius piscatorius</td>
</tr>
<tr>
<td>10</td>
<td>Large monkfish</td>
<td>Atlantic coust (PT)</td>
<td>Lophius piscatorius</td>
</tr>
<tr>
<td>11</td>
<td>Canned tuna</td>
<td>Portugal</td>
<td>Katsuwonus pelamis</td>
</tr>
<tr>
<td>12</td>
<td>Canned sardine</td>
<td>Portugal</td>
<td>Sardina pilchardus</td>
</tr>
<tr>
<td>13</td>
<td>Mackerel</td>
<td>Channel (FR)</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>14</td>
<td>Mussel</td>
<td>Channel (FR)</td>
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</tr>
<tr>
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<td>North Sea (DK)</td>
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</tr>
<tr>
<td>16</td>
<td>Farmed salmon</td>
<td>Scotland</td>
<td>Salmo solar</td>
</tr>
<tr>
<td>17</td>
<td>Mussel</td>
<td>Inshot (ND)</td>
<td>Mytilus edulis</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Origin</td>
<td>Genus</td>
</tr>
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<td>---</td>
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<td>-----------------</td>
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<tr>
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<td>Brown crab</td>
<td>North Sea (DK)</td>
<td>Cancer pagurus</td>
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<tr>
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<td>Pacific ocean</td>
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</tr>
<tr>
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<td>Tuna small</td>
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<td>Nile Perch</td>
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<td>Pangasius bocourti</td>
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<td>India</td>
<td>Litopenaeus vannamei</td>
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<td>25</td>
<td>Mackerel</td>
<td>Atlantic coast (ES)</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>26</td>
<td>Mussel</td>
<td>Mediterranean Sea</td>
<td>Mytilus galloprovincialis</td>
</tr>
</tbody>
</table>
Table 5 - Seafood species from Round II (captured in September-January, 2015) for BPA and TBBPA analysis and their respective capture location.

<table>
<thead>
<tr>
<th>Code</th>
<th>Matrix</th>
<th>Location</th>
<th>Specie</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 raw</td>
<td>Mackerel</td>
<td>Goro (IT)</td>
<td><em>Scomber scombrus</em></td>
</tr>
<tr>
<td>27 cooked</td>
<td>Mackerel, cooked</td>
<td>Goro (IT)</td>
<td><em>Scomber scombrus</em></td>
</tr>
<tr>
<td>28 raw</td>
<td>Farmed seabrem</td>
<td>Greece</td>
<td><em>Sparus aurata</em></td>
</tr>
<tr>
<td>28 cooked</td>
<td>Farmed seabrem, cooked</td>
<td>Greece</td>
<td><em>Sparus aurata</em></td>
</tr>
<tr>
<td>29 raw</td>
<td>Mussels</td>
<td>Goro (IT)</td>
<td><em>Mytilus galloprovincialis</em></td>
</tr>
<tr>
<td>29 cooked</td>
<td>Mussels, cooked</td>
<td>Goro (IT)</td>
<td><em>Mytilus galloprovincialis</em></td>
</tr>
<tr>
<td>30</td>
<td>Drip Loss</td>
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<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td><em>Scomber scombrus</em></td>
</tr>
<tr>
<td>32</td>
<td>Atlantic cod</td>
<td>North Sea (DK)</td>
<td><em>Gadus morhua</em></td>
</tr>
<tr>
<td>33</td>
<td>Farmed salmon</td>
<td>Norway</td>
<td><em>Salmo solar</em></td>
</tr>
<tr>
<td>34</td>
<td>Mussels</td>
<td>Limfiord (DK)</td>
<td><em>Mytilus edulis</em></td>
</tr>
<tr>
<td>35</td>
<td>Canned sardine</td>
<td>Portugal</td>
<td><em>Sardina pilchardus</em></td>
</tr>
<tr>
<td>36 raw</td>
<td>Small monkfish</td>
<td>Atlantic coast (PT)</td>
<td><em>Lophius piscatorius</em></td>
</tr>
<tr>
<td>36 cooked</td>
<td>Small monkfish, cooked</td>
<td>Atlantic coast (PT)</td>
<td><em>Lophius piscatorius</em></td>
</tr>
<tr>
<td>37 raw</td>
<td>Large monkfish</td>
<td>Portugal</td>
<td><em>Lophius piscatorius</em></td>
</tr>
<tr>
<td>37 cooked</td>
<td>Large monkfish, cooked</td>
<td>Portugal</td>
<td><em>Lophius piscatorius</em></td>
</tr>
<tr>
<td>38</td>
<td>Canned tuna</td>
<td>Portugal</td>
<td><em>Katsuwonus pelamis</em></td>
</tr>
<tr>
<td></td>
<td>Canned mackerel</td>
<td>Portugal</td>
<td>Scomber sp.</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 raw</td>
<td>Mussel</td>
<td>Channel (FR)</td>
<td>Mytilus edulis</td>
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<tr>
<td>40 cooked</td>
<td>Mussel, cooked</td>
<td>Channel (FR)</td>
<td>Mytilus edulis</td>
</tr>
<tr>
<td>41</td>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>42</td>
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<td>Inshot (ND)</td>
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</tr>
<tr>
<td>42 steamed</td>
<td>Mussel, steamed</td>
<td>Inshot (ND)</td>
<td>Mytilus edulis</td>
</tr>
<tr>
<td>43</td>
<td>Brown crab</td>
<td>North Sea (DK)</td>
<td>Cancer pagurus</td>
</tr>
<tr>
<td>43 steamed</td>
<td>Brown carb, steamed</td>
<td>North Sea (DK)</td>
<td>Cancer pagurus</td>
</tr>
<tr>
<td>44</td>
<td>Imported tuna small</td>
<td>-</td>
<td>Katsuwonus pelamis</td>
</tr>
<tr>
<td>45</td>
<td>Imported tuna large</td>
<td>-</td>
<td>Katsuwonus pelamis</td>
</tr>
<tr>
<td>46</td>
<td>Shrimp vannamei</td>
<td>India</td>
<td>Litopenaeus vannamei</td>
</tr>
<tr>
<td>47</td>
<td>Shrimp vannamei</td>
<td>India</td>
<td>Litopenaeus vannamei</td>
</tr>
<tr>
<td>48 raw</td>
<td>Mussel</td>
<td>Atlantic coast (ES)</td>
<td>Mytilus galloprovincialis</td>
</tr>
<tr>
<td>48 cooked</td>
<td>Mussel, cooked</td>
<td>Atlantic coast (ES)</td>
<td>Mytilus galloprovincialis</td>
</tr>
<tr>
<td>49 raw</td>
<td>Mackerel</td>
<td>Mediterranean Sea</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>49 cooked</td>
<td>Mackerel, cooked</td>
<td>Mediterranean Sea</td>
<td>Scomber scombrus</td>
</tr>
</tbody>
</table>
3.2. **Seaweed**

BPA and TBBPA were also studied in the wild seaweeds (*Saccharina latissima* and *Laminaria digitata*) species supplied from Hortimare. The samples were collected in the Netherlands (Texel) in August/September/October 2014 and transferred to tanks for acclimation at Hortimare facilities. After 14 days of cultivating in suitable conditions, the macroalgae samples were collected at different times (see Table 6), homogenized and freeze-dried for quantification of the target contaminants. One control, corresponding to macroalgae cultivated in water without any level of contaminants, was also analysed.

<table>
<thead>
<tr>
<th>Seaweed (<em>n</em>=1)</th>
<th><strong>Spiking concentration (µg/L)</strong></th>
<th><strong>Sampling time (hours)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (control I)</td>
<td>0</td>
</tr>
<tr>
<td><em>Saccharina latissima</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>0 (control I)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

4. **Sample preparation**

Once arrived at the laboratory, lyophilized samples had similar treatments, although given the specificity of the samples under study some adjustments were needed for each kind of matrix (fish, mussels and seaweeds).

The sample preparation is schematized in Figure 21, entailing the following steps: (1) weigh 2 g of thoroughly homogenized sample into a 40 mL glass vial tube; (2) add 80 µL of BPAd$_{16}$ at 1000 ng/mL (BPB at 1000 ng/mL for seaweeds) and 80 µL of TBBPA$_{13}$C$_{12}$ at 1000 ng/mL working solution (I.S.); (3) add 7 mL of ultra-pure MilliQ water and seal the tube, handshaking it for 2 min; (4) add 10 mL of MeCN, 100 µL of HCl 10M (pH=4), 4 g of anhydrous MgSO$_4$ and 1 g of NaCl; (7) seal the tube and shake vigorously by hand for 10 min; (8) centrifuge the tube at 2000 g for 5 min. Then, a LLE procedure was performed: (1) transfer 3 mL of the MeCN extract to a 15 mL glass vial tube with 7 mL of ultra-pure MilliQ water; (2) add 4 mL of *n*-hexane:MTBE (3:1, v/v) to the MeCN extract and handshak
vigorously; (3) transfer 3 mL of the upper layer to a new 15 mL vial; (4) add 4 mL of \(n\)-hexane:benzene (3:1, v/v) to the MeCN extract and hand shake vigorously; (5) transfer 3 mL of the upper layer to the 15 mL glass vial, with a total volume of 6 mL extracted. The final extracts were concentrated under a stream of nitrogen (SBH CONC/1 sample concentrator from Stuart® (Staffordshire, OSA, USA), reconstituted in mobile phase - 100 µL of 5mM ammonium acetate and 900 µL of MeOH - and transferred to a 2 mL glass vial before LC-MS/MS analysis.

For water or aqueous samples, the procedure was the same, accomplished with 2 g of the water/aqueous sample.

![Diagram of sample preparation](image)

**Figure 21** - Sample preparation QuEChERS and LLE procedures steps prior to LC-MS/MS analysis.
5. **LC-MS/MS equipment and conditions**

Separation and quantification of the target analytes were performed by using a liquid chromatograph Waters 2695 Separations Module interfaced to a triple quadrupole mass selective detector Micromass Quattro micro API™, both equipments purchased from Waters (Milford, MA, USA). The injection volume was set at 20 µL. The chromatographic separation was carried out with a Kinetex® Phenomenex® C18 column (2.6 µm, 100 mm x 4.60 mm (i.d.)) from Phenomenex (Torrance, CA, USA) at a flow rate of 0.20 mL/min. The column temperature was kept at 30ºC. The mobile phases were (A) 5mM ammonium acetate (pH=5) and (B) MeOH in an isocratic gradient (10% solvent A and 90% solvent B).

![Figure 22 - LC-MS/MS apparatus.](image_url)
III. Results and Discussion
1. Optimization of the analytical methodology to quantify BPA and TBBPA by LC-MS/MS

1.1. Optimization of chromatographic conditions

LC-MS/MS settings and ESI source parameters were initially optimized by manual infusion of each compound (BPA, TBBPA, BPAd16, TBBPA\(^{13}\)C\(_{12}\) and BPB) at 200 ng/mL, using a syringe pump.

Mass analysis was performed with an ESI source in the negative ion mode (ESI\(^-\)) for all the analytes because of its higher sensitivity compared with positive ion mode (ESI\(^+\)). Nitrogen was used as the nebuliser gas. The optimum MS parameters were: capillary, 3.00 kV; extractor, 2 V; RF Lens, 0.5 V; Source Temperature, 150ºC; Desolvation Temperature, 350ºC; Desolvation Gas Flow, 350.0 L/h; Cone Gas Flow, 60.0 L/h; LM Resolution, 13.0; Ion energy, 1.0; Entrance, 1; Exit, 2; Multiplier, 650. All analyses were performed in multiple reaction monitoring (MRM) mode. The optimized parameters are summarized in Table 7.

<table>
<thead>
<tr>
<th></th>
<th>BPA</th>
<th>BPAd16</th>
<th>TBBPA</th>
<th>TBBPA(^{13})C(_{12})</th>
<th>BPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>7.89</td>
<td>7.82</td>
<td>9.03</td>
<td>9.11</td>
<td>7.80</td>
</tr>
<tr>
<td>Precursor ion (Da)</td>
<td>227.26</td>
<td>241.15</td>
<td>542.87</td>
<td>554.92</td>
<td>242.33</td>
</tr>
<tr>
<td>Product ions (Da)</td>
<td>133.14</td>
<td>142.18</td>
<td>419.87</td>
<td>428.84</td>
<td>212.32</td>
</tr>
<tr>
<td>Cone energy (V)</td>
<td>40</td>
<td>40</td>
<td>35</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Collision energy (kV)</td>
<td>30</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td>Dwell time (ms)</td>
<td>0.3</td>
<td>0.05</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The optimal flow rate of the mobile phase was found to be 0.2 mL/min. Once this value was determined, two different transitions were selected (precursor-product ion) for each analyte, one for quantification (the ion quantifier) and another for confirmation (the ion qualifier).
For BPA, the 211.43 m/z ion was selected as the qualifier owing to the low signal-to-noise (S/N) ratio with high peak intensity (SIR transition 227.86 > 211.43). The 133.14 m/z ion was selected as the quantifier based on the higher S/N ratio (SIR transition 227.86 > 133.14) (Figure 23). BPAd_{16} was used as I.S. for BPA, with the 223.25 m/z ion selected as the qualifier (SIR transition 241.15 > 223.25) and the 142.18 m/z ion was selected as the quantifier (SIR transition 241.15 > 142.18) (Figure 25). BPB was also used as I.S: for BPA, with the 226.83 m/z ion selected as the qualifier (SIR transition 242.33 > 226.83) and the 212.32 m/z ion was selected as the quantifier (SIR transition 242.33 > 212.32) (Figure 27).

For TBBPA, the 446.00 m/z ion was selected as the qualifier owing to the low S/N with high peak intensity (SIR transition 542.87 > 446.00). The 419.87 m/z ion was selected as the quantifier based on the higher S/N ratio (SIR transition 542.87 > 419.87) (Figure 24). This two daughter ions correspond to the loss of one methyl group [M–CH$_3$]$^-$, and the subsequent loss of one bromine [M–CH$_2$–Br$^-$. TBBPA$^{13}$C$_{12}$ was used as I.S. for TBBPA, with the 457.92 m/z ion selected as the qualifier (SIR transition 554.92 > 457.92) and the 428.84 m/z ion was selected as the quantifier (SIR transition 554.92 > 428.84) (Figure 26). These ions were monitored using the MRM mode and this is summarized in Table 7. In both spectrums (TBBPA and TBBPA$^{13}$C$_{12}$) it can be seen (Figure 24 and 26) that the most predominant ions are the 79.0 and 81.0 m/z fragments, corresponding to the bromo ion which was expected since bromo natural abundance is 50% for 79.0 m/z and 50% for 81.0 m/z.
Figure 23 - Ion spectrum of BPA after direct infusion in the MS/MS system with collision at 30 kV. The product ions selected from BPA confirmation and quantification were 211.43 m/z and 133.14 m/z, respectively.

Figure 24 - Ion spectrum of TBBPA after direct infusion in the MS/MS system with collision at 40 kV. The product ions selected from TBBPA confirmation and quantification were 446.00 m/z and 419.87 m/z, respectively.
Figure 25 - Ion spectrum of BPA\textsubscript{16} after direct infusion in the MS/MS system with collision at 20 kV. The product ions selected from BPA confirmation and quantification were 223.25 m/z and 142.18 m/z, respectively.

Figure 26 - Ion spectrum of TBBPA\textsuperscript{13}C\textsubscript{12} after direct infusion in the MS/MS system with collision at 40 kV. The product ions selected for TBBPA\textsuperscript{13}C\textsubscript{12} confirmation and quantification were 457.92 m/z and 428.84 m/z, respectively.
The use of two stable isotope-labeled I.S. allowed a clear identification of the signals for both BPA and TBBPA. These fragmentation patterns were included in the acquisition method, and quantitation of BPA and TBBPA are reported relative to the I.S..

When preparing the gradient, the first attempt was an isocratic gradient of ultra-pure MilliQ water as solvent A and MeOH as solvent B. It was observed that the addition of ammonium acetate to ultra-pure MilliQ water improved sensitivity and peak shapes owing to the optimum ionization of the compounds. The best separation, peak shapes and ionization of the compounds were obtained with an isocratic gradient: 10% of a mixture of ammonium acetate aqueous solution at pH 5 as solvent A, and 90% MeOH as solvent B.

Figure 27 - Ion spectrum of BPB after direct infusion in the MS/MS system with collision at 26 kV. The product ions selected for BPB confirmation and quantification were 226.83 m/z and 212.32 m/z, respectively.
1.2. Optimization of extraction conditions

BPA is almost ubiquitous in the environment; thus, special attention should be given to all factors that could interfere with the analysis such as plastic tubes, contamination of glassware and other materials that come in contact with analytical samples or standards. In this study, only glass vials were used, all of them previously treated for 2 h at 500 °C in a muffle furnace before use, and quality control blanks were periodically prepared and analysed.

Three methodologies were initially tested for BPA and TBBPA extraction and determination, all the experiments have been performed in duplicated. The method reported by Gallart-Ayala et al. (2013) was the first tested. In this method, the extraction of BPA and TBBPA from seafood is achieved with the addition of 6 mL of ethyl acetate to a previously homogenised large monkfish sample fortified with 200 µL a mixture solution of BPA and TBBPA (both at 1000 ng/mL). After, centrifugation the extract that was evaporated and reconstituted with mobile phase and added with 80 µL of each I.S. BPAd_{16} (1000 ng/mL) and TBBPA^{13}C_{12} (1000 ng/mL). Although the analytes has been successfully eluted from the column, the recovery of BPA for this procedure was only 11.9% when compared to the same extracted sample spiked with 200 µL of mixture solution of BPA and TBBPA (both at 1000 ng/mL) in the final step of extraction (reconstitution phase).

In an attempt to improve recovery, 6 mL of a mixture of ethyl acetate/MeOH (1:1, v/v) was added in substitution of the ethyl acetate. A homogenized large monkfish sample fortified with 200 µL a mixture solution of BPA and TBBPA (both at 1000 ng/mL), was submitted to a clean-up step with an ultrasounds bath for 10 min prior to a salting-out extraction step using 4 g of MgSO_{4} and 1 g of NaCl. Followed the evaporation, the extract was added with 80 µL of each I.S. BPAd_{16} (1000 ng/mL) and TBBPA^{13}C_{12} (1000 ng/mL) and reconstitution with MeOH/ 5mM ammonium acetate (9:1, v/v). The recoveries were also lower (22.0% for BPA and 3.0% for TBBPA), when compared to the same extracted sample but with the addition of mixture solution of BPA and TBBPA (both at 1000 ng/mL) in the reconstitution phase.
Last, we modified and tested the extraction method reported by Cunha et al. (2012) to determination of BPA and BPB in canned seafood by GC-MS for this work. Thus, 2 g of homogenized large monkfish sample was weight and fortified with 200 µL a mixture solution of BPA and TBBPA (both at 1000 ng/mL). Then was added 10 mL of MeCN, 7 mL of ultra-pure MilliQ water 4 g of anhydrous MgSO₄ and 1 g of NaCl. After shaking for 15 min and centrifuge at 2000 rpm for 5 min, 3mL of the supernatant was transferred to a new vial with 7 mL of ultra-pure MilliQ water. Then a LLE was applied procedure instead of DLLME: 4 mL of the mixture of n-hexane/MTBE (3:1, v/v) was added, shaken by hand and 3 mL of the top-layer was transferred to a new vial. Then, a second LLE with 4 mL of the mixture n-hexane/benzene (3:1, v/v) were made, shaken by hand and another 3 mL of the top-layer
was transferred to the vial. The final extract (6 mL) was finally evaporated to dryness, added with 80 μL of each I.S. BPAd_{16} (1000 ng/mL) and TBBPA^{13}C_{12} (1000 ng/mL), and reconstituted in mobile phase (100 μL of 5mM ammonium acetate and 900 μL MeOH) and 20.0 μL injected in the LC-MS/MS system. This procedure was compared with one similar where the fortification was performed in the last step (reconstitution in mobile phase). With this procedure, recoveries of 28.0 % for BPA and 51.8% for TBBPA were achieved.

**Figure 30** - LC-MS/MS product ions chromatogram of a solution of 100 ng/mL BPA and TBBPA and 40 ng/mL BPAd_{16} and TBBPA^{13}C_{12} after extraction with MeCN followed by a salting-out with the addition of 4 g MgSO_{4} and 1 g NaCl and 7 mL ultra-pure MilliQ water and clean-up with n-hexane/MTBE (3:1, v/v) and n-hexane/benzene (3:1, v/v).
With the aim of achieving a better performance with this methodology, namely better recoveries of the analytes, the last tested method was improved with the addition of 100 µL HCl 10M to the extraction solvent (MeCN). The acidification of samples suppresses the dissociation of phenols and prevents the ionization of the analytes, which increased the efficiency of the extraction. Otherwise, either decrease retention on the analytical column or interactions of the analytes and the stationary phase would be obtained, resulting in lower separation efficiencies (Halaleh et al., 2001). This acidification promoted a better extraction of both BPA and TBBPA with recoveries of 71.3% and 89.9% respectively.

**Figure 31** - LC-MS/MS product ions chromatogram of a solution of 100 ng/mL BPA and TBBPA and 40 ng/mL BPAd16 and TBBPA$^{13}$C$_{12}$ after extraction with MeCN followed by a salting-out with the addition of 4 g MgSO$_4$ and 1 g NaCl and 7 mL ultra-pure MilliQ water with 100 µL of HCl 0.01 M and clean-up with n-hexane/MTBE (3:1, v/v) and n-hexane/benzene (3:1, v/v).
2. BPA and TBBPA determination in SEAFOOD

2.1. Validation

The appropriate validation of analytical methods has become an essential part of an experimental work in order to prove that the analytical method is able to provide reliable data. Validation of a method involves using experimental design to prove that the method can produce accurate and precise results within the scope of its intended use. Understanding the application and limitations of the test method will allow for accurate assessment of sample information. The validation was achieved through the analysis of several analytical parameters as linearity, intra-day and inter-day precision, recovery and LODs and LOQs.

Since the method developed in this work was applied to three different matrices (seafood, mussels and seaweed) it was essential to make a validation of the method for all of them in order to test and demonstrate its applicability.

2.1.1. Linearity

Matrix effect can strongly affect chromatographic performance; therefore initially the slopes of the calibration curves obtained from standard solutions were compared with those obtained from matrix-matched standards. An enhancement of analytical response for TBBPA and BPA with matrix was observed. Therefore, the use of matrix-matched calibration was required for a reliable quantification.

The linearity study was performed by analysing the determination coefficient of the calibration curves obtained by injection of spiked extracts of large monkfish from the Round I and canned sardine from the Round II. These two samples were selected since none of them presented neither BPA nor TBBPA traces, when analysed prior to validation tests. Additionally, large monkfish is a representative sample of the Round I and canned sardine a representative of the Round II.

The levels of BPA and TBBPA were quantified using I.S. calibration. Each set of samples was analyzed with mixed calibration standards with 0.00, 1.00, 2.50, 5.00, 10.0, 15.0, 20.0, 40.0, 100, 150, 400 and 600 ng/g dw of BPA and 0.00, 1.00, 2.50, 5.00, 10.0, 15.0, 20.0, 40.0, 100, 150, 400, 600 and 1000 ng/g dw of TBBPA. Each calibration standard was fortified with BPAd_{16} and TBBPA^{13}C_{12} at 40.0 ng/g dw, added to 2.00 g of selected BPA
free and TBBPA free samples prior to the extraction methodology described before in duplicate and finally subjected to LC-MS/MS.

The calibration standards were analyzed before and after each set of validation samples and the results obtained were used to construct the calibration curve. Calibration curves were constructed by plotting the analyte/I.S. ratio obtained against the concentration of each analyte as can be seen in Figure 32 for large monkfish sample from Round I as an example. The results obtained demonstrated a good linearity within the tested interval, with coefficients of determination ($R^2$) always higher than 0.98 in the two distinct matrices and the two chemicals studied (Table 8).

![Figure 32 - Calibration curve with large monkfish from Round I samples extracted TBBPA standard solutions (n=2).](image)

<table>
<thead>
<tr>
<th>Equation</th>
<th>Coefficient of determination ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large monkfish Round I</strong></td>
<td></td>
</tr>
<tr>
<td>BPA</td>
<td>$y = 0.0039x + 0.232$</td>
</tr>
<tr>
<td>TBBPA</td>
<td>$y = 0.0203x - 0.0228$</td>
</tr>
<tr>
<td><strong>Canned Sardine Round II</strong></td>
<td></td>
</tr>
<tr>
<td>BPA</td>
<td>$y = 0.0023x + 0.0787$</td>
</tr>
<tr>
<td>TBBPA</td>
<td>$y = 0.0104x + 0.4495$</td>
</tr>
</tbody>
</table>
2.1.2. Precision

The efficacy of the analytical method developed was calculated as the relative standard deviation (%RSTD) for intra and inter-day precision. These values were calculated from the peak area obtained from six replicates of a large monkfish (Round I) spiked sample at two concentration levels (1 and 10 ng/g of both BPA and TBBPA) before the extraction methodology. The values obtained ranged from 6 to 26 % for large monkfish sample intra-day repeatability and from 12 to 22 for inter-day repeatability, shown in Tables 9 and 10 respectively.

Table 9 - Intra-day precision in RSTD (%) of BPA and TBBPA after extractive procedure in large monkfish sample (n=6).

<table>
<thead>
<tr>
<th>Large monkfish (Round I)</th>
<th>1 ng/g</th>
<th></th>
<th>10 ng/g</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>STD</td>
<td>RSTD (%)</td>
<td>Average</td>
</tr>
<tr>
<td>BPA</td>
<td>0.0227</td>
<td>0.0366</td>
<td>16</td>
<td>0.122</td>
</tr>
<tr>
<td>TBBPA</td>
<td>1.21</td>
<td>0.0737</td>
<td>6</td>
<td>0.338</td>
</tr>
</tbody>
</table>

Table 10 - Inter-day precision in RSTD (%) of BPA and TBBPA after extractive procedure in large monkfish sample (n=6).

<table>
<thead>
<tr>
<th>Large monkfish (Round I)</th>
<th>1 ng/g</th>
<th></th>
<th>10 ng/g</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>STD</td>
<td>RSTD (%)</td>
<td>Average</td>
</tr>
<tr>
<td>BPA</td>
<td>0.0220</td>
<td>0.0374</td>
<td>17</td>
<td>0.0232</td>
</tr>
<tr>
<td>TBBPA</td>
<td>0.0135</td>
<td>0.0296</td>
<td>22</td>
<td>0.262</td>
</tr>
</tbody>
</table>

2.1.3. Recovery

To evaluate the accuracy of the analytical method recovery tests were performed. The recovery was determined by comparing the analytical response of the analytes in spiked large monkfish sample from Round I before and after the extraction step, for two concentration levels (40.0 and 100 ng/g BPA or TBBPA), being each level performed six times. The mean recovery values present higher recovery of BPA compared to TBBPA, ranging from 84 to 94% in the determination of BPA and from 75 to 79% in the determination of TBBPA, both in large monkfish samples (Table 1).
Table 11 - Recovery (%) of BPA and TBBPA and respective STD after extractive procedure in a large monkfish sample from Round I (n=6).

<table>
<thead>
<tr>
<th>Concentration (ng/g)</th>
<th>Recovery (%) ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BPA</td>
</tr>
<tr>
<td>40.0</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>100</td>
<td>94 ± 15</td>
</tr>
</tbody>
</table>

2.1.4. Method detection limit (MDL) and method quantification limit (MQL)

The MDL is the lowest concentration of analyte that can be detected but not necessarily quantified. The MDL of the method were determined by successive analyses of sample extracts with decreasing amounts of the compounds until a 3:1 signal-to-noise ratio was reached.

The MQL is defined as the smallest amount of analyte in a sample that can be measured with acceptable accuracy and precision (coefficients of variation <20%) under the same analytical conditions as those in the samples. The MQL were established as the lowest concentration assayed with a 10:1 signal-to-noise ratio.

The obtained values showed a MQL of the method of 1.00 ng/g dw (0.17 ng/g ww) for both BPA and TBBPA and a MDL of 0.30 ng/g dw (0.05 ng/g ww) equally for both analytes. These values are slightly smaller than those reported in literature, with levels ranging from 0.05 to 1.00 ng/g ww (EFSA, 2011).

Table 12 - MDL and MQL (ng/g dw) for both BPA and TBBPA determined in large monkfish sample from Round I.

<table>
<thead>
<tr>
<th></th>
<th>MDL (ng/g dw)</th>
<th>MQL (ng/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>0.30</td>
<td>1.00</td>
</tr>
<tr>
<td>TBBPA</td>
<td>0.30</td>
<td>1.00</td>
</tr>
</tbody>
</table>
2.2. **Levels of BPA and TBBPA in Seafood**

**Table 13** - BPA and TBBPA levels (ng/g dw) found in seafood samples from Round I (n=2). nd=not detected

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Sampling site</th>
<th>[BPA] ± STD, ng/g dw</th>
<th>[TBBPA] ± STD, ng/g dw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackerel</td>
<td>Goro (IT)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed seabream</td>
<td>Greece</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Atlantic Cod</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed salmon</td>
<td>Norway</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Canned mackerel</td>
<td>Portugal</td>
<td>150.8 ± 12.6</td>
<td>nd</td>
</tr>
<tr>
<td>Small monkfish</td>
<td>Portugal</td>
<td>724.7 ± 24.2</td>
<td>nd</td>
</tr>
<tr>
<td>Large monkfish</td>
<td>Portugal</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Canned tuna</td>
<td>Portugal</td>
<td>83.8 ± 9.0</td>
<td>52.6 ± 1.3</td>
</tr>
<tr>
<td>Canned sardine</td>
<td>Portugal</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Channel (FR)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>North Sea</td>
<td>nd</td>
<td>107.6 ± 29.2</td>
</tr>
<tr>
<td>Farmed salmon</td>
<td>Scotland</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Brown crab</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cod</td>
<td>Pacific ocean</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Tuna small</td>
<td>Indonesia (Pacific ocean)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Tuna large</td>
<td>Indonesia (Pacific ocean)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Nile Perch</td>
<td>Indonesia (Pacific ocean)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed pangasius</td>
<td>Vietnam</td>
<td>nd</td>
<td>144.2 ± 8.1</td>
</tr>
<tr>
<td>Farmed shrimp</td>
<td>India</td>
<td>nd</td>
<td>84.2 ± 3.8</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Atlantic coast (ES)</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MDL</th>
<th>0.30</th>
<th>0.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQL</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
### Table 14 - BPA and TBBPA levels (ng/g dw) found in seafood samples from Round II (*n*=2). *nd*=not detected

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Sampling site</th>
<th>[BPA] ± STD, ng/g dw</th>
<th>[TBBPA] ± STD, ng/g dw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackerel</td>
<td>Goro (IT)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel, cooked</td>
<td>Goro (IT)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed seabream</td>
<td>Greece</td>
<td>151.3 ± 1.9</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed seabream, cooked</td>
<td>Greece</td>
<td>21.6 ± 1.2</td>
<td>nd</td>
</tr>
<tr>
<td>Drip Loss</td>
<td>-</td>
<td>513.3 ± 51.1</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>200.7 ± 1.5</td>
</tr>
<tr>
<td>Atlantic cod</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Farmed salmon</td>
<td>Norway</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mussels</td>
<td>Limfiord (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Canned sardine</td>
<td>Portugal</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Small monkfish</td>
<td>Atlantic coast (PT)</td>
<td>31.7 ± 2.6</td>
<td>nd</td>
</tr>
<tr>
<td>Small monkfish, cooked</td>
<td>Atlantic coast (PT)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Large monkfish</td>
<td>Portugal</td>
<td>22.9 ± 2.0</td>
<td>nd</td>
</tr>
<tr>
<td>Large monkfish, cooked</td>
<td>Portugal</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Canned tuna</td>
<td>Portugal</td>
<td>21.6 ± 4.5</td>
<td>nd</td>
</tr>
<tr>
<td>Canned mackerel</td>
<td>Portugal</td>
<td>41.9 ± 19.7</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Brown crab</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Brown crab, steamed</td>
<td>North Sea (DK)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Imported tuna small</td>
<td>-</td>
<td>143.0 ± 12.9</td>
<td>nd</td>
</tr>
<tr>
<td>Imported tuna large</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Shrimp vannamei</td>
<td>India</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Shrimp vannamei</td>
<td>India</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Mediterranean Sea</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Mackerel, cooked</td>
<td>Mediterranean Sea</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

| MDL                  | 0.30                   | 0.30                  |
| MQL                  | 1.00                   | 1.00                  |
Mean results and corresponding STD are detailed in Tables 13 and 14 for seafood samples from Round I and Round II, respectively.

In Round I, only three of the twenty one analysed species (about 14%) showed the presence of BPA with levels ranging from 83.8 to 724.7 ng/g dw. Canned tuna sample was the only sample presenting both BPA (83.8 ng/g dw) and TBBPA (52.8 ng/g dw) levels detected above the MQL (also 1.00 ng/g for TBBPA). The other two species positive for BPA were small monkfish and canned mackerel.

The values of BPA found are in accordance with Geens et al. that found 163.3 ng/g ww BPA in canned tuna in oil (Geens et al., 2010). However, slightly lower values were obtained by Noonan et al. and Cunha et al. with levels of 4.5 ng/g ww in tuna in oil and 21.2 ng/g ww in tuna conserved in vegetable oil, respectively.

TBBPA was present in 19% of samples of Round I: farmed pangasius from Vietnam (144.2 ng/g dw), mackerel from the North Sea (107.6 ng/g dw), canned tuna (52.6 ng/g dw), and farmed shrimp from India (84.2 ng/g dw). The levels found are similar to those report in literature for species of fish collect in different countries (EFSA, 2011), with content ranging from 0.005 to 13.7 ng/g ww of TBBPA.

The presence of TBBPA was not expected in the farmed species due the controlled environment of their growth that should prevent contamination of waters in which habitat for products resulting from the pollution of rivers, seas and oceans.

In Round II, BPA was detected in more samples (32%) than in Round I, the levels ranging from 21.6 to 513.3 ng/g dw. The canned tuna sample from Portugal is one more time BPA+, although presented a lower level than the sample analysed in Round I (83.8 ng dw of BPA per g of canned tuna was detected in Round I and 21.6 ng dw of BPA per g of canned tuna in Round II). This last concentration has better accordance with the levels reported in previous studies cited above (Geens et al., 2010; Noonan et al., 2011; Cunha et al., 2012). In addition to these canned samples, where the presence of BPA is expected since this ED is present in the epoxy resin coating the metal can and can migrate to its content, BPA is also found in the imported tuna small sample (143.3 ng/g dw). In this round, BPA was found in canned mackerel samples again from Atlantic coast (collected in Portugal) with 41.9 ng/g dw despite no traces were detected in Round I (Table 13). In the study of Cunha et al. (2012), mackerel conserved in different sauces was analysed (Table 2) and variable levels were reported ranging from 9.9 to 40.4 ng/g ww BPA of canned mackerel in vegetable oil and in tomato oil, respectively.
Additionally to the first round, in this trial some of the species were analysed in the raw form and also cooked/steamed in the case of mackerel from Goro, farmed seabream from Greece, small monkfish from Atlantic cost, large monkfish from Atlantic cost, brown crab from North Sea and mackerel from Mediterranean Sea. In all these samples, BPA levels decreased with the thermal treatment. Once again it was detected BPA in a farmed seafood sample, which was not expected for the reasons explained above. In both small and large monkfish samples an annulation of the detection of BPA was observed when the samples were cooked, from 31.7 ng/g dw in small monkfish and 22.9 ng/g dw in large monkfish to not detected in both cooked samples.

When observing the results for TBBPA detection, mackerel from the North Sea (collected in Denmark) was the only positive sample in twenty five seafood samples analysed with 200.7 ng of TBBPA per g dw of mackerel detected. This value is comparable to the one obtained from Morris et al. (2004) which detected a mean level of 209.05 ng/g dw TBBPA in harbor porpoise samples. Also in whiting samples, the TBBPA levels reached a maximum of 245.00 ng/g dw TBBPA. Both these fishes were collected from the North Sea, as well as samples positive TBBPA sample in this work. However, other seafood from the same local, as the Atlantic cod, another mackerel and brown crab, did not presented any trace levels of this BFR. To the best of our knowledge, no more data concerning such higher amounts of TBBPA in seafood were reported in the literature reaching the other values found in the order of the decimals (from 0.04 to 0.98 ng/g ww TBBPA), units (from 1.00 to 8.48 ng/g ww TBBPA) or a few tens of ng/g TBBPA (from 13.0 to 66.0 ng/g ww TBBPA) (EFSA, 2011).

It does not appear to be a continuous profile from the Round I to the Round II. Between these two different moments of samples collection, there is a great variability of BPA and TBBPA levels found between different species and even in samples from the same species fact that is totally acceptable since the season of the year can affect the level of contaminants in seafood.

Despite the developed method presented low MDL and MQL values, an optimization of the extraction methodology in order to obtain cleaner and fatty free samples to inject (namely with fat separation) would improve the LC-MS/MS response.
3. BPA and TBBPA determination in **MUSSELS**

3.1. **Validation**

3.1.1. **Linearity**

When analyzing the appearance of the mussel samples and observing the difficulty in the extraction methodology already validated, it was necessary to make a new validation of the method developed. The linearity study was performed by analysing the determination coefficient of the calibration curves obtained by injection of an extract of mussel sample from the Round II, which was applied to all mussels samples from Round I and II.

The levels of BPA and TBBPA were quantified using I.S. calibration. Each set of samples was analyzed with mixed calibration standards 0.00, 10.0, 40.0, 100, 150, 200, 400 and 600 ng/g dw of BPA and 0.00, 5.00, 40.0, 100, 150, 200, 400 and 600 ng/g dw of TBBPA. Each calibration standard was fortified with BPAd_{16} and TBBPA_{13}C_{12} at 40.0 ng/g dw, added to 2.00 g of selected BPA free and TBBPA free samples prior to the extraction methodology described before in duplicate and finally subjected to LC-MS/MS.

The calibration standards were analyzed before and after each set of validation samples and the results used to construct the calibration curve. Calibration curves were constructed by plotting the analyte/I.S. ratio obtained against the concentration of each analyte. The results obtained demonstrated a good linearity within the tested interval, with coefficients of determination ($R^2$) always higher than 0.98 for the two analytes in studied (Table 15).
### Results and Discussion

#### Table 15 - Calibration curves equations obtained from the analysis of mussels sample fortified with BPA and TBBPA at crescent concentrations and respective coefficient of determination ($R^2$). I.S. at 40 ng/mL.

<table>
<thead>
<tr>
<th>Mussel Round II</th>
<th>Equation</th>
<th>Coefficient of determination ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>$y = 0.0054x + 0.211$</td>
<td>0.9876</td>
</tr>
<tr>
<td>TBBPA</td>
<td>$y = 0.0139x + 0.365$</td>
<td>0.9930</td>
</tr>
</tbody>
</table>

#### 3.1.2. Recovery

The recovery was determined by comparing the analytical response of the analytes in samples spiked before and after the extraction step, for two concentration levels (40.0 and 100 ng/g BPA or TBBPA), being each level performed six times. The mean recovery values present higher recovery of TBBPA compared to BPA, ranging from 57 to 78% in the determination of BPA and from 73 to 86% for TBBPA, both in mussels samples (Table 16).

#### Table 16 - Recovery (%) of BPA and TBBPA and respective STD after extractive procedure in mussel samples (n=6).

<table>
<thead>
<tr>
<th>Recovery (%) ± STD</th>
<th>Mussels (Round II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/g)</td>
<td>BPA</td>
</tr>
<tr>
<td>40.0</td>
<td>78 ± 18</td>
</tr>
<tr>
<td>100</td>
<td>57 ± 15</td>
</tr>
</tbody>
</table>

These results are similar to the ones obtained from seafood samples other than mussels, which was “surprising” since the mussels’ matrix fatter and more complex than the seafoods matrix and lower recoveries were expected.
3.1.3. **MDL and MQL**

MDL in mussels was higher to those obtained for fish (Table 17) for both BPA and TBBPA, with 10.0 ng/g dw (2.15 ng/g ww) and 5.00 ng/g (1.08 ng/g ww), respectively. Similar behaviour was observed for MDL with 3.03 ng/g dw (0.65 ng/g ww) for BPA and 1.52 ng/g dw (0.33 ng/g ww) for TBBPA.

<table>
<thead>
<tr>
<th></th>
<th>MDL (ng/g dw)</th>
<th>MQL (ng/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>3.03</td>
<td>10.0</td>
</tr>
<tr>
<td>TBBPA</td>
<td>1.52</td>
<td>5.0</td>
</tr>
</tbody>
</table>

When comparing to MDL and MQL values obtained with the data from literature, it is possible to conclude that the values achieved with the method employed in this work are slight higher. Cunha et al. (2012) reports a MDL of 0.2 ng/g ww and a MQL of 1.0 ng/g ww in their work assessing BPA and BPB levels in canned seafood. Although the sample preparation was quite similar to the one of this work, the detection and quantification equipment was different (GC-MS) (Cunha et al., 2012).
3.2. Levels of BPA and TBBPA in Mussels

After validation, five samples of mussels collected in the first round and nine from the second round were analysed for the determination of the presence and quantification of BPA and TBBPA (Table 18).

In Round I, only the mussel sample from the Mediterranean Sea was positive for TBBPA with 60.4 ng/g dw (12.99 ng/g ww). The EFSA report in 2011 reports two smaller TBBPA determinations in mussels: < 0.6 ng/g ww in mussels from North Sea (Van Leeuwen, 2009) and < 0.26 ng/g ww in mussels from North Europe (Papke et al., 2010).
In Round II, as well as for seafood, some mussel samples were also analysed in the raw and cooked form. In this second trial, only the blue mussels from Limfiord (Denmark) presented BPA levels although in a low extent: 18.66 ng/g dw (4.01 ng/g ww). Cunha et al. (2012), in addition to the canned seafood, also determined the migration of BPA from the can to its content in canned mussels samples, founding once again high variable values for conservation in different sauces: 1.4 ng/g ww BPA in mussels conserved in pickled sauce and 49.2 ng/g ww BPA in mussels conserved in escabeche sauce. None of these two data can be compared to the results obtained in this work, since all mussel samples were collected and analysed in fresh (raw or cooked) but no conservation in cans was employed.
4. BPA and TBBPA determination in **SEAWEEDS**

4.1. **Validation**

4.1.1. **Linearity**

The seaweed matrix is similar to the seafood, since these two members of the aquatic family are rich in fatty tissues. However, is important to make a new validation for the extraction method in this matrix. The linearity study was performed by analysing the determination coefficient of the calibration curves obtained by injection of spiked extracts of seaweed free of both BPA and TBBPA. This could be a control sample, but in the absence of sufficient quantity of seaweed to obtain 8 or 10 points for the calibration curve, it has been decided to analysed a sample collected after 360 hours in culture in a 10.0 ng/mL selected since it did not present neither BPA nor TBBPA traces when analyzed prior to validation tests.

The levels of BPA and TBBPA were quantified using I.S. calibration. Each set of samples was analyzed with mixed calibration with 0.00, 10.0, 15.0, 20.0, 40.0 and 100 ng/g dw of BPA and 0.00, 5.00, 10.0, 15.0, 20.0, 40.0 and 100 ng/g dw of TBBPA. Each calibration standard was fortified with BPB and TBBPA$^{13}$C$_{12}$ at 80 ng/g, added to 2.00 g of selected BPA free and TBBPA free samples prior to the extraction methodology described before in duplicate and finally subjected to LC-MS/MS. BPB was used as an I.S. for seaweeds instead of BPAd$_{16}$ since this I.S. could not be detected in the LC-MS/MS probably because it could not be extracted from the seaweeds matrix with the developed method.

The calibration standards were analyzed before and after each set of validation samples and the results used to construct the calibration curve. Calibration curves were constructed by plotting the analyte/I.S. ratio obtained against the concentration of each analyte. The results obtained demonstrated a good linearity within the tested interval, with determination coefficients ($R^2$) always higher than 0.99 in the two chemicals studied (Table 19).
Table 19 - Calibration curves equations obtained from the analysis of seaweed sample fortified with BPA and TBBPA at crescent concentrations and respective coefficient of determination ($R^2$). I.S. at 80 ng/mL.

<table>
<thead>
<tr>
<th>Seaweed after 360h</th>
<th>Equation</th>
<th>Coefficient of determination ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>$y = 0.0147x + 0.0121$</td>
<td>0.9923</td>
</tr>
<tr>
<td>TBBPA</td>
<td>$y = 0.0139x + 0.046$</td>
<td>0.9947</td>
</tr>
</tbody>
</table>

4.1.2. Recovery

The recovery was determined by comparing the analytical response of the analytes in spiked seaweed samples before and after the extraction step, for two concentration levels (40.0 and 100 ng/g BPA or TBBPA), being each level performed six times. The mean recovery values ranged from 49 to 66% in the determination of BPA and from 60 to 61% for TBBPA, both in seaweed samples (Table 20).

Table 20 - Recovery (%) of BPA and TBBPA after extractive procedure in seaweed samples.

<table>
<thead>
<tr>
<th>Concentration (ng/g)</th>
<th>Seaweed</th>
<th>BPA</th>
<th>TBBPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>40.0</td>
<td>100</td>
</tr>
<tr>
<td>BPA</td>
<td>66 ± 6</td>
<td>61 ± 24</td>
<td></td>
</tr>
<tr>
<td>TBBPA</td>
<td>49 ± 6</td>
<td>60 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

Differently from the results obtained in the seafood and mussels samples, where the higher recovery was achieved for TBBPA, in the seaweed samples both analytes have similar recoveries and smaller than those achieved for the two matrices analyzed before.
4.1.3. MDL and MQL

For BPA and TBBPA, the MQL is 10.0 ng/g dw (3.25 ng/g ww) and 5.00 ng/g dw (1.62 ng/g ww) respectively. The obtained values showed that the MDL is 3.03 ng/g dw (0.98 ng/g ww) for BPA determination and 1.51 ng/g dw (0.49 ng/g ww) for TBBPA.

<table>
<thead>
<tr>
<th></th>
<th>MDL (ng/g dw)</th>
<th>MQL (ng/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>3.03</td>
<td>10.0</td>
</tr>
<tr>
<td>TBBPA</td>
<td>1.52</td>
<td>5.00</td>
</tr>
</tbody>
</table>

As this matrix is fatty as like the mussels samples, was expected that the MDL and MQL achieved in this case were similar than the ones obtained for mussels.
4.2. **Levels of BPA and TBBPA in Seaweed**

Table 22 - BPA and TBBPA levels (ng/g dw) found in seaweed samples with different spiking and collected at different times (n=2) from the 10 ng/mL BPA and TBBPA concentrated media. nd=not detected

<table>
<thead>
<tr>
<th>Seaweed (n=1)</th>
<th><strong>Spiking concentration (µg/L)</strong></th>
<th><strong>Sampling time (hours)</strong></th>
<th><strong>Levels ng/g dw</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TBBA</td>
<td>BPA</td>
</tr>
<tr>
<td>Saccharina latissima</td>
<td>0 (control I)</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>240</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>360</td>
<td>nd</td>
</tr>
<tr>
<td>Laminaria digitata</td>
<td>1</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>240</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>360</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>240</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>360</td>
<td>nd</td>
</tr>
</tbody>
</table>
As reported in Table 22 neither BPA nor TBBPA were found in both seaweed species tested (Saccharina latissima and Laminaria digitata) during all the experiment. The seaweeds grew well on the medium with two concentrations of contaminants, but no demonstrate capacity to bioaccumulate persistent organic pollutants from marine habitats.
In a previous study, it was found that brown seaweed macroalgae, *Laminaria japonica*, could remove PAHs from medium (Wang and Zhao, 2008). Phycoremediation of TBBPA or BPA by macroalgae, however, was as far as we know not reported.

The phycoremediation of BPA has been successfully reported by Hirooka *et al.* (2005) using the *Chlorella Fusca*. This microalga could remove 90% of 40 µM BPA under 8:16 h light dark condition (Hirooka *et al.*, 2005). Biotransformation of TBBPA was recently verified by Peng *et al.* (2014) using *Scenedesmus quadricauda* and *Coelastrum sphaericum* following 10 day incubation. Five transformation products were positively identified by mass spectrometry: TBBPA sulfate, TBBPA glucoside, sulfated TBBPA glucoside, TBBPA monomethyl ether, and tribromobisphenol-A. The mechanisms involved in the biotransformation of TBBPA include sulfation, glucosylation, O-methylation, and debromination, which could be an important step for its further degradation (Peng *et al.*, 2014).
IV. Conclusions
This dissertation intended to develop an effective method for the simultaneous determination of BPA and TBBPA in seafood, mussels, and seaweeds, as part of the European Project ECsafeSEAFOOD. The samples were collected in different rivers, seas or aquaculture spots along the European region at different times of the year.

After optimization of the chromatographic conditions to LC-MS/MS detection, an optimization of the sample preparation was accomplished in order to achieve the high sensitivity. Thus, three procedures published in literature were primarily compared for recovery. The selected method based on a QuEChERS extraction followed by a LLE extraction showed the best recoveries results for the analytes in study.

In order to certify its sensibility and accuracy, the analytical method developed was validated, demonstrating to be robust and appropriate for the intended analysis. The selected method performance was validated for the three matrices under study given its great diversity in terms of composition. The optimized method showed to be accurate (>49% recovery), precise (<26 % relative standard deviation) and sensitive for the target analytes (higher MDLs found of 3.03 ng/g dw for BPA and 1.51 ng/g dw for TBBPA). Between the three matrices, the method showed to be less accurate for seaweeds and the higher detection and quantification limits were found for mussels. Then, this method should be upgraded in futures experiments for these two matrices.

When applying the method for BPA and TBBPA determination in seafood and mussels samples collected in different regions of Europe at different times of the year, it is possible to conclude that there is not a continuous profile. For the same sample collected in the same region but in different times of the year, the BPA levels detected are quite variable (for example, canned mackerel from Portugal with 41.9 ng/g dw BPA in Round I and 150.8 ng/g dw in Round II). The higher value observed was 724.7 ng/g dw BPA in monkfish small from Round I, another variable value for Round II (31.7 ng/g dw BPA). In case of TBBPA, the higher value obtained was 200.7 ng/g dw in mackerel from North Sea in Round II, while in Round I was not detected any trace of this BFR on the same specie caught in the same spot.

In mussels, only two samples revealed the presence of one of these contaminants: the blue mussel from Limfiord with 60.4 ng/g dw BPA and the Mediterranean mussel with 18.66 ng/g dw TBBPA.

For both seaweed species tested (*Saccharina latissima* and *Laminaria digitata*) neither BPA nor TBBPA were found in during all the experiment. The seaweeds were cultivated on the medium with two concentration of contaminants (1.0 and 10.0 ng/mL),
but did not demonstrated capacity to bioaccumulate these two persistent organic pollutants from marine habitats.

In consideration to the actual apprehensions about these two EDs, the amounts found in seafood and mussels samples are generally low and within the regulated and safety limits. However contamination of the environment should not be ignored since there are researchers who consider that these chemical are malignant even in small quantities. The absence of the both EDs in the seaweeds subjected to study may indicate that these contaminants are not absorbed and metabolized by these two macroalgae but the lack of other studies do not allow more confident conclusions.

These findings should be a starting point for future studies. More studies should be developed concerning these contaminants in foodstuff contaminated. For more consistent and sustained conclusions, it would be important to:

- Develop better extraction techniques, especially for fat removal prior to analysis (removal of triglycerides, for instance) should be advanced in order to have better precision and recovery;
- Evaluate BPA and TBBPA regarding other parameters besides local of origin and time of the year, as the fat and protein content which can affect these contaminants determination;
- Apply this method more optimized for simultaneous detection of BPA ad TBBPA in commercial foodstuff, other than seafood, as a technique for Quality Control.
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