SIMULTANEOUS DETERMINATION OF PCDD/Fs, PCBs, PBDEs AND PBDD/Fs IN FISH AND MUSSELS FROM THE MEDITERRANEAN SEA

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Introduction

Brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), have been largely used in a variety of consumer products for many decades in order to inhibit the initial phase of a developing fire and prevent human injury. Furthermore, incineration of waste containing BFRs can also generate persistent environmental contaminants such as polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs). The structural similarity of PBDD/Fs and PBDEs to dioxins and polychlorinated biphenyls (PCBs) and their consistent detection in biota and environmental samples are evidence of the persistent nature of these contaminants. In addition, these contaminants are chemicals of concern because of their persistence, bioaccumulation, and potential for toxicity, both in animals and in humans.[1-4]

Human exposure to these contaminants can occur from a variety of routes, including consumption of contaminated food. Therefore is important to evaluate the occurrence of these contaminants in food, in order to estimate the human dietary exposure to these compounds.

Aim of this study was to evaluate the levels of PCDD/Fs, dioxin-like PCBs (DL-PCBs), non-dioxin-like PCBs (NDL-PCBs), PBDEs and PBDD/Fs in fish samples. This study is part of a larger investigation that is still ongoing.

Materials and methods

Sampling

A total of 60 samples (including eggs, meat, milk, fish and mussels) were randomly collected between January 2014 and February 2016 by the regional veterinary services, covering the national territory. The samples were stored frozen prior to analysis. Up to now, 21 samples of fish and mussels have been analyzed for PCDD/Fs, DL-PCBs, NDL-PCBs, PBDEs and PBDD/Fs. Details on the fish and mussel species are reported in Table 1. Fish samples were collected from the Mediterranean sea off the coast of Sicily and Apulia. Samples of mussels were collected from aquaculture industry in mussel farming located near the industrial area of Taranto, known to be contaminated by PCDD/Fs and PCBs.

Analytes and standards

The following analytes were determined: the **17**, 2378-chloro-substituted PCDD/Fs; non-*ortho*-substituted PCBs (IUPAC numbers **77**, **81**, **126**, **169**); mono *ortho*-substituted PCBs (IUPAC numbers **28**, **52**, **101**, **105**, **114**, **118**, **123**, **138**, **153**, **156**, **157**, **167**, **180**, **189**); PBDEs (IUPAC numbers **28**, **47**, 66, 85, **99**, **100**, **153**, **154**, **183**); and 14 PBDD/Fs (**2,3,7,8-TeBDF**, 2,4,6,8-TeBDF, 1,2,3,7,8-PeBDF, **2,3,4,7,8-PeBDF**, **1,2,3,4,7,8-HxBDF**, 1,**2,3,4,6,7,8-HpBDF**, **OBDF**, **2,3,7,8-TeBDD**, **1,2,3,7,8-PeBDD**, **1,2,3,4,7,8-HxBDD**, **1,2,3,6,7,8-HxBDD**, 1,2,3,7,8,9-HxBDD, **1,2,3,4,6,7,8-HpBDD**, **OBDD**).

Reference standards, native as well as ^{13}C labelled, were obtained either from Wellington Laboratories Inc. Ontario, Canada or from Cambridge Isotope Labs, MA, USA ($^{13}C_{12}$ labelled standards used as internal standards are given in bold). All internal standard solutions were prepared in iso-octane. The internal standard solution for the PCDD/Fs contained nominal concentrations of 5 ng/ml for tetra to hepta-substituted congeners and 10 ng/ml for octa-substituted congeners. The internal standard solution for the PCBs and the PBDEs contained nominal concentrations of 25 ng/ml of $^{13}C_{12}$ labelled PCBs and 20 ng/ml of $^{13}C_{12}$ labelled PBDEs. The internal standard solution for the PBDD/Fs contained nominal concentrations of 10 ng/ml for tetra-penta-substituted congeners, 25 ng/ml for hexa-hepta-substituted congeners and 50 ng/ml for octa-substituted congeners.

Extraction and clean-up

All equipment was cleaned and thoroughly rinsed with dichloromethane prior to use. Vials were kept capped while flasks and concentration tubes were covered with cleaned aluminium foils to avoid direct sunlight and airborne contamination of containers.
Samples were tested by a validated and accredited method (EN ISO/IEC 17025) routinely used for PCDD/Fs, DL-PCBs, NDL-PCBs and PBDEs analysis in food and, successfully tested in a number of inter-laboratory studies.
This method was slightly modified in the automated clean-up procedure and instrumental analysis to optimize the performance for the determination of PBDD/Fs. Before analysis all samples were spiked with the specific PCDD/Fs, PCBs, PBDEs and PBDD/Fs standard solutions, a mixture of $^{13}C_{12}$-labelled congeners. All samples (except milk) were extracted by accelerated solvent extraction (ASE) using an ASE 350 Thermo Scientific Dionex (Sunnyvale, California, USA) instrument with a mixture of n-hexane and acetone 80:20 (v/v). An ethyl alcohol and ammonia solution was first added to the milk samples, and then fat was extracted by a mixture of diethyl ether and petroleum ether 1:1 (v/v). After solvent evaporation, gravimetric lipid determination was performed. The extracted fat was dissolved in hexane and the clean-up procedure was carried out in two steps. First a double liquid-liquid partitioning process with sulphuric acid was performed to remove the lipid component. Then the extract was dissolved in hexane and purified by means of an automated clean-up process with a Power-Prep® system (Fluid Management System, Massachusetts, USA) using disposable columns (multilayer silica, alumina and carbon). The fraction containing PCBs and PBDEs was collected after elution from the alumina column (140 ml hexane:dichloromethane 95:5, v/v), while the fraction containing PCDD/Fs and PBDD/Fs was eluted from the carbon column (80 ml toluene).
The two fractions were concentrated, first under vacuum and then under nitrogen stream and the remainders were dissolved in the corresponding recovery standards solutions ($^{13}C_{12}$-labelled congeners). Separate runs were performed for each class of contaminants. A laboratory blank and a control sample were analysed for each batch of samples.

Instrumental analysis

Aliquots of 1 µL of the final sample extracts were introduced into gas-chromatography/high resolution mass spectrometry (GC/HRMS) system. The GC-HRMS system consisted of a GC Trace Series 2000 coupled with a MAT 95 XP (Thermo Fisher, Bremen, Germany).
The GC/MS interface was set to 280°C. Injections were made with a split/splitless injector operated in splitless mode for PCDD/Fs, PCBs and PBDEs and surge splitless mode for PBDD/Fs. In the surge splitless injection a pressure of 100 kPa was applied for the 4.0 minutes injection (splitless) time. The target compounds moved through the inlet rapidly thus reducing the time to interact with the inside walls of the liner and minimised the eventual amount of breakdown products for brominated compounds.
PCDD/Fs and PBDEs were separated on a DB-5 MS capillary column (60 m x 0.25 mm, 0.10 µm film thickness (J&W Scientific, California, USA) while a shorter DB-5 MS capillary column (15 m x 0.25 mm, 0.10 µm) was used for PBDD/Fs. The determination was carried out by high resolution mass spectrometry (HRMS), at a resolution of 10000 operating with electron ionisation (EI) at 40 eV, in the selected ion monitoring (SIM) mode. PCBs were separated by HRGC on a HT-8 capillary column (60 m x 0.25 mm, 0.25 µm film thickness, SGE Analytical Science Pty, Ltd. Victoria, Australia) and determined by HRMS, in the same operating conditions adopted for PCDD/Fs.

Quantification

For PCDD/Fs and DL-PCBs toxic equivalent (TEQ) values were calculated using the World Health Organization Toxic Equivalency Factors established in 2005 (WHO-$TEFs_{05}$). As suggested by van de Berg et al.[5] the TEFs for human risk assessment of chlorinated analogues have been used for PBDD/Fs. For NDL-PCBs and PBDEs the analytical sum of six and nine congeners was calculated. WHO-TEQs and the sum of six NDL-PCBs and nine PBDEs were expressed as upper bound (UB) concentrations, assuming that all values of specific PCDD/F, PBDD/F, PCB and PBDE congeners below the limit of quantification (LOQ) are equal to their respective LOQ.

Results and discussion

Results of chemical analysis of PCDD/Fs, DL-PCBs, and PBDD/Fs, expressed as WHO-TEQ_{05} on a whole weight basis (pg WHO-TEQ/g), NDL-PCBs and PBDEs, expressed as sum of the congeners analysed (ng/g), are shown in Table 1.

PCDD/Fs, DL-PCBs and NDL-PCBs

Contamination levels of PCDD/Fs and DL-PCBs in fish ranged from 0.00329 to 0.273 and from 0.0240 to 3.54 pg WHO-TEQ/g fat respectively while, in mussels ranged from 0.250 to 2.30 and from 0.615 to 5.43 pg WHO-TEQ/g respectively. Regarding NDL-PCBs, contamination levels were in the interval 0.190-17.9 ng/g for fish and 9.32-95.7 ng/g for mussels. In general, mussels were more contaminated than fish samples. This result is consistent with the presence of a large industrial area in Taranto.[6,7] Additionally, the contamination in mussels showed a seasonal trend with highest levels in summer. This results may be explained by the life cycle of mussels, in fact in summer the bivalves species reach sexual maturity increasing the filtering capacity and the growth of lipid content in the body.

Table 1. Occurrence levels of PCDDD/Fs, DL-PCBs, NDL-PCBs PBDEs and PBDD/Fs in fish and mussel (fresh weight basis).

SPECIES	**PCDD/Fs** (pg WHO_{05}-TEQ/g)	**DL-PCBs** (pg WHO_{05}-TEQ/g)	**PBDD/Fs** (pg WHO_{05}-TEQ/g)	**NDL-PCBs** (ng/g)	**PBDEs** (ng/g)
Sea bream (*Sparus aurata*)	0.0670	0.151	0.0248	2.10	0.396
Sea bream (*Sparus aurata*)	0.0500	0.0900	0.0295	1.52	0.839
Sardina (*Sardina pilchardus*)	0.0730	1.19	0.0444	11.5	0.456
Sardina (*Sardina pilchardus*)	0.174	0.798	0.0312	5.94	0.453
Sardina (*Sardina pilchardus*)	0.0380	0.655	0.0295	8.12	0.322
Sand steenbras (*Lithognathus mormyrus*)	0.148	0.402	0.0357	6.73	0.431
Red mullet (*Mullus barbatus*)	0.00329	0.0240	0.0392	0.190	0.334
Mussel (*Mytilus galloprovincialis*)	1.06	1.47	0.0263	25.0	0.232
Mussel (*Mytilus galloprovincialis*)	0.759	0.966	0.0225	17.0	0.175
Mussel (*Mytilus galloprovincialis*)	0.250	1.32	0.0245	14.7	0.276
Mussel (*Mytilus galloprovincialis*)	0.625	1.38	0.0250	15.1	0.245
Mussel (*Mytilus galloprovincialis*)	2.30	5.43	0.0358	95.7	0.203
Mussel (*Mytilus galloprovincialis*)	0.415	3.23	0.0228	20.5	0.280
Mussel (*Mytilus galloprovincialis*)	0.317	0.945	0.0337	9.32	0.197
Mussel (*Mytilus galloprovincialis*)	0.773	1.49	0.0255	27.4	0.268
Mussel (*Mytilus galloprovincialis*)	0.386	0.615	0.0600	12.0	0.184
Mullet (*Mugil cephalus*)	0.148	0.981	0.0488	15.9	0.642
Hake (*Merlucius vulgaris*)	0.130	0.333	0.0496	2.84	0.305
Bogue (*Boops boops*)	0.135	2.36	0.0871	17.9	0.379
Mackerel (*Scomber scombrus*)	0.0250	3.27	0.0312	3.89	0.723
Mackerel (*Scomber scombrus*)	0.273	3.54	0.0562	14.2	1.03

PBDEs

PBDEs were detected in all the fish and mussel species. Higher levels of PBDEs were observed in mackerel and sea bream (1.03and 0.839 ng/g respectively) compared to the other species, with the lowest levels being observed in mussels (mean value 0.229 ng/g). In general, low and mid-brominated congeners (BDE# 47, 99, 100) were most abundant in fish. The predominance of BDE-47, 49, 99 and 100 and to a lesser extent BDE-154 is similar to that observed in other studies.[8]

PBDD/Fs

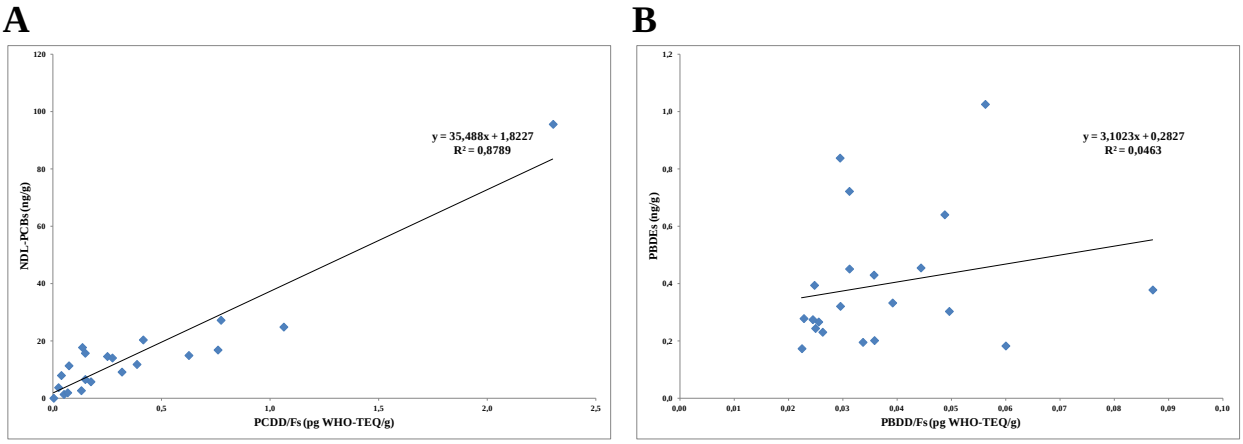
In general, relatively low contamination levels were recorded among species with a high proportion of congeners below the LOQ. Tetra and hepta-brominated dioxins and furans were also detected in a number of samples. Other non-2,3,7,8 bromo substituted congeners were also observed although due to lack of standards (except for 2,4,6,8-TBDF) it was not possible to quantify these.

Correlations

Comparing PCDD/Fs and DL-PCBs WHO-TEQs, and the NDL-PCBs sum among different species, data showed a good overlap, in particular mussels where the levels are higher and the contribution of not detected congeners is negligible. As an example, Figure 1A shows the relationship between the levels of PCDD/Fs and NDL-PCBs.

Considering the brominated compounds, no correlation in the levels of PBDD/Fs WHO-TEQ and PBDEs sum were found (Figure 1B); however these outcomes could be influenced by the low contamination levels of PBDD/Fs and more data should be collected for further clarification.

Figure 1. Correlation between PCDD/F-TEQ and NDL-PCB sum (A), and PBDD/F-TEQ and PBDE sum in fish and mussel samples



This study represents the first survey on the occurrence of brominated dioxins in foods from Italy. A larger number of different food types will be analysed for this set of contaminants and, this data will allow a more realistic estimate of human dietary intake and provide a better assessment of the health risk arising from the ingestion of these contaminants.

Acknowledgements

This work was funded by the Italian Ministry of Health (Grant IZSAM 08/13 RC).

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