Brominated and Chlorinated contaminants in Food (PCDD/Fs, PCBs, PBDD/Fs PBDEs): Simultaneous Determination and Occurrence in Italian Produce

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HIGHLIGHTS

- Simultaneous, validated, determination of PCDD/Fs, PCBs, PBDEs & PBDD/Fs in food
- PCB TEQ was greater than PCDD/F or PBDD/F TEQ, in most marine/animal products
- No correlation was seen between chlorinated and brominated contaminant occurrence

Abstract

Validated methodology for the simultaneous determination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/Fs) and polybrominated diphenyl ethers (PBDEs) in foods of animal origin is presented. Method performance indicators were equivalent or better than those required for the control of EU regulated (EU 2017/644) PCDD/F and PCB congeners in these foods, and for risk assessment through dietary intake. The method uses a high (>90 %) proportion of ^{13}Carbon-labelled surrogates for internal standardisation combined with high resolution mass spectrometry that allow accurate quantitation, and this was confirmed by multiple successful participations in proficiency testing for PCDD/Fs, PCBs and PBDEs in food. The same validation and method performance requirements as used for PCDD/Fs were followed for PBDD/Fs. The analysis of a range of food samples (eggs, milk, fish, shellfish, pork, beef and poultry), showed the occurrence of all four classes of contaminants at varying concentration ranges. In general, PCBs were the most prominent contaminant, both, in terms of dioxin-like toxicity, as well as in the occurrence of non-dioxin-like congeners, an observation that concurs with those made in other studies on Italian foods. The levels of PCDD/F and PCB occurrence are consistent with a gradual decline in contamination as reported by some other similar studies. Although all the determined contaminants were detected in the sampled foods, there was poor correlation between the occurrences of the brominated and chlorinated contaminants, and between PBDEs and PBDD/Fs, but better associations were observed between the occurrences of the chlorinated contaminants.

1.0 Introduction

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) along with polychlorinated biphenyls (PCBs) are now widely recognised and well-studied environmental and food contaminants. Lateral chlorine substitution on PCDD/F molecules initiates a range of biological effects (van den Berg et al., 1994; Mandal, 2005) from lethality in rodent offspring at concentrations as low as 50 ng/kg/day (Bell et al., 2007), to teratogenesis, reproductive effects, carcinogenesis, thymic atrophy, immunotoxicity, etc. Many of these pleiotropic responses are mediated through the ability of these compounds to bind potently to the aryl hydrocarbon receptor, which among other effects, promotes early pre-carcinogenesis (Denison et al., 2003; Moennikes, et al., 2004). A number of PCB congeners, in particular, those with a predominance of laterally substituted chlorines, elicit similar toxic responses as those listed for PCDD/Fs and are referred to as dioxin-like PCBs (DL-PCBs). Additionally, polybrominated dibenzo-p-dioxins and dibenzofurans (PBDD/Fs) which are brominated structural analogues of the PCDD/Fs also show similar toxic responses in humans and animals (WHO, 1998; Birnbaum et al., 2003; Samara et al., 2009; Wall et al., 2015). However, despite the commonality in the biological effects observed, there are important differences in the current state of knowledge on these three groups of closely related contaminants. PCDD/Fs and PCBs are regulated in some regions and countries, such as Japan, the US and the EU (US-EPA, 1976; MEJ, 1999; European Commission, 2011), and a number of other food exporting countries such as Turkey and Brazil have monitoring facilities for these contaminants. The regulation also extends to non-dioxin-like PCB congeners (NDL-PCBs) that show other effects such as phenobarbitone-type induction and endocrine disruption (Denomme et al., 1983; McGovern, 2006) and are also commonly seen as an indicator of the total PCB content. Regulation in some regions and the increased awareness of control on PCDD/Fs and PCBs has resulted in increased monitoring and a larger volume of data on these contaminants. On the other hand, despite increasing documentation (Fernandes et al., 2008, 2014; Fernandes and Falandysz, 2021; Hagburg et al., 2011; Pratt et al., 2013; Zacs et al., 2016; Diletti et al., 2020; Deshmukh et al., 2020) on the occurrence of PBDD/Fs in a range of everyday foods and in human tissues, levels in food are not regulated.

Another persistent organic pollutant (POP), the polybrominated diphenyl ethers (PBDEs), has also come to prominence in the last two decades. PBDEs were volume manufactured industrial chemicals that were used for fire-retarding a wide range of industrial, transport and household goods, until their use was restricted in the early 2000s, because of increasing awareness of their toxicity. PBDEs are now known to target the brain, and neurotoxic effects such as delayed cognition and motor skills in humans and rats, and synaptic plasticity in mice have been reported (Kodavanti et al., 2005; Dishaw et al., 2014), as well as effects on thyroid regulation and reproduction. There is global awareness of the need to minimise human exposure to these three classes of contaminants as PCBs and PBDEs are listed in Annex A, and PCDD/Fs (and PCBs) are listed in Annex C, of the Stockholm Convention as undesirable POPs whose inadvertent production should be controlled. On the other hand, relatively little is known about the occurrence of PBDD/Fs in the environment or biota, despite having similar mechanisms of inadvertent by-production during the manufacture of industrial chemicals, or formation during incineration processes, as the PCDD/Fs (Fernandes and Falandysz, 2021). Biogenic formation of some congeners, through enzymatically (bromoperoxidases) mediated processes involving natural precursors such as bromophenols has been documented (Haglund et al., 2007; Haglund, 2010), but contamination from these is seen primarily in marine species such as shellfish, sponges and to a lesser extent, fish (Fernandes et al., 2009; Fernandes and Falandysz, 2021).

To some extent, this lack of information on PBDD/Fs may arise from the lower levels of reported occurrence – in comparison to the PCDD/Fs, there are very few publications on food occurrence and human exposure – and these, in most cases report lower concentrations than PCDD/Fs. However, not all of the relevant PBDD/F congeners are reported (standards for some congeners are still not commercially available), which contributes to the lower levels observed. There is however, another and more practical reason for the scarcity of data, and this relates to analytical methodology. In addition to the absence of some analytical standards, the analysis of PBDD/Fs requires more care than PCDD/Fs, particularly during the measurement stage, where their tendency to adsorb on the internal surfaces of the instrumentation can result in poorer analytical sensitivity (Fernandes and Falandysz, 2021).

It is necessary from a risk assessment point of view to have occurrence data in foods for all of these contaminants in order to estimate the dietary intake of different population groups. The monitoring of food samples to allow these estimates needs to be done with care so that common food types and consumption patterns are represented and this carries the burden of cost, as does the separate analysis of these four groups of contaminants. This burden can be reduced significantly if the analyses were combined. PCDD/Fs and PCBs are commonly analysed within the same analytical method (Fernandes et al., 2004; CEN, 2012; L'Homme et al., 2015; Diletti et al., 2018; Kedikoglou et al., 2018), and in recent years some methods have demonstrated the ability to include PBDEs within this methodology. However, the ability to determine all four sets of contaminants simultaneously is rare and represents expediency as well as cost savings. It also minimises the inherent variability in data that arises from using different procedures or different laboratories to analyse the same samples.

This study aimed to investigate the occurrence of PCDD/Fs, PBDD/Fs PCBs and PBDEs in foods produced in different regions of Italy. As these contaminants have been reported to occur in foods of animal origin (Scortichini et al., 2004; Diletti et al., 2018), 60 samples, including eggs, meat, milk, fish and mussels were selected for monitoring. A well-validated methodology for the analysis of PCDD/Fs and PCBs that has been used successfully in a range of different monitoring projects over many years (Diletti et al., 2007, 2018) was used as the basis for method development. The method was adapted to include PBDEs and PBDD/Fs, and the basic analytical characteristics of the method with respect to the new analytes was established. The method was then validated and successfully applied to the determination of PCDD/Fs, PCBs, PBDD/Fs and PBDEs in the selected food samples. The data generated will provide an update (PCDD/F and PCBs) as well as a baseline for future monitoring of the levels of these contaminants in Italian foods.

2.0 Experimental

The analytical methodology used for the determination was based on isotope dilution (or internal standardisation, where labelled surrogates were not available), using $^{13}C_{12}$ labelled surrogates. The analytes were isolated by oxidative degradation of the food matrices, chromatographically purified, concentrated and measured by high resolution gas chromatography coupled to high resolution mass spectrometry (HRGC-HRMS). The methodology incorporated a high level of automated handling through all stages.

2.1 Samples

As part of the ongoing surveillance of the food supply in Italy, a set of 61 samples of food that are routinely consumed were analysed using the newly developed methodology. This initial stage of testing concentrated on the type of foods that were known to show the occurrence of some of these contaminants, from earlier studies (Scortichini et al., 2004; Diletti et al., 2018). Thus foods of marine origin such as shellfish and fish as well as foods obtained from terrestrial animals such as eggs (n=6), poultry (n=5), pork (n=6), beef (n=6) and milk from cows (n=6) and sheep (n=4) were selected. Other foods which tend to contain lower PCDD/F and PCB concentrations, such as bread, cereals, fruit and vegetables etc. will be investigated in later studies. The samples of terrestrial animal products were collected by the veterinary service from all regions of Italy and included samples from individual farms and breeders, slaughterhouses, and food processing sites between January 2014 and February 2016.

Of the marine products, mussel samples (n=9) were collected from mussel farms in the Taranto area which form part of one of the largest shellfish aquaculture sites in Europe. Fresh fish (n=19) were sourced from the Mediterranean Sea coasts of Abruzzo, Apulia and Sicily and included hake, mullet, sea bream, bogue, red mullet mackerel, sardines and sand steenbras.

2.2 Chemicals

Toluene, dichloromethane, isooctane, ethyl ether, hexane, petroleum ether and ethanol were obtained as analytical grade solvents from Honeywell Burdick & Jackson in Seelze, Germany, as were other reagents such as sulphuric acid, ammonia solution, anhydrous sodium sulphate and sodium chloride. A Purelab option-Q system (ELGA, High Wycombe, Bucks., U.K.) was used to generate ultra-pure water on demand within the laboratory.

2.3 Analytes and reference standards

Primary standards for the individual brominated and chlorinated analyte groups that were targeted, were prepared by the dilution and combination of commercial standards which were obtained as certified concentration solutions, either from Cambridge Isotope Labs, MA, USA, or from Wellington Laboratories Inc. Ontario, Canada. In support of the isotope dilution technique, the majority of the standards (53 out of 58) were also obtained as $^{13}C_{12}$ surrogates and these are marked in bold in the listing below:

Brominated analytes: Eight PBDE congeners: IUPAC numbers, **BDE-28, BDE-47**, BDE-66, BDE-85, **BDE-99, BDE-100, BDE-153, BDE-154, and BDE-183.**
Thirteen 2,3,7,8-bromo substituted PBDD/F congeners: **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**, **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**.

Chlorinated analytes: The **seventeen 2,3,7,8-chloro substituted PCDD/Fs**, twelve dioxin-like PCBs (**CB-77, CB-81, CB-126, CB-169, CB-105, CB-114, CB-118, CB-123, CB-156, CB-157, CB-167 and CB-189**) and

six NDL-PCBs (**CB-28, CB-52, CB-101, CB-138, CB-153, CB-180**) that are regulated in European commission regulation No 1259/2011 (European Commission, 2011).

2.4 Analytical Methodology - Extraction and purification

Normal precautions for avoiding contaminations involved the cleaning and rinsing of extraction and purification equipment and glassware with dichloromethane prior to use. Additionally, in order to prevent photo-degradation of the brominated analytes and airborne contamination in general, all flasks and concentration tubes were covered with cleaned aluminium foil.

All samples apart from milk, were cut into small segments where required and homogenised to yield representative portions. Aliquot weights equivalent to 1 to 5 g of fat (depending on the sample) were fortified with the internal standard solutions (equivalent to 0.2 - 0.4 ng of PCDD/Fs, 1.0 ng of DL-PCBs, 1.0 ng of NDL-PCBs, 0.8 ng of PBDEs and 0.4 - 3.0 ng of PBDD/Fs). Diatomaceous earth (amount equivalent to between one third to a half of the sample weight) was added to those aliquots with a high moisture contents, mixed and allowed to equilibrate before extraction. An ASE 350 Thermo Scientific Dionex (Sunnyvale, California, USA) accelerated solvent extraction system using a mixture of n-hexane and acetone 80:20 (v/v) was used to extract the samples (three extraction cycles at a temperature of 125 ^{0}C and a pressure of 1500 psi). Aliquots of the milk samples (up to 200 ml depending on the fat content) which were similarly fortified with the internal standard solutions, were mixed with an ethyl alcohol and ammonia solution and the fat was extracted using 300 ml of a mixture of diethyl ether and petroleum ether 1:1 (v/v). Extracts for all samples were filtered through anhydrous sodium sulphate and rotary evaporated to exclude the extraction solvent followed by gravimetric determination of the lipid content.

In the first stage of the purification process, the extracted lipid was dissolved in 40 ml hexane and partitioned against 40 ml of concentrated H_2SO_4 in a separation funnel. The partition was repeated until the aqueous layer was no longer coloured. The extract was washed with 40 ml of a 5% NaCl solution to remove any residual acid and also to prevent the formation of emulsions. It was then purified on an automated Power-Prep^{TM} system (Fluid Management System (FMS) Massachusetts, USA) using multilayer silica, activated carbon and alumina columns (obtained directly from FMS). The three columns, first alumina then carbon (which was eluted in forward and reverse directions) and finally silica were conditioned using 170 ml hexane. The sample was loaded into the system, passing firstly through the silica column where any residual co-extractives were hydrolysed, then directly through to the alumina column where the targeted contaminants were trapped. 140 ml of hexane:dichloromethane 95:5, v/v, was used to elute a fraction (A), which contained DL-PCBs, NDL-PCBs and PBDEs (except for BDE 28) from the alumina column. A mixture of hexane:dichloromethane 50:50, v/v was used to transfer the remaining contaminants from alumina to the carbon column in the forward direction. The first 30 ml were discarded while the following fraction (50 ml) which included BDE 28 was added to fraction A. A further 200 ml completed the transfer from alumina to carbon.

The carbon column was then reverse eluted with 80 ml toluene to recover fraction B which contained the PCDD/Fs and PBDD/Fs. Both fractions, A and B, were concentrated, initially under vacuum to approximately 1 ml and transferred to vials. The final concentration to incipient dryness was carried out under a gentle stream of nitrogen, followed by re-dissolution in 20 µl of the respective recovery (syringe) standard solutions in isooctane. As recovery standards, $^{13}C_{12}$-1,2,3,7,8-PeBDF and $^{13}C_{12}$-1,2,3,7,8,9-HxBDD were used for PBDD/Fs, while $^{13}C_{12}$-1,3,7,8-TeCDD, $^{13}C_{12}$-1,2,4,7,8-PeCDD, $^{13}C_{12}$-1,2,3,4,6,8-HxCDD, and $^{13}C_{12}$-1,2,3,4,6,7,9-HpCDD were used for PCDD/Fs. For DL-PCBs and NDL-PCBs, $^{13}C_{12}$-PCB 70, $^{13}C_{12}$-PCB 111 and $^{13}C_{12}$-PCB 170 surrogates were used, while for PBDEs, the recovery standards were $^{13}C_{12}$-BDE 77 and $^{13}C_{12}$-BDE 138.

2.5 Instrumental Analysis and Quantitation

The four sets of contaminants were measured in separate instrument sequences, but some conditions were common to all four. All analyses were performed by HRGC-HRMS using a Trace Series 1310 GC, coupled to a DFS (Thermo Fisher Scientific, USA). The procedure used for PCDD/Fs and PCBs has been described earlier in greater detail (Diletti et al., 2007). For all analytes, a volume of 1 µl of the sensitivity standardised sample extracts was injected into a split/splitless injector held at 280°C. The injector was operated in splitless mode for PCDD/Fs, PCBs and PBDEs, and in surge splitless mode for PBDD/Fs. This latter injection mode uses a pressure of 100 kPa over a four minutes splitless injection period. In conjunction with the injector temperature, this causes volatilisation and rapid transfer of the PBDD/Fs through the injector liner reducing interaction with the liner walls and thus minimising any degradation of the analytes during injection. Helium (1 ml/min) was used as the carrier gas for all measurements and the GC/MS interface was set to 280°C.

PCBs were separated on a 60 m x 0.25 mm (0.25 µm film thickness) HT-8 capillary column (SGE Analytical Science, Victoria, Australia), while the separation for PBDE and PCDD/F congeners was made using a DB-5 MS capillary column (60 m x 0.25 mm, 0.10 µm film thickness) supplied by J&W Scientific, California, USA who also supplied the shorter DB-5 MS capillary column (15 m x 0.25 mm, 0.10 µm film thickness) that was used for PBDD/F separation. The oven temperature control programmes were as follows:

- PCBs: 120°C for 0.5 min, ramped to 180°C at 20°C/min, ramped to 260°C at 2°C/min, ramped to 300°C at 5°C/min, ramped to 310°C at 1.6°C/min.
- PBDEs: 140°C for 2 min, ramped to 315°C at 11°C/min, held at 315°C for 7 min.
- PCDD/Fs: 120°C for 2 min, ramped to 220°C at 10°C/min, held at 220°C for 11 min, ramped to 235°C at 3°C/min, held at 235°C for 5 min, ramped to 315°C at 3.5°C/min.
- PBDD/Fs: initial hold at 100°C for 4 min, ramped to 200°C at 40°C/min, held at 200°C for 3.5 min, ramped to 320°C at 10°C/min, held at 320°C for 2 min.

The mass spectrometer was tuned to a resolution of 10,000 using perfluorotributylamine (FC-43) and high boiling perfluorokerosene (PFK) to calibrate the mass axis for chlorinated and brominated analytes, respectively. Mass filtering in the selected ion monitoring (SIM) mode was carried out using electron ionisation (EI) at 50 eV. For each of the analyte groups, the two most intense ions in each molecular ion cluster were measured for each ^{13}C-labelled and native homologue group. Further details of the conditions used for monitoring have been reported for individual analytes (Diletti et al., 2007, 2020)

In all cases the mass spectrometric data was imported to Excel™ for calculation of analyte amounts in each of the samples followed by conversion to concentration data. Toxic equivalents (TEQs) for PCDD/Fs DL-PCBs were calculated using the World Health Organization Toxic Equivalency Factors (WHO-TEFs) (van den Berg et al., 2006). The PCDD/F TEF values were also used for the analogous PBDD/F congeners that were measured. For PBDEs and NDL-PCBs, congener concentrations were summed, and all reported concentrations and TEQ values were expressed in lower and upper bound terms (upper bound includes the limit of quantitation (LOQ) values for those congeners that were below the LOQ; lower bound excludes these values).

2.6 Quality Control and Method performance parameters

PCDD/F, DL-PCB and NDL-PCB analyses of food within the EU are required to follow regulated analytical guidelines (European Commission, 2017) with quality control requirements and these requirements were followed for these analyses. Similarly, harmonised analytical criteria (Fernandes et al., 2022) supported by a working group on brominated contaminants were followed to ensure reliable and validated PBDE measurements. The regulation and criteria specify the use of HRMS, use of ^{13}C labelled surrogates or internal standards for quantitation and control of analytical recovery with

limits for satisfactory performance. They also provide specifications on sensitivity, trueness and precision and guidance on estimation of LOQs through the use of procedural blanks, as well as the estimation of measurement uncertainty (MU). All of these conditions were followed for the analysis of PCDD/Fs, DL-PCBs, NDL-PCBs and PBDEs and the available PT (proficiency testing) for these analytes (in multiple tests conducted by the Norwegian institute of Public Health and European Union reference laboratory) has shown good performance with Z-scores below 2.

However, there are no specific guidelines for PBDD/F analytical method performance, so the same validation and method performance requirements as used for PCDD/Fs were followed. The performance parameters such as linearity of measurement, LOQ, precision, recovery and MU were very similar to other contemporary studies on PBDD/Fs (Zacs et al., 2016; Fernandes et al., 2008, 2009, 2018, 2019).

Procedural blanks and reference materials (BCR-607 milk powder, food samples with consensus or assigned analyte values from previous PTs and an in-house reference material "fortified bovine fat") were also analysed with every batch of samples. The performance of the method for all analytes is summarised in Table 1 which lists the validation parameters and the performance achieved for these.

3.0 Results and Discussion

There are a number of benefits in simultaneously determining PCDD/Fs, PCBs, PBDD/Fs and PBDEs in the same sample of food – all are regulated or near-regulated contaminants which require to be monitored, there are obvious savings in cost of sampling and analysis, and from a human exposure point of view, the similarity in effects for the dioxin-like contaminants allows a fuller risk assessment through dietary intake. The existing comprehensive criteria for reliable determination of PCDD/Fs, PCBs and PBDEs (European Commission, 2017; Fernandes et al., 2022) are also an advantage - due to the similarities in chemical structures (all are stable, halogenated, diaromatic molecules), environmental and toxicological behaviour, levels of occurrence and methods of determination, the criteria may be applied, with some modification, to all four sets of contaminants. It was on this basis that the method was validated, for initial investigation of contaminant occurrence, but more importantly, for cost-effective and expedient future monitoring studies.

3.1 Analytical Method performance

The main parameters of method performance that were evaluated were the LOQs, the analytical recovery, precision and accuracy, the dynamic range of reliable measurement and estimation of MU. The commercial availability of a relatively large number of surrogate ^{13}C labelled standards across all four analyte groups was exploited advantageously. The extensive use (labelled standards were used for 53 out of 58 analytes) allows an inherent ability to control and measure method performance. The combination of this resource with the sensitivity and specificity of HRMS, results in reliable data that underpins risk assessments based on dietary intake.

The limits of quantitation were evaluated in conjunction with the most recent guidelines for determining this parameter (European Commission, 2017B), and incorporate daily instrument performance along with current procedural blank concentrations. The range of LOQ values that were achieved are listed in Table 1. For the regulated contaminants (PCDD/Fs and PCBs), these values are in agreement or better than the limits required for regulatory requirements. For PBDEs and PBDD/Fs, the range of LOQs achieved was similar or better, depending on the congener, than that achieved in other current literature. The intermediate precision based on replicate analysis of the same matrices at different times was found to lie within 20% for all contaminant groups. The concentration ranges chosen for establishing linearity of measurement for all contaminants were based on the observed levels of food occurrence in the literature, but were also guided by the maximum limits specified in the regulations for PCBs and PCDD/Fs. The analytical recovery of the ^{13}C labelled internal standards was measured against the recovery (syringe) standards added just prior to measurement and ranged

typically from 50 - 90% for all analytes. However, in the case of OBDD/F, the lower limit of recovery was much lower (~15%) due to the greater lability of these molecules and this also resulted in higher LOQ values. The estimation of MU was based on the recommended criteria described in the guidance document (European Commission, 2017C), and used the empirical approach. For individual analytes, MU ranged between 18 – 46% for concentrations within the working range, but was found to increase at lower concentrations, particularly at levels approaching the LOQ. As reported for the same set of analytes, MU could be as high as 200% at very low concentrations approaching the limits of detection (Fernandes et al., 2018, 2019). For summed parameters such as PCDD/F and DL-PCB WHO-TEQs and the sum of six NDL-PCBs, the MU was of the order of ± 20%.

3.2 Occurrence levels in food

The levels of occurrence of PCDD/Fs, PBDD/Fs, PCBs and PBDEs determined in the food samples are summarised in Table 2. The units used to express the concentrations follow conventional reporting in the literature, thus allowing easy comparability between studies. The units are also in agreement with regulatory requirements for PCDD/Fs and dioxin-like PCBs and are reported in toxic equivalents of pg/g lipid (apart from fish and shellfish which are reported in pg/g whole weight, as required). As PBDD/Fs show a similar mode of toxicity, the same units have been used as the other dioxin-like compounds. The PBDD/F TEQ values were calculated using the analogous PCDD/F TEF values that have been used in other studies (Fernandes et al., 2009, 2014, 2018, 2019; Pratt et al., 2013; Zacs et al., 2016). As noted in some of these studies, this approach assumes a wide margin of error on the TEQ and is seen as an interim measure for any human risk assessment (van den Berg et al., 2013; Fernandes and Falandysz, 2021). Non-dioxin-like PCBs and PBDEs are reported as the sum of the concentrations of the relevant congeners in ng/g. In agreement with European regulation (European Commission, 2011), the concentrations for NDL-PCBs are given on a lipid weight basis for the animal products (eggs, milk and meat) and on a whole weight basis for fish and shellfish. For PBDEs, concentrations for all foods are reported on a whole weight basis as per convention and recommendations (Fernandes et al., 2022).

The chlorinated contaminants, PCDD/Fs and PCBs were detected in all the studied samples at varying frequencies and magnitudes. PCBs were more frequently detected and at higher TEQ concentrations in most of the measured food groups. The mean values ranged from 0.05 to 1.51 pg PCB TEQ/g fat for the animal products and 0.9 to 2.0 pg PCB TEQ/g whole weight for the marine products. The differences between upper and lower bound values were negligible at < 5%, easily meeting a key requirement for good quality of data (European Commission, 2017). These differences for PCDD/F TEQ were similarly low for the marine products, but considerably higher for the animal products (e.g. 0.02 vs 0.05 pg TEQ/g fat for pork), perhaps reflecting the lower levels of PCDD/F contamination. Mean values ranged from 0.05 to 0.3 pg PCDD/F TEQ/g fat for the animal products and 0.1 to 0.81 pg PCDD/F TEQ/g whole weight for the marine products. Apart from a single sample of mussels which was taken from an aquaculture farm near the industrial area of Taranto, a location of known PCDD/F and PCB contamination (Diletti et al., 2020), all samples were within the regulatory limits specified for these contaminants (European Commission, 2011) within the EU. Mean TEQs for the PBDD/Fs ranged from 0.15 to 0.48 pg/g fat upper bound for the animal products and 0.03 to 0.05 pg/g whole weight for shellfish and fish. These were comparable to PCDD/F TEQs, although the difference between upper and lower bound values were considerably greater. This difference arises despite the low LOQs which were within the same range as the PCDD/Fs and is primarily due to the higher TEF values associated with some PBDDs that tend to occur at very low levels in foods or are not detected. The resulting consequence on the expression of the TEQ is a large difference between upper and lower bound estimates, even if the analytical method is very sensitive as has been noted in the literature (Fernandes and Falandysz, 2021). The other reason for lower PBDD/F TEQ values is that these are underestimated because not all congeners that contribute to the TEQ can currently be measured because of the non-availability of the full set of PBDD/F standards. In relative terms, DL-PCBs made the highest contribution to the summed TEQ as seen in Figure 1, with generally, lower levels of contribution from

PCDD/Fs and PBDD/Fs, although these varied depending on food group. This proportion may change depending on the outcome of the re-evaluation of PCB TEQ (EFSA 2018) currently underway, and is likely to increase for PBDD/Fs when all relevant TEQ-contributing congeners are reported. The proportion may also change if more specific relative potency factors are used to estimate the PBDD/F TEQ (Fernandes and Falandysz, 2021).

As mass produced and extensively utilised chemicals, PBDEs and PCBs, particularly the NDL-PCBs were expected to occur at higher levels in these foods, which is reflected in the units used for reporting. NDL-PCB concentrations ranged from 1.05 to 8.97 ng/g fat in the animal products and 7.25 to 28 ng/g whole in the marine products, with no differences between upper and lower bound values as all analytes were detected. PBDE occurrence was lower than the NDL-PCBs, with upper bound values ranging from 0.03 to 0.18 ng/g and 0.23 to 0.38 ng/g whole in animal and marine products respectively, for the sum of the nine measured PBDE congeners.

3.3 Comparative evaluation with recent data.

In recent years, there have been reports of a trend to lower concentrations in some of these contaminants such as PCDD/Fs and PBDEs (Fernandes et al., 2014, 2018; Bramwell et al., 2017 Lupton et al., 2017), so it is important to view the data from this study in relation to more recent literature. There are a limited number of recent reports on PCDD/Fs and PCBs in food, but these have generally targeted particular foods (Perello et al., 2015., Piskorska-Pliszczynska et al., 2019; Fernandes et al., 2018, Pajurek et al., 2019) or particular causes of food contamination (Lake et al., 2015, Squadrone et al., 2015). Very few have investigated the larger range of foods that are commonly associated with the human diet (Shen et al., 2017; Wang et al., 2017; Diletti et al., 2018; Barone et al., 2019) and even fewer have analysed PCDD/Fs, PCBs, PBDEs and PBDD/Fs in the same foods (Bramwell et al., 2017; Fernandes et al., 2012, 2019).

In general, these studies (Fernandes et al., 2012; Wang et al., 2017; Diletti et al., 2018), show a relatively narrow range of concentrations (0.1 to 0.34 pg/g fat) for PCDD/F TEQ in foods of animal origin i.e. eggs, poultry, milk, beef pork, and a similar range of 0.08 to 0.58 pg TEQ/g whole, in foods of marine origin. These data are consistent with the gradual decline in food concentrations of PCDD/Fs over the last two decades, and for some foods such as fish, eggs, pork, beef, poultry and milk, this is also seen in Italian produce, relative to an earlier study (Fattore et al., 2006) on PCDD/Fs and PCBs which indicates an average decline of up to 65% in concentrations. However, two recent studies (Barone et al., 2019; Shen et al., 2017) continue to show a higher range of PCDD/F concentrations, at 0.79 to 2.41 pg TEQ/g fat in foods of animal origin from Italy and China. The corresponding range reported for DL-PCBs was 0.05 to 0.77 pg TEQ/g fat and 0.21 - 0.58 pg TEQ/g whole for foods of animal and marine origin respectively, is similar if perhaps a little lower than the concentrations observed in this study. Surprisingly, the range for NDL-PCBs in animal products in the present study was considerably lower than those reported (33-53 ng/g fat) by the earlier Italian study (Barone et al., 2019), although the meat products analysed were sourced regionally, rather than the national sampling used in the present study. Notwithstanding, occurrence levels of NDL-PCBs given here are in agreement with results reported in other studies conducted in different European countries (Fattore et al., 2008, Arnich et al., 2009, Sirot et al., 2012, Cimenci et al., 2013, Mihats et al. 2015).

The range of PBDE concentrations reported here are similar to other studies for Italian foods (Martellini et al., 2016) as well as foods from other countries (Fernandes et al., 2016, Bramwell et al., 2017; Boucher et al., 2018). PBDD/F concentrations documented for a range of foods in other studies (Fernandes et al., 2012, 2014; Fernandes and Falandysz, 2021; Bramwell et al., 2017) report detection in all food groups with a similar predominance of PBDFs over PBDDs as seen in this study, as well as a similar range of occurrence. Some food matrices such as shellfish and fish can display the full range of PBDF congeners from tri- to hepta-BDF but only tri- and tetra-BDDs (Fernandes and Falandysz, 2021). This was confirmed in the present study where in the case of shellfish, the occurrence of up to six (out

of seven) measured PBDF congeners was noted, but only two PBDDs (tetra- and hepta-BDD) out of six were detected. The most frequently detected congeners were 2,3,7,8-TetraBDF, 2,3,7,8-TetraBDD as well as 1,2,3,4,6,7,8-hepta-BDD/F. 2,3,4,7,8-PentaBDF, 1,2,3,4,7,8-HexaBDF and OctaBDF were also detected in some samples. In general, this pattern is similar to those reported for PBDD/Fs occurrences in different foods which have been reviewed recently (Fernandes and Falandysz, 2021).

3.4 Relative occurrences between chlorinated and brominated contaminants

Although all of the contaminants discussed here are of anthropogenic origin, the historical periods when these occurred at levels of maximal environmental abundance, were different. PCB and PBDE production were major sources of PCDD/Fs and PBDD/Fs, along with the incineration of chlorine and bromine containing products and wastes. PCBs were manufactured and extensively used during the early and mid-20th century until recognition of their adverse effects during the 1970s and subsequent restrictions on use - in contrast to PBDEs (and other BFRs) which saw peak production and utilisation following fire safety regulation in the latter part of the 20th century, until their manufacture and use was restricted in the US and Europe between 2003 and 2009. More than a million tons of both these chemicals was produced world-wide (Jinhui et al., 2017; Mao et al., 2021). However, the relative occurrences of PCBs and PBDEs in retail food matrices and in human tissues (non-occupationally exposed) shows differences, with PCBs generally occurring to a greater extent than PBDEs (Fängström et al., 2005; Covaci et al., 2008; Schiavone et al., 2010 Fernandes et al., 2012, 2018; Zhang et al., 2013; Tlustos et al., 2013), despite the later production and use of the brominated product. To some extent, the lower occurrence is due to the greater environmental lability of PBDEs through different environmental pathways (e.g. degradation and de-bromination by photo-ionisation in the atmosphere and in surface sediments and water, microbial degradation in soils and sediments, etc.) due to the greater lability of the carbon-bromine bond, but also due to the type of utilisation (open-ended, and additive rather than chemically bonded). In the present study, apart from pork, the occurrence of PBDEs is generally at least an order of magnitude lower than PCBs. Despite the similarity in production volumes and use, there is poor correlation ($r < 0.25$) between occurrences of PCBs and PBDEs (Figure 2C) even in the more contaminated fish/seafood samples. This poor correlation ($r=0.5$) is also observed for PBDEs and PBDD/Fs (Figure 2D) in this study as well as in other studies on marine foods (Fernandes et al., 2009), despite manufactured PBDEs being a major source of PBDD/Fs (Fernandes and Falandysz, 2021). Similarly, no correlation was observed between PBDE inputs and PBDD/F emissions from some combustion processes indicating that in some cases, PBDEs did not act as precursors for PBDD/F formation (Drage et al., 2014). However there is a better association within the chlorinated contaminants ($r=0.82$ for PCB TEQ and NDL PCBs, and $r= 0.73$ for PCB and PCDD/F TEQ – Figure 2 A and B). The associations would have been considerably stronger ($r=0.99$ and 0.93, respectively), but were affected by a set of four samples (three of fish and one of shellfish, all from different locations) that appear to have been more strongly contaminated by PCBs (Fig 2).

4.0 Conclusions

The well-validated methodology described here, allows the simultaneous determination of four sets of contaminants, PCDD/Fs, PCBs, PBDD/Fs and PBDEs in food. Apart from the obvious advantages of the savings in cost and effort arising from separate sampling and analysis, the resulting data would also allow a fuller assessment of dietary intake of dioxin-like toxicity, as well as that of PBDEs. The method validation parameters achieved are similar or better than those specified in analytical criteria for the regulated contaminants (European Commission, 2017), and the same criteria guided the validation of PBDEs and PBDD/Fs.

When applied to the determination of food samples, the data showed that all four classes of contaminants occurred in the foods at different concentration ranges. In general, PCBs were the most prominent contaminants, both, in terms of dioxin-like toxicity, as well as in the occurrence of non-dioxin-like congeners, which agrees with observations that have been made in other recent studies on Italian foods (Esposito et al., 2014; Squadrone et al., 2015; Diletti et al., 2018). In general PCBs made the highest contribution to dioxin-like toxicity in the samples, and although the lowest proportion of dioxin-like toxicity was attributed to PBDD/Fs, not all relevant congeners were measured due to the lack of commercially available standards. Although all of the determined contaminants were detected in the sampled foods, there did not appear to be a correlation in the occurrences of the brominated and chlorinated contaminants despite the similar production volumes of PCBs and PBDEs which are important sources of PCDD/Fs and PBDD/Fs. Better associations were observed between the occurrences of the chlorinated contaminants.

The human exposure to Italian population groups arising from dietary intake of the dioxin-like contaminants has been reported before for PBDD/Fs (Diletti et al., 2020) and for PCDD/Fs and PCBs (Diletti et al., 2018) in conjunction with a wider selection of other foods, in order to allow better estimation of the exposure. These estimations showed that some population sub-groups such as young children, would exceed the revised tolerable dioxin-like dietary intake (of 2 pg/kg bodyweight per week) estimated by EFSA (EFSA, 2018). Continued monitoring of the food supply would therefore be prudent.

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Table 1: Summary of method performance indicators for PCDD/F, PCB, PBDE and PBDD/F congeners achieved during simultaneous determination

Performance Indicator	**PCDD/Fs**	**DL - PCBs**	**PBDD/Fs**	**PBDEs**	**NDL- PCBs**
LOQ[a] (pg/g)	0.001 – 0.01	0.02 – 1.3	0.001 – 0.05; 7[b]	0.16– 15	2.0 – 10
Linearity (pg)	0.05 – 50	0.5 – 800	0.4 – 500	1 – 500	10 – 5000
Precision[a] (%)	9 – 19	5 – 14	7 – 20	6 – 17	6 – 10
Trueness[a] (%)	93 – 107	87 – 116	90 – 120	105 – 113	92 – 109
Recovery range[c] %	60 – 90	60 – 90	50 – 70 [d]	50 – 70	60 – 90
Measurement uncertainty[e] (%)	25 to 45 (at 0.1 to 4.0 pg/g)	22 to 38 (at 10 to 40 pg/g)	29 to 46 (at 1.0 to 4.0 pg/g)	18 to 37 (at 50 to 200 pg/g)	19 to 25 (at 3 to 12 ng/g)

[a]All relevant congeners, unless specified [b]For OBDD/Fs [c]Based on labelled internal standards
[d]Recovery for $^{13}C_{12}$-OBDF and $^{13}C_{12}$-OBDD was lower at ~15%. [e]Expanded uncertainty (95% confidence level)

Table 2: Concentration ranges and mean values of PCDD/Fs, PCBs, PBDEs and PBDD/Fs in Italian foods.

Food Type	Fat	Chlorinated						Brominated				Chlorinated + Brominated TEQ	
		ΣPCDD/F-WHO-TEQ LB	ΣPCDD/F-WHO-TEQ UB	ΣPCB WHO-TEQ LB	ΣPCB WHO-TEQ UB	ΣNDL-PCB LB	ΣNDL-PCB UB	ΣPBDD/F TEQ LB	ΣPBDD/F TEQ UB	ΣPBDE LB	ΣPBDE UB	ΣPCDD/F, PCB, PBDD/F TEQ LB	ΣPCDD/F, PCB, PBDD/F TEQ UB
[1]Units→	(%)	pg/g	pg/g	pg/g	pg/g	ng/g	ng/g	pg/g	pg/g	ng/g	ng/g	pg/g	pg/g
Eggs													
Mean	10	**0.27**	**0.30**	**0.47**	**0.49**	**8.97**	**8.97**	**0.008**	**0.34**	**0.01**	**0.03**	**0.74**	**1.13**
Range		0.05 - 0.62	0.08 - 0.63	0.01 - 1.93	0.04 - 1.93	0.59 - 38	0.59 - 38	<0.001 - 0.05	0.25 - 0.51	<0.01 - 0.03	0.03 - 0.05	0.06 - 2.6	0.37 - 3.07
Poultry													
Mean	11	**0.11**	**0.13**	**0.22**	**0.22**	**2.64**	**2.64**	**<0.001**	**0.21**	**0.07**	**0.07**	**0.32**	**0.56**
Range		0.02 - 0.25	0.04 - 0.26	0.002 - 0.72	0.017 - 0.72	0.31 - 8.89	0.31 - 8.89	N.D.	0.1 - 0.56	0.02 - 0.22	0.02 - 0.22	0.02 - 0.97	0.16 - 1.54
Mussels													
Mean		**0.77**	**0.81**	**2.03**	**2.03**	**28.10**	**28.10**	**0.006**	**0.03**	**0.23**	**0.23**	**2.80**	**2.87**
Range		0.21 - 2.24	0.25 - 2.30	0.94 - 5.43	0.95 - 5.43	9.32 - 96	9.32 - 96	0.002 - 0.01	0.02 - 0.036	0.18 - 0.28	0.18 - 0.28	1.16 - 7.68	1.45 - 8.22
Marine Fish													
Mean		**0.10**	**0.10**	**0.89**	**0.90**	**7.25**	**7.25**	**0.009**	**0.05**	**0.38**	**0.38**	**1.00**	**1.05**
Range		<0.001 - 0.37	0.003 - 0.37	0.022 - 3.54	0.022 - 3.54	0.19 - 20	0.19 - 20	0.001 - 0.03	0.03 - 0.09	0.01 - 1.03	0.03 - 1.03	0.02 - 3.94	0.05 - 4.01
Cows milk													
Mean	4	**0.09**	**0.12**	**0.64**	**0.65**	**2.74**	**2.74**	**<0.001**	**0.48**	**0.04**	**0.04**	**0.73**	**1.25**
Range		0.04 - 0.18	0.07 - 0.2	0.14 - 1.53	0.14 - 1.53	0.63 - 5.09	0.63 - 5.09	N.D.	0.26 - 0.70	0.02 - 0.08	0.02 - 0.08	0.18 - 1.71	0.47 - 2.43
Sheep milk													
Mean	6	**0.26**	**0.29**	**1.51**	**1.51**	**6.21**	**6.21**	**0.006**	**0.26**	**0.03**	**0.03**	**1.77**	**2.06**
Range		0.07 - 0.38	0.09 - 0.41	0.85 - 2.24	0.85 - 2.24	1.09 - 12.9	1.09 - 12.9	<0.001 - 0.02	0.18 - 0.33	<0.01 - 0.05	0.01 - 0.05	0.92 - 2.64	1.12 - 2.98
Beef													
Mean	18	**0.09**	**0.15**	**0.65**	**0.67**	**3.92**	**3.92**	**0.002**	**0.35**	**0.07**	**0.07**	**0.75**	**1.17**
Range		0.002 - 0.15	0.028 - 0.27	0.11 - 1.45	0.13 - 1.45	0.93 - 6.72	0.93 - 6.72	<0.001 - 0.01	0.062 - 0.63	0.03 - 0.21	0.03 - 0.22	0.11 - 1.61	0.22 - 2.35
Pork													
Mean	19	**0.02**	**0.05**	**0.05**	**0.05**	**1.05**	**1.05**	**0.002**	**0.15**	**0.17**	**0.18**	**0.06**	**0.25**
Range		<0.001 - 0.04	0.027 - 0.07	0.004 - 0.17	0.009 - 0.18	0.5 - 1.88	0.5 - 1.88	<0.001 - 0.01	0.06 - 0.40	0.02 - 0.36	0.07 - 0.36	0.004 - 0.22	0.1 - 0.65

[1]Concentrations for PCDD/Fs, PBDD/Fs and PCBs are given on a lipid weight basis for the animal products and on whole weight basis for marine fish and mussels. TEQ values are calculated using WHO-TEF_{2005} values. For PBDEs, levels are reported on a whole weight basis for all food types.

Figures 1 and 2

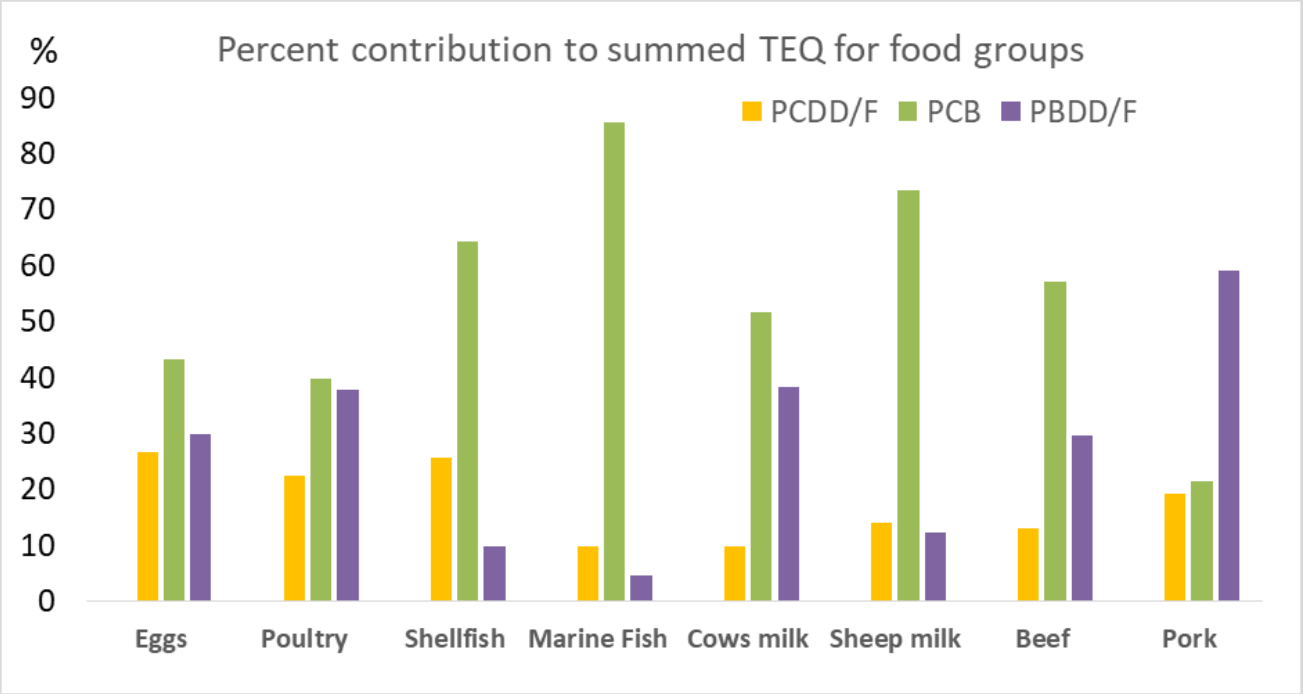

Figure 1: Percentage contribution of the individual classes (PCDD/F, PCB and PBDD/F) of contaminants to the summed dioxin-like TEQ (upper bound basis)

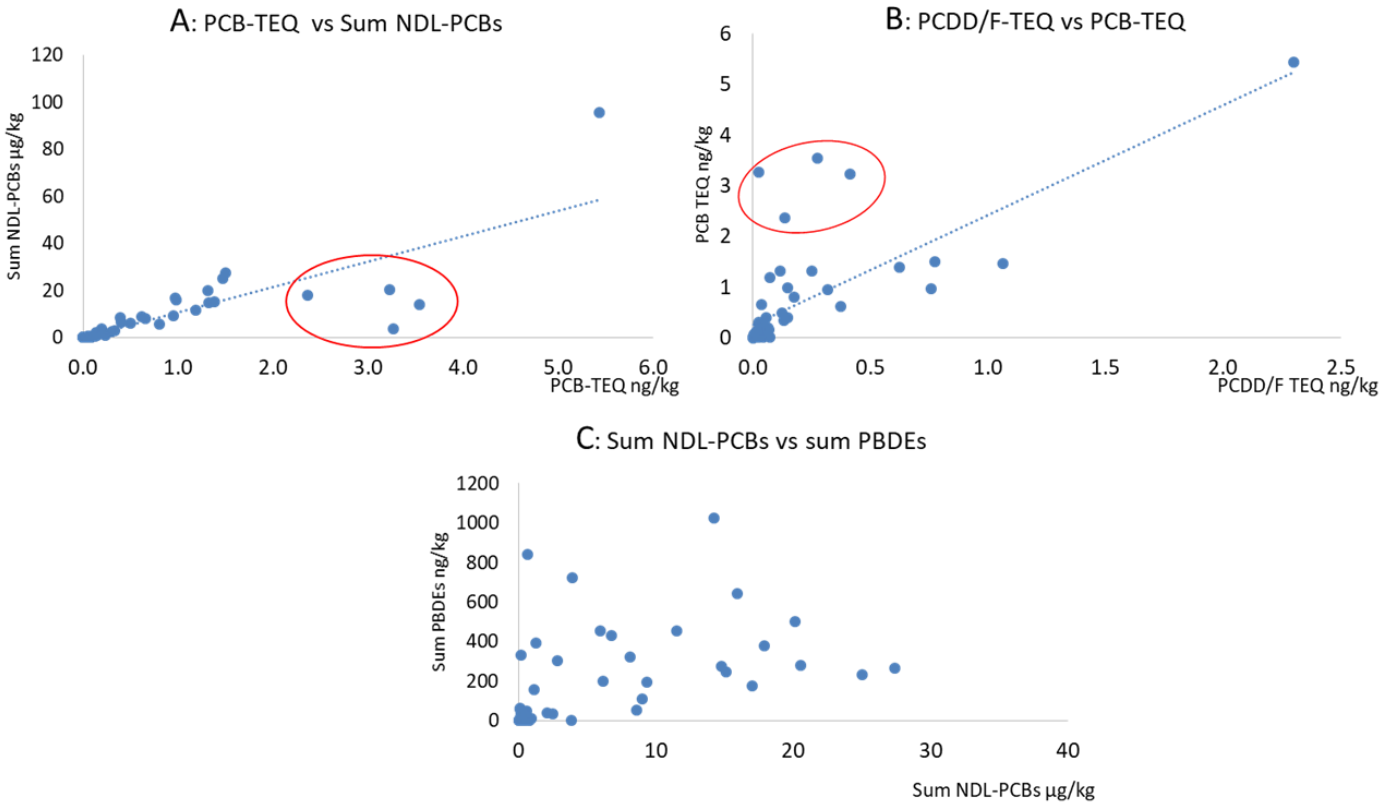

Figure 2: Associations between different contaminant groups in individual food samples. The cluster of the same four samples affecting the correlations in graphs A and B showed high PCB occurrence levels.