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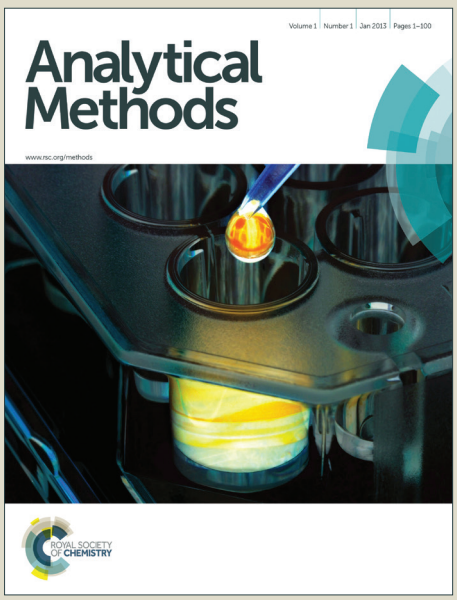

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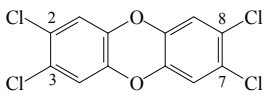

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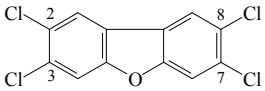

2,3,7,8-TCDF

An isotope dilution method, validated in accordance with specific requirements defined by European Commission, for quantification of 17 polychlorinated dioxins and furans in fishes from Brazil is described.

Method validation and occurrence of dioxins and furans (PCDD/Fs) in fish from Brazil

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An isotope dilution method for quantification of 17 polychlorinated dioxins (PCDDs) and furans (PCDFs) in fish is described. The method uses pressurized liquid extraction (PLE) followed by clean up steps (elution of extracts into two sequential columns filled with distinct stationary phases: acid silica-gel and Florisil®.) and gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) analysis. $^{13}C_{12}$-labelled PCDD/Fs were used as internal and injection standards. The method was validated in accordance with specific requirements defined by European Commission as described in the regulations 2012/252/EU and 2011/1259/EU. The EPA 1613 method was also used as reference. Precision and recovery were evaluated at three levels for each PCDD/PCDF by means of spiked samples. Trueness was checked by analysis of a certified reference material (CRM). The following figures of merit were also assessed: linearity, selectivity, limit of detection (LOD), limit of quantification (LOQ) and measured uncertainty. Consistent results within the guidelines established were achieved for all of the parameters evaluated. Finally, 132 samples of 25 different species of fish were collected from different regions of Brazil over a 13 months period. No sample presented concentration higher than the maximum permitted level. The following dioxins and furans were found in some of these samples: 2,3,7,8-TCDF (19 samples), OCDD (2 samples) and 2,3,4,7,8-PeCDF, 1,2,3,7,8 PeCDD, 1,2,3,4,6,7,8-HpCDF (1 sample each) at levels higher than their LOQs. This validated analytical method has been used by the Ministry of Agriculture, Livestock and Food Supply of Brazil.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are classes of compounds that are always studied together because of their quite similar chemical and toxicological properties. There are 75 PCDDs and 135 PCDFs, but only 7 PCDDs and 10 PCDFs present toxicological concern. These compounds induce a common spectrum of responses, and have a common mechanism of action.[1] The harmful effects in human health are well documented and include a combination of toxic responses, such as embryotoxicity, hepatotoxicity, immunotoxicity, teratogenicity, and carcinogenicity.[2] Because of their high lipophilicity and low biodegradability, PCDD/Fs are persistent in the environment and are bioaccumulated in the adipose tissues of animals and humans, where they are similarly resistant to metabolism.[3,4] These compounds are unintentionally-produced contaminants, generated by different processes, including paper bleaching, synthesis of chemical compounds, as well as burning of wastes and forest fires[5-7]. In Brazil the major sources of PCDD/Fs releases are production of ferrous and non-ferrous metals (38.2%), followed by open burning (22.8%).[8]

Dietary intake is considered as the main pathway of PCDD/Fs to human beings, contributing more than 90% of the daily exposure.[9,10] The main sources are food of animal origin (i.e. dairy products, poultry and fish), and the rest might be attributed to inhalation and skin exposure to contaminated soils.

Over recent decades there has been a notable promotion of fish consumption in many countries due to the low levels of saturated fats, as well as high amounts of omega-3 polyunsaturated fatty acids which seem to cause health benefits.[11,12] Currently wide-ranging efforts have been made to protect the consumer health. Hence, the EU Council set a maximum level for PCCD/Fs in fish and fishery products at 3.5 pg WHO-TEQ g^{-1} wet weight.[13] Specific criteria concerning the performance of analytical methods have also been documented and revised.[14, 15]

Gas chromatography (GC) in combination with high resolution mass spectrometry (HRMS) has been commonly used for the identification and quantification of PCDD/Fs.[5] However, as usual for chromatographic methods, the sample preparation and the extraction steps are crucial to ensure reliable results. One of the most promising and recent sample preparation techniques is the pressurized liquid extraction (PLE) which offers the advantages

of reducing solvent consumption and automating sample handling and extraction time.[16-19] Many authors have described the application of PLE in the analysis of PCDD/Fs and POPs (persistent organic pollutants) in fish samples.[20-21] Its performance has usually been compared with the Soxhlet extraction, another classical procedure.[22,23] Automated extraction and clean up systems have been widely described.[24] In spite of their suitability for PCDD/Fs analyses, their main drawbacks are related to the need of using disposable columns and their limited supply.

Method validation evaluates the fitness for purpose of an analytical method.[25] It is an essential component among the measures that laboratories should implement within their quality management systems in order to guarantee reliability, traceability and comparability among the results issued.[26,27]

There are few reports in the literature that fully describes the performance of an analytical method developed by improvements of a well-established procedure.[14] The aim of this work is therefore to validate and apply an isotope dilution analytical method, which applies the PLE extraction procedure and makes use of neither carbon column nor toluene as solvent. GC-HRMS analyses were conducted to quantify by the first time the 17 most toxic PCDD/Fs in samples of fish from the Brazilian territory. The method described intends to be an integrant part of the monitoring as recommended by the National Residue and Contaminant Control Plan from the Brazilian government.[28,29]

2. Experimental

2.1. Standards

The EPA-1613 Stock solution, purchased from Wellington Laboratories (USA), contained the 17 native PCDD/Fs standards (Table 1) at the following concentrations: tetra at 400 ng mL^{-1} (2,3,7,8-TCDD, 2,3,7,8-TCDF); penta at 2000 ng mL^{-1} (1,2,3,7,8-PeCDD, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF); hexa at 2000 ng mL^{-1} (1,2,3,4,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF); hepta at 2000 ng mL^{-1} (1,2,3,4,6,7,8-HpCDD, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF); and octa at 4000 ng mL^{-1} (OCDD, OCDF). The EDF-8999 solution, supplied by Cambrigde Isotope Laboratories (USA), contained 15 $^{13}C_{12}$-labelled PCDD/Fs (excepting $^{13}C_{12}$-OCDF and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD, see Table 1) that were used as internal standards at the following concentrations: 100 ng mL^{-1} (tetra, penta, hexa and hepta $^{13}C_{12}$-PCDD/Fs) and 200 ng mL^{-1} ($^{13}C_{12}$-OCDD). The following injection standards were used: $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9-HxCDD (Table 1). These standards were the constituents of the EPA-1613ISS solution, purchased from Wellington Laboratories (USA), each one at a concentration of 200 ng mL^{-1}. For evaluation of selectivity the window defining standard supplied by Cambrigde isotopes was used.

2.2. Solvents, reagents and other materials

Nonane (99% purity), *n*-hexane (for PCDDs and PCDFs analysis, minimum 95% purity) and dichloromethane (Pestanal analysis) were purchased from Sigma-Aldrich (Germany). Silica gel 60 for column chromatography was purchased from Macherey-Nagel (Germany). Florisil®. 60-100 mesh was acquired from SorbLine (Brazil). Sulfuric acid (95-97 %, ACS reagent) was obtained from Merck (Germany). Granular anhydrous sodium sulfate (pesticide residue grade) was acquired from Fisher Scienctific (UK). All glassware was washed with an appropriate soap (Extran, Merck, Germany). After completely dry, the glass materials were rinsed with dichloromethane and *n*-hexane. This procedure minimizes the presence of interfering compounds that could affect the subsequent analyses.

2.3. Samples

For the validation experiments blank samples were selected after checking for PCDD/Fs contamination. Two different pools of samples, covering freshwater fish, *Hypophytaimus edentates*, as well as marine fishes, *Phycis phycis* and *Prionotus sp*, were prepared. The samples were grinded and freeze-dried for 36 hours at -60 ^{o}C under pressure of 10.66 Pa until constant mass. Subsequently, the samples were homogenized, stored at room temperature and protected from light and heat. In order to assess the method trueness, a certified reference material (CRM, WMF1-01 Reference Fish Tissue for Organic Contaminant Analysis) from Wellington Laboratories Inc. (Canada) was analyzed. The CRM consisted of a Chinook salmon *(Oncorhynchus tshawytscha)* lyophilized and naturally contaminated.

2.4. Apparatus

GC-HRMS analyses

PCDD/Fs were quantified by using a GC (gas chromatograph) (Agilent 6890 N, Agilent Technologies, Palo Alto, CA, USA) coupled to a HRMS (high resolution mass spectrometer) (Autospec Premier, Micromass, Manchester, UK). The mass spectrometer operated at a resolution greater than 10,000 (10% valley) in the EI mode with an ionization energy of 35 eV, a trap current of 650 µA and an acceleration voltage of 7,950 V. The analyses were conducted using a HP-5MS capillary column (60 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, USA). Ultrapure helium (purity higher than 99.999%) was used as carrier gas at a flow rate of 1.0 mL min^{-1}. Samples were injected (2 µL) in the splitless mode (1 min) using an autosampler (Agilent Technologies, USA). The GC oven program consisted of an initial temperature of 140 ºC (hold for 4 min), temperature ramp of 8 ºC min^{-1} up to 220 ºC, temperature ramp of 1.4 ºC min^{-1} up to 260 ºC, temperature ramp of 5 ºC min^{-1} up to 310 ºC (hold for 7 min). The injector, transfer line and source temperatures were set at 280 ºC, 310 ºC and 280 ºC, respectively. Isotope dilution mass spectrometry was utilized for the quantification of the target PCDD/Fs. Two isotopic ions were monitored for qualitative and quantitative purposes for each analyte, including internal and injection standards (Table 1). The retention time achieved for each compound is also indicated in Table 1. The toxicity equivalent quantity (TEQ) was calculated as previously described.[30]

Table 1. Monitored ions used for the identification and quantification of each dioxin and furan.

Target PCDD/Fs	Monitored Ions (Isotope)	Retention Time (min)
Native Standards		
2,3,7,8-TCDD	319.8965 (M); 321.8936 (M+2)	30.18
2,3,7,8-TCDF	303.9016 (M); 305.8987 (M+2)	30.23
1,2,3,7,8-PeCDD	355.8546 (M+2); 357.8516 (M+4)	38.48
1,2,3,7,8-PeCDF 2,3,4,7,8-PeCDF	339.8597 (M+2); 341.8567 (M+4)	36.37 37.77
1,2,3,4,7,8-HxCDD 1,2,3,7,8,9-HxCDD 1,2,3,6,7,8-HxCDD	389.8157 (M+2); 391.8127 (M+4)	45.55 46.30 45.73
1,2,3,4,7,8-HxCDF 1,2,3,6,7,8-HxCDF 2,3,4,6,7,8-HxCDF 1,2,3,7,8,9-HxCDF	373.8208 (M+2); 375.8178 (M+4)	43.92 44.15 45.25 46.35
1,2,3,4,6,7,8-HpCDD	423.7766 (M+2); 425.7737 (M+4)	51.02
1,2,3,4,6,7,8-HpCDF 1,2,3,4,7,8,9-HpCDF	407.7818 (M+2); 409.7789 (M+4)	49.47 51.56
OCDD	457.7377 (M+2); 459.7348 (M+4)	55.17
OCDF	441.7428 (M+2); 443.7399 (M+4)	55.32
Internal Standards		
$^{13}C_{12}$-2,3,7,8-TCDD	331.9368 (M); 333.9339 (M+2)	31.16
$^{13}C_{12}$-2,3,7,8-TCDF	315,9419 (M); 317.9389 (M+2)	30.22
$^{13}C_{12}$-1,2,3,7,8-PeCDD	367.8949 (M+2); 369.8919 (M+4)	38.45
$^{13}C_{12}$-1,2,3,7,8-PeCDF $^{13}C_{12}$-2,3,4,7,8-PeCDF	351.9000 (M+2); 353.8970 (M+4)	36.35 37.74
$^{13}C_{12}$-1,2,3,4,7,8-HxCDD $^{13}C_{12}$-1,2,3,6,7,8-HxCDD[a]	401.8559 (M+2); 403.8529 (M+4)	45.54 45.71
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF $^{13}C_{12}$-1,2,3,6,7,8-HxCDF $^{13}C_{12}$-2,3,4,6,7,8-HxCDF $^{13}C_{12}$-1,2,3,7,8,9-HxCDF	383.8639 (M); 385.8610 (M+2)	43.90 44.13 45.25 46.64
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	435.8169 (M+2); 437.8140 (M+4)	51.00
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDF $^{13}C_{12}$-1,2,3,4,7,8,9-HpCDF	417.8253 (M); 419.8220 (M+2)	49.45 51.55
$^{13}C_{12}$-OCDD[b]	469.7779 (M+2); 471.7750 (M+4)	55.15
Injection Standards		
$^{13}C_{12}$-1,2,3,4-TCDD	331.9368 (M); 333.9339 (M+2)	30.90
$^{13}C_{12}$-1,2,3,7,8,9 HxCDD	401.8559 (M+2); 403.8529 (M+4)	46.28

[a] Used also as internal standard for 1,2,3,7,8,9-HxCDD; [b] Used also as internal standard for OCDF.

2.5. Extraction

Two different procedures for the extraction of PCDD/Fs from fish samples were accomplished: pressurized liquid extraction (PLE) and Soxhlet. For the PLE extraction, a given mass of lyophilized fish (equivalent to 20.0 g of wet weight) was placed in a 34-mL cell and spiked with the ^{13}C-labeled standards (50 µL of a solution prepared from a 100-fold dilution of the EDF-8999 stock solution with nonane). The sample was transferred to an automated pressured liquid extraction system (ASE 350 Accelerated Solvent Extraction, Dionex Corporation, Sunnyvale, CA, USA) and submitted to the extraction procedure using *n*-hexane as solvent. The extraction conditions were as follows: temperature (100 ºC), static time (15 min), number of cycles (3), rinse (90 %), purge time (120 s). For the Soxhlet extraction, a given mass of lyophilized fish (equivalent to 20.0 g of fresh fish) was placed in a cellulose cartridge and spiked with the ^{13}C-labeled standards. The sample was extracted with 300 mL of *n*-hexane for 16 hours, with 5 to 7 cycles per hour. The extracts obtained from both extraction methods were reduced in an evaporator (Multivapor P-6, Buchi, Flawil, Switzerland) at a temperature of 50 ºC and pressure of ca. 1.8 x 10^4 Pa and afterward submitted to the cleanup steps using two distinct columns: acid silica-gel and Florisil®.

2.6. Clean-up

A silica-gel column was made by adding sequentially the following stationary phases into a glass cylindrical tube (25 mm diameter, 25 cm height): 30 g of acidic silica-gel (roughly 10 cm-long layer) and 3 g of silica-gel activated overnight at 130°C (roughly 1 cm-long layer). Acidic silica-gel was prepared by mixing concentrated H_2SO_4 (95 % w/w minimum) and activated silica-gel in a proportion of 3:2 w/w. The column was conditioned with 50 mL of *n*-hexane. The sample was eluted with 150 mL of *n*-hexane and the volume of the eluate reduced as previously described. The resulting extract was further purified on a Florisil® column, which was prepared by placing sequentially 0.5 g of granular anhydrous sodium sulfate (3 cm-long layer), 1.5 g of Florisil® activated overnight at 130 ºC (9 cm-long layer) and again 0.5 g of granular anhydrous sodium sulfate into a glass cylindrical tube (10.5 mm diameter, 20 cm height). The stationary phase was conditioned by adding 15 mL of *n*-hexane. Possible interfering compounds in the extract were eluted with *n*-hexane (20 mL) whereas the PCDD/Fs were eluted with 35 mL of dichloromethane. The eluate (dichloromethane fraction) was firstly reduced to 0.5 mL in an evaporator (TurboVap II, Caliper, California City, CA, USA) and sequentially lead to almost dryness under a stream of nitrogen (Reacti-therm, Pierce, Kent City, MI, USA). The purified extract was then reconstituted with 20 µL of a diluted solution of the injection standards, which was prepared from a 10-fold dilution of the EPA-1613ISS stock solution with nonane.

2.7. Method validation

The present method was validated for linearity, recovery, precision, trueness, selectivity, limit of detection (LOD), limit of quantification (LOQ) and measured uncertainty in order to meet the performance criteria required by the European Commission for the analysis of PCDD/Fs as described in Commission Regulations, 2011/1259/EU, 2012/252/EU and US-EPA 1613 method.[13-15]

Linearity

Linearity was investigated by the injection of three standard calibration curves for three different days. Each point of the curve was randomly injected. The concentration levels evaluated were: 0.05, 0.35, 2.00, 3.5, 10.00 and 100.00 pg g^{-1} of fish for tetra

PCDDs and PCDFs. For penta, hexa and hepta PCDDs and PCDFs, 025, 1.75, 10.00, 17.5 50.00 and 500.00 pg g^{-1} of fish. Finally, for OCDD and OCDF, 0.50, 3.50, 20.00, 35.00, 100.00 and 1000.00 pg g^{-1} of fish.

Calibration curves were constructed considering the relative response factor (RRF) for each compound as a function of concentration. RRF was obtained from Equation 1:[14]

$$RRF = \frac{A_N \bullet Q_I}{A_I \bullet Q_N} \qquad \text{Equation (1)}$$

where A_N is the chromatographic area of the native analyte; A_I is the chromatographic area of the internal standard, Q_I is the amount of the internal standard injected; Q_N is the amount of the native standard injected. Chromatographic response corresponded to the sum of the peak areas regarding the quantifier and qualifier ions.

Recovery and precision

Recovery and precision studies were conducted using spiked blank samples at three concentration levels (SL), which were selected based on the maximum levels of these compounds permitted in fish and fishery products as fixed by European Commission[13] (SL1: 0.7, 3.5 and 7.0 pg g^{-1} for the tetra PCDD/Fs; SL2: 3.5, 17.5 and 35.0 pg g^{-1} for the penta, hexa and hepta PCDD/Fs; SL3: 7.0, 35.0 and 70.0 pg g^{-1} for OCDD/F). The studies were conducted by three different analysts that individually repeated the extraction procedure in six replicates per level, resulting in 18 extractions per batch, over three different days. The total number of samples used in each parameter was therefore 54.

Precision was assessed from the relative standard deviations (RSD) obtained from the analysis performed in the recovery trials under repeatability (r) and intra-laboratory reproducibility (R) (intermediate precision) conditions.

Selectivity

Selectivity was checked by evaluation of ion abundance ratios as described in EPA 1613 method.[14] In addiction, two standard mixes were used for evaluation of chromatographic separation: one containing five TCDD congeners (window defining standard) and other containing two $^{13}C_{12}$-HxCDF (1,2,3,6,7,8 and 1,2,3,4,7,8). The 2,3,7,8-substituted congeners were individually separated (< 25% peak to peak).[14,15]

LOD, LOQ and measurement uncertainty

The detection limits of the instrument were appraised by sequentially injecting successive diluted solutions of the native standards. The limit of quantification was tested with samples spiked at the potential LOQ (n = 10).

Uncertainty was estimated using the top-down approach which considers the values of intermediate precision and the uncertainties of calibration curves.[27] Standard uncertainty was then obtained by combining both values. The combined standard uncertainty was multiplied by the coverage factor (k = 2) to obtain the expanded uncertainty (U).

2.8. Real samples analyses

The validated method was applied to quantify the 17 PCDD/Fs as well as their sum (TEQ) using the 2005 WHO-TEF values.[13] These analyses are part of the National Control Plan for Residues and Contaminants (PNCRC) of the Ministry of Agriculture Livestock and Food Supply of Brazil.[28,29] Hence, 132 samples of fish (from rivers and sea) were collected in 12 different states of the Brazilian territory, from August/2012 to September/2013. The samples included wild-caught and farmed fishes of 25 different species. In every batch of analysis a blank and spiked sample procedures were performed as part of the QA/QC. The following species (and number of samples) were analyzed: *Oreochromis niloticus* (23), *Urophycis brasiliensis*, (2) *Pomatomus saltator* (1), *Pellona castelnaeana* (1), *Tunnus spp* (1), *Auxis thazard* (1), *Micropogonias furnleri* (7), *Brachyplatystoma flavicans* (2), *Hexanematichthys parkeri* (1), *Paulicea lutkeni* (1), *Paralichthys spp* (1), *Merluccius hubbsi* (3), *Priacanthus spp*.(1), *Chloroscombrus chrysurus* (1), *Pagrus pagrus* (6), *Ptionotus spp*.(1) , *Lophius gastrophysus* (1), *Balistes spp* (1), *Cynoscion acoupa* (1), *Pseudoplatystoma coruscans* (16), *Branchyplatystoma vaillant* (18), *Arapaima gigas* (1), *Sadinella pilchardus* (3), *Colossoma macropomum* (15), *Thunnus alalunga* (1) and unknown species (22).

3. Results and discussion

3.1. Validation

In the following sections the results concerning the validation of the entire analytical method to quantify PCDD/Fs in samples of fish are presented and discussed.

Linearity

Linearity was evaluated by the variation of the Relative Response Factor, obtained from Equation 1. The RRF value was found to be constant over the calibration levels, showing a maximum variation, expressed as RSD, of 10.07 %. These values are below the 20 % maximum variation recommended in the EPA 1613 method.[14] The low values of RSD evidence that the instrument can maintain the linearity over the calibration range. Finally, another evidence for the adequate method linearity comes from the absence of a tendency as observed in the plot of residual values as a function of concentration (not shown). A positive response might disturb the results at a certain level.

Recovery and Precision

Table 2 summarizes the Measured Concentration (MC), percent recovery (PR), relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_R). Recovery ranged from 95.57 % (OCDF) up to 108.28 % (1,2,3,4,7,8-HxCDD) and are within the permitted range, from 80% to 120%, for confirmatory methods according to the European regulation 2012/252/EU.[15]

Precision results assessed are also complying. As the criteria indicated in the European regulation 2012/252/EU is a RSD_R < 15% for confirmatory methods, the values found herein (< 12.15%) meets the requirements of the directive.[15] Moreover, the RSD values are below 10%, excepting for OCDF. In the absence of a performance criteria in the regulations consulted, it was adopted herein that RSD_r must be 2/3 of RSD_R or 10 % as a maximum accepted value. Again the results were considered acceptable.[31]

Since the application of an alternative extraction procedure (Soxhlet) caused the attainment of no different recovery and precision rates, both approaches can be indistinctly applied.

Table 2. Measured concentrations (MC), percent recoveries (PR), relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_R) resulting from the analysis of PCDD/Fs at three distinct spiked levels (SL).

Analyte	SL ($pg.g^{-1}$)	MC ($pg.g^{-1}$)	PR (%)	RSD_r (%)	RSD_R (%)
2,3,7,8-TCDD	0.70	0.70	99.42	4.34	6.04
	3.5	3.58	102.27	9.39	5.54
	7.0	7.24	103.43	3.58	4.29
2,3,7,8-TCDF	0.7	0.69	98.24	5.65	3.70
	3.5	3.54	101.17	8.67	2.98
	7.0	7.22	103.11	3.36	2.28
1,2,3,7,8-PeCDD	3.5	3.42	97.61	4.71	3.05
	17.5	17.63	100.74	8.75	3.40
	35.0	35.32	100.92	2.65	2.20
1,2,3,7,8-PeCDF	3.5	3.42	97.85	3.90	2.99
	17.5	18.11	103.48	8.04	4.02
	35.0	35.99	102.81	4.08	1.57
2,3,4,7,8-PeCDF	3.5	3.43	97.96	4.54	2.61
	17.5	17.97	102.69	7.32	3.96
	35.0	35.65	101.87	3.79	1.72
1,2,3,4,7,8-HxCDD	3.5	3.43	95.57	5.17	2.61
	17.5	17.97	102.67	7.51	3.96
	35.0	35.62	101.47	3.55	1.72
1,2,3,4,7,8-HxCDF	3.5	3.40	97.12	4.83	3.15
	17.5	17.93	102.45	8.01	3.98
	35.0	35.75	102.13	3.68	1.49
1,2,3,6,7,8-HxCDD	3.5	3.37	96.26	5.46	3.64
	17.5	18.05	103.17	7.59	6.28
	35.0	35.55	101.58	4.43	2.67
1,2,3,6,7,8-HxCDF	3.5	3.37	96.34	4.57	3.01
	17.5	17.72	101.26	8.84	2.78
	35.0	35.48	101.38	4.09	2.66
1,2,3,7,8,9-HxCDD	3.5	3.48	99.43	4.86	3.33
	17.5	18.48	105.60	8.42	6.76
	35.0	36.27	103.64	5.05	3.02
1,2,3,7,8,9-HxCDF	3.5	3.37	96.39	4.51	2.57
	17.5	17.75	101.40	8.45	3.13
	35.0	35.81	102.33	3.79	2.01
2,3,4,6,7,8-HxCDF	3.5	3.37	96.32	4.71	2.68
	17.5	17.64	100.79	7.82	2.01
	35.0	35.57	101.63	3.32	2.39
1,2,3,4,6,7,8-HpCDD	3.5	3.45	98.70	4.62	3.28
	17.5	17.71	101.21	8.08	4.34
	35.0	34.92	99.77	3.07	1.68
1,2,3,4,6,7,8-HpCDF	3.5	3.39	96.90	5.30	3.86
	17.5	18.25	104.26	8.13	4.56
	35.0	35.79	102.24	2.39	2.97
1,2,3,4,7,8,9-HpCDF	3.5	3.35	95.78	4.57	1.77
	17.5	17.64	100.83	7.95	4.91
	35.0	35.39	101.11	3.10	2.01
OCDD	7.0	6.82	97.47	4.41	6.61
	35.0	34.40	98.30	9.36	5.45
	70.0	67.55	96.50	2.93	2.33
OCDF	7.0	7.05	100.68	6.03	6.97
	35.0	37.90	108.28	7.38	11.97
	70.0	72.74	103.92	7.01	12.15

Trueness

The results of the CRM analysis are shown in Figure 1, which compares the certified and measured concentrations for each PCDD/F. The values obtained, considering the method uncertainty,-are in accordance with the certified values and within the range of the CRM uncertainty. Some compounds, as hepta and octa PCDD/Fs, are notably problematic as they shown uncertainties higher than the other congeners. One possible cause, according to the EPA 1613 Method, would be the glassware contamination that could affect the analysis.[14] However, even for these compounds results close to the mean value were attained.

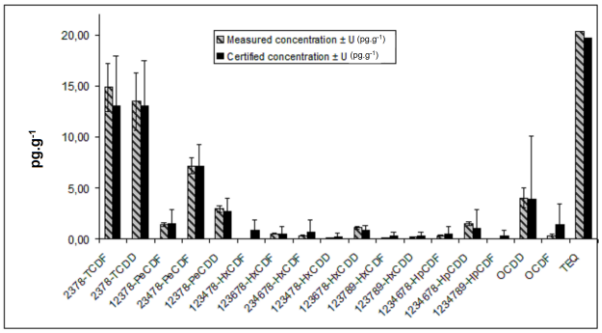

Figure 1. Certified and measured concentrations obtained for the CRM material.

Selectivity

The ratios between the chromatographic peak areas of the isotopic ions, i. e. (M)/(M+2) and (M+2)/(M+4) (Table 1), were consistent with the theoretical isotopic distributions.

The chromatographic separation between 2,3,7,8-TCDD from the other TCDD congeners (1,2,3,4; 1,2,3,7; 1,2,3,8; 1,2,3,9) was found to be < 25% peak to peak[14] (13 %), as shown in Figure 2. The isomer-specific determination is an important criteria used to avoid erroneous quantification related to the influence of the non-2,3,7,8 substituted congeners. Moreover, the chromatographic separation between $^{13}C_{12}$-HxCDF (1,2,3,6,7,8 and 1,2,3,4,7,8) was also found to be accordance with EU criteria.[15]

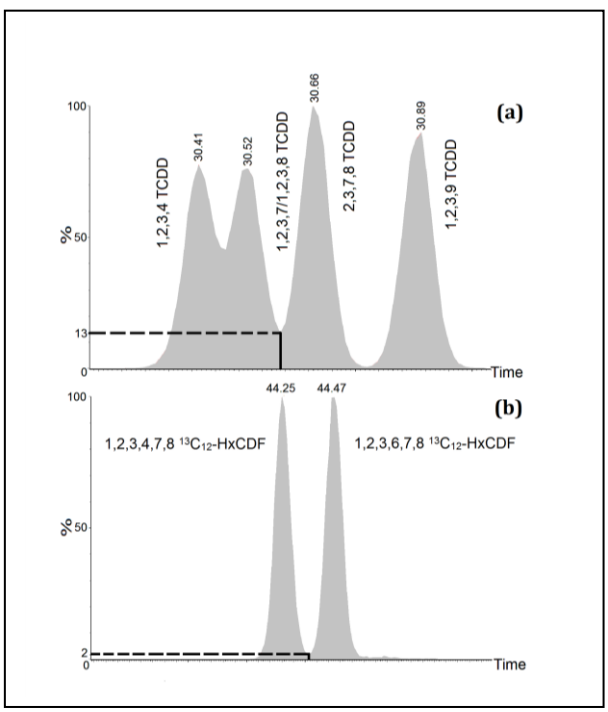

Figure 2. Chromatograms of two distinct mixtures: (a) five TCDDs (2,3,7,8 and non-2,3,7,8 substituted congeners); (b) $^{13}C_{12}$-1,2,3,6,7,8-HxCDF and $^{13}C_{12}$-1,2,3,4,7,8-HxCDF.

Specific studies to evaluate possible matrix effects were not conducted herein because isotopic dilution methodologies can overcome these effects.[32,33] Furthermore, the method accuracy was indicated by using both CRM and spiked samples.

Limits and measurement uncertainty

The instrument detection limits were set at the dilution level that provided a chromatographic response with a signal-to-noise ratio greater than 10 and that simultaneously yielded an isotopic distribution consistent with the theoretical profile. The limit of quantification was tested with samples spiked at the potential LOQ (n = 10). The limits attained are summarized in Table 3. Expressed as the upperbound level, the method LOQ is 0.37 pg WHO-TEQ g^{-1}. This value was considered adequate since confirmatory methods must furnish LOQs about one fifth of the maximum permitted level.[15]

Table 3. Instrument limit of detection (LOD), method limit of quantification (LOQ) and measurement uncertainty.

Compound	LOD (fg)	LOQ (pg g^{-1})	U, k=2 (%)
2,3,7,8-TCDF	20	0.05	15.84
2,3,7,8-TCDD	40	0.05	20.66
1,2,3,7,8-PeCDF	50	0.20	13.40
2,3,4,7,8-PeCDF	100	0.20	11.10
1,2,3,7,8-PeCDD	100	0.10	11.05
1,2,3,4,7,8-HxCDF	200	0.20	11.39
1,2,3,6,7,8-HxCDF	100	0.20	9.27
2,3,4,6,7,8-HxCDF	100	0.20	9.32
1,2,3,4,7,8-HxCDD	50	0.20	10.83
1,2,3,6,7,8-HxCDD	200	0.20	14.32
1,2,3,7,8,9-HxCDF	50	0.20	11.12
1,2,3,7,8,9-HxCDD	200	0.20	17.67
1,2,3,4,6,7,8-HpCDF	200	0.20	13.12
1,2,3,4,6,7,8-HpCDD	50	0.20	12.79
1,2,3,4,7,8,9-HpCDF	100	0.20	12.37
OCDD	100	0.40	23.64
OCDF	200	0.40	28.84
WHO-TEQ	-	0.37[1]	-

[1]Expressed in pg WHO-TEQ 2005 g^{-1} (upperbound value)

The uncertainty was estimated for each level studied in the validation experiments. In a practical approach, the higher value, in relative terms, was used as the compound uncertainty. The results are summarized in Table 3. The congeners OCDD/F presented higher values as a consequence of the higher RSD_R.

3.2. Real samples analysis

Among the 132 samples evaluated, only 23 (17.4 %) contained quantifiable levels (higher than the LOQ) of at least one PCDD/Fs. For these samples, curiously the congener 2,3,7,8-TCDF was found in 19 samples (82.6 %) with a maximum of 0.03 pg WHO-TEQ g^{-1} or 0.39 pg WHO-TEQ g^{-1} expressed in upperbound value, fairly lower than the adopted maximum level for these contaminants in fish, 3.5 WHO-TEQ g^{-1}.[13] The congeners OCDD (2 samples) and 2,3,4,7,8-PeCDF, 1,2,3,7,8 PeCDD, 1,2,3,4,6,7,8-HpCDF (1 sample each) were also found at levels higher than their LOQs. All the results were, however, below the permitted values. Moreover, it must be emphasized that there were no differences between the farmed and wild-caught fishes. Finally, it is important to mention that there were no previous reports regarding the contamination of fishes by PCDD/Fs in Brazil. The data in the literature for these contaminants in Brazil are restricted to environment,[34-38] human milk[39] and inventory of releases.[8] Even the major sources of dioxin and furans in Brazil, as metal production and uncontrolled combustion processes[8], seem to be insufficient to induce relevant fish contamination. Data from other countries usually indicates similar or higher contamination values for fish tissue. The average TEQ concentration for freshwater fish from South Korea was reported as 0.32 pg WHO-TEQ g^{-1}, although the individual concentration was up to 1.31 pg WHO-TEQ g^{-1}.[23] Mean concentrations of 0.33 pg WHO-TEQ g^{-1} was reported for Tilapia (*Oreochromis niloticus)* from Africa[21] and up to 0.79 pg WHO-TEQ g^{-1} for the same species from China.[40] A screening study from Spain reported TEQ concentrations <1.0 pg WHO-TEQ g^{-1} for ocean and farmed fishes.[24] Finally, it must be said that an analogous study was previously conducted by Hites and coworkers who verified that farmed salmons from Europe were significantly more contaminated than those from South and North America.[41]

4. Conclusions

An analytical method based on PLE extraction and analyses by GC-HRMS was successfully validated fulfilling the criteria described in the directives of the European Commission Decision.[13,15] It must be emphasized that the PLE extraction procedure demands about 1 hour whereas the traditional Soxhlet approach requires about 16 hours. Besides this important advantageous aspect, the PLE procedure consumes three times less solvent. The method has been applied to quantify PCDD/Fs in 132 fish samples collected in 12 different regions of Brazil. These results constitute an important part of the National Residue and Contaminant Control Plan and indicated that fishes from Brazil have not been subjected to contamination by 17 polychlorinated dioxins and furans over a period of 13 months.

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