IN-HOUSE METHOD VALIDATION FOR SIMULTANEOUS QUANTIFICATION OF PCDD/PCDFs AND DIOXIN-LIKE PCBs IN FISH USING PRESSURIZED LIQUID EXTRACTION AND GC-HRMS ANALYSIS

Augusti DV, Nunes CM, Castro DS, dos Santos EV, Prates RGV, Pussente IC, Pissinatti R*

Laboratório Nacional Agropecuário (LANAGRO-MG), Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Avenida Rômulo Joviano, s/n, CP 35 e 50, Pedro Leopoldo, MG, Brazil

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like biphenyls (dl-PCBs) are well-known persistent organic pollutants. The harmful effects on human health are well-documented and include a combination of toxic responses, such as embryotoxicity, hepatotoxicity, immunotoxicity, teratogenicity, and carcinogenicity.[1] Dietary intake is considered the main pathway of PCDD/Fs and PCBs to human beings, contributing to more than 90% of the daily exposure.[1] Therefore, a continuous monitoring of these compounds in foodstuffs is needed. For ultra-trace analysis, method validation is a key element in the assessment of a laboratory's competence in producing reliable analytical data. To ensure the attainment of high-quality results, an isotope dilution method for quantification of 17 PCDD/Fs and 12 dl-PCBs in fish was optimized and validated according to specific requirements.

Materials and methods

Extraction. Pressurized Liquid Extraction was accomplished using an automated pressured liquid extraction system (ASE 350 Accelerated Solvent Extraction, Dionex Corporation, Sunnyvale, CA, USA). 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 pg tetra, penta, hexa and hepta PCDD/Fs; 100 pg OCDD; 250 pg non-ortho PCBs and 2000 pg mono-ortho). The sample was extracted with *n*-hexane (Sigma-Aldrich, Germany) in the following conditions: temperature (100 ºC), static time (15 min), number of cycles (3), rinse (90 %), purge time (120 s). The resulting extract was reduced in an evaporator (Multivapor P-6, Buchi, Flawil, Switzerland) at a temperature of 50 ºC and pressure of 1.8 x 10^4 Pa and afterward submitted to the cleanup and fractioning steps.

Clean up. A silica-gel column was prepared by adding sequentially the following stationary phases into a glass cylindrical tube (25 mm diameter, 25 cm height): 30 g of acidic silica-gel, prepared by mixing concentrated H_2SO_4 (95 % w/w minimum. Merck, Germany) and activated silica-gel in a proportion of 3:2 w/w; and 2 g of silica-gel activated overnight at 130°C. After conditioning (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.

Separation of PCDD/Fs and PCBs. The resulting extract was purified on a Florisil® column, which was prepared by placing sequentially 0.5 g of granular anhydrous sodium sulfate activated overnight at 130°C , 1.5 g of Florisil® (60-100 mesh. US Silica company, USA) activated overnight at 600 ºC and 0.5 g of granular anhydrous sodium sulfate activated into a glass cylindrical tube (10.5 mm diameter, 20 cm height). The stationary phase was conditioned by adding 15 mL of *n*-hexane:dichloromethane (95:5, v:v). PCBs and possible interfering compounds for PCDD/Fs were eluted with 20 mL of *n*-hexane:dichloromethane (95:5, v:v). PCDD/Fs were eluted with 35 mL of dichloromethane and reserved.

Separation of mono and non-ortho PCBs. A further separation in the PCB fraction was conducted by using a column (10.5 mm diameter, 20 cm height) filled with 0.50 g of a Carbopack C (80-100 mesh. Supelco, USA) and celite (545 AW. Sigma-Aldrich, Germany) mixture (18/82 w/w) and 1.50 g of activated Florisil®. The column was conditioned by adding 20 mL of dichloromethane followed by 30 mL of *n*-hexane. The first fraction, containing mono-ortho PCBs, was eluted with 20 or 25 mL of *n*-hexane and collected apart. The non-ortho PCBs were eluted with 35 mL of dichloromethane and joined with the PCDD/Fs fraction.

Concentration and injection into the GC-HRMS system. *(a) PCDD/Fs and non-ortho PCBs fraction:* the eluate 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 extract was then reconstituted with 20 µL of a diluted solution containing the injection standards (160 pg); *(b) Mono-ortho PCB fraction:* the purified extract was reduced to 0.5 mL in an TurboVap II evaporator and then

100 µL of a diluted solution containing the injection standards (800 pg) was added and homogenized. The extract was again reduced to a final volume of 0.5 mL. The purified extracts were analyzed on a GC-HRMS (AutoSpec® - Waters) instrument in selective ion mode (SIM), operating positive EI ionization at a resolution > 10,000 with HP-5MS capillary column (60 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, USA). Quantification was conducted considering the relative response factor (RRF).

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 and dl-PCBs, as described in Commission Regulations 2012/252/EU, 2011/1259/EU and US-EPA methods (1613 and 1668).

Linearity. It was investigated by construction of three standard calibration curves in three different days. The concentration levels evaluated were: 0.05, 0.40, 2.00, 3.5, 10.00 and 100.00 pg g^{-1} of fish for tetra PCDD/Fs; 0.25, 2.00, 10.00, 17.5 50.00 and 500.00 pg g^{-1} of fish for penta, hexa and hepta PCDD/Fs; 0.50, 4.00, 20.00, 35.00, 100.00 and 1000.00 pg g^{-1} of fish for OCDD and OCDF; 0.25, 2.50, 12.50, 25.00, 250.00, 1000.00 pg g^{-1} of fish for non-ortho PCBs; 2.00, 4.00, 80.00, 200.00, 400.00 and 800.00 pg g^{-1} of fish for mono-ortho PCBs.

Recovery and Precision. Recovery and precision studies were conducted using spiked samples at three concentration levels, which were selected based on the maximum permitted levels of these compounds in fish and fishery products as fixed by Commission Regulation 2011/1259/EU. For PCB-77 and PCB-81 the natural contamination of the samples was summed to the spiked concentration. 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 (n = 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.

Trueness and Selectivity. In order to assess the method trueness, a certified reference material (CRM, WMF1-01 Reference Fish Tissue for Organic Contaminant Analysis, Wellington Laboratories Inc., Canada) was analyzed. The CRM consisted of a freeze-dried *Chinook* salmon *(Oncorhynchus tshawytscha)* naturally contaminated. Selectivity was checked by evaluation of ion abundance ratios as described in the EPA 1613 method. In addition, the chromatographic separation of PCB 169 e 1,2,3,7,8 PeCDD was evaluated.

LOD, LOQ and Measurement Uncertainty. The detection limits of the instrument were appraised by sequentially injecting successive diluted solutions of the native standards. The LOQ was tested with samples spiked at the potential LOQ ($n \geq 10$). Uncertainty was estimated using the top-down approach which considers the values of intermediate precision and the uncertainties of calibration curves. Standard uncertainty was then obtained by combining both values. The combined standard uncertainty was multiplied by the coverage factor (k = 2) to be obtained the expanded uncertainty (U).

Real Sample analysis. The validated method was applied to quantify real samples as part of the National Control Plan for Residues and Contaminants of the Ministry of Agriculture, Livestock and Food Supply of Brazil.[2] A total of 13 samples of farmed and wild-caught fishes were analysed.

Results and discussion

Linearity. Linearity was demonstrated by the evaluation of the Relative Response Factor (RRF). The RRF value was found to be constant over the calibration levels, showing a maximum variation, expressed as RSD, of 10.38 %. These values are below the 20 % maximum variation recommended. 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 can be observed by 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 1 summarizes the mean recovery, relative standard deviations for repeatability (RSD_r) and for intermediate precision (RSD_R). Recoveries ranged from 91.8 % to 118.4 % are within the permitted range (80 % - 120 %) for confirmatory methods according to the European regulation 2012/252/EU. Precision results are also complying with the criteria adopted by the European regulation 2012/252/EU (RSD_R < 15% for confirmatory methods). Moreover, the RSD_r values are below 10 %, excepting for PCB-77, PCB-81 and OCDF.

Table 1. Mean recovery, relative standard deviations for repeatability (RSD_r) and for intermediate precision (RSD_R), Limit of detection (LOD), limit of quantification (LOQ) and uncertainty (U), obtained in method validation.

	Mean recovery (%)			RSD_R (%)			RSD_r (%)			LOQ	LOQ	U
Analyte	**L1**	**L2**	**L3**	**L1**	**L2**	**L3**	**L1**	**L2**	**L3**	**(fg)**	**(pg g^{-1})**	**(%)**
2,3,7,8-TCDD	97.1	98.9	100.0	4.1	2.4	2.9	6.1	4.6	4.8	20	0.05	20.64
2,3,7,8-TCDF	99.8	98.2	99.7	3.2	2.1	3.0	3.2	3.6	3.3	40	0.05	16.12
1,2,3,7,8-PeCDD	98.6	98.2	100.5	2.4	1.8	2.7	4.2	4.1	4.6	50	0.20	17.28
1,2,3,7,8-PeCDF	98.2	97.9	100.3	1.8	1.1	2.4	2.9	3.2	4.2	100	0.20	17.36
2,3,4,7,8-PeCDF	103.6	97.7	100.6	3.8	2.4	2.9	7.1	6.2	6.3	10	0.10	19.29
1,2,3,4,7,8-HxCDD	94.2	95.1	97.4	3.5	1.9	2.9	4.3	4.9	6.0	200	0.20	16.13
1,2,3,4,7,8-HxCDF	98.5	99.0	100.5	2.6	1.5	2.4	3.4	5.1	6.3	100	0.20	16.44
1,2,3,6,7,8-HxCDD	97.7	99.1	101.1	2.2	1.7	2.9	3.8	4.0	4.9	100	0.20	14.13
1,2,3,6,7,8-HxCDF	97.6	97.6	100.2	2.4	2.1	3.5	5.6	3.4	4.0	200	0.20	18.03
1,2,3,7,8,9-HxCDD	97.7	98.7	99.8	1.7	2.0	3.6	4.6	3.8	5.2	50	0.20	17.00
1,2,3,7,8,9-HxCDF	95.4	97.8	99.0	3.0	2.8	2.8	6.0	5.9	6.7	50	0.20	19.50
2,3,4,6,7,8-HxCDF	97.2	97.1	99.4	3.6	2.3	3.4	5.2	4.8	6.3	200	0.20	16.12
1,2,3,4,6,7,8-HpCDD	95.3	96.5	97.7	3.1	1.9	2.5	5.4	5.1	7.1	200	0.20	17.59
1,2,3,4,6,7,8-HpCDF	94.1	96.3	97.5	3.0	2.5	3.1	5.4	5.2	6.7	100	0.20	19.01
1,2,3,4,7,8,9-HpCDF	97.4	98.0	100.3	3.2	2.1	2.9	4.3	4.1	4.9	50	0.20	14.17
OCDD	93.4	93.5	93.6	6.4	4.1	4.6	9.4	7.0	6.4	200	0.40	25.44
OCDF	112.4	111.1	112.9	12.8	12.3	7.8	13.2	12.5	10.3	100	0.40	35.89
PCB-81	118.4	102.7	91.8	13.1	2.9	3.5	13.5	7.5	7.6	100	2.72	28.03
PCB-77	107.9	104.7	93.4	11.0	3.0	4.2	14.8	3.2	4.2	200	5.77	37.19
PCB-126	115.1	107.4	96.2	6.0	2.9	4.6	7.0	4.4	6.0	100	2.50	23.72
PCB-169	107.1	106.1	94.2	5.6	2.9	6.6	5.8	4.0	6.6	200	2.50	19.43
PCB-123	96.2	97.0	100.4	3.1	2.6	1.6	7.1	2.6	1.7	125	50	16.92
PCB-118	99.2	96.7	100.6	6.2	2.5	1.1	9.7	2.5	1.6	50	50	21.99
PCB-114	97.1	97.8	102.4	3.8	2.6	1.1	7.2	2.7	1.3	25	50	17.07
PCB-105	96.7	97.2	100.8	4.3	2.6	1.3	6.9	2.6	1.8	50	50	16.47
PCB-167	94.5	96.7	100.3	2.1	2.6	1.3	3.8	2.6	1.6	25	50	10.10
PCB-156	95.0	97.2	101.5	2.3	2.4	1.1	3.4	2.4	1.6	50	50	9.56
PCB-157	94.3	96.7	100.2	1.9	2.3	1.2	3.7	2.4	1.7	25	50	9.84
PCB-189	93.5	95.5	99.8	2.3	1.8	1.2	3.2	1.8	1.6	25	50	10.91

Levels: TCDD/F (L1: 0.7; L2: 3.5; L3: 7 pg.g^{-1}); Pe/Hx/HpCDD/F (L1: 3.5; L2: 17.5; L3: 35 pg.g^{-1}); OCDD/F (L1: 7; L2: 35; L3: 70 pg.g^{-1}); PCB-81 (L1: 2.72; L2: 250.22; L3: 1000.22 pg.g^{-1}); PCB-77 (L1: 5.77; L2: 253.27; L3: 1000.27 pg.g^{-1}); PCB-126/PCB-169 (L1: 2.50; L2: 250; L3: 1000 pg.g^{-1}); mono-ortho PCBs (L1: 50; L2: 1000; L3: 5000 pg.g^{-1}).

Trueness and Selectivity. The concentrations of each PCDD/F and dl-PCB in the CRM material, shown in Figure 1, are accordance with the certified values and within the range of the CRM uncertainty, except for PCB 167. It is known the non-dioxin-like PCB-128 is a co-elutent of PCB-167 when using the HP-5MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness).[3] The present method reports the sum of these two analytes, considering the TEF of PCB-167. By this conservative approach, the value of ΣTEQ may be overestimated. However, as verified in the CRM analysis, it is not expected this value to be significantly disturbed. Furthermore, when such co-elution takes place, a DB-5 column (60 m; 0.32 mm i.d.; 0.25 µm film thickness) can be conveniently used.[2] When assessing selectivity, the ratios between the chromatographic peak areas of the isotopic ions, i. e. (M)/(M+2) and (M+2)/(M+4), 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% by checking the window defining standard (Cambrigde Isotopes Laboratories). The pairs PCB-169 and 1,2,3,7,8 PeCDD as well as PCB-156 and PCB-157 were found to be well resolved. Specific studies to evaluate possible matrix effects were not conducted herein because isotopic dilution methodologies can overcome these effects.[4] Furthermore, the method accuracy was indicated by using both CRM and spiked samples.

LOD, LOQ and Measurement Uncertaint. 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 also yielded an isotopic distribution consistent with the theoretical profile. The method limit of quantification (LOQ) was tested with samples spiked at the potential LOQ ($n \geq 10$). For dl-PCBs the lower studied level in recovery and precision experiments was considered as the method LOQ. The limits attained are summarized in Table 1. Expressed as the upperbound level, the method LOQ is 0.706 pg WHO-TEQ g^{-1}. This value was considered adequate since confirmatory methods must furnish LOQs about one fifth of the maximum permitted level (2012/252/EU). 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 1. The congeners OCDF, PCB-81 and PCB-77 presented higher values as a consequence of the higher RSD_R.

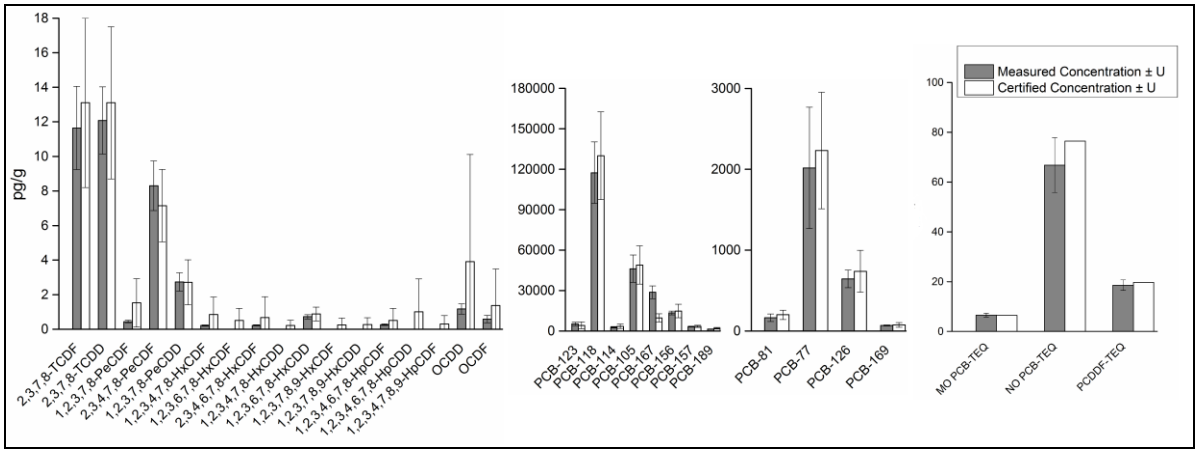

Figure 1. Certified and measured concentrations obtained for the CRM material.

Real Sample analysis. No sample presented contamination higher than the maximum permitted level (6.5 pg TEQ-OMS g^{-1}). Moreover the concentrations of all samples were found to be lower than the LOQ. The most commonly detected compounds were PCB-118 and PCB-77, present in 92.3 % of samples. The same PCBs where predominant in other studies. [5,6]

An analytical method based on PLE extraction and analysis by GC-HRMS was successfully validated fulfilling the reference criteria and applied to real samples. Since there are no significant data on PCDD/F and dl-PCB contamination in fish from Brazil, this study can indicate on a preliminary basis that levels of contamination of fish intended for human consumption are low. The method is being applied by the Ministry of Agriculture, Livestock and Food Supply of Brazil to enable a comprehensive monitoring.

Acknowledgements

The authors acknowledge financial support from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Storelli MM, Storelli AD, Addabbo R, Barone G, Marcotrigiano GO. (2004); *Environ. Int.* 30: 343–349.
2. Mauricio AQ, Lins ES, Alvarenga MB. (2009); *Anal. Chim. Acta*. 637: 333–336.
3. Wiberg K, Sporring S, Haglund P, Bjorklund E. (2007); *J. Chromatogr. A.* 1138: 55–64.
4. Sargent M, Harte R, Harrington C. (2002); Guidelines for achieving high accuracy in isotope dilution mass spectrometry (IDMS), *Royal Society of Chemistry*, Cambridge, 1st ed.
5. Bhavsar Rs, Fletcher R Hayton A, Reiner, E Jackson D. (2007); *Environ. Sci. Technol.* 41: 3096-3102.
6. European Food Safety Authority (2012); Update of the monitoring of dioxins and PCBs levels in food and feed, *EFSA Journal* 10 (7): 2832: 1-82.