METHOD VALIDATION TO RESIDUES AND CONTAMINANTS IN FOOD IN AGREEMENT WITH THE BRAZILIAN LEGISLATION: A CASE STUDY TO ORGANOCHLORINE PESTICIDES AND POLYCHLORINATED BIPHENYLS IN ANIMAL FAT

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Introduction

One of the pillars of the food security is to control the residue of pesticides and veterinary drugs in food. Since 1976, the European Community has introduced several directives establishing more than 45,000 MRLs for pesticides in a wide range of commodities and foodstuffs of animal origin.[1] Consequently, the development of methods to determine residues and contaminants in this MRLs levels was necessary as well the establishment of criteria to method validation and quality control. In this context, since 1999, the European Community has published documents to help the member countries to uniformize this laboratorial process. [2]

In Brazil context, the 2000s was marked by the consolidation of Brazil as a producer and exporter of beef.[3] Among others advances, action has been taken from the Brazilian government to promote the safe production of food.[4] One of this is the National Residue Control Plan (PNCRC) created in 1979.[5] This document has as main objectives to highlight the potential risk to which the population is exposed and to function as a guidance parameter for the adoption of National Policies of Health and Inspection.[6] In 2011, in agreement with the European tendency, the MAPA had published a guide called Brazilian Manual of Analytical Quality Assurance–Residues and Contaminants in Food.[7] In the Part 6 of this document, subsection II, specifics aspects to validation of pesticides are described. This document is highly influenced by the SANCO document[2], which can be noted by the similarity of the text, procedures and criteria.

To comply with the PNCRC program, the National Agricultural and Livestock Laboratory of São Paulo (Lanagro-SP) has been analyzed organochlorine pesticides (OCPs) since 1980´s. However, the method was not validated before because the definition of the parameters validation is from 2011. Recently, the polychlorinated biphenyls (PCBs) were included in the PNCRC list. So, modification on the chromatographic parameters were done to include this compounds in the same method. This paper outlines this validation process according to the Brazilian Manual of Analytical Quality Assurance–Residues and Contaminants in Food.

Material and Methods

Standard solution

Pesticide standard solution for □-hexachlorocyclohexane (□-HCH), hexachlorobenzene (HCB), □-hexachlorocyclohexane (□-HCH), □- hexachlorocyclohexane (LIN), heptachlor (HEP), aldrin (ALD), *cis*-chlordane (*c*CLD), p,p'-DDE (ppE), dieldrin (DLD), endrin (END), p,p'-DDD (ppD), o,p'-DDT (opT), p,p'-DDT (ppT), methoxychlor (ppX) and mirex (MRX) were purcjased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). *Trans*-chlordane (*t*CLD) and heptachlor epoxide (HPX) were obtained from Supelco (Belleforte, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Standard solution for PCBs 28, 52, 101, 118, 138, 153 and 180 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock standard solutions of OCPs and PCBs were prepared in isooctane. Finally, a working standard solution containing each compound in the concentration of 1,0 MRL was prepared.

Reagents and material

N-Hexane, isooctane and ethylic ether were obtained from Vetec (Rio de Janeiro, RJ, Brazil). Anhydrous sodium sulfate and aluminium oxide were obtained from Merck (Darmstadt, Germany). 4-Methoxy-azobenzol (MAB) was obtained from Fluka (Buchs SG, Switzerland). Helium (99.999% purity) and nitrogen (99.999%) were supplied by White Martins (Rio de Janeiro, Brazil).

Sample preparation

Blank bovine fat samples were obtained from establishments that have a register in the Federal Inspection Service (SIF) and were participatin of the PNCRC. All the samples were storage under controlled temperature to the analysis. In brief, pieces of samples were cut, homogenized and kept for 12 hours at 80 ºC. They were transferred to a funnel with anhydrous sodium sulfate. So, 0.125 g of these samples were weighed and transferred to the top of a chromatographic column containing n-hexane and 10 g of neutral alumina previously deactivated. To elute the analytes from the fat was used a mix of n-hexane and ethylic ether (249:1,

v/v). The eluate was evaporated and dissolved in 1 mL of n-hexane. 1 µL of the extract was injected in the GC-ECD or GC-MS systems, according to the study.

Instrumentation

The validation was performed using a GC-ECD system (Trace GC Ultra, Thermo Scientific, Madison, WI) fitted with a OV-5MS column (Ohio Valley Specialty Chemical, Marietta, Ohio), 25 m x 0.25 mm i.d., containing 5% phenylmethylpolysiloxane with a phase thickness of 0.25 µm. The split-splitless injector was operated in the splitless mode. The injector and detector temperatures were 250 ^{o}C and 300 ^{o}C, respectively. Helium was used as carrier gas at a flow rate of 0.8 mL min^{-1} and nitrogen was the make-up gas at 30 mL min^{-1}.

To the confirmation, it was used a GC-MS system (GCMS-QP 2010 quadrupole mass spectrometer, Shimadzu, Tokyo, Japan). The separation was carried out using an OPTIMA 5 (Macherey-Nagel, Duren, Germany) (30 m x 0.25 mm x 0.25 μm) and the injector and interface temperature were 250 ^{o}C while the ion source was 220 ^{o}C. Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. Full-scan mass spectra were obtained in the electron ionization mode (70 eV). After, the analysis was performed in selected ion monitoring (SIM) mode and the selected ions were monitored. In both systems, the oven temperature was programmed as follows: 80 ºC (hold for 1.5 min), then 40 ºC min^{-1} to 170 ºC (hold for 0 min), then 6.5 ºC min^{-1} to 220 ºC (hold for 15 min), then 8 ºC min^{-1} to 245 ºC min^{-1} (hold for 0 min) and 8 ºC min^{-1} to 265 ºC min^{-1} (hold for 4 min).

Validation of the analytical method

The validation of the method was been done in agreement to the Brazilian Manual of Analytical Quality Assurance–Residues and Contaminants in Food as described below:

Selectivity: It was evaluated through the analysis of ten blank samples, in both systems, considering the criteria that the area of the blank matrix should not be greater than 30% of the minimum calibration level.

Limit of equipment detection (LOD): It was obtained through successive dilutions of the standards until a signal/noise to 3.

Limit of quantification (LOQ): It was defined as the lowest validated spike level, which the acceptability criteria were kept (mean recovery between 70 and 120%, with an RSD ≤ 20%). In this study, the LOQ was the value correspondent to 0.5 MRL.

Linearity: It was carried out spiking the extract samples in 5 levels (0.5, 0.75, 1.0, 1.5 and 2.0 MRL) with six replicates of injection. The acceptability criterion to the linear regressions was $r^2 \geq 0.980$. To the residues dispersion of the analytical curve, the acceptability criterion was deviation (RSD) lower than 20% to 0.5 and 0.75 MRL or 10 % when MRL was approximated or exceeded.

Precision (repeatability and reproducibility): The repeatability was obtained spiking the matrix in two levels (0.5 and 1.0 MRL), in six replicates. The within-laboratory reproducibility was carried out for the same experiments, for two different people, in different days, using the criteria of RSD ≤ 20%.

Accuracy: It was estimated to the analysis of 6 replicates of blank samples spiked in two concentration: 0.5 and 1.0 MRL. It was considered to the acceptability criteria a recovery between 70 and 120 %.

Mass confirmation: The identity of the analytes was confirmed in the GC-MS by the analysis of a matrix spiked with 1.0 MRL of all the compounds.

Uncertainty of measurement: It was calculated based on the top-down methodology, taking into account the recovery, the precision, and the calibration curve. The expanded uncertainty should be lower or equal than 50% according the criteria (corresponding to a 95% confidence level and a coverage factor of 2).

Results and Discussion

The quantitative measurements in real samples normally require applying the standard addition technique. So, to perform the validation steps, non contaminated samples were spiked with the mixture of OCPs and PCBs congeners in the concentration around the MRL, according to the experiment.

The method showed selectivity to the majority of the analytes, however, in the retention time of the □HCH (peak 3), PCB 28 (peak 5) and PCB 52 (peak 7) were found peaks with area ≥ 30% of the lower calibrated level (0.5 MRL). So these analytes were not considered to the next steps of the validation.

A significant correlation was found in the range of 0.5 to 2.0 MRL showing r^2 between 0.998 (αHCH, PCB 101 and ppD) and 1.000 (PCB 180). The residues dispersion analysis showed a random distribution of the experiments of each analyte. The LODs were between 0.06 (HCB) and 0.52 □g kg^{-1} (END). The considered LOQs was 0.5 MRL so it were between 10.0 □g kg^{-1} (LIN) and 125.0 to DDTs. The repeatability values were between 6.9 (ALD and HPX) and 16.0% (□HCH) to 0.5 MRL. Only ppX was not approved in this criteria with the variation coefficient of 51.5% to this level. To 1.0 MRL level, the results were between 4.7 (ppE) and 11.4% (tCLD) to 1.0 MRL. The within-laboratory reproducibility was in the range of 5.9 (opT) and 19.6% (□-HCH) to

0.5 MRL and between 5.0 (MRX) and 14.6% (HCB) to 1.0 MRL. In both cases repeatability and within-laboratory reproducibility, the results were in accordance to the Guide criteria of 20%. The recoveries achieved to 0.5 MRL were between 84.4 (LIN) and 107.7% (DLD) and to 1.0 MRL were between 93.4 (LIN) and 115.5% (DLD). The Figure 1 shown a chromatogram of a sample spiked with 1.0 MRL of each organochlorine.

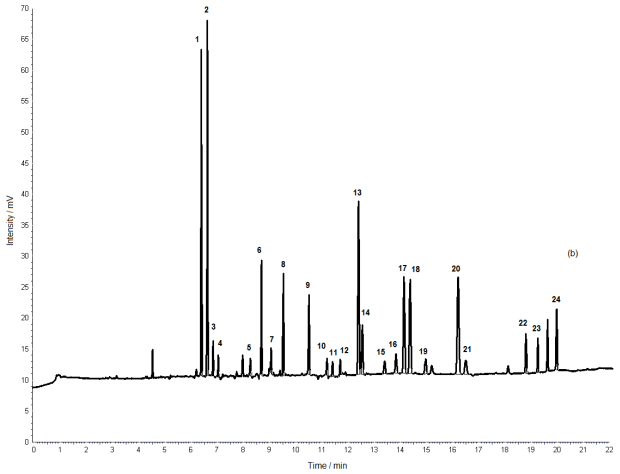

Figure 1. Chromatogram of a sample spiked with the concentration of 1.0 MRL of the followed compounds: 1. α-HCH (6.47min), 2. HCB (6.61min), 3. β-HCH (6.84 min), 4. LIN (7.02min), 5. PCB 28 (8.31min), 6. HEP (8.75min), 7. PCB 52 (9.11min), 8. ALD (9.60min), 9. HPX (10.53min), 10. tCLD (11.21min), 11. PCB 101 (11.45min), 12. cCLD (11.78min), 13. ppE (12.41min), 14. DLD (12.59min), 15. END (13.41min), 16. PCB 118 (13.96min), 17. ppD (14.27min), 18. opT (14.51min), 19. PCB 153 (15.08min), 20. ppT (16.42min), 21. PCB 138 (16.64min), 22. ppX (19.05min), 23. PCB 180 (19.42min) e 24. MRX (20.09min).

When this same experiment was conducted in the GC-MS, LIN and END were not confirmed probably because the fragmentation energy was higher than the necessary to generate diagnostic fragmentations of these molecules. For this, these two analytes were also removed of the validation set.

As well as the SANCO document, the Mapa Guide considers that the laboratory should have sufficient repeatability/reproducibility data from method validation, inter-laboratory studies and in-house quality control tests, which can be used to estimate the uncertainty.[2,7] In this study it was observed that the within-laboratory reproducibility was the main responsible to the uncertainty of all analyte. The expanded uncertainties and the validation data are described in Table 1.

Conclusions

The present study allowed validating thirteen OCPs and five PCBs in bovine fat using the Brazilian Manual of Analytical Quality Assurance–Residues and Contaminants in Food. This validation was a demand of the MAPA to the laboratory to be able to comply with PNCRC. Finally, the Guide was an important tool of the Brazilian Government to guarantee a uniformity of the validation procedures between the range of official and accredited laboratories that provide services to the PNCRC program.

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Table 1. Resume of the validation data.

Compound	Selectivity	Linearity	Limit of detection (LOD)	Limit of quantification (LOQ)	Repeatability		Reproducibility				Accuracy		GC/MS Confirmation	Expanded uncertainty
	%	r^2	□g kg^{-1}	0.5 MRL (□g Kg^{-1})	0.5 MRL	1.0 MRL	0.5 MRL Day 2	0.5 MRL Day 3	1.0 MRL Day 2	1.0 MRL Day 3	0.5 MRL	1.0 MRL	Yes/No	%
□HCH	< 30%	0,998	0,25	100.0	16.0	7.6	16.0	19.6	13.6	8.6	89.9	105.6	Yes	23.35
HCB	< 30%	0.999	0.06	100.0	8.7	8.3	18.4	13.9	14.6	10.0	95.4	111.9	Yes	38.69
HEP	< 30%	0.999	0.09	50.0	8.1	6.4	10.8	9.1	7.8	9.5	96.4	106.8	Yes	23.38
ALD	< 30%	0.999	0.10	50.0	6.9	8.3	9.0	11.4	7.7	5.4	98.5	109.6	Yes	22.89
HPX	< 30%	0.999	0.07	50.0	6.9	5.7	12.2	10.1	7.6	5.6	97.8	106.0	Yes	22.85
tCLD	< 30%	0.999	0.14	12.0	10.3	11.4	7.2	10.2	10.0	7.2	103.0	115.9	Yes	24.65
PCB 101	< 30%	0.998	0.13	16.0	11.3	7.9	11.1	12.4	6.5	8.0	98.1	117.2	Yes	31.35
cCLD	< 30%	1.000	0.14	12.0	11.7	4.8	16.9	7.3	8.0	9.4	88.6	98.2	Yes	24.49
ppE	< 30%	0.999	0.25	125.0	7.9	4.7	11.1	6.7	7.2	7.9	94.3	102.9	Yes	22.40
DLD	< 30%	0.999	0.31	50.0	11.4	11.0	12.0	7.8	6.2	9.6	107.7	115.4	Yes	25.04
PCB 118	< 30%	0.999	0.07	16.0	7.3	5.0	10.8	8.2	7.7	13.0	94.3	103.0	Yes	24.05
ppD	< 30%	0.998	0.19	125.0	8.6	5.0	12.0	6.0	8.6	9.4	95.0	102.7	Yes	24.97
opT	< 30%	0.999	0.22	125.0	8.4	5.0	12.1	7.1	8.5	9.0	94.0	102.8	Yes	24.37
PCB 153	< 30%	0.999	0.25	16.0	8.2	5.6	19.1	6.7	9.8	8.0	96.1	105.4	Yes	29.33
ppT	< 30%	0.999	0.25	125.0	8.7	5.1	5.9	11.3	7.9	14.3	94.4	103.8	Yes	21.32
PCB 138	< 30%	0.999	0.16	16.0	9.2	5.9	8.8	7.6	7.0	13.3	88.1	106.7	Yes	24.78
PCB 180	< 30%	1.000	0.07	16.0	8.1	6.3	10.5	6.2	6.2	10.3	95.4	106.9	Yes	22.73
MRX	< 30%	1.000	0.09	50.0	8.2	6.2	10.4	12.1	6.0	5.0	92.6	107.4	Yes	23.45