DETERMINATION OF PCDD/Fs IN COW MILK USING TWO CONVENTIONAL METHODS OF EXTRACTION

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Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), known generally just as dioxin and furans (PCDD/F), are two classes of chemicals with high environmental persistence and potent toxicity which include them in the Stockholm Convention list as well recognized Persistent Organic Pollutants – POPs[1]. Depending on the number and position of chlorine atoms in their chemical structure, PCDDs and PCDFs present a total of 75 and 135 possible congeners, respectively[2]. A group of dioxins and furans comprising 17 substituted PCDD/F with chlorine atoms in 2,3,7,8 positions induce similar toxic responses, which has been used to establishing the toxic equivalency factors (TEF) and the total toxic equivalent (TEQ) concepts[3].

Once released to the atmosphere, PCDD/Fs are subsequently deposited in soils and sediments getting accumulated in the environment. Furthermore, because of their high lipophilicity they enter in food chain and are bioconcentrated in organisms. Therefore, the main source of human exposure to these chemical residues is the consumption of contaminated food, including cow milk and dairy products that represent a considerable fraction of total dietary exposure throughout the world. It is estimated that 90% of human exposure to PCDD/Fs residues results of the consumption of fatty food of animal origin[4].

Based on that, several studies have been conducted in different countries aiming to assess human exposure to these contaminants. Authors demonstrated the occurrence of PCDD/Fs in foodstuffs like fish and other seafood, meat, egg and milk. However, milk has been pointed out as a tough matrix of working because of its high fat and protein content that can interfere in analytical determinations, making milk extraction usually long and tedious, involving several clean-up steps to reduce possible matrix interferences[4]. In general, studies of milk contamination that present good extraction efficacy employ liquid-liquid extraction (LLE) or conventional Soxhlet. Althogh other extraction techniques have been cited, the best results were usually found using these two types of extraction. In Brazil, there is a lack of information about food contamination by PCDD/Fs. Thus, this study aims to establish the best extraction for determining dioxins and furans concentrations in raw cow milk, once it is considered a nearly complete food as an important source of fat, protein and minerals, besides representing a very good media for PCDD/Fs dissolution because of its fat content.

Materials and Methods

- *Milk Sampling*

Milk sampling was performed in 20 different dairy cattle farms located in southeastern of Brazil. From each farm, a total of 10 liters of raw cow milk were collected from refrigerated tanks with homogenization. Aliquots of 2 liters of each sample were transferred into brown grass bottles which were stored in laboratory at -20 ºC until subsequent steps of sample preparation. Samples were individually analyzed but each one corresponded to a pool of dozens of cow milk.

- *Chemical Analysis*

All Solvents, Silica and Florisil® used in this study were of trace analysis quality and were purchased from Merck Co., Sigma Aldrich Co. or TediaBrazil. PCDD/Fs ^{13}C-labeled standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) or Wellington Laboratories (Guelph, Ontario, Canada).

Determination of PCDD/Fs was carried out by High Resolution Gas Chromatography/High Resolution Mass Spectrometry (Magnetic Sector Thermo/Dual Focused System - DFS) using isotopic dilution methods. Gas chromatographic separation was performed on a DB 5-MS 60 m column (Agilent Technologies). The total of PCDD/F concentrations in each sample was expressed as Toxic Equivalents (TEQ), which were calculated by multiplying the individual congeners concentrations by their respective toxic equivalency factor (TEF), as established by the World Health Organization in 1998[5] and were subsequently summed up to give the total concentrations as WHO-TEQ.

Milks were unfrozen and homogenized before sample processing. Half of the samples was submitted to lyophilization for total water removal. Dried milk and liquid crude milk were extracted using Soxhlet and liquid-liquid extraction (LLE) respectively. Samples were spiked with all seventeen 2,3,7,8-substituted PCDD/PCDF congeners with a solution containing the ^{13}C-labelled internal standards. A mixture of toluene:ethanol (4:1, v/v) was used for the Soxhlet extraction that was runned for 16 hours. LLE was carried out using sodium oxalate and methanol followed by successively extractions with n-hexane. All extracts were evaporated to dryness and the fat amount was gravimetrically determined at constant weight. The fat was dissolved in n-hexane, transferred into a clean-up column containing acidic silica and eluted using n-hexane. This fraction was partitioned in a Florisil column eluted with dichloromethane. The fraction containing PCDD/Fs was evaporated and resuspended in 20 µL of nonane containing 15 pg μL^{-1} ^{13}C-labelled internal standards.

- *Quality accuracy and quality control*

Response factors were determined by seven-point calibration. Precision and accuracy were evaluated for each method. Studies for precision were performed using real samples and accuracy studies carried out using spiked samples. The relative uncertainty for the sum of PCDD/Fs was between 10% and 15%. Recovery of the internal standards was acceptable and varied between 90% and 110%. Analytical reliability was guaranteed by the analysis of frequent five replicates and blanks. Limit of detection (LOD) was calculated at a signal to noise ratio of 3:1 on the corresponding mass trace of the analyte and the limit of quantification (LOQ) was calculated at a signal to noise ratio of 6:1. The quantification criteria included confirmation of retention times and isotope ratios of the labeled standards and respective analytes and the mass fragment with the highest intensity of the molecular or fragment ion cluster. Results bellow LOQ were assigned with a zero value. Concentrations were given as pg g^{-1} fat and all analyses were performed five times each.

Results and Discussion

The mean concentrations of each PCDDs and PCDFs congener found in 20 different cow milk samples, extracted by LLE and Soxhlet, are reported in Table 1. All concentrations were calculated with respect to the lipid content of each sample by dividing the whole weight concentration by the lipid in each sample.

Our results indicated that in general, LLE was able to extract a larger amount of the analytes than the Sohxlet extraction (Table 1) and total PCDD concentration in the milk was in average 58% higher when LLE was used. Non-target PCDD concentration found using LLE were also higher (73%) than using Sohxlet extraction. LLE also rendered 51% higher dl-PCDF levels, and 46% higher levels of total-PCDF. TEQ values were 49% higher when LLE was used. Our results indicated that LLE of liquid milk was more efficient than the Sohxlet extraction of dried milk for determination of PCDD/Fs in raw cow's milk.

The profile of congeners (Figures 1), showed a clear predominance of 2,3,4,7,8-PeCF and 2,3,7,8-TCDD. However, the congeners profile was not clearly affected by the extraction method.

The TEQ values obtained were in the same order of magnitude of those previously reported in other countries. For example they ranged from 0,3 to 1,3 pg WHO-TEQ g^{-1} fat (PCDD/Fs = 0,60 pg WHO-TEQ g-1 fat in Spain, from 1,67 pg. WHO-TEQ g^{-1} fat in Italy and 0,70 – 1,27 pg. WHO-TEQ g^{-1} fat in United Kingdom)[6,7,8].

Table 1. Mean, median and range (minimun-maximun) concentrations of PCDD/Fs (pg g^{-1} fat) in 20 milk samples extracted by liquid-liquid extraction and by Soxhlet.

	LIQUID-LIQUID EXTRACTION (n=20)				SOXHLET EXTRACTION (n=20)			
Congeners	**Mean**	**Median**	**(min-max)**	**D***	**Mean**	**Median**	**(min-max)**	**D***
PCDDs								
2,3,7,8-TCDD	0,3976	0,3798	(0,3186-0,4672)	8	0,2838	0,2724	(0,2113-0,3434)	6
1,2,3,7,8-PeCDD	-	-	-	0	-	-	-	0
1,2,3,4,7,8-HxCDD	0,6422	0,6224	(0,5679-0,7006)	19	0,0525	0,1091	(0,0396-0,1783)	15
1,2,3,6,7,8-HxCDD	0,5326	1,3122	(0,2173-1,0949)	20	0,3248	0,3709	(0,0838-0,6580)	20
1,2,3,7,8,9-HxCDD	1,2042	1,2264	(1,0167-1,4342)	18	0,0798	0,1202	(0,0599-0,1375)	10
1,2,3,4,6,7,8-HpCDD	3,0463	3,2325	(2,0693-4,1550)	20	1,3488	1,3598	(1,2460-1,5104)	20
OCDD	5,5763	6,7232	(4,0583-8,0080)	20	3,3737	4,4214	(2,2800-5,0186)	20
TCDD (non-targeted)	0,4292	0,3986	(0,2723-0,5862)	20	0,9106	0,6786	(0,3854-1,1937)	20
PeCDD (non-targeted)	1,4678	1,7422	(0,5736-2,3616)	20	1,7299	1,5429	(0,1587-3,3012)	20
HxCDD (non-targeted)	2,0306	2,3506	(1,5421-2,5193)	20	1,4066	1,9973	(0,5675-2,6843)	20
HpCDD (non-targeted)	3,6612	4,3747	(2,2255-5,2693)	20	1,5173	1,7621	(1,0598-1,9452)	20
PCDFs								
2,3,7,8-TCDF	0,4752	0,5547	(0,3467-0,7416)	20	0,3038	0,3537	(0,2424-0,4385)	20
1,2,3,7,8-PeCDF	0,6569	0,9815	(0,4417-1,1892)	20	0,2126	0,2462	(0,1314-0,2763)	20
2,3,4,7,8-PeCDF	1,5647	2,1273	(0,8869-3,1392)	20	0,7753	1,2453	(0,2811-1,7092)	20
1,2,3,4,7,8-HxCDF	0,9387	1,1035	(0,7815-1,2934)	20	0,3692	0,4867	(0,1773-0,6258)	20
1,2,3,6,7,8-HxCDF	0,8575	0,9672	(0,6398-1,3426)	20	0,3745	0,4286	(0,1744-0,6630)	20
2,3,4,6,7,8-HxCDF	1,0679	1,1095	(0,7734-1,4023)	20	0,3623	0,3845	(0,2263-0,4969)	20
1,2,3,7,8,9-HxCDF	1,5178	1,3985	(0,9862-1,7832)	10	0,7557	0,6348	(0,4327-0,8347)	12
1,2,3,4,6,7,8-HpCDF	1,1914	1,2962	(0,8997-1,5021)	20	0,5739	0,5213	(0,3892-0,7965)	17
1,2,3,4,7,8,9-HpCDF	1,0037	1,0956	(0,8934-1,2874)	15	0,3443	0,3742	(0,2564-0,4216)	10
OCDF	5,4055	5,1534	(4,6314-6,1875)	18	3,3592	3,9954	(2,9172-5,1007)	20
TCDF (non-targeted)	6,0688	6,8354	(5,5323-7,2316)	20	2,9382	3,0062	(0,9745-3,3682)	20
PeCDF (non-targeted)	3,1657	2,9042	(2,3675-3,4429)	20	0,6317	0,5992	(0,3226-0,9309)	20
HxCDF (non-targeted)	0,8828	0,9235	(0,7243-1,0413)	20	0,3396	0,3873	(0,2627-0,4394)	20
HpCDF (non-targeted)	0,8939	0,7654	(0,5598-1,2361)	20	0,5205	0,6364	(0,3452-0,8858)	16
TOTAL	*pg.g^{-1}*		*pg WHO-TEQ g^{-1}*		*pg.g^{-1}*		*pg WHO-TEQ g^{-1}*	
PCDD/Fs (targeted)	26,0785	29,2839	1,9898		12,8942	15,3245	0,9676	
PCDD/Fs (non-targeted)	18,6000	20,2950			9,9944	10,6100		

*D = numbers of samples above LOD

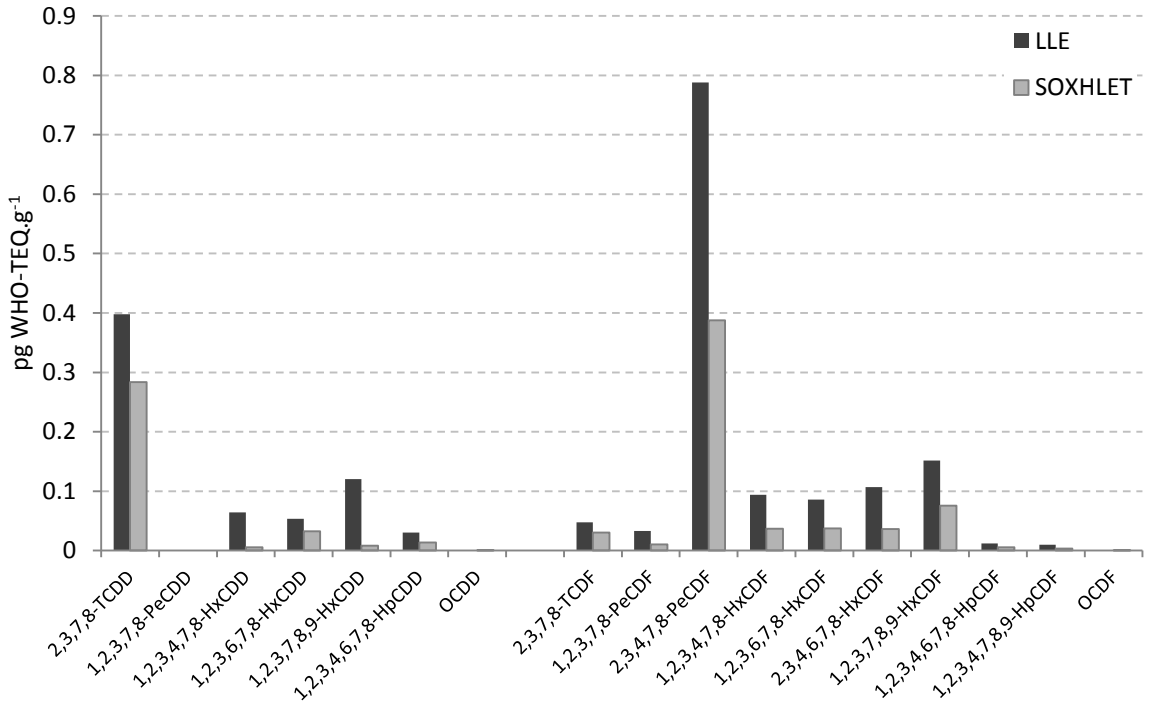

Figure 1. WHO-TEQ values of each PCDD/Fs congener (pg WHO-TEQ g^{-1} fat) in cow milks extracted by liquid-liquid extraction (LLE) and Soxhlet.

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References

1. UNEP (2001). *Stockholm Protocol on Persistent Organic Pollutants.* United Nations Environmental Programme - Chemicals, Geneva, Switzerland.

2. Baird, C. & Cann, M. (2011). Química Ambiental. 4ª Edição. Porto Alegre. Bookman. 844 pp.

3. Van Den Berg, *et al*., (1998). *Environmental Health Perspective*. 106: 775-791.

4. FAO/WHO (2009). *Environmental Health Criteria 240*. FAO/WHO Publication. Ch1: 1-24 pp.

5. Aguilera-Luiz, M.M., Plaza-Bolaños, P., Romero-González, R., Vidal, J.L.M., Frenich,A.G. (2011). *Anal. Bioanal. Chem*. 399: 2863–2875.

6. Van Den Berg, *et al*. (2006). *Toxicol. Sci.* 93(2) 223–241 .

7. Lake, I.R., Foxal, C.D., Fernandes, A., Lewis, M., Rose, M., White, O., Dowding, A. (2013). *Chemosphere.* 90: 72-79.

8. Marin, S., Villalba, P., Diaz-Ferrero, J., Font, G., Yusàn, V. (2011). *Chemosphere.* 82: 1253-1261.

9. Esposito, M. Cavallo, S., Serpe, F. P. D'Ambrosio, R., Gallo, P., Pellicanò, R., Baldi, L., Guarino, A., Serpe, L. (2009). *Chemosphere*. 77: 1212-1216.