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Fast automated extraction and clean-up of biological fluids for polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls analysis

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Abstract

A fast automated extraction and clean-up procedure for low-level analysis of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (cPCBs) in biological fluids is presented. Online extraction of prepared fluids is carried out using disposable octadecyl bonded (C_{18}) solid-phase extraction columns. Extracts are then cleaned up through disposable multi-layer silica (acidic, basic and neutral) and dispersed PX-21 carbon columns. This new methodology is compared with classical Soxhlet extraction and manual solid-phase extraction in terms of repeatability, reproducibility, accuracy and recovery rates for reference and certified materials. Robustness is evaluated on different matrices, such as cow's milk, breast milk and human serum. As a consequence of the reduced number of reusable glassware used, as well as lowering of solvent consumption, recorded blank levels are decreased in favor of limits of detection (LODs). Total analysis time and cost are further reduced using simultaneous sample preparation units and the sample throughput is increased compared to classical methods. As a result, this new approach appears to be suitable for the fast sample preparation often required for such fluids in case of emergency foodstuffs analysis or during large epidemiological studies.

Keywords: Polychlorinated dibenzo-*p*-dioxins; Polychlorinated dibenzofurans; Polychlorinated biphenyls

1. Introduction

Unintentionally produced organic pollutants, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and associated coplanar polychlorinated biphenyls (cPCBs), have attracted the interest of environmental scientists for over 30 years. These persistent, toxic and bioaccumulative compounds represent the most toxic class of persistent organic pollutants (POPs) [1]. For several years, major concern has been dedicated to these compounds because they represent a potential human risk via the environment and food consumption. Even if most countries have banned the production and use of PCBs for many years, they are

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still present in the environment and constitute a dormant source for PCDD/Fs. In addition, well-established PCDD/F sources, such as iron and steel metallurgy and incineration, are not yet reduced to a sufficiently low level to be dismissed. The lipophilic character of these POPs allows them to bio-accumulate in the food chain, thus consumption of fatty food has become the most important route of exposure.

Analytical aspects of dioxin analysis have been extensively reviewed [2–5], pointing out not only the importance of final gas chromatography–mass spectrometry (GC–MS) determination, but also the necessity of rigorous sample pre-treatment (extraction) and clean-up. Extraction of dioxins and related compounds, which traditionally implied non-instrumental approaches such as Soxhlet or liquid–liquid partitioning, has lately evolved towards techniques allowing treatment of increased number of samples in less time and reduction of solvent consumption, but requiring more sophisticated apparatus [6–8]. Instrumentation for such techniques as pressurized fluid extraction (PFE), supercritical fluid extraction (SFE) or microwave-assisted extraction (MAE) is still expensive however, and does not really allow on-line coupling with following clean-up steps.

Considering liquid samples, reversed-phase solid-phase extraction (SPE) is probably the simplest alternative to conventional techniques, such as Soxhlet and liquid–liquid extraction, used to separate analytes of interest from aqueous matrix interferences [9]. The use of such sorbents as silica-based hydrophobic octadecyl bonded (C_{18}) has been presented in recent years as a valuable tool for extraction of some POPs [10–12]. After each type of extraction, highly specific and efficient clean-up has to be carried out in order to produce clean sample extracts, for which separation of congeners can be achieved using GC–MS.

Among the broad range of purification methods, column chromatography using packing materials such as polymer beads for gel permeation, alumina, silica gel, Florisil and activated carbon have proven to be well suited for the purification of dioxins and related compounds [13–15]. Due to repetitive manipulations and the laborious character of these purification steps, several research groups have been working on automation process development in order to increase sample throughput. Already at the beginning of the 1970s, Tindle and Stalling [16] reported cost-effective automated gel permeation clean-up.

Since the demand for measurements of dioxins and related compounds in human populations dramatically increased in the 1980s, the National Center for Environmental Health at the Centers for Disease Control and Prevention (CDC) had to decrease the time required for sample clean-up and adapt it to a broader range of analytes. As blood and adipose tissues are some of the most easily available matrices used in assessing exposure to dioxins [17], Lapeza et al. [18] at CDC developed an automated apparatus for enrichment of dioxins in serum. This enabled the reduction of the time required for the clean-up procedure by half [19]. Fluid Management Systems, Inc. (FMS, inc.) upgraded and patented the system (Dioxin-Prep™) that, due to a modular design, was able to process multiple samples simultaneously using disposable PTFE columns [20]. The latest upgrades added to the current version of the system (Power-Prep™), have made it entirely PC controlled by software operating under Windows and capable of cleaning-up up to 10 samples in parallel in less than 2 h. The system is not only dedicated to serum analysis [21], but is commonly used for the analysis of a broad range of environmental [22] and food-type matrices [23]. Several groups have also reported the use of such a system for the isolation of PCBs and pesticides [24,25].

Following the trend of sample preparation time reduction, we investigated the possibility to avoid time-consuming classical extraction steps (such as Soxhlet and LLE) using SPE. Furthermore, we integrated the SPE step into the automated clean-up process. In this paper the comparison between Soxhlet, manual SPE and automated on-line (integrated) SPE for PCDD/F and cPCB analysis in different types of biological fluids samples is outlined. Repeatability, reproducibility, accuracy, robustness and recovery rates are evaluated for reference and certified materials.

2. Experimental

2.1. Chemicals and reagents

Water, hexane, pentane, toluene, ethyl acetate, acetonitrile, methanol and dichloromethane were

Pestanal reagents (Riedel-de Haën, Seelze, Germany). Nonane puriss analytical-reagent-grade standard for GC was purchased from Fluka (Steinheim, Germany). Sodium sulfate anhydrous was obtained from Baker (Deventer, Netherlands), silica gel 60 (0.063–0.200 mm) was column chromatography grade (Merck, Darmstadt, Germany), glass fiber thimbles (43×123 mm) were from Schleicher and Schuell (Dassel, Germany) and borosilicate solids glass beads (3 mm) were from Aldrich (Milwaukee, WI, USA).

The $^{13}C_{12}$-labeled internal standard solutions containing PCDDs, PCDFs and cPCBs were from Cambridge Isotope Laboratories (Andover, MS, USA). All details concerning standard solutions have been listed in a previous paper [23].

2.2. Samples

Long-life pasteurized cow's milk samples were issued from high delivery rate supermarkets and were full-fat grade (3%). Breast milk samples issued from primi and multiparae mothers were collected at different times of lactation from volunteers living in the area of Liege (Belgium). Samples were stored less than 1 day at 4 °C before freezing at −20 °C until analysis. Milk sample sizes ranged between 20 and 100 ml.

Reference materials, milk powder RM 532, RM 533, RM 534 and the certified reference material, milk powder CRM 607 were obtained from the Institute of Reference Materials and Measurements of the European Commissions Joint Research Center (IRMM, Gell, Belgium) [26,27]. These represent various levels, below and above regulation values. An ''in-house'' serum quality control (QC) sample was used. It consisted of fetal bovine serum fortified with 17 PCDD/Fs and four cPCBs to have a content of about 230 pg TEQ/l and 380 pg TEQ/l, respectively. The toxic equivalent (TEQ) acronym represents the translation of a given concentration of PCDDs, PCDFs and PCBs in terms of toxicity, relative to the most toxic congener (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). This TEQ calculation rests on the concept of toxic equivalency factors (TEFs) correlating the toxicity of each 2,3,7,8 congener to 2,3,7,8-TCDD [28]. The concentrations present in the serum QC samples were similar to those currently found in general European populations.

2.3. Extraction

2.3.1. Soxhlet extraction

Glass fiber thimbles were extracted 2 h with hexane before use. Milk powder samples were Soxhlet extracted using pentane–dichloromethane (1:1) as solvent. Extraction with 400 ml of solvent containing borosilicate solid glass beads was carried out overnight on 10 g of spray-dried milk slurry with 10 g of water in order to increase accessibility of the solvent [27]; 10 g of sodium sulfate as well as 10 g of silica gel were also mixed into the slurry. Resulting extracts were dried on sodium sulfate and the extraction solvent was removed using a rotary evaporator. Lipid content was determined gravimetrically. The required amounts of lipids (up to 4 g) were then dissolved in hexane prior to further clean-up.

2.3.2. Manual solid-phase extraction

Cow's milk and breast milk samples (20–100 ml) were pre-treated using a modified version of an AOAC method [29]. Briefly, milk fat globule membranes were disrupted by potassium oxalate (20 mg/g milk) and acetonitrile that is added to the milk (1:1) prior to water (1:1). Between 50 and 300 ml of treated sample is then loaded on Isolute Flash C_{18} cartridges (International Sorbent Technology, Hengoed, UK). These cartridges consisted of polypropylene syringe-barrels of 150 ml filled with 25 g of non-endcapped C_{18} bonded silica sorbent (average particle size 50 μm, 60 Å porosity). Cartridges were used on a Flashvac® extraction vacuum manifold (IST). C_{18} sorbent was solvated using two cartridge volumes of acetonitrile and two cartridge volumes of water at a flow of 20 ml/min prior to the addition of sample at a maximum flow-rate of 10 ml/min. The SPE cartridge was washed twice with 10 ml of water and 2 ml of methanol were added (to make drying easier) prior to 1 h of drying under vacuum. PCDD/Fs and PCBs were eluted using four times 15 ml of hexane at a flow-rate of 5 ml/min. Extracts were concentrated to 15 ml using a Turbovap II Concentration Workstation (Zymark, Hopkinton, MA, USA) prior to further clean-up.

Spray dried reference (RM 532, 533, 534) and certified (CRM 607) materials were similarly treated after reconstitution in warm (50 °C) water in order to produce the equivalent of a full fat (3%) cow's milk.

Serum samples were extracted following a modified CDC protocol [20]. Sample sizes were 30–60 ml. Isolute 10 g/70 ml C_{18} non-endcapped cartridges (IST) were used. A mixture containing sample, formic acid and water (1:1:1) was loaded on the C_{18} SPE cartridge (previously solvated using two volumes of methanol and two volumes of water) at a maximum flow-rate 10 ml/min. The SPE cartridge was washed twice with 10 ml of water and 2 ml of methanol prior to 1 h of drying under vacuum. PCDD/Fs and PCBs were eluted using three times 15 ml of hexane at a flow-rate of 5 ml/min. Extracts were concentrated to 15 ml using a Turbovap II.

2.4. Clean-up

Automated multi-column clean-up was carried out on the Power-Prep System (FMS Inc.). Details concerning the system have been previously published [22,23]. Briefly, hexane serum extracts (low fat content) were processed through a set of disposable columns consisting of a multi-layer silica column (4 g acid, 2 g base and 1.5 g neutral), a basic alumina column (8 g) and a carbon column (2 g dispersed PX-21). Purified extracts (60 ml of toluene) were concentrated to approximately 150 μl, using the optical sensor and time options available on the Turbovap II workstation, and transferred to conical vials containing 4 μl of nonane used as keeper. The remaining toluene was slowly evaporated at room temperature by placing the vial in a dust-free evaporation box connected to the hood, prior to GC–HRMS injection.

Milk extracts (high fat content) were purified in a similar automated way, but using additional high capacity disposable silica columns (HCDS) (28 g acidic, 16 g basic, 6 g neutral) required to eliminate higher amounts of lipids. The use of this set of four columns has been described elsewhere [23].

2.5. Integrated extraction and clean-up

The modified plumbing diagram of the system is presented in Fig. 1.

In the present study, we used various C_{18} column sizes (10–40 g) as well as various silica column sizes, depending on the amount of fat present in the samples. A classical set of columns for cow's milk was 20 g C_{18} column, HCDS and carbon column, although 10 g C_{18} column, small silica column and carbon column were sufficient for serum samples. Events occurring during the automated extraction and clean-up step are schematized in Fig. 2.

Prior to loading on the system, samples were prepared with adequate solvents to allow lipid membrane disruption and protein precipitation, as in manual SPE. After the sample loading, during which most of the matrix components are eliminated (F1 to aqueous waste), the C_{18} column was flushed with 50 ml of water at a flow-rate of 10 ml/min to wash out the remaining milk from the column (F2 to aqueous waste). After a drying step of 30 min using nitrogen (F3 to aqueous waste), elution of C_{18}-retained compounds was carried out to the waste (F4 to organics waste) via the multi-layer silica and the carbon columns using 100 ml of hexane at a flow-rate of 10 ml/min. Lipid degradation occurred in the silica column which is further washed with 150 ml of hexane to ensure that all dioxins and related compounds were loaded onto the carbon column (F5 to organic waste). Most of the non-planar organohalogen compounds are eliminated during this step. Two wash steps using a mixture of hexane–dichloromethane (1:1) (F6 to organic waste) and a mixture of ethyl acetate–toluene (1:1) (F7 to organic waste) were finally performed to ensure removal of any remaining organic interferences prior a back-flush elution (60 ml of toluene at a flow-rate of 5 ml/min) of the carbon column, producing the F8 PCDD/F and cPCB fraction. At the end of the process, the system was automatically decontaminated via a simple solvent program. As in the case of the classical clean-up, the toluene solution (60 ml) containing PCDD/Fs and cPCBs was concentrated to 150 μl and transferred to conical vials containing 4 μl of nonane used as keeper. The vial was then capped when remaining toluene was evaporated, leaving analytes in nonane in the conical vial.

2.6. Analysis

A set of seven PCDDs, 10 PCDFs and four cPCBs was analyzed by GC–HRMS using a MAT95XL high-resolution mass spectrometer (Finnigan, Bremen, Germany) and a Hewlett-Packard (Palo Alto,

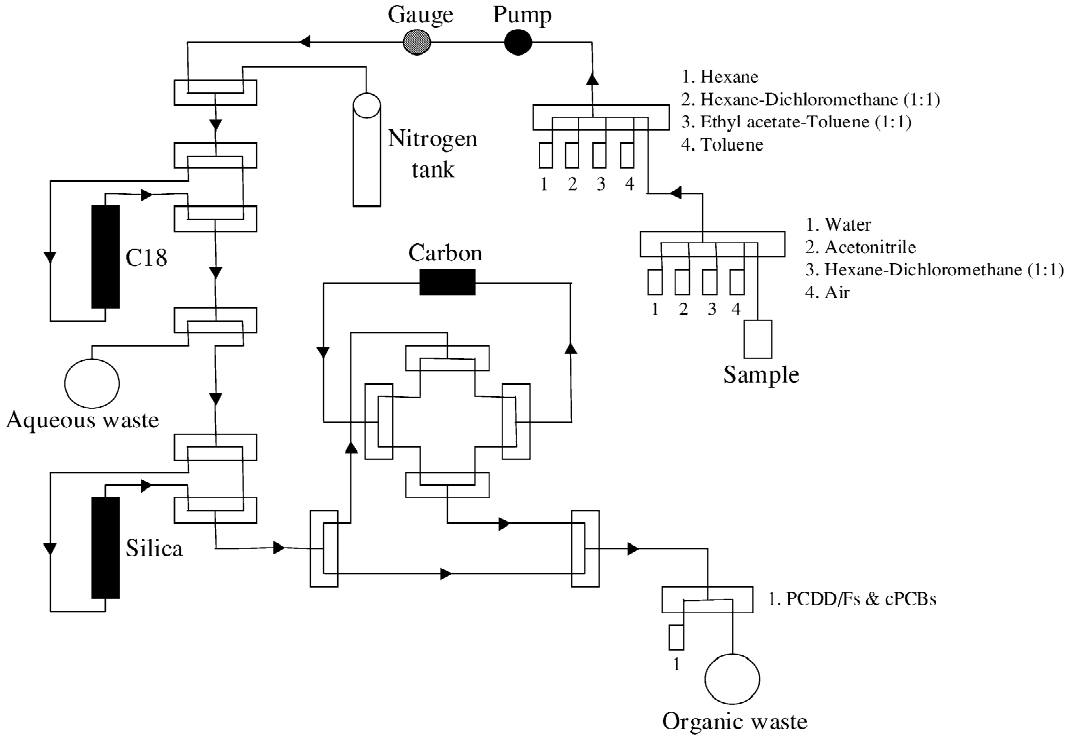

Fig. 1. Modified plumbing diagram of the integrated extraction and clean-up system. The presented set of solvents is used for preparation of milk samples; if serum samples are treated, acetonitrile is replaced by methanol. PC-controlled electrostatic valves are responsible for the solvent selection and travel inside the system, in accordance with a prepared flow program.

CA, USA) 6890 Series gas chromatograph. All details of physico-chemical analyses have been described in a previous report [23]. Briefly, the column was a RTX-5SIL-MS (30 m×0.25 mm I.D., 0.25 μm film thickness) capillary column (Restek, Evry, France). The mass spectrometer operated at a minimum resolution of 10 000 (10% valley), in the electron impact (EI) ionization mode, using selected ion monitoring (SIM) in isotopic dilution. Samples were spiked with $^{13}C_{12}$-labeled internal standard solutions containing PCDDs, PCDFs and cPCBs prior to the extraction step. Isotope ratios, MS sensitivity and relative response factor (RRF) of each congener were monitored to ensure that the system was permanently under control. Percentages of ^{13}C recoveries were calculated using a recovery standard solution that was added to the conical vial containing the nonane prior to the injection on the GC. TEQs of all congeners were calculated using 2,3,7,8-TCDD TEFs reported by WHO [30].

2.7. Quality control

In addition to RMs, CRMs and ''in house'' QC samples, procedural blanks (both instrumental and method) were included to ensure that the analytical system was free of interfering contamination. For Soxhlet, thimbles were filled with 10 g of sodium sulfate, 10 g of water and 10 g of silica gel; extracts were further processed as real samples. For SPE, water was used instead of milk or serum. The $^{13}C_{12}$-labeled multi-analyte internal standard solution (10 μl of nonane) was spiked directly in water.

3. Results and discussion

3.1. Practical aspects

Although the syringe-barrel cartridge format is usually the favored one for manual SPE of biological

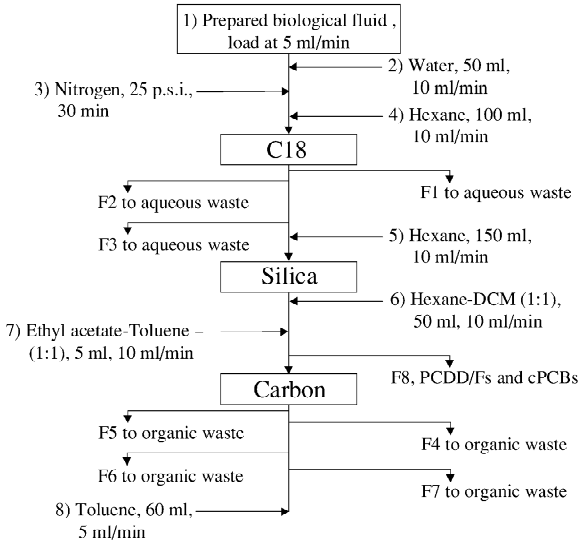

Fig. 2. Flow chart of events occurring during the automated extraction and clean-up steps. Each step takes place sequentially from sample load (step 1) to final back-flush elution of carbon using toluene (step 8), producing the PCDD/F and cPCB fraction (F8). Solvation and conditioning steps not shown for clarity.

fluids, this format was not really suitable for on-line LC coupling with clean-up columns. The column format, which does not present dead-volume problems, was more versatile in the case of automation. In addition, two important parameters encouraged us to use a column format. Firstly, considering that quite large amounts of samples were necessary to ensure that enough dioxins will be present to be detected, unusually large quantities of C_{18} were required. Secondly, we observed that excessive shaking of such treated samples could result in strong protein precipitation and plug the C_{18} bed, especially in the case of some protein-rich breast milk samples. Since it was recently reported [31] that a non-negligible fraction of organochlorine compounds could be present in some protein fraction of biological fluids, we decided to avoid any filtration step prior to analysis. A combination of a soft controlled agitation of samples, to reduce precipitation problems, and the use of a larger C_{18} particle size of 120–200 μm instead of classical 40–60 μm size allowed samples to be loaded in good condition on the C_{18}. We then ended up with a completely automated system able to accommodate aqueous and organic solvents required for the use of C_{18} columns, high capacity disposable multi-layer silica columns (HCDS) and carbon columns.

During the present study, special attention was given to the monitoring of cleanliness and the quality of extracts produced. As the new method was developed to be used in routine analysis of a large number of samples, efforts were carried out to ensure stability and robustness of the whole analysis, including the delicate GC–HRMS step. The most challenging part of the method development consisted of the coupling of the aqueous media extraction step to the organic solvent media clean-up step inside a single automated apparatus. A major problem was to ensure an efficient drying step of the C_{18} sorbent prior to hexane elution through the multi-layer silica column (Fig. 2). The first attempt did not really succeed with the consequence of partial deactivation of the acidic silica and production of acidic fluids inside the system, resulting in reduced clean-up efficiency. The insertion of a small disposable sodium sulfate column (2.5 g) between C_{18} and silica columns was a partially working solution, but the use of nitrogen gas (25 p.s.i. for 30 min) was the best alternative. The final configuration of the system produced extracts of same quality as the classical Power-Prep automated clean-up system, as illustrated in Fig. 3.

Required GC–MS criteria, such as isotopic ratio check, signal-to-noise ratio value and peak resolution were fulfilled, and neither GC column nor MS source required additional downtime maintenance.

3.2. Milk powder reference materials

The applicability of the new procedure was tested on milk powder reference materials presenting various concentrations (naturally contaminated and spiked) of PCDD/Fs (RM 532, RM 533, RM 534) [26]. Since Soxhlet extraction is still considered as the reference method in many applications, Soxhlet was used as a well-established technique against which the new method was compared in order to demonstrate its efficiency. In addition to this reference method, manual SPE was carried out as an intermediate method between Soxhlet and integrated SPE. Figs. 4 and 5 represent mean values for low

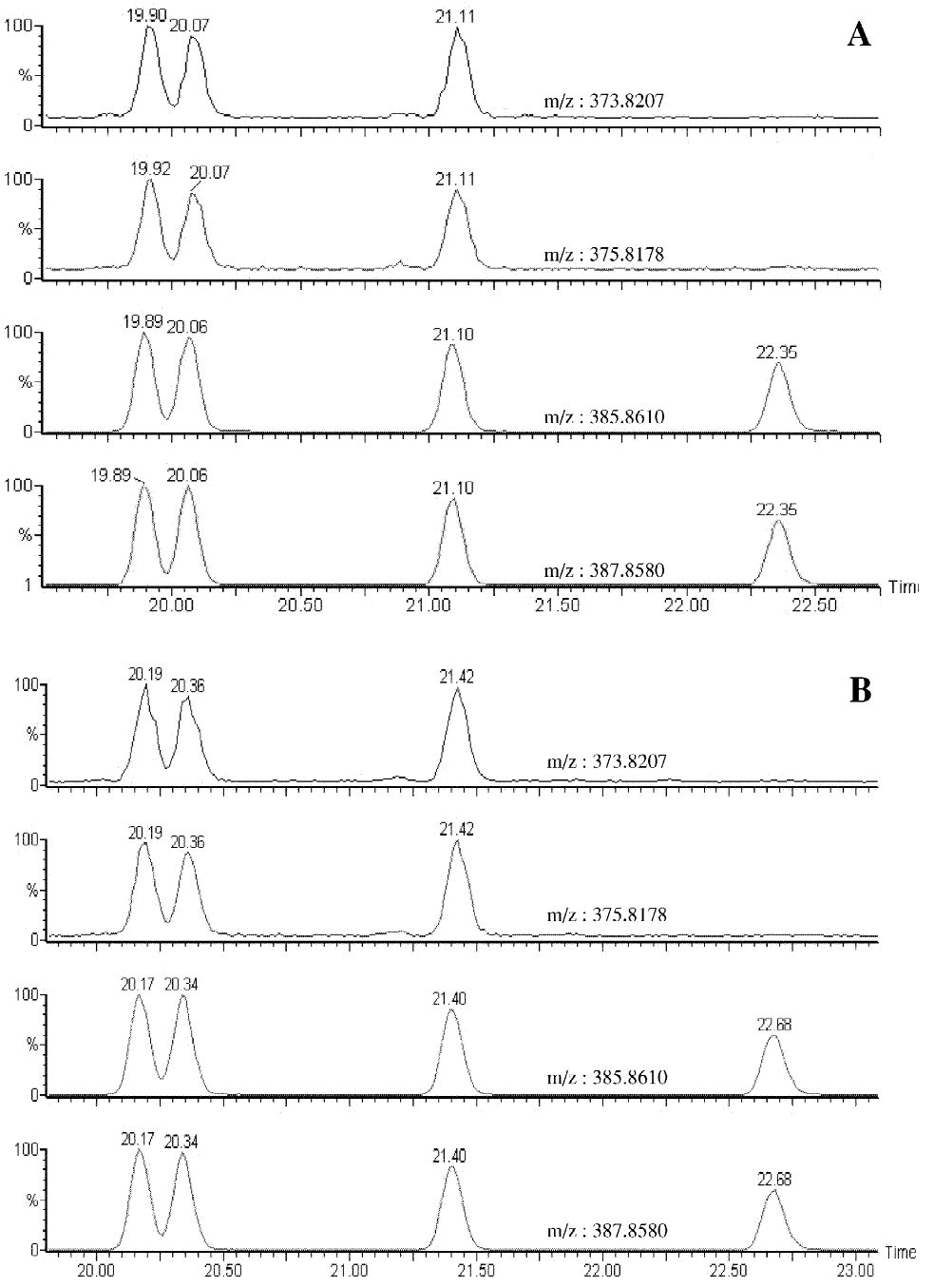

Fig. 3. GC-IDHRMS chromatogram (non-smoothed peaks) of four HxCDFs congeners using Soxhlet extraction followed by automated clean-up (A) and integrated SPE clean-up (B) determined in the milk powder reference material RM 533. Peaks from left to right, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF and 1,2,3,7,8,9-HxCDF. In both chromatograms, the first two windows represent native congeners (m/z: 373.8207 and m/z: 375.8178), the second and third windows account for ^{13}C-labeled congeners (m/z: 385.8610 and m/z: 387.8580) used for isotopic dilution. The 1,2,3,7,8,9-HxCDF congener is not present as native in RM 533. Time scale in min.

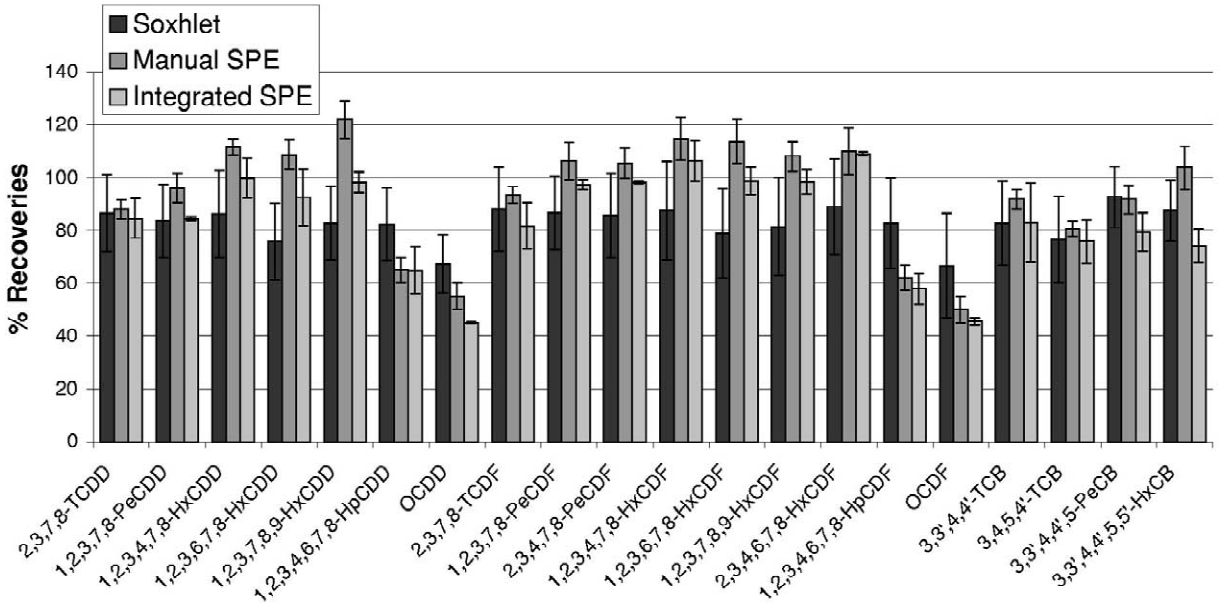

Fig. 4. Recoveries of ^{13}C-labeled PCDD/F and cPCB congeners for milk powder (RM 533) samples (10 μl of a nonane solution containing analytes in concentrations ranging between 25 and 125 pg/μl added to the milk prior extraction). These values are representative of the whole extraction and clean-up process.

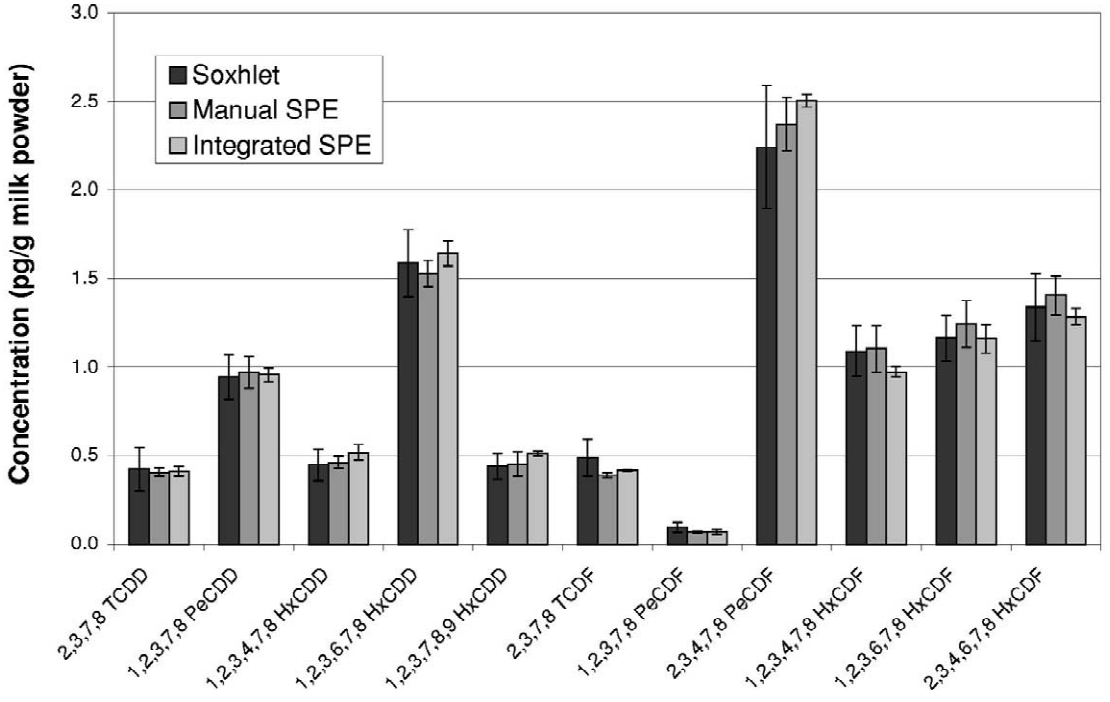

Fig. 5. Comparison between concentration values obtained using Soxhlet, manual SPE and integrated SPE for milk powder reference material RM 533.

level spiked milk powder reference material (RM 533, $n>10$ for each method).

Fig. 4 illustrates PCDD/F and cPCB recoveries (^{13}C-labeled congeners) of the three investigated methods. Labeled standards (10 μl of a nonane solution containing analytes in concentrations ranging between 25 and 125 pg/μl) were added to the milk prior to extraction, and recoveries were measured after the complete process, including clean-up. Recovery values for both SPE methods were higher or equal to Soxhlet for most PCDD/F congeners. However, in the case of hepta- and octa-chlorinated congeners, recoveries were 15–20% lower for SPE. A reason for that might be the lower capability of these congeners to be retained on the C_{18} through non-polar interactions that are a primary retention factor on such modified silica sorbents. The potential for secondary polar interaction via free silanol groups (non-endcapped) was insignificant because of the predominant effect of the long C_{18} hydrocarbon chain.

Focusing on SPE methods, it was observed that integrated SPE recovery rates were always slightly lower than manual SPE ones. This resulted from the loss of the opportunity of flow adjustment during the automated experiment compared to manual processing during which a skilled operator always has a possibility of intervention. Nonetheless, integrated SPE still produced satisfactory results. Considering SDs, it appeared that SPE methods were more stable than Soxhlet. Surprisingly, the manual SPE method presented lower RSD than the integrated one. Again, a skilled operator always has access to fine-tuning following his own experience, while the automated system executes a pre-defined sequence of events.

In Fig. 5, congener-specific concentration values are plotted for a relative comparison between methods. All triplicate runs were carried out by the same operator using the same amount of powder (8 g). Inter-method reproducibility was shown to be very good with a maximum difference amplitude of 10% between concentrations of congeners present in the material. It is interesting to note that for this RM 533 milk, as in the case of recoveries, Soxhlet extraction exhibited higher intra-method RSD values.

In order to evaluate the accuracy of the method, we have analyzed a certified (low level naturally contaminated) reference material (CRM 607) dedicated to method validation [27]. As for reference (non-certified) RM 533, only five PCDDs and six PCDFs were present in the milk. In addition, since: (1) we were still using 8 g of powder and (2) 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF were in very low concentrations (0.05 and 0.054 pg/g powder, respectively), no data were obtained for these particular congeners. This, however, was the best available certified material at the time of the study. Measured PCDD and PCDF concentrations are given in Table 1.

These results (triplicate analyses) were obtained by the same operator in a short time period. It appeared that repeatability (short-term standard deviation) for Soxhlet extraction of investigated congeners was slightly better (RSDs below 10%) than in

Table 1
Repeatability and accuracy of Soxhlet, manual SPE and integrated SPE for determination of PCDD/F concentrations in certified reference milk powder material CRM 607

	Certified values	Soxhlet			Manual SPE			Integrated SPE		
	Conc. (pg/g)	Conc. (pg/g)	RSD (%)	Accuracy (%)	Conc. (pg/g)	RSD (%)	Accuracy (%)	Conc. (pg/g)	RSD (%)	Accuracy (%)
2,3,7,8-TCDD	0.25±0.03	0.29	3	116	0.27	13	108	0.34	6	134
1,2,3,7,8-PeCDD	0.79±0.04	0.81	7	102	0.94	2	119	1.03	4	130
1,2,3,4,7,8-HxCDD	0.42±0.07	0.46	8	110	0.47	15	113	0.40	10	95
1,2,3,6,7,8-HxCDD	0.98±0.11	1.09	7	115	0.90	10	92	0.98	15	100
1,2,3,7,8,9-HxCDD	0.34±0.05	0.38	8	113	0.33	3	98	0.37	8	110
2,3,4,7,8-PeCDF	1.81±0.13	1.81	5	101	1.98	1	109	2.23	6	123
1,2,3,4,7,8-HxCDF	0.94±0.04	0.92	8	101	0.86	15	91	0.87	8	93
1,2,3,6,7,8-HxCDF	1.01±0.09	1.08	3	109	1.14	8	113	1.10	6	109
2,3,4,6,7,8-HxCDF	1.01±0.05	1.07	8	100	1.18	2	110	1.18	8	110
Sum	7.61±0.61	7.92	4	103	8.08	2	105	8.49	4	110

the case of both SPE methods (RSDs up to 15%). These rather high RSDs might be a consequence of the combination of a very low level of contamination and a quite fast extraction step (a matter of minutes for SPE, compared to hours for Soxhlet). Any small variation in parameters such as solvent volume, solvent flow or C_{18} bed homogeneity can therefore have a significant impact on so short a process from a time scale viewpoint. Accuracy in terms of recovery of native congeners regarding the certified values was acceptable for all methods with a 110% value for the sum of congeners in the case of integrated SPE.

3.3. Method blanks

As one would expect, recorded blank levels were much lower for the integrated SPE method than for classical Soxhlet extraction. Among congeners constituting the background concentration for Soxhlet, levels were reduced and OCDF was not detected any more using the new method. Concentrations of remaining congeners decreased by one or two orders of magnitude. For total PCDD/Fs, the blank level decreased from 18.1±8.3 pg/g fat in the case of Soxhlet to less than 2 pg/g fat (1.2±1.4 pg/g fat) for the integrated SPE method. PCB 77 concentrations were more than 70 times lower, reducing the mean cPCB level (sum of four congeners) to 14.7±4.6 pg/g fat instead of 486.6±83.2 pg/g fat.

Knowing that estimations of LODs for a designated method are directly related to the blank levels (and related SDs) of a selected matrix in a defined method, the reduction of the background signal allowed to significantly reduce LODs. LODs were evaluated using either average method blank values or values of smaller added concentration giving a signal with a signal-to-noise ratio (S/N) greater than 3 when method blank values were too low (n.d.). Congeners were recorded as ''non-detected'' when S/N for a given peak was lower than 3. LODs were defined as this $S/N>3$ value plus three times the standard deviation (SD) of the blank. LOQs were defined as this $S/N>3$ value plus 10 times the SD of the blank. LODs for methods based on both Soxhlet and integrated SPE system are listed in Table 2.

No differences in LOD values were observed for congeners that were not present in the blank since

Table 2
Comparison of measured blank levels and calculated LODs for Soxhlet and integrated SPE in the case of milk samples

	Soxhlet			Integrated SPE		
	Blank pg/g fat	LOD pg/g fat	LOQ pg/g fat	Blank pg/g fat	LOD pg/g fat	LOQ pg/g fat
2,3,7,8 TCDD	n.d	0.10	0.20	n.d	0.10	0.20
1,2,3,7,8 PeCDD	n.d	0.15	0.29	n.d	0.15	0.29
1,2,3,4,7,8 HxCDD	n.d	0.17	0.34	n.d	0.17	0.34
1,2,3,6,7,8 HxCDD	n.d	0.15	0.29	n.d	0.15	0.29
1,2,3,7,8 HxCDD	n.d	0.16	0.32	n.d	0.16	0.32
1,2,3,4,6,7,8 HpCDD	**3.15**	**4.25**	**6.81**	**0.11**	**0.69**	**2.04**
OCDD	**9.61**	**13.42**	**22.30**	**0.86**	**5.32**	**15.74**
2,3,7,8 TCDF	n.d	0.12	0.25	n.d	0.12	0.25
1,2,3,7,8 PeCDF	n.d	0.10	0.20	n.d	0.10	0.20
2,3,4,7,8 PeCDF	n.d	0.14	0.27	n.d	0.14	0.27
1,2,3,4,7,8 HxCDF	n.d	0.11	0.23	n.d	0.11	0.23
1,2,3,6,7,8 HxCDF	n.d	0.11	0.23	n.d	0.11	0.23
1,2,3,7,8,9 HxCDF	n.d	0.11	0.23	n.d	0.11	0.23
2,3,4,6,7,8 HxCDF	n.d	0.13	0.26	n.d	0.13	0.26
1,2,3,4,6,7,8 HpCDF	**2.56**	**4.51**	**9.04**	**0.25**	**0.69**	**1.72**
OCDF	**2.74**	**3.78**	**6.20**	n.d	0.19	0.39
PCB 77	**443.37**	**692.78**	**1274.74**	**6.00**	**18.88**	**48.92**
PCB 81	**35.80**	**46.92**	**72.87**	**8.10**	**8.47**	**9.32**
PCB 126	**7.42**	**10.27**	**16.94**	**0.57**	**1.58**	**3.93**
PCB 169	n.d	0.10	0.20	n.d	0.10	0.20

Differences in LOD values only appeared when congeners were detected in the blank. Major improvements are in bold.

LODs were then mainly based on the mass spectrometry instrument itself. However, for all congeners present in the blank, a significant reduction of LODs and LOQs were observed. The larger improvement in LODs was observed inside the cPCB family in which LODs for PCB 77 and PCB 81 were strongly reduced.

In addition, since lower bound (contribution of n.d. congeners equal to zero) and upper bound (contribution of n.d. congeners equal to the LOQ) strategies tend to develop in the dioxin field, and as EU proposed maximum limits of PCDD/Fs in food have been set on the basis of upper bound limits, the proposed integrated sample preparation technique well fit with the need of developing analytical methods that exhibit sufficient sensitivity to avoid overestimation of analyte concentrations. In fact, since no standardized official method exists so far for the determination of PCDD/Fs in foodstuffs, expert committees recommend that the difference between lower bound values and upper bound values should not exceed 20%. Reduced LOQs observed in the present study can help to minimize this difference and reduce overestimation problems due to the use of an upper bound approach.

The use of disposable low background level columns in parallel with a significant reduction of amount of glassware involved in the sample preparation represent the main reasons for reduction of LODs. Risk of cross-contamination is also reduced to a minimum after a simple wash of the extraction and clean-up apparatus. Solvent volume reduction already reported for the use of the classical automated clean-up system [23] is further increased by integrating the extraction step in the process. Large volumes of solvent required for Soxhlet were reduced by half, including C_{18} solvation and conditioning steps. Additionally, for biological fluids, the time-consuming lyophilisation step is no longer required prior to SPE. These improvements also yield a reduction of cost per sample and simplification of operating procedures, which then require less personnel training.

The proposed integrated SPE method has a final attractive advantage over Soxhlet extraction. In the case of an emergency situation, the time between sample reception and report can be reduced down to 1 day. This is valuable in the case of food samples suspected of contamination for which reduction of the waiting period prior to voicing of results can be crucial.

3.4. Serum QC samples

In addition to high fat content cow's milk, serum samples represent another important biological fluid that also requires intensive labor prior to analysis. For the general population, however, serum presents a much lower lipid content than milk (0.5% in weight). In terms of sample preparation, 40 ml of serum are generally required to produce PCDD/F and cPCB values for samples issued from industrialized countries. We evaluated the new method on this amount of serum samples used as QCs in routine analysis of real samples. Fig. 6 illustrates typical QC charts routinely used to monitor QC levels and ensure control of the method accuracy.

These charts were built up on the basis of a measured mean concentration for the ''in-house'' QC material. The mean value was recalculated each time a new value was made available following a QC sample analysis. The confidence interval was established based on SD values. The 95 and 99% intervals were set as mean$\pm$2SD and mean$\pm$3SD, respectively. In practice, the 95% limit is the one outside of which measures have to be taken to re-establish a stable system, and the 99% interval defines the ultimate limit outside of which the method has to be considered as defective and requires adequate improvement. In these charts, manual (validated routine method) SPE values account for a 3-month period and integrated SPE values were recorded in a 2-month period. Reproducibility (long-term standard deviation) of the new method was demonstrated with different operators over time. Although a contamination problem occurred (run number 13) for manual SPE, none of the recorded concentrations were ever outside the 95% control limit for the integrated SPE method. Recoveries ranged between 85 and 50% (heptachlorinated- and octachlorinated-congeners) for PCDD/Fs and were around 80% for cPCBs.

3.5. Real human and animal milk samples

The robustness and applicability of the integrated SPE method were evaluated through analysis of real

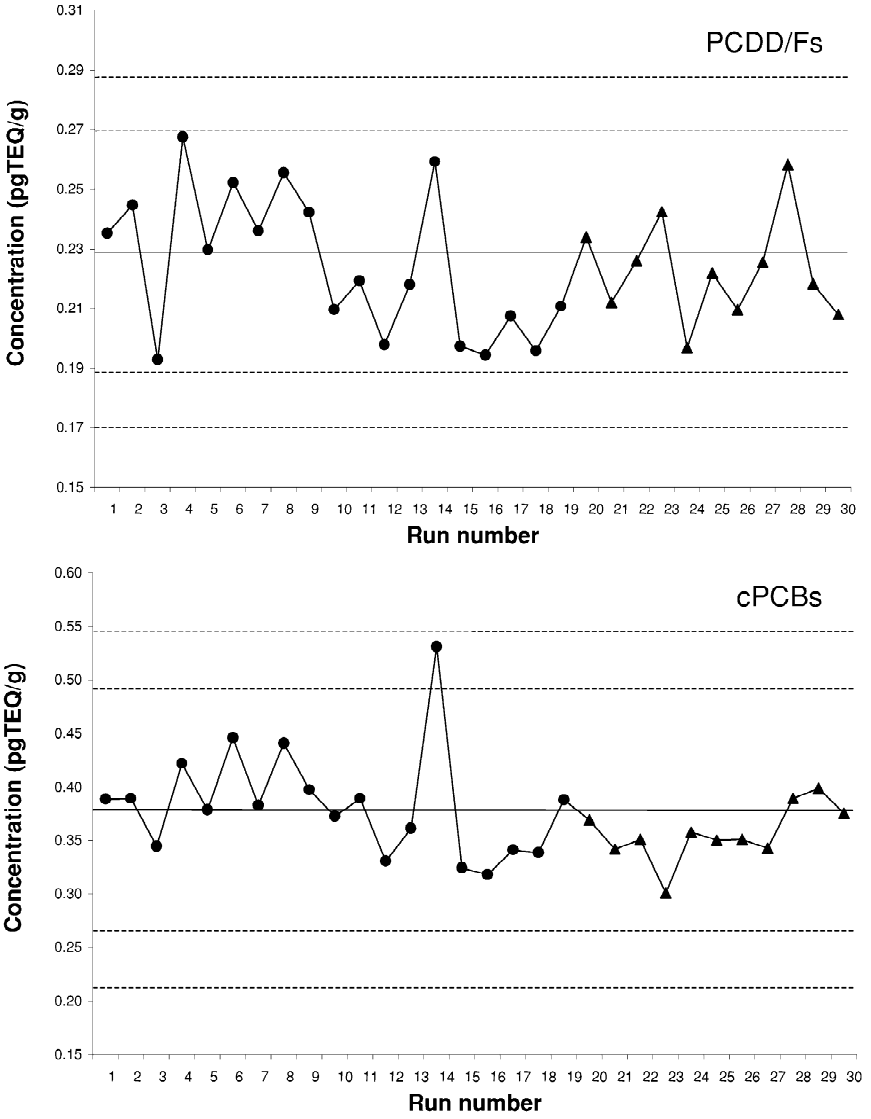

Fig. 6. Quality control chart for determination of PCDD/Fs (top) and cPCBs (bottom) in the ''in-house'' QC pool of fortified bovine serum; ●, manual SPE values; ▲, automated extraction and clean-up values. Dotted lines account for 95 and 99% confidence interval.

breast milk and cow's milk samples. Measured concentrations are listed for each congener in Table 3.

It appeared that recovery rates for breast milk were somewhat lower than one could have expected from reference material study. This might be partially due to the fluctuations in composition of investigated breast milks. The lipid content of samples ranged between 0.5 and 5.9%, depending on when the sampling was carried out during the lactation period. In addition, since collected samples were aged between week 1 and week 128 after delivery, the protein content was also very different from one sample to another. Higher sample turbidity was always observed with early-collected colostrum milks, mainly due to their higher protein (mainly casein) concentration. As mentioned in Section 2.5, careful agitation of such samples did allow their

Table 3
Congener-specific concentrations of PCDD/Fs and PCBs and recovery rates of corresponding ^{13}C-labeled PCDD/Fs and cPCBs (10 μl of a nonane solution containing analytes in concentrations ranging between 25 and 125 pg/μl added to the milk prior to extraction) in breast and cow's milk samples analyzed by the integrated SPE method

Matrix compounds	Breast milk ($n=20$)		Cow's milk ($n=35$)	
	Conc. (pg/g fat)	Recovery (%)	Conc. (pg/g fat)	Recovery (%)
2,3,7,8-TCDD	2.31	49	0.08	71
1,2,3,7,8-PeCDD	8.29	55	0.32	72
1,2,3,4,7,8-HxCDD	8.01	60	0.11	75
1,2,3,6,7,8-HxCDD	28.82	58	0.54	72
1,2,3,7,8, 9-HxCDD	4.68	60	0.14	73
1,2,3,4,6, 7,8-HpCDD	26.57	40	0.58	70
OCDD	223.20	42	1.81	55
2,3,7,8-TCDF	1.33	53	0.11	74
1,2,3,7,8-PeCDF	0.78	54	0.04	74
2,3,4,7,8-PeCDF	25.20	52	0.97	72
1,2,3,4, 7,8, -HxCDF	5.43	62	n.d	78
1,2,3,6,7,8-HxCDF	6.45	57	0.39	76
1,2,3,7,8,9-HxCDF	0.12	56	n.d	70
2,3,4,6,7,8-HxCDF	3.16	64	0.53	77
1,2,3,4,6,7,8-HpCDF	5.32	36	0.02	71
OCDF	n.d	38	n.d	54
3,3′,4,4′-TCB (PCB 77)	45.94	54	12.26	73
3,4,4′,5-TCB (PCB 81)	<LOQ	54	<LOQ	74
3,3′,4,4′,5-PeCB (PCB 126)	107.67	51	10.56	74
3,3′,4,4′,5,5′-HxCB (PCB 169)	68.16	56	1.41	68

treatment in acceptable conditions, but such samples remained difficult to handle and always produced lower recovery values. Using this method in a routine epidemiological study context should eliminate such problems since it is generally recommended by WHO to perform the collection of breast milk samples between the second week and the second month after delivery [32]. Such samples would therefore consist of mature milk for which protein content will be lower and much more constant. This study was in any case a nice occasion to test the robustness of the method, which finally produced good quality results for all investigated milks.

Cow's milk was full-fat grade (3%) long-life pasteurized commercial samples. They were easily processed in various quantities ranging between 40 and 100 ml. Recovery rates were in good agreement with recommendations on ^{13}C-labeled congener recovery rates for foodstuffs analysis (50 to 120%) and permitted quantification in good conditions. In terms of toxicity, measured TEQ concentrations were, respectively 40.8 pg TEQ/g fat and 2.2 pg TEQ/g fat for PCDD/Fs and cPCBs in breast and cow's milk. These concentrations were comparable to those generally reported in the literature [33,34]. Details on these studies have been published elsewhere [35,36].

4. Conclusions

Simplification of PCDD/F and cPCB analysis in biological fluids such as milk and serum has been studied using a system in which the extraction step was integrated within the automated clean-up procedure. This results in a fast and reliable extraction and clean-up technique suitable for the treatment of a large number of cow's milk, breast milk and human serum samples. The on-line use of disposable silica-based C_{18} columns prior to further silica and carbon columns clean-up appeared to be efficient and stable. Accuracy, repeatability, reproducibility and robustness of the new method were investigated. The entire analysis time was greatly reduced in comparison with classical Soxhlet extraction, allowing faster response in the case of emergency. Due to lowered

blank levels, the method is well suited to accommodate expert committees' recommendations concerning analysis of such analytes in foodstuffs. Finally, the presented method appears to be appropriate to simultaneously prepare several (up to five samples in parallel) biological fluid samples that, together with substantially reduced analysis time, yield to high throughput PCDD/F and cPCB analysis, as often required in large epidemiological studies.

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