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RESEARCH PAPER

Multi-residue method for the determination of 57 Persistent Organic Pollutants in human milk and colostrum using a QuEChERS-based extraction procedure

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Abstract Human breast milk represents the best choice for the nutrition of infants. However, in addition to containing beneficial nutrients and antibodies, it can also be considered the best indicator of infant exposure to contaminants. We developed a multi-residue method using a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure and capillary gas chromatography–triple quadrupole mass spectrometry for the determination of 57 persistent organic pollutants, including 23 organochlorine pesticides, 18 polychlorinated biphenyl (PCB) congeners, and 16 polycyclic aromatic hydrocarbons in human milk and colostrum samples. We have used primary secondary amine in the clean-up step as it gave a more efficient separation of the analytes from fat and superior removal of the co-extracted substances compared with gel permeation chromatography. No significant matrix effect was observed for the tested pollutants, and therefore matrix-matched calibration was not necessary. The average recoveries from spiked samples were in the range of 74.8–113.0 %. The precision was satisfactory, with relative standard deviations below 16 %, while values of 0.1–0.4 μg L^{-1} were established as the limit of quantification for all the target analytes (0.05 and 100 μg L^{-1}). The method was successfully applied to the analysis of 18 human colostrum and 23 mature milk samples. All the samples tested were positive for at least nine different residues, with some samples containing up to 24 contaminants. Remarkably, the contaminants hexachlorobenzene, p,p'-DDE, PCB 138, PCB 180, phenanthrene, fluoranthene, and pyrene were present in 100 % of the colostrum and mature milk samples analyzed.

Keywords Organochlorine pesticides · Polychlorinated biphenyls · Polycyclic aromatic hydrocarbons · Breast milk · QuEChERS · GC-tandem MS

Introduction

Anthropogenic contaminants include many chemicals that are resistant to degradation in the environment and biota. Over the last 30 years, a number of these substances have been highlighted as a cause for concern [1] and have been the subject of extensive study and international regulation. Due to their stable structures and lipophilic properties, persistent organic pollutants (POPs), such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), often concentrate and magnify in the food chain, particularly in fat sources. Other contaminants, such as polycyclic aromatic hydrocarbons (PAHs), cannot be considered POPs sensu strictu because they are efficiently metabolised; however, due to their prevalence in the environment and their lipophillicity, they are frequently

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categorised as and studied together with POPs. It is widely accepted that food consumption is the primary source of non-occupational human exposure to these contaminants, as opposed to alternative exposure routes such as inhalation and dermal contact. The ingestion of contaminated food constitutes over 90 % of total human exposure, and foodstuffs of animal origin are recognised as one of the main contributors [2–5].

OCPs were widely used prior to the recognition of their toxicity and persistence. Pesticide exposure has been associated with arthritis, various types of cancer and diabetes [6–9]. Organochlorine exposure has been associated with neurobehavioral and developmental changes and DNA hypomethylation [10]. Furthermore, PCBs were widely used in electrical systems and hydraulic fluids. Their production was banned worldwide in the 1970s. Nonetheless, PCBs are still detectable in wildlife and humans [11]. PCBs have been associated with adverse neurological development, including decreases in motor skills and cognitive development [12]. Prenatal exposure to PCBs and related chemicals, such as chlorinated dibenzofurans and dioxins, in highly exposed populations has been associated with altered pubertal timing and growth abnormalities, including decreased height and birth weight [13, 14]. Cancer and endocrine disruption have been associated with adult exposure [1, 15, 16]. Finally, PAHs are widespread chemical pollutants that are introduced into the environment from a number of different sources. They are mainly produced by pyrolysis but can also be of petrogenic origin from crude oils or refinery products. PAHs can enter the environment through atmospheric deposition, road run-off, industrial discharges and oil spills. More than 50 % of the PAH emitted into the atmosphere comes from car emissions, and 28 % results from residential and industrial combustion [17, 18]. PAHs have been identified in biological samples from wildlife [19] and humans [20]. Many PAHs are toxic, mutagenic and carcinogenic [21, 22].

Human milk not only contains nutrients and antibodies but can also be used as an indicator of the level of organic pollutants in human bodies [20, 23]. The presence of organochlorines and other contaminants is strongly correlated with maternal adipose tissue, plasma, cord blood and breast milk, demonstrating both placental and lactational transfer [24, 25]. A greater elimination of persistent organic pollutants from the maternal body occurs during breastfeeding [26, 27], contributing, along with the prenatal exposure of the foetus [28], to the newborn's bodily burden. Therefore, breast milk is a sensitive matrix for monitoring the maternal bodily burden of pesticides [23]. Furthermore, the sampling of milk is non-invasive and therefore generally accepted by mothers [29]. Within the lactation period, colostrum is secreted by women for 7 days following parturition. Because it contains a higher percentage of fat, it may contribute to the bioaccumulation of lipophilic contaminants in the newborn's body [23]. For this reason, it is important to develop rapid and sensitive analytical methods to identify and determine POP residues in human colostrum at trace levels.

Numerous methods for the extraction, purification and quantification of organic contaminants in fatty samples such as milk have been described in the literature. Since it was first developed, the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction and clean-up method has become an important and widely used technique in the analysis of multiple chemical residues in a variety of matrices, including fatty tissues and foods such as milk [30–34]. Recently, the efficacy of this methodology has been proven for the extraction of pesticides, including some organochlorines [35]. Excellent recovery and repeatability has been obtained with this technique for a wide range of contaminants. Therefore, QuEChERS can be an effective, flexible and inexpensive choice for the multi-residue analysis of POPs in human milk and colostrum. In addition, tandem mass spectrometry (MS/MS) has been increasingly utilised in the final determining step of contaminant residue analysis and is considered a practical means of circumventing the challenges associated with the identification of target analytes in matrices containing excessive quantities of potentially interfering substances, such as the fat in human milk [36]. To our knowledge, the use of GC–MS/MS with an electron ionisation (EI)-QqQ analyser has not been applied to the analysis of POPs in human milk and colostrum, where the low levels of these residues necessitate a powerful technique with a low LOD and a confirmatory approach.

The aim of the present work was to develop and optimise a multi-residue method based on QuEChERS extraction and GC–MS/MS for the simultaneous determination of 23 OCPs, 18 PCBs and 16 PAHs in human colostrum and milk samples. The performance parameters were determined by the GC–MS/MS analysis of standard solutions, standard reference materials and spiked real samples. The proposed method was successfully applied to the characterisation of 41 human milk samples (18 of colostrum and 23 of mature milk) from volunteers recruited in the Insular Materno-Infantil University Hospital (Gran Canaria, Canary Islands, Spain; CHUIMI).

Materials and methods

Chemicals and reagents

Acetonitrile, hexane and cyclohexane were of the highest purity available (>99.9 %) and purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure water was produced from a Milli-Q Gradient A10 (Millipore, Molsheim, France). The OCPs (hexachlorobenzene, α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, δ-HCH, heptachlor, aldrin, endrin, dieldrin, dicofol, *o*,*p*′-DDT, *p*,*p*′-DDT, *o*,*p*′-DDE, *p*,*p*′-DDE, *o*,*p*′-DDD, *p*,*p*′-DDD, *cis*-chlordane, *trans*-chlordane, α-

endosulfan, β-endosulfan, endosulfan sulphate and metoxychlor and mirex), PCB congeners (IUPAC numbers 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180 and 189), surrogates (PCB 12, PCB 202, *p*,*p*′-DDE-d8 and acenaphtylene D8) and internal standards (tetrachloro-*m*-xylene, heptachloro epoxide *trans* and benzo[a]pyrene D12) were purchased from Dr. Ehrenstorfer Reference Materials (Augsburg, Germany). The PAHs (naphthalene, acenaphtylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene and benzo[ghi]perylene) were purchased from Absolute Standards, Inc. (Connecticut, USA). All standards were neat compounds (purity from 97 to 99.5 %). Stock solutions of each compound at 1 mg mL^{-1} were prepared in cyclohexane and stored at −20 °C. Diluted solutions ranging in concentration from 0.05 to 100 ng mL^{-1} were used to prepare the calibration curves. The QuEChERS bulk materials (anhydrous magnesium sulphate, sodium chloride, di-sodium hydrogen citrate 1.5 hydrate, tri-sodium citrate 2 hydrate and primary secondary amine (PSA)) were purchased from Panreac Ibérica (Barcelona, Spain). The standard reference material SRM 1953 (organic contaminants in human milk—non-fortified) was purchased from NIST (Gaithersburg, USA).

Extraction and clean-up procedure

A QuEChERS extraction procedure was developed for the milk and colostrum samples. A 5-mL volume of milk or colostrum was sampled in 50 mL polypropylene centrifuge tubes. A 5-mL volume of ultrapure water was then added and shaken. A 10-mL volume of acetonitrile saturated in *n*-hexane and 50 μL of the surrogates solution at 1 μg mL^{-1} were added, and the mixture was allowed to stand at room temperature for 30 min to allow the swelling of the matrix, vortexing it every 10 min. Subsequently, a mixture of 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 0.5 g of di-sodium hydrogen citrate and 1 g of tri-sodium citrate salts was added directly to the tube; the mixture was immediately manually shaken for 1 min to prevent the agglomeration of salts. A centrifugation (5,000 rpm, 5 min, 20 °C) was performed, and the upper phase was removed and transferred to a glass tube. A second extraction was performed adding 5 mL of acetonitrile saturated in n-hexane to the remaining pellet, which was then vigorously shaken for 1 min and centrifuged a second time (5,000 rpm, 5 min, 20 °C) to separate the organic-upper layer, which was added to the previously obtained organic layer.

Finally, an additional clean-up step was performed. The acetonitrile phase was transferred to a 15 mL polypropylene centrifuge tube containing 0.9 g of anhydrous magnesium sulphate and 0.5 g of PSA. The mixture was vigorously shaken for 1 min and centrifuged (5,000 rpm, 5 min, 20 °C). The sample solution was filtered through a 0.2-μm PTFE filter. The filtered extract was evaporated to dryness under a gentle nitrogen stream. The residue was then reconstituted in 1 mL cyclohexane, 10 μL of the solution of internal standards at 1 μg mL^{-1} was added and the mixture was transferred to a GC vial that was used for the chromatographic analysis.

GC-QqQ-MS/MS analysis

The gas chromatography separations were performed on a Thermo Trace GC Ultra equipped with a TriPlus autosampler and a split/splitless injector with electronic pressure control (Thermo Fisher Scientific Inc., Waltham, MA, USA). A fused silica capillary column BPX5 (cross-linked 5 % phenyl methylpolysiloxane, SGE Inc., USA) with a length of 30 m, a 0.25 mm i.d. and a film thickness of 0.25 μm was used as the stationary phase. Helium (99.999 %) at a constant flow rate of 1.0 mL min^{-1} was used as the carrier gas. The temperatures were programmed as follows: the initial oven temperature of 60 °C was maintained for 1 min, ramped at 12 °C/min to 210 °C, then raised at 8 °C/min to 320 °C with a 6 min hold time. The total run time was 61 min. The injector and transfer line were set to 270 and 310 °C, respectively. The standards and samples were injected (1 μl) in the splitless mode.

The GC was interfaced with a TSQ Quantum Max QqQ mass spectrometer (mass range, *m*/*z*, from 10 to 1,050) for the detection of the contaminants included in this study. The instrument data system also contained an EI-MS/MS library, which was specially created for the target analytes under our experimental conditions. The mass spectrometer scale was calibrated weekly with perfluorotributylamine. ThermoFisher Xcalibur Software (Ver. 2.0.1) was used for the instrument control, data acquisition and data analysis.

After the retention times were determined in full scan mode (range *m*/*z* 45–650), a timed-selected reaction monitoring (SRM) method was developed to analyse the 57 target compounds plus four surrogates and three internal standards in one single run. A calibration curve was constructed from 0.05 to 100 ng mL^{-1} with all the compounds, with the exception of the surrogates and internal standards, contained in each calibration standard mixture. Argon (99.99 %) was used as the collision gas, and the collision cell pressure was set to 0.2 Pa. The QqQ mass spectrometer was operated under the following conditions: ionisation with electron impact at 70 eV in MRM with an emission current of 50 μA. The ionisation source temperature was set to 220 °C. A filament multiplier delay of 5 min was established to prevent instrument damage. The electron multiplier voltage was set to 1,500 V. The scan width was 0.15, and the scan time was 0.05 s. Peak widths of *m*/*z* 0.7 Da were set for both the first (Q1) and third quadrupole (Q3).

Validation

Eighteen samples of human colostrum and 23 samples of mature breast milk were collected as a sub-sample set from a cross-sectional study based on 103 women aged 18–40 years who gave birth at the CHUIMI in 2010. The samples selected were from primiparous mothers ranging from 17 to 37 years in age. The samples were stored at −20 °C until analysis. Standard reference material SRM 1953 was used for the validation experiments. The certified values of SRM 1953 were firstly assessed in our laboratory using a previously described method [37], and later with this QuEChERS-based method. The results with our method revealed a relative standard deviations (RSD) <17 %, which was considered acceptable (Electronic Supplementary Material, Table S1). We also used pooled colostrum samples, selected among those with the lowest contamination levels (e.g. <0.1 μg L^{-1}), in the validation experiments. To a 5-mL volume of the SRM 1953 or pooled colostrum, 100 μL of a 1 μg mL^{-1} or 50 ng mL^{-1} working standard solution in acetone was added to obtain concentrations of 20 and 1 μg L^{-1}, respectively. The samples were thoroughly mixed and allowed to stand at room temperature for 4 h to ensure that the analytes were homogenously distributed throughout the sample. The recoveries were determined in quintuplicate by comparing the obtained concentrations for the spiked SRM 1953 and the colostrum samples with the same concentrations of contaminants prepared in the solvent. The same experiments were also used to determine the intra-day and inter-day precision (five successive days).

To evaluate the possibility of a matrix effect, we also compared the spiked-extracted samples with the SRM 1953 samples spiked at the same concentration following the QuEChERS extraction.

The limit of quantification (LOQ) of the method was designated as the analyte concentration that produced a peak signal of ten times the background noise from the chromatogram. The quantification was based on peak area. Ten-point calibration curves were constructed using a least-squares linear regression from the injection of samples spiked with solutions to provide final concentrations ranging from 0.05 to 100 μg L^{-1}.

Quality control

In each batch of samples, two controls were included for every 12 samples, comprising a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of melted butter spiked at 20 ng g^{-1} with each of the analytes processed by the same

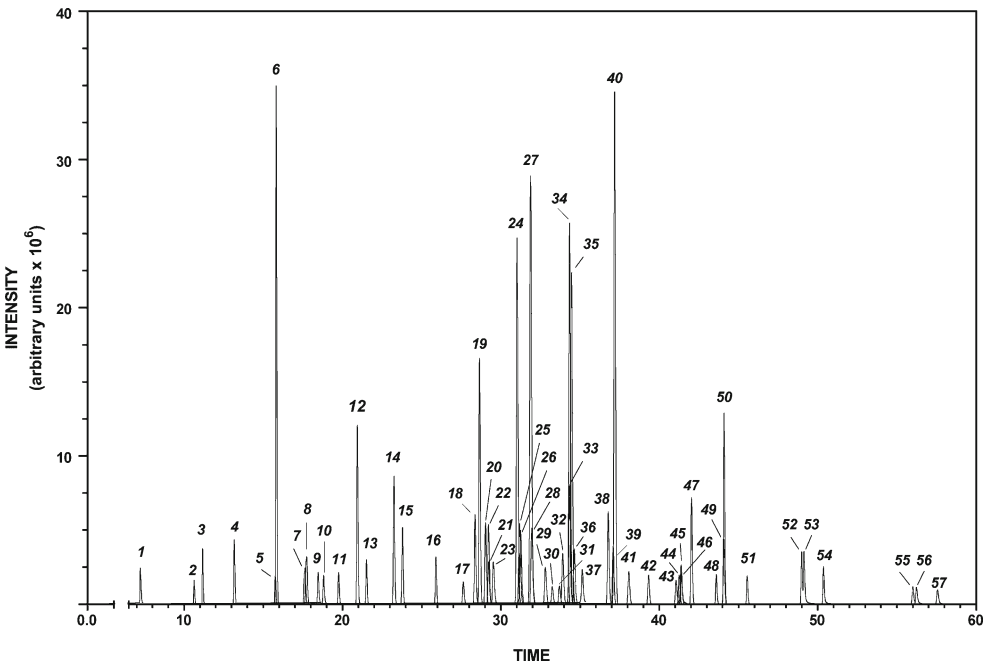

Fig. 1 Total ion chromatograms of the 57 MS/MS transition filters of a single mixture of contaminants prepared in cyclohexane (100 μg L^{-1}). *1* Naphthalene, *2* acenaphtylene, *3* acenaphtene, *4* fluorene, *5* α-HCH, *6* HCB, *7* β-HCH, *8* γ-HCH, *9* anthracene, *10* phenanthrene, *11* δ-HCH, *12* PCB 28, *13* heptachlor, *14* PCB 52, *15* aldrin, *16* dicofol, *17* fluoranthene, *18* chlordane (*trans*), *19* *o,p′*-DDE; *20* PCB 101, *21* α-endosulfan, *22* chlordane (*cis*), *23* pyrene, *24* *p,p′*-DDE, *25* PCB 81, *26* dieldrin, *27* *o,p′*-DDD, *28* PCB 77, *29* endrin, *30* PCB 123, *31* PCB 118, *32* β-endosulfan, *33* PCB 114, *34* *o,p′*-DDT, *35* *p,p′*-DDD, *36* PCB 153, *37* PCB 105, *38* endosulfan sulphate, *39* PCB 138, *40* *p,p′*-DDT, *41* PCB 126, *42* PCB 167, *43* benzo[*a*]anthracene, *44* PCB 156, *45* chrysene, *46* PCB 157, *47* PCB 180, *48* PCB 169, *49* metoxychlor, *50* mirex, *51* PCB 189, *52* benzo[*b*]fluoranthene, *53* benzo[*k*]fluoranthene, *54* benzo[*a*]pyrene, *55* indeno[*1,2,3-cd*]pyrene, *56* dibenzo[*a,h*]anthracene, *57* benzo[*ghi*]perylene

Table 1 Conditions of the optimised GC-MS/MS method

Name	t_R/min	precursor ion (m/z)	Product ions, m/z (collision energy/eV)	IPs
Polycyclic aromatic hydrocarbons (PAHs)				
Naphtalene	7.24	128	103 (15), 78 (15)	4
Acenaphtylene	10.63	152	151 (10), 126 (10)	4
Acenaphthene	11.16	154	153 (10), 152 (10)	4
Fluorene	13.18	166	165 (15), 163 (15)	4
Anthracene	18.46	178	176 (30), 152 (30)	4
Phenanthrene	18.81	178	176 (30), 152 (30)	4
Fluoranthene	27.62	202	201 (10), 200 (10)	4
Pyrene	29.52	202	201 (10), 200 (10)	4
Benzo[a]anthracene	41.05	228	226 (20), 202 (20)	4
Chrysene	41.37	228	226 (20), 202 (20)	4
Benzo[b]fluoranthene	48.66	252	250 (30), 226 (30)	4
Benzo[k]fluoranthene	48.82	252	250 (30), 226 (30)	4
Benzo[a]pyrene	50.36	252	250 (30), 226 (30)	4
Indeno[1,2,3-cd]pyrene	55.99	276	274 (35), 250 (35)	4
Dibenzo[a,h]anthracene	56.23	278	276 (35), 226 (35)	4
Benzo[ghi]perylene	57.56	276	274 (35), 250 (35)	4
Organochlorine pesticides (OCs)				
Hexachlorocyclohexane (alpha)	15.75	216	181 (15), 183 (15)	4
Hexachlorobenzene	15.81	284	214 (20), 249 (20)	4
Hexachlorocyclohexane (gamma)	17.50	217, 219	181 (15), 183 (15)	5
Hexaclorocyclohexane (beta)	17.65	217	181 (15), 183 (15)	4
Hexachlorocyclohexane (delta)	19.75	217	181 (15), 183 (15)	4
Heptachlor	21.51	258, 339	186 (22), 304 (15)	5
Aldrin	23.79	263	193 (32), 228 (26)	4
Dicofol	25.85	139, 251	111 (15), 139 (15)	5
Chlordane (*trans*)	28.36	373, 375	266 (15), 268 (17)	5
o,*p*′-DDE	28.65	318	246 (20), 248 (20)	4
Endosulfan (alpha)	29.23	196	159 (17), 161 (15)	4
Chlordane (*cis*)	29.72	373, 410	266 (18), 375 (5)	5
p,*p*′-DDE	31.02	318	246 (20), 248 (20)	4
Dieldrin	31.24	277	207 (20), 241 (10)	4
o,*p*′-DDD	31.72	235	165 (20), 199 (18)	4
Endrin	32.80	263	191 (25), 193 (27)	4
Endosulfan (beta)	33.91	196	159 (16), 160 (15)	4
o,*p*′-DDT	34.40	235	165 (20), 199 (18)	4
p,*p*′-DDD	34.57	235	165 (15), 199 (15)	4
Endosulfan sulphate	35.49	274	237 (10), 239 (15)	4
p,*p*′-DDT	37.15	235	165 (20), 199 (15)	4
Metoxychlor	44.08	274	239 (20), 259 (20)	4
Mirex	44.23	270, 272	235 (15), 237 (15)	5
Polychlorinated biphenyls (PCBs)				
PCB 28	20.93	256, 258	186 (22), 186 (42)	5
PCB 52	23.09	290, 292	220 (22), 220(20)	5
PCB 101	29.03	324, 326	254 (20), 256 (25)	5
PCB 81	31.16	290, 292	220 (22), 220 (20)	5
PCB 77	31.95	290, 292	220 (22), 220 (20)	5
PCB 123	33.33	324, 326	254 (20), 256 (25)	5

Table 1 (continued)

Name	t_R/min	precursor ion (m/z)	Product ions, m/z (collision energy/eV)	IPs
PCB 118	33.64	324, 326	254 (20), 256 (25)	5
PCB 114	34.33	324, 326	254 (20), 256 (25)	5
PCB 153	34.55	358, 360	288 (30), 290 (22)	5
PCB 105	35.12	324, 326	254 (20), 256 (25)	5
PCB 138	37.08	358, 360	288 (30), 290 (22)	5
PCB 126	38.07	324, 326	254 (20), 256 (25)	5
PCB 167	39.32	358, 360	288 (30), 290 (22)	5
PCB 156	41.19	358, 360	288 (30), 290 (22)	5
PCB 157	41.45	358, 360	288 (30), 290 (22)	5
PCB 180	42.03	392, 394	322 (30), 324 (20)	5
PCB 169	43.60	358, 360	288 (30), 290 (22)	5
PCB 189	45.54	392, 394	322 (30), 324 (20)	5

method used for the samples. The batch analyses were considered valid when the values of the analytes in the QC were within a 10 % of the deviation of the theoretical value.

Results and discussion

Optimisation of the instrumental method

Gas chromatography coupled with a triple quadrupole mass spectrometer was demonstrated to provide low detection limits and successfully enabled the identification and confirmation of the peak identities. Combining the SRM transitions with their retention times allowed us to positively confirm the contaminant identity. Occasionally, a chromatographic separation is not critical to the development of a multi-residue method with QqQ analysers because the high QqQ acquisition speed permits the monitoring of co-eluted compounds with a high number of transitions simultaneously in the SRM mode [38]. However, to achieve a good separation, various alterations to the temperature program were assayed. The selected GC operating conditions and optimised oven temperature programs are explained fully in the "Materials and methods" section. A chromatogram of the mixture of 57 contaminants with good analyte separation was successfully obtained under these optimised conditions in 61 min (Fig. 1).

To optimise the triple quadrupole MS/MS conditions, relevant considerations included the choices of precursor ions and product ions as well as the optimisation of the collision energies to achieve the best response from each target compound. After obtaining full scan spectra, the precursor ion for every analyte was selected and subjected to collision energy voltages (the potential on the second quadrupole) to generate MS/MS product ions; in this work, collision energies from 5 to 35 eV were evaluated (Table 1). The final aim was to develop a timed SRM method with two reactions or transitions per compound. Moreover, the peak shapes were highly related to the scan time, dwell time, scan rate and the number of monitored transitions [39, 40]. To obtain low detection limits and well-shaped chromatographic peaks, the dwell time was adjusted to allow 10 cycles per second throughout the chromatographic run, providing a sufficient number of chromatographic points for all the compounds. The final MS/MS conditions used in this study are detailed in Table 1.

According to the European Commission Decision 2002/657/EC [41], which introduced the concept of identification points (IPs) for the confirmation stage, the confirmation of the analytes included in this work involved monitoring two product ions from the same precursor ion, which resulted in four IPs, or two product ions derived from two different precursor ions, which resulted in five IPs. Therefore, the timed-SRM method used in this work met the requirements of the aforementioned regulation. The resulting number of IPs for each analyte is outlined in the Table 1.

Optimisation of the sample extraction and clean-up

In the present study, the QuEChERS [42] principle was adopted for the extraction of contaminants from human milk and colostrum. The classic QuEChERS method, initially developed for vegetables and fruits, involves an extraction with acetonitrile due to the ability of this solvent to separate from water upon the addition of an appropriate mixture of salts. Nevertheless, the QuEChERS approach allows considerable flexibility and can be modified to accommodate the specific analyte properties, matrix composition, equipment and analytical techniques available in the laboratory [43]. After a careful review of the bibliography in this study, we chose to use acetonitrile and citrate salts because several authors have demonstrated that this combination is the best choice for fatty

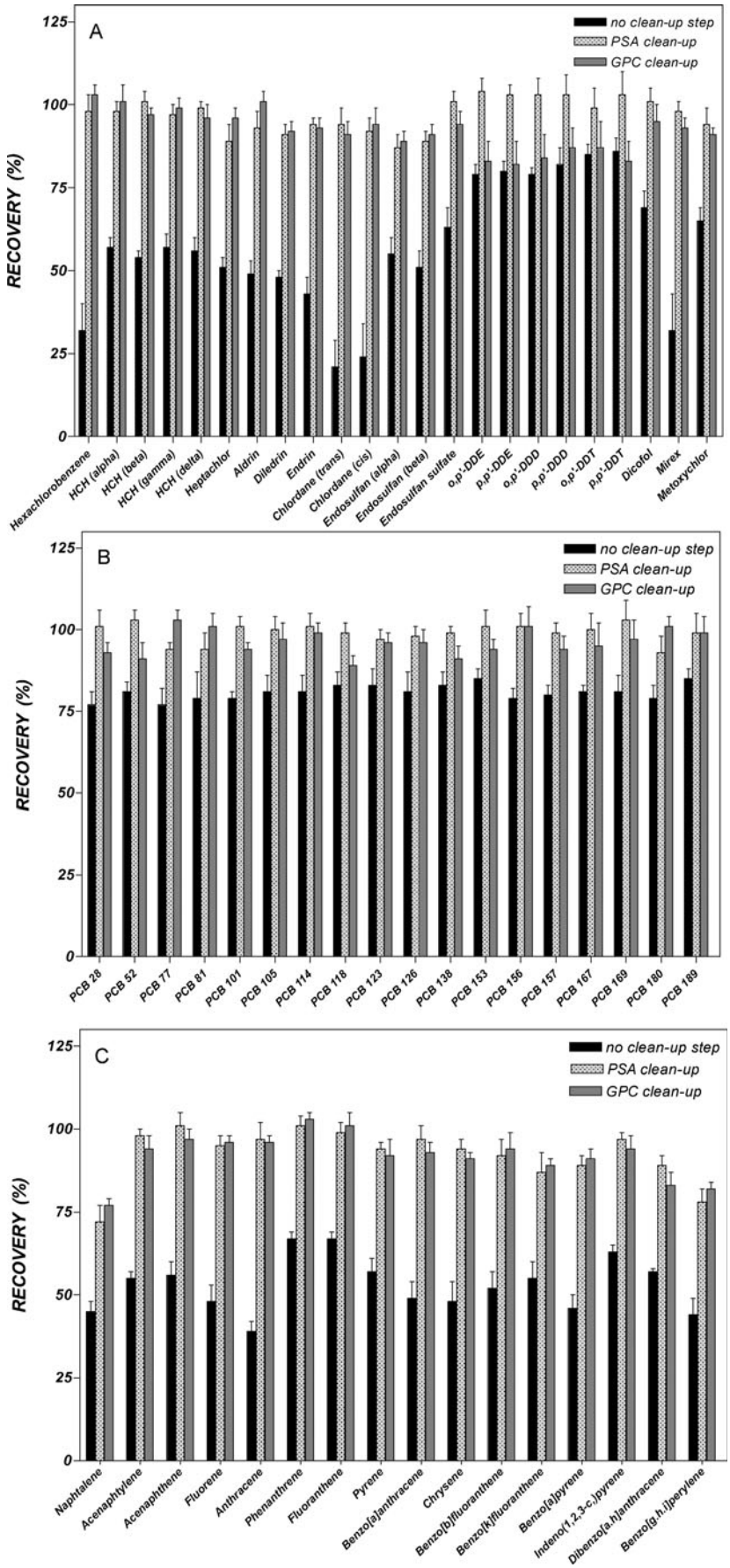

Fig. 2 Effect of the clean-up step by PSA or GPC on the extraction efficiency of the compounds from human colostrum spiked with 57 contaminants (20 μg L^{-1}). **A**. Organochlorine pesticides; **B**. Polychlorinated byphenils; **C**. Polycylic aromatic hydrocarbons

Table 2 Validation parameters ($n=5$) obtained for the 57 contaminants at two concentration levels in colostrum

Compound	Average recovery, % (RSD[a,b])		LOQ (μg L^{-1})	RSD for retention time, %	R^2
	1 μg L^{-1}	20 μg L^{-1}			
Organochlorine pesticides (OCs)					
Hexachlorobenzene	98 (6, 7)	98 (8, 10)	0.1	0.009	0.9980
α-HCH	98 (10, 13)	97 (6, 9)	0.5	0.004	0.9962
β-HCH	101 (9, 11)	99 (7, 11)	0.2	0.011	0.9977
γ-HCH	97(12, 15)	96 (11, 14)	0.2	0.009	0.9963
δ-HCH	99 (11, 9)	97 (8, 12)	0.3	0.018	0.9977
Heptachlor	89 (5, 8)	93 (8, 10)	0.4	0.021	0.9837
Aldrin	93 (14, 11)	99 (8, 7)	0.4	0.019	0.9985
Dieldrin	91 (13, 13)	98 (9, 11)	0.05	0.024	0.9935
Endrin	94 (12, 9)	94 (4, 7)	0.5	0.022	0.9972
Chlordane (*trans*)	94 (7, 11)	101 (9, 13)	0.4	0.033	0.9954
Chlordane (*cis*)	92 (8, 5)	88 (4, 8)	0.4	0.035	0.9995
α-Endosulfan	87 (16, 12)	96 (8, 11)	0.2	0.021	0.9826
β-Endosulfan	89 (11, 10)	87 (11, 9)	0.2	0.018	0.9813
Endosulfan sulphate	76 (13, 16)	82 (9, 14)	0.3	0.011	0.9971
o,p′-DDE	104 (11, 8)	95 (12, 7)	0.05	0.009	0.9995
p,p′-DDE	103 (6, 12)	93 (14, 11)	0.05	0.011	0.9981
o,p′-DDD	103 (5, 8)	99 (5, 9)	0.1	0.020	0.9985
p,p′-DDD	105 (9, 5)	101 (3, 6)	0.1	0.014	0.9987
o,p′-DDT	99 (11, 13)	94 (6, 11)	0.1	0.014	0.9996
p,p′-DDT	102 (9, 9)	97 (7, 11)	0.1	0.009	0.9997
Dicofol	95 (12, 9)	89 (11, 8)	0.1	0.023	0.9960
Mirex	101 (5, 7)	92 (11, 14)	0.1	0.021	0.9985
Metoxychlor	94 (12, 8)	97 (7, 6)	0.4	0.008	0.9915
Polychlorinated biphenyls (PCBs)					
PCB 28	101 (10, 8)	93 (4, 11)	0.2	0.009	0.9985
PCB 52	103 (12, 8)	91 (14, 10)	0.2	0.013	0.9824
PCB 77	94 (5, 9)	103 (4, 8)	0.2	0.017	0.9959
PCB 81	94 (11, 12)	101 (5, 7)	0.2	0.016	0.9816
PCB 101	101 (4, 7)	94 (9, 13)	0.1	0.017	0.9904
PCB 105	100 (9, 6)	97 (6, 9)	0.1	0.009	0.9934
PCB 114	101 (6, 9)	99 (9, 6)	0.1	0.013	0.9813
PCB 118	99 (11, 8)	89 (12, 9)	0.1	0014	0.9890
PCB 123	97 (8, 12)	96 (6, 9)	0.1	0.018	0.9992
PCB 126	98 (9, 8)	91 (11, 11)	0.1	0.019	0.9899
PCB 138	99 (5, 8)	94 (7, 10)	0.1	0.021	0.9972
PCB 153	101 (5, 9)	101 (3, 7)	0.05	0.009	0.9948
PCB 156	101 (4, 8)	94 (8, 9)	0.05	0.021	0.9982
PCB 157	99 (7, 11)	101 (5, 9)	0.05	0.023	0.9929
PCB 167	100 (4, 6)	99 (6, 4)	0.05	0.025	0.9994
PCB 169	103 (5, 9)	97 (9, 4)	0.05	0.027	0.9856
PCB 180	93 (10, 7)	99 (6, 8)	0.05	0.025	0.9908
PCB 189	99 (4, 8)	93 (8, 11)	0.05	0.028	0.9873
Polycyclic aromatic hydrocarbons (PAHs)					
Naphtalene	77 (8, 10)	77 (6, 9)	0.2	0.019	0.9878
Acenaphtylene	98 (11, 11)	94 (8, 12)	0.1	0.008	0.9877
Acenaphthene	101 (7, 9)	97 (6, 8)	0.1	0.015	0.9887

Table 2 (continued)

Compound	Average recovery, % (RSD[a,b])		LOQ (μg L^{-1})	RSD for retention time, %	R^2
	1 μg L^{-1}	20 μg L^{-1}			
Fluorene	95 (11, 9)	96 (10, 11)	0.1	0.009	0.9964
Anthracene	97 (14, 12)	96 (9, 11)	0.09	0.021	0.9987
Phenanthrene	101 (10, 14)	97 (7, 7)	0.05	0.017	0.9945
Fluoranthene	113 (9, 12)	99 (8, 11)	0.09	0.008	0.9976
Pyrene	103 (14, 11)	94 (18, 12)	0.07	0.011	0.9995
Benzo[a]anthracene	98 (6, 8)	93 (12, 8)	0.09	0.013	0.9883
Chrysene	100 (8, 9)	94 (12, 14)	0.07	0.017	0.9978
Benzo[b]fluoranthene	94 (12, 10)	92 (11, 13)	0.1	0.019	0.9889
Benzo[k]fluoranthene	96 (8, 11)	87 (12, 8)	0.1	0.026	0.9887
Benzo[a]pyrene	95 (6, 8)	89 (7, 11)	0.1	0.023	0.9856
Indeno[1,2,3-cd]pyrene	99 (11, 14)	87 (9, 13)	0.2	0.019	0.9819
Dibenzo[a,h]anthracene	101 (9, 12)	89 (11, 13)	0.2	0.019	0.9881
Benzo[ghi]perylene	98 (7, 9)	78 (9, 12)	0.2	0.023	0.9883
Surrogates					
PCB 12	97 (4, 6)	94 (6, 11)	0.2	0.005	0.9948
PCB 202	95 (8, 7)	96 (4, 9)	0.05	0.022	0.9932
p,*p*′-DDE-d8	101 (5, 9)	99 (6, 11)	0.05	0.018	0.9921
Acenaphtylene-d8	96 (6, 10)	94 (7, 5)	0.1	0.011	0.9904

[a] Intra-day

[b] Inter-day

matrices, minimising the co-extraction of lipids due to the low solubility of the lipids in this solvent [30, 44, 45].

Nevertheless, as human milk and colostrum in particular are matrices with a relatively high fat content, a clean-up step was presumed necessary to eliminate lipids that could reduce signal or cause column damage. In the QuEChERS methodology, the separation of the acetonitrile and aqueous phases upon the addition of sodium chloride, anhydrous $MgSO_4$ and citrate salts is usually followed by a dispersive solid-phase extraction clean-up step using a small quantity of SPE sorbents (PSA, GCB and/or C18). Because apolar compounds can be adsorbed on solid phases such as C18 and GCB, we decided to use PSA in this work and compare it with the traditional approach for lipid removal by gel permeation chromatography (GPC). Thus, we compared the chromatographic signals of the extracts of 57-contaminant-spiked (20 ng mL^{-1}) human milk and colostrum samples obtained either without an additional clean-up step or with a clean-up using 500 mg PSA or GPC. For each spiked concentration, the results were expressed as the percentage of the signal of the contaminant prepared in solvent. As displayed in Fig. 2, the chromatographic results revealed that a clean-up step was necessary for the human colostrum samples, which displayed weaker signals when a clean-up step was not performed. Figure 2 also demonstrates that the clean-up using 500 mg d-SPE produced very similar results (or even cleaner extracts, as in the case of DDTs, see Fig. 2) to those obtained by the GPC method. We performed a statistical comparison of the mean result values for all the analytes in each of the three experiments (no clean-up, PSA clean-up and GPC clean-up). For the PAHs and PCBs, there were statistically significant differences between "no clean-up" and "clean-up" as well as between the two types of clean-up. In all instances, the p value was below 0.001. For the OCPs, we also found statistically significant differences ($p < 0.001$) between performing and not performing the clean-up step, but not between the two types of clean-up evaluated. Similar results were obtained for the human milk samples (data not shown). Because d-SPE is a much less solvent- and time-consuming method, we concluded that using d-SPE with PSA was the better clean-up procedure for removing co-extracted material from human milk and colostrum samples.

Analytical performance

After optimising the clean-up procedure and analysis program, the confirmation criteria, precision, linearity, LOQs and repeatability were studied in order to evaluate the usefulness of the method for the quantitative determination of contaminants in milk and colostrum.

Compounds were identified as target analytes only when their chromatographic peaks satisfied all of the following criteria: (1) the retention time (tR) of the candidate was within three standard deviations (SD) of the average tR (tR±3SD) obtained when six blank samples spiked at the second level of calibration were injected; (2) the ion ratios matched those of the standard with a tolerance of ±30 % in absolute ion abundances, and (3) the S/N ratio of the target analytes was >10 for the sample extract.

To assess the extraction efficiency of the proposed method, recovery studies were conducted by spiking human milk and colostrum with the mixture of 57 analytes included in this work. As previously described, we used SRM 1953 and a pool of previously screened colostrum samples that exhibited the lowest numbers and concentrations of contaminants. The recoveries were determined from five replicates at two spiking concentrations (1.0 and 20.0 μg L^{-1}). The recovery values and the relative standard deviations were calculated for each concentration by comparing the areas of the analytes in the extracted, spiked samples with those of the same concentrations in a solvent (Table 2). The results ranged from 72.0 to 113.0 %, with the majority of the recoveries greater than 90 % at both concentrations. The precision was satisfactory, with the majority of unfavourable RSDs below 16 %. We also evaluated the recoveries and precision over five consecutive days (inter-day measurement), and the RSD was again observed to be below 16 %. Table 2 demonstrates that all the results were within the acceptable range and indicates the adequate precision of the method, with RSD values of 4–16 % for all the analytes.

To determine the presence of a matrix effect, the same comparison was performed using SRM 1953 or a colostrum "blank" spiked after the QuEChERS extraction; no significant differences were observed. Thus, we concluded that there were no significant matrix effects, and the remainder of the studies and calculations were therefore performed against the calibration curves of standards prepared in solvent.

The quantifications of the measurements were performed over ten different concentrations in solvent, ranging from 0.05 to 100 μg L^{-1} (three replicates for each concentration were analysed). Calculations were performed using the peak areas. The calibration curves were constructed without including the origin point. The calibration graphs obtained by plotting the concentration versus the average peak area are summarised in Table 2. The calibration curves were found to exhibit good linearity, with correlation coefficients (r^2) of more than 0.9813 for all the analyses (Table 2). The residual analyses

Table 3 Levels of organochlorine pesticides levels detected in samples of human colostrum (n=18) and mature milk (n=23)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
Hexachlorobenzene	0.75	100	0.22–0.54	0.76	100	0.18–1.76
α-HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-HCH	2.24	72.22	1.05–3.18	0.81	86.96	0.20–2.87
γ-HCH	2.49	77.78	0.67–5.25	0.76	60.87	0.57–2.49
δ-HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aldrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dieldrin	2.58	50.00	1.75–3.58	1.21	73.91	0.74–3.78
Endrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlordane (*trans*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlordane (*cis*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α-Endosulfan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-Endosulfan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endosulfan sulphate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
o,p′-DDE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDE	8.84	100	2.90–110.34	9.14	100	2.64–72.78
o,p′-DDD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDD	0.55	22.22	0.12–0.88	0.28	43.48	0.1–0.47
o,p′-DDT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDT	0.19	11.11	0.11–0.23	0.12	13.04	0.12–0.14
Dicofol	0.35	27.78	0.12–0.59	n.d.	n.d.	n.d.
Mirex	0.13	61.11	0.1–0.29	0.19	43.48	0.1–0.44
Metoxychlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 4 Levels of polychlorinated biphenyls detected in samples of human colostrum ($n=18$) and mature milk ($n=23$)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
PCB 28	0.49	61.11	0.22–0.60	0.23	43.48	0.13–0.58
PCB 52	0.15	44.44	0.10–0.20	0.12	21.74	0.10–0.32
PCB 77	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 81	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 101	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 105	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 114	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 118	0.16	61.11	0.10–0.32	0.14	91.30	0.10–0.31
PCB 123	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 126	0.15	66.67	0.10–0.42	0.13	52.17	0.10–0.21
PCB 138	1.19	100	0.39–6.89	0.62	100	0.15–3.34
PCB 153	0.98	100	0.39–5.68	0.53	82.61	0.18–2.77
PCB 156	0.21	83.33	0.11–0.40	0.13	82.61	0.10–0.22
PCB 157	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 167	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 169	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 180	1.31	100	0.45–7.35	0.56	100	0.10–3.27
PCB 189	0.11	72.22	0.10–0.37	0.00	30.43	0.10–0.16

revealed values within the −9.588 to 7.765 range, indicating that the linear regression method can be used to accurately calculate the concentrations of the analytes included in this study within the concentration range investigated.

Application to real samples

The validated method was applied to the routine contaminant analysis of real samples. Eighteen human colostrum and 23

Table 5 Levels of polycyclic aromatic hydrocarbons detected in samples of human colostrum ($n=18$) and mature milk ($n=23$)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
Naphtalene	2.33	77.78	1.27–9.82	1.04	82.61	0.45–2.96
Acenaphtylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acenaphthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluorene	1.12	83.33	0.60–2.76	1.07	100	0.24–1.22
Anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenanthrene	11.38	100	7.15–13.51	5.33	100	4.21–14.44
Fluoranthene	0.79	100	0.49–0.89	0.41	100	0.34–0.80
Pyrene	2.15	100	1.22–2.66	1.06	100	0.81–3.71
Benzo[a]anthracene	0.61	11.11	0.36–0.86	0.30	13.04	0.27–0.32
Chrysene	0.34	61.11	0.1–0.62	0.17	30.43	0.13–0.21
Benzo[b]fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[k]fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[a]pyrene	0.19	16.67	0.16–0.19	n.d.	n.d.	n.d.
Indeno[1,2,3-cd]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[a,h]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[ghi]perylene	0.11	5.56	0.11–0.11	n.d.	n.d.	n.d.

mature milk samples were analysed using the developed method. Each batch of samples was processed in duplicate together with an SRM 1953 aliquot that was subjected to the entire procedure. Each field sample and QC sample was spiked with the surrogates at 50 μg L^{-1} and internal standards at 10 μg L^{-1}.

As observed in Table 3, of the 23 OCPs analysed, we identified nine residues in both colostrum and mature milk samples. The remaining OCPs were either absent or present at much lower levels that were below the detection limits of the method. Hexachlorobenzene and *p,p′*-DDE were present in 100 % of the analysed samples, with median concentrations of 0.75 and 8.84 μg L^{-1} in colostrum and 0.76 and 9.14 μg L^{-1} in milk, respectively. The contaminant present at the highest concentrations was *p,p′*-DDE (ranging from 2.64 to 110.34 μg L^{-1}), followed by dieldrin, β-HCH, γ-HCH and hexachlorobenzene. As displayed in Table 3, the pattern of contamination was similar for both types of samples, although the colostrum samples exhibited higher contaminant levels overall than the mature milk samples. This result is likely due to the higher level of fat in colostrum as well as to a progressive "purging" of contaminants from the mothers over the course of the lactation period.

Detailed data for the PCB residues detected in the colostrum and milk samples are displayed in Table 4. The results revealed that the most frequently detected congeners were the highly chlorinated marker-PCBs nos. 138 and 180, which were identified in 100 % of both types of samples, and no. 153, which was present in 100 % of the colostrum samples and in 82.61 % of the milk samples. In total, we identified nine of the 18 congeners analysed, with six of them being marker-PCBs (nos. 28, 52, 118, 138, 153 and 180); marker-PCB no. 101 was not detected in any of the analysed samples. Remarkably, the dioxin-like and highly toxic PCB no. 126 was found in 66.67 % of the colostrum samples and in 52.17 % of the milk samples. The concentrations of the PCBs were typically low (with the average concentrations of the individual congeners ranging from 0.11 to 1.94 μg L^{-1}), although some colostrum samples exhibited concentrations as high as 7.35 μg L^{-1} for some of the congeners (Table 4).

Finally, Table 5 displays the analytical figures for the PAH residue levels in the analysed samples. The results indicate the presence of naphthalene, fluorene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene and chrysene in both types of samples. In colostrum, we also identified benzo[a]pyrene and benzo[ghi]perylene in 16.67 and 5.56 % of the samples, respectively. A frequency of detection of 100 % was found for phenanthrene, fluoranthene and pyrene. Phenanthene was also the compound present at the highest concentrations (Table 5).

Conclusions

The applicability of a QuEChERS-based extraction procedure combined with triple quadrupole GC-MS/MS for the simultaneous detection and quantification of 23 OCPs, 18 PCBs and 16 PAHs was demonstrated. The results revealed satisfactory validation parameters. The studied contaminants were detected at very low concentrations, and highly linear calibration curves were developed within the investigated calibration range (0.05–100 μg L^{-1}, with r^2>0.98). The LOQ values of the instrument varied from 0.05 to 0.4 μg L^{-1}. The recovery rates were between 72 to 113 % with very good precision (RSD<16 %). The proposed method is recommended for routine monitoring applications due to its simplicity, sensitivity and utility. The applicability of the optimised method was demonstrated by monitoring contaminant residues in human milk samples (colostrum and mature milk) collected from women who gave birth at the CHUIMI in 2010. The results indicated a high presence of contaminant residues in this important newborn food supply, with a minimum of nine out of 57 contaminants detected. These results demonstrate the necessity of regularly monitoring persistent pollutants in human milk over extended time periods.

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