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Optimized determination of polybrominated diphenyl ethers and polychlorinated biphenyls in sheep serum by solid-phase extraction–gas chromatography–mass spectrometry

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ABSTRACT

We describe a solid-phase extraction (SPE) method, followed by gas chromatography–mass spectrometry (GC–MS), for the simultaneous determination of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in sheep serum samples. The denaturation of serum proteins by formic acid, water-1-propanol and water-2-propanol were compared and optimized. Seven different solid-phase sorbents were tested and it was found that Strata-X cartridge (200 mg, 6 mL) gave the best recoveries (92–106%, SD < 6%, $n = 3$) for all the target analytes. The different extraction solvents (iso-hexane and dichloromethane), either alone or in combination, were used to extract these persistent organic compounds from spiked serum samples by SPE. Removal of co-extracted biogenic materials was achieved using adsorption chromatography with acid modified silica and activated silica. Iso-hexane was found to be the most appropriate solvent for clean-up providing good recoveries and clear chromatographic separation; its use is preferable to that of DCM because it is less environmentally toxic. The limits of detection (LOD) of the proposed method were 47–105 pg g^{-1} and 16–24 pg g^{-1} for the different PBDEs and PCBs studied, respectively. The developed method was linear over the range from 0.05 to 30 ng g^{-1}, for all PBDEs except PBDE 183 (0.10–30 ng g^{-1}), and from 0.02 to 30 ng g^{-1} for all tested PCB congeners. The established method was successfully applied to sheep serum samples from Scotland, UK, for the determination of the target PBDEs and PCBs.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of flame-retardant chemicals, first produced commercially in the 1970s and widely used in electronic equipment, plastics, construction materials, polyurethane foam padding, coatings and textiles [1,2]. PBDEs are similar in structure to polychlorinated biphenyls (PCBs) and consist of 209 possible substances, called congeners (PBDE = $C_{12}H_{10-x}Br_xO$ ($x = 1, 2, \ldots, 10 = m + n$)). Like PCBs, they persist in the environment and, because they are lipophilic, they bioaccumulate in aquatic and terrestrial organisms where they exert endocrine disrupting effects [3–6]. For most of the human population, dietary intake is probably the main route of exposure to PBDEs, as for PCBs, especially through food of animal origin [7].

Since the early 1990s PBDEs have been measured in the serum of humans all over the world [8–11]. Concentrations of these compounds, both in human and in the environment, have increased during recent decades [11–13]; this is of concern, because of their possible adverse health effects, including their potential for endocrine disruption [11,14,15]. Toxicological studies using animal models suggest that the effects of PBDEs are similar to those of PCBs, being associated with increased risk of reproductive and endocrine disruption [16]. Even low doses have been found to be associated with a reduction in thyroxine (T4) concentrations in rats [11,17]. Other negative health effects seen in rodents include immunotoxicity and behavioural problems [11,18].

Farm animals, including sheep and other ruminants may be exposed to varying concentrations of endocrine-disrupting pollutants for extended periods, through their feed or inhaled air, and potential effects of exposure on the fertility of livestock are of commercial concern [19]. Sheep can accumulate limited amounts of pollutants in tissue (a component of human diet) and the animals themselves are susceptible to their effects [20–26]. However, although PBDEs and PCBs have been found in a number of investigations on human serum, there is little information available on the presence or concentrations of these pollutants in sheep serum. Knowledge of changes in concentrations of these pollutants in the blood, over time, is essential to understanding of factors influencing the pattern of target organ

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exposure and associated physiological effects since neither adult nor fetal tissue concentrations of selected endocrine disrupting compounds (EDCs) have been found to differ with increased rates of environmental exposure, when measured at slaughter, following periods of environmental exposure [26,27], even although animal physiology was perturbed. Measurements of serum concentrations may reveal transient changes in circulating levels and EDC burdens.

Identification of PBDEs and PCBs, in sheep serum specimens for example, presents a challenge to the bioanalytical investigator since they are present in serum at low-ng and sub-ng per gram levels. Also, because serum is a complex matrix composed of lipids, proteins, and inorganic salts, extensive cleanup procedures are required prior to analysis to reduce interferences [4,6,28,29]. Thus, the appropriate extraction method and pretreatment of serum is critical, prior to their instrumental analysis. Liquid–liquid extraction (LLE) has been employed for serum sample extraction [30,31] but the LLE-based procedure is laborious and requires greater volumes of solvent than, for example, solid-phase extraction (SPE). Loconto and his colleagues [32,33] used solid-phase disk extraction (SPDE) and stir bar sorptive extraction (SBSE) methods for PBDE determination in sheep serum samples but with lower recoveries (some down to ~20%). For SPE a number of approaches have been taken: Tran et al. [6] and Conka et al. [29] used cartridge C18; Ramos et al. [28] and Focant et al. [4] preferred Oasis HLB cartridges while Thomsen et al. [34] suggested that Strata-X yielded a higher recovery than the other two SPE cartridges (Isolute and Oasis HLB) but often encountered pressure problems (caused difficulties with serum throughput) when performing extractions with serum due to the relative small particle and pore size of this sorbent. Denaturation of serum proteins before sample extraction by SPE is necessary to ensure that analytes are fully released from the sample matrices and to increase the extraction efficiency of the target compounds by SPE sorbent [29,35]. Different denaturing reagents have been used previously. Thomsen et al. [34] applied the mixture of 2-propanol and formic acid denaturants, Ramos et al. [28], Tran et al. [6] and Focant et al. [4] used formic acid and Zhu et al. [10] and Bradman et al. [36] preferred 2-propanol. However, Conka et al. [29] chose 1-propanol for denaturing the serum samples because the use of 2-propanol resulted in low recoveries for PCB and organochlorine pesticides (OCPs). There are also some differences in choice of solvent for SPE. While Sandau et al. [37] chose methanol:dichloromethane (DCM) (1:9, v/v), Conka et al. [29] concluded that hexane:DCM (1:1, v/v) gave better recoveries for PCBs/OCPs than hexane as the eluting solvent but Martinez et al. [38] preferred hexane, while Covaci and Voorspoels [39], Focant et al. [4] and Sjodin et al. [9] preferred to use DCM for SPE eluting. The relative merits of all of the above variations in analytical method parameters, as well as other parameters (e.g. clean-up option, amount of sample, length of denaturing time, etc.), with respect to extraction efficiency, accuracy of measurement and method feasibility are not known.

The aim of this work was to develop and optimize an efficient and simple analytical method for simultaneous determination of the environmentally important PBDE compounds (tri- to hepta-BDEs, BDE 28, 47, 99, 100, 153, 154, 183) and the International Council for the Exploration of the Sea (ICES) 7 set of PCB congeners (28, 52, 101, 118, 138, 153, 180) in sheep serum by SPE and gas chromatography–mass spectrometry (GC–MS). Specifically, we aimed to optimize the SPE cartridge, solvent type, clean-up, sample size, denaturing reagent and duration of denaturing time. A further objective was to determine whether or not the method that we developed was suitable for quantification of these pollutants in real sheep serum samples.

2. Material and methods

2.1. Reagents and materials

All the solvents used were of HPLC grade, including methanol, DCM and iso-hexane (Rathburns, Walkerburn, UK). Seven PBDE compounds were combined to produce a stock solution (approximately 20 $\mu g\,mL^{-1}$ for each component) including 2,4,4′-tribromodiphenyl ether (PBDE 28), 2,2′,4,4′-tetrabromodiphenyl ether (PBDE 47), 2,2′,4,4′,5-pentabromodiphenyl ether (PBDE 99), 2,2′,4,4′,6-pentabromodiphenyl ether (PBDE 100), 2,2′,4,4′,5,5′-hexabromodiphenyl ether (PBDE 153), 2,2′,4,4′,5,6′-hexabromodiphenyl ether (PBDE 154), 2,2′,3,4,4′,5′,6-heptabromodiphenyl ether (PBDE 183) and an internal standard (^{13}C-2,2′,4,4′,5-pentabromodiphenyl ether, ^{13}C-PBDE 99: 50 $\mu g\,mL^{-1}$); all were all supplied by AccuStandard (New Haven, CT, USA). Six PCB compounds (stock solution, approximately 10 $\mu g\,mL^{-1}$ for each component) including 2,4,4′-trichlorobiphenyl (PCB 28), 2,2′,5,5′-tetrachlorobiphenyl (PCB 52), 2,2′,4,5,5′-pentachlorobiphenyl (PCB 101), 2,2′,3,4,4′,5′-hexachlorobiphenyl (PCB 138), 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153), 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (PCB 180) plus 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118, neat, prepared stock solution; approximately 3 $\mu g\,mL^{-1}$) and an internal standard (^{13}C-2,2′,3,4,4′,5′-hexachlorobiphenyl, ^{13}C-PCB 138: 50 $\mu g\,mL^{-1}$) were also supplied by AccuStandard (New Haven, CT, USA). These test solutions were stored at 4 °C and diluted in DCM before use. The various SPE cartridges including SampliQ C18 (SOC, silica reversed phase, 200 mg/3 mL) and SampliQ OPT (OPT, polymers, 150 mg/6 mL) (Agilent Technologies, Stockport, UK), Strata-SI-1 Silica (SSI, silica-based silica sorbent, 1000 mg/6 mL), Strata-NH_2 (SNH, silica-based NH_2 sorbent, 1000 mg/6 mL) and Strata-X (STX, polymer-based sorbent, 200 mg/6 mL), Strata-CN (SCN, silica-based CN sorbent, 1000 mg/6 mL) (Phenomenex, Macclesfield, UK) and Oasis HLB (Oasis, poly(divinylbenzene-co-N-vinylpyrrolidone), 200 mg/3 mL) (Waters, Herts, UK) were used for serum samples extraction tests. Silica gel (70–230 mesh; VWR, Leicestershire, UK) used for SPE clean-up was washed in DCM, by Soxhlet, for 6 h and baked in a muffle furnace at 550 °C overnight before use. Acid-modified silica gel was made at a ratio of 1:2 (98%) sulfuric acid (Fisher Scientific, Loughborough, UK):silica gel, and then mixed on a roller for 1 h and used immediately. Sodium sulfate, also used in the clean-up columns, was heated in a muffled furnace at 550 °C overnight to remove water. Cotton wool used to pack the columns was washed by Soxhlet extraction in DCM. Ultrapure water was supplied by a Milli-Q system (Millipore, Watford, UK). All glassware used throughout the experiments was machine washed and then baked at 450 °C for 12 h to eliminate the potential for any organic contamination.

2.2. Solid-phase extraction

The target compounds were extracted from serum samples using various SPE cartridges. All the cartridges were first conditioned with DCM (5 mL), followed by 5% methanol in 0.1 M hydrochloric acid (5 mL). Triplicate batches of 1.00 g of sheep serum were each spiked with 10 ng of each of the compounds for the recovery test. Then, serum samples (after denaturation of serum proteins, e.g. by 1 mL of formic acid) were slowly loaded to the column at a flow rate of 1 mL min^{-1}. After the extraction, the cartridges were dried under vacuum, with the analytes being eluted to 20 mL vials from the sorbents with 15 mL of solvents (e.g. DCM) at a flow rate of 1 mL min^{-1}. The solvents were reduced to near dryness under a gentle flow of nitrogen at less than 35 °C. The sample was redissolved in 0.5 mL iso-hexane and then subjected to further clean-up and analysed by GC–MS (see below).

2.3. Clean-up

Two clean-up methods were compared:

1. Glass columns (i.d. 3 cm, length 22 cm, 50 mL) with Teflon stopcocks, plugged with cotton wool were packed with 15 g of acid-silica/silica (10 g:5 g) and 0.5 g sodium sulfate. After extraction with SPE, the extract was concentrated under nitrogen flow and transferred to the top of an acid modified silica/silica column, pre-rinsed with 40 mL iso-hexane. The column was eluted with iso-hexane (first 20 mL discarded; next 80 mL collected). The eluates from clean-up were concentrated to 1 mL by rotary evaporation, and then taken to near dryness under a nitrogen stream. The residue was redissolved in about 0.1 mL iso-hexane for GC–MS analysis.
2. An empty SPE cartridge (6 mL) plugged with cotton wool was packed with silica/acid-silica (0.2 g:1.8 g) and 0.5 g sodium sulfate. In clean-up solvent elution optimization experiments, different solvents or their mixture were investigated using the spiked serum SPE. The concentrated eluate obtained from the SPE cartridge was then transferred to the top of an acid modified silica/silica column (pre-conditioned by 5 mL of iso-hexane). The cartridge was eluted with solvents (e.g. iso-hexane) and was evaporated to near dryness under a nitrogen stream. The residue was dissolved in 0.1 mL iso-hexane for GC–MS analysis.

2.4. GC–MS analysis

An Agilent 5975C MSD (mass spectrometer detector) linked to 7890A GC with an autosampler (7683B), was used for PBDE and PCB analyses with selected ion mode. The capillary column was ZB-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Phenomenex, Macclesfield, UK). The operating temperature for PBDEs was programmed from 70 °C (1 min) to 170 °C at 30 °C min^{-1}, then ramped to 300 °C at 8 °C min^{-1} and held for 5 min. The operating temperature of the column oven for PCB analysis started each analysis sequence at 120 °C for 1 min; the temperature was then ramped at 4 °C min^{-1} to 280 °C and held for 1 min and then ramped to 320 °C at 30 °C min^{-1} and held for 5 min. The carrier gas was helium and samples were injected in splitless mode. The injector and mass spectrometer were held at 250 °C and 200 °C, respectively. The electron impact energy was set 70 eV for mass spectrometer. The ions monitored for each compound were as follows: *m/z* 256 (PCB 28), *m/z* 220 (PCB 52), *m/z* 326 (PCB 101 and 118), *m/z* 360 (PCB 138 and 153), *m/z* 394 (PCB 180), *m/z* 372 (^{13}C-PCB 138), *m/z* 406 (PBDE 28), *m/z* 326 (PBDE 47), *m/z* 566 (PBDE 99 and 100), *m/z* 644 (PBDE 153 and 154), *m/z* 722 (PBDE 183) and *m/z* 416 (^{13}C-PBDE 99). Response factors relative to the internal standard were calculated for PCB 28, 52, 101, 118, 153, 154, 180 and PBDE 28, 47, 99, 100, 153, 154 and 183; these were used for quantification.

Before sample analysis, relevant standards were analysed to check instrumental performance, peak height and resolution. With each set of samples to be analysed, reference standard mixtures, quality control samples, and procedural blanks were run in sequence to check for contamination, instrumental performance, peak identification and quantification. Since the type of matrix effect, experimental variation or machine response variation could affect the analytical data, procedural blanks were used in the study (e.g. 1 mL ultrapure water) these were subject to the same processes as experimental samples. The PBDE/PCB values in procedural blanks were subtracted from the concentrations detected in the serum samples. This procedural blank was included in each batch of samples analysed and was used to correct results throughout the experiments. Compounds were identified mainly by selected ion and by their retention times. All results were reported on the serum sample weight basis.

3. Results and discussion

3.1. Effect of cartridge type on extraction recovery rates

The optimization of an appropriate SPE cartridge with different sorbent materials plays a key role in the achievement of high and reproducible recovery for contaminants. The most commonly used sorbents are porous silica particles, surface-bonded with C18 or other hydrophobic alkyl groups and polymeric sorbents, such as styrene–divinylbenzene and activated carbon. Furthermore, some hydrophilic groups, i.e. sulfonic acid and N-vinylpyrrolidone group are often added into the polymeric sorbents to enhance water movement which makes the sorbent more effective. In this study, seven types of cartridges from three different manufacturers were selected for the evaluation of extraction efficiency of PBDEs and PCBs. Recoveries of PBDE 183 were relatively poor (45%, 69% and 74%) for Strata-NH_2 (1000 mg, 6 mL), Strata-CN (1000 mg, 6 mL) and Strata-SI-1 Silica (1000 mg, 6 mL), respectively, compared with the other cartridges. Much higher recovery (114%) was observed on SampliQ OPT (150 mg, 6 mL) but this cartridge showed poor reproducibility for PBDE 183 (standard deviation (SD): 42%). Overall, cartridge Strata-X (200 mg, 6 mL) gave the best recoveries for both PCBs (98–106%) and PBDEs (92–105%), followed by SampliQ C18 (200 mg, 3 mL) and Oasis HLB (200 mg, 3 mL) which showed recoveries as 83–119% for PBDEs and 92–130% for PCBs. In addition, when the serum samples were loaded onto these two cartridges (SampliQ C18 and Oasis HLB), it was observed that the flow speed was relatively slow and it took longer to complete the sample extraction by SPE.

Sandau et al. [37] compared three SPE sorbents (Bondesil ENV, Abselut Nexus and Oasis HLB) for persistent organic pollutant (POPs: PCBs, PBDEs and OCPs, etc.) extraction. All three sorbents performed well, resulting in high recoveries for all analytes of interest and none of the sorbents showed large differences in recoveries for any of the analytes tested although Oasis HLB provided the highest recoveries and was chosen for their method [37]. Covaci and Voorspoels [39] tested four SPE sorbents (Empore™ C18, Isolute Phenyl, Isolute ENV+ and Oasis HLB) and also found that Oasis HLB gives the highest absolute recoveries (between 64% and 95%, RSD < 17%, $n = 3$) for PBDEs. However, when Thomsen et al. [34] evaluated three sorbents for extractions of the POPs from human serum, i.e. Isolute 101, Oasis HLB and Strata-X, the highest absolute recoveries were obtained using the Strata-X column, followed by Oasis HLB and Isolute 101. Thomsen et al. [34] noted that pressure problems were often encountered when performing extractions of serum using Strata-X columns (60 mg, 3 mL), as in our experiments when using this size of SPE cartridge; this caused difficulties with serum throughput but the problem was resolved by using the bigger size of Strata-X columns (200 mg, 6 mL). The Strata-X cartridge (200 mg, 6 mL) was therefore chosen as the best SPE sorbent for the further testing in this study.

3.2. Effect of solvent volume on extraction recovery rate

Sample amount (volume) is also a critical component of the analytical procedure of POPs in sheep serum which can influence the results provided by the instrumental techniques in quantitative determination, the final step of the analysis. Classical methods usually need large volumes of sample, i.e. 10–200 mL, use large volumes of solvents [30,40] and limit the application of these methods due to the difficulty of obtaining enough sample (e.g. only possible for adult sheep). More recently, new methods using automated sample preparation procedures have been developed for serum samples of up to 5 mL [29], 4 mL [4], and even to 1.5 mL and 1 mL but only for predominant PBDEs/PCBs or those present at relatively high concentrations [28,41]. In this study, we inves-

Table 1
Linearity, limits of detection (LOD) and limits of quantification (LOQ) for PBDEs and PCBs of the proposed method.

Chemicals	LOD (pg g^{-1})	LOQ (pg g^{-1})	Linear range (ng g^{-1})	Correlation coefficients (r)
PBDE 28	50	167	0.05–30	0.999
PBDE 47	54	180	0.05–30	0.997
PBDE 99	52	174	0.05–30	0.999
PBDE 100	47	155	0.05–30	0.999
PBDE 153	53	177	0.05–30	0.994
PBDE 154	53	176	0.05–30	0.993
PBDE 183	105	350	0.10–30	0.991
PCB 28	24	81	0.02–30	0.999
PCB 52	16	52	0.02–30	0.995
PCB 101	18	60	0.02–30	0.998
PCB 118	21	69	0.02–30	0.998
PCB 138	16	54	0.02–30	0.998
PCB 153	21	69	0.02–30	0.999
PCB 180	19	63	0.02–30	0.998

Table 2
Recoveries (%) for different spiked levels of PBDEs and PCBs in sheep serum samples (n = 3, RSD: 2–11%).

Spiked level (ng g^{-1})	PBDE 28	PBDE 47	PBDE 99	PBDE 100	PBDE 153	PBDE 154	PBDE 183	PCB 28	PCB 52	PCB 101	PCB 118	PCB 138	PCB 153	PCB 180
0.02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	96	135	85	85	154	98	110
0.05	131	105	117	96	103	99	N/A	84	120	121	97	144	115	113
0.10	114	106	105	120	112	100	89	103	140	140	138	164	139	155
0.20	116	100	111	120	102	109	86	101	138	142	137	166	145	142
0.50	137	138	107	101	78	77	68	86	82	89	91	97	94	89
1.00	112	119	106	94	77	72	74	97	93	99	87	99	102	93
5.00	131	124	101	96	71	69	43	98	104	111	105	110	101	93
10.0	127	125	101	99	73	72	54	103	103	106	105	109	101	90
20.0	148	134	100	94	71	66	53	93	100	94	92	96	97	88
30.0	121	121	102	98	82	77	61	92	90	96	91	95	99	95

tigated the SPE recoveries using three different volumes of sheep serum (1 mL, 2 mL and 5 mL). The results showed good recoveries for all target PBDE/PCB compounds in 1 mL (92–106%), 2 mL (69–124%) and 5 mL (87–129%) except for PBDE 100 (140%) and PCB 180 (56%) in 5 mL serum samples. It is concluded that Strata-X SPE cartridges (200 mg, 6 mL) could be used for analysis of 1–5 mL of sheep serum and the volume of 1 mL was chosen for further tests.

3.3. Clean-up method options following SPE

The non-selective nature of the exhaustive extraction procedures and the complexity of the sample matrices result in complex extracts that require further purification, in order to avoid contamination of the instrument, as well as meeting the quantitative and qualitative requirement for the chromatographic resolution and mass spectrum identification. When the sheep serum samples were extracted by SPE, there was a need to develop and optimize clean-up protocols because any loss of analyte in the clean-up step, or incomplete purification, could imply, perhaps wrongly, incomplete extraction or result in unsatisfactory chromatograms. Due to its efficient removal of organic matter, sulfuric acid treatment (e.g. sulfuric acid activated silica gel) is one of the most commonly used lipid removal methods for PCB/PBDE compound analysis in biotic samples [9,35,42]. In this study, we compared two clean-up methods by glass column (50 mL) and SPE cartridges (6 mL) both filled with acid-silica/silica in-house, as described above (Section 2.3). The results (data not presented) showed that clean up using 15 g silica (glass column) [26,43] was slightly better than 2 g silica (SPE cartridge) [39] for either PCBs or PBDEs, in terms of extraction efficiency. However, the use of glass columns not only resulted in the use of more silica than with the SPE cartridge but also consumed 140 mL of solvent, for both conditioning and elution of the column, while the SPE cartridge filled with silica only required 13 mL of the solvents, in total. Moreover, the recoveries for both target compounds (PCB: 105–129% and PBDE: 73–121%) by SPE cartridge were still satisfactory when compared with glass column (PCB: 98–106%, PBDE: 92–105%). The chromatograms for these two protocols both produced well-resolved peaks, with low contamination and background noise. It is concluded that the use of the SPE cartridge reduced both silica gel and solvent usage and so it was as the preferred clean-up method.

3.4. Optimal solvent for SPE cartridges elution

In general, physico-chemical properties such as polarity and specific density (influences penetration into the matrix), as well as toxicity (a workplace hazard), are considered when determining

Table 3
PBDE and PCB content of sheep serum samples collected from Hartwood, Lanarkshire, UK.

Concentration (ng g^{-1})	PBDE 28	PBDE 47	PBDE 99	PBDE 100	PBDE 153	PBDE 154	PBDE 183	PCB 28	PCB 52	PCB 101	PCB 118	PCB 138	PCB 153	PCB 180
A	0.12	0.15	0.05	0.05	<0.05	<0.05	<0.10	0.27	0.09	0.06	0.11	0.07	0.06	0.06
B	0.09	0.66	0.09	<0.05	0.05	0.09	0.25	0.19	0.08	0.03	0.02	0.04	0.04	<0.02
C	<0.05	0.56	<0.05	<0.05	0.08	<0.05	0.22	0.21	0.05	0.02	<0.02	<0.02	<0.02	<0.02
D	<0.05	0.94	<0.05	<0.05	<0.05	<0.05	0.42	0.11	0.04	<0.02	<0.02	<0.02	<0.02	<0.02
E	0.11	0.12	0.08	0.05	0.09	<0.05	<0.10	0.22	0.08	0.07	0.06	0.07	0.06	0.04
F	0.08	0.17	0.10	0.08	0.09	0.10	<0.10	0.27	0.05	0.07	0.21	0.14	0.09	0.13
G	0.23	0.14	0.18	0.09	0.13	0.13	0.11	0.21	0.07	0.05	0.10	0.06	0.05	0.07
H	0.10	0.09	<0.05	<0.05	<0.05	<0.05	0.14	0.14	0.02	<0.02	<0.02	<0.02	<0.02	<0.02

the extraction solvent of choice. The recovery of target compounds by SPE is highly dependent on the polarity of the eluents. In view of the polarity of the target compounds (PCB and PBDE) and the results of a previous study [43], iso-hexane, iso-hexane:DCM (1:1, v/v), DCM, and DCM:methanol (9:1, v/v) were tested as eluents for the recovery of PCBs and PBDEs from SPE cartridges. The data showed that iso-hexane and iso-hexane:DCM produced lower recoveries for PBDE 183 (51 and 53%, respectively). Improved recoveries were observed with methanol:DCM (62%) and especially with DCM (73%). The result suggested that DCM gave satisfactory recoveries (73–129%, RSD < 9%, $n = 3$) for both PCBs and PBDEs; the use of this single solvent was considered likely to reduce the amount of co-extract relative to that which might be obtained using the mixture of methanol and DCM. DCM was therefore the SPE cartridge elution solvent of choice for PCB and PBDE extraction and was used to investigate further parameters.

3.5. Optimal solvent for clean-up

The use of the SPE procedure replaced the LLE step and so allowed a reduced solvent consumption together with parallel processing of a higher number of samples. The tested SPE adsorbents were highly hydrophobic, a characteristic needed for the successful retention of highly lipophilic compounds such as PBDEs and PCBs. However, the hydrophilic compounds present in serum were co-eluted from SPE and were also present in the sample extract. Since the co-extracted material included biogenic material (e.g. cholesterol) interference from this was likely to increase the background level present in the chromatograph generated by instrumental analysis [9,39]. Thus, silica/acid-silica was used to eliminate cholesterol and other polar biogenic constituents of serum samples. This produced a sample extract virtually free of any biogenic background and very well suited for trace GC–MS analysis.

As discussed in Section 3.3, the use of 2 g silica/acid-silica was proposed for the SPE clean-up. The previous studies suggested that hexane, DCM or mixture of DCM and hexane [35,38,43–45] were generally the preferred solvents for PCB and PBDE elution from classic sorbents (such as silica gel) and so DCM, iso-hexane and mixture of DCM and iso-hexane were tested for the clean-up elution in this study. The results showed that iso-hexane resulted in better recoveries (120–121%) for PBDE 28 and 47 than the other two solvents or mixtures (130–144%) comprising DCM and iso-hexane:DCM (1:1, v/v), although they were similar for all of these solvents with respect to PBDE 99, 100 and 153. The use of DCM resulted in a higher recovery for PBDE 183 but the reproducibility was poor (SD: 23%). In addition, the PCB chromatograms showed that DCM was associated with greater contamination and background noise than the other two solvents. Thus, iso-hexane was chosen as the eluting solvent for the SPE cartridge (filled with silica/acid-silica) clean-up method.

3.6. Denaturation of serum proteins and the choice of denaturing time

The choice of denaturant was crucial, because it is responsible for destroying any protein/analyte interactions and disrupting micelle formation, both of which decrease recoveries of analytes [37]. In previous studies, various denaturants including formic acid and organic solvents (water-1-propanol, water-2-propanol) were assessed [4,9,28,29,36,37,46,47]. Some authors [4,9,28,37] preferred the use of formic acid for denaturation of human serum or plasma proteins; formic acid is added to the serum to ensure the analytes are fully released from the sample matrices. The reduced pH, due to formic acid, inhibits protein binding and increases the extraction efficiency of the analytes by the SPE sorbent [46]. While organic solvents (water-1-propanol, water-2-propanol) were favoured for denaturation of human serum in other laboratories [29,36,47], Conka et al. [29] compared both water-2-propanol and water-1-propanol mixtures for denaturation of human serum proteins. The recoveries achieved for PCB congeners and organochlorine pesticides using water-1-propanol were higher than with the use of water-2-propanol [29]. However, it was not clear which was more suitable for sheep serum denaturation prior to PBDE/PCB extraction analysis and so we compared all of the above reagents for sheep serum denaturation. The result showed that better recoveries were achieved using formic acid for PBDE congeners (73–121%) and for PCBs (105–129%) while the use of water-2-propanol and water-1-propanol (both 85:15, v/v) resulted in relatively poor recoveries (34–133% PBDEs, 73–143% PCBs). Also, we investigated the effect of denatured serum samples under different condition (ultra-sonication for 10 min or left capped and in a refrigerator overnight). There was little difference between these two methods using formic acid, in terms of the extraction efficiency (data not shown), and so formic acid was chosen as the denaturant with 10 min ultra-sonication.

3.7. Validation and application of the developed method

The limits of detection (LOD) were calculated as three times the signal-to-noise ratio (S/N = 3), whereas the limits of quantification (LOQ) were determined as the analyte concentration corresponding to a signal/noise ratio of 10. As shown in Table 1, LOD varied from 47 pg g^{-1} to 105 pg g^{-1} for PBDEs and from 16 to 24 pg g^{-1} for PCBs. No LOD or LOQ data were available for sheep serum analysis and so it was compared with the human serum analytical method. In our study, these values were similar to the LOD reported by other authors that were in the ranges 0.01–0.30 ng mL^{-1} for PCBs [48], 14–56 pg g^{-1} [34], 0.01–0.02 ng mL^{-1} [29] or 0.1–0.3 ng g^{-1} for PCBs [6] and 25 pg mL^{-1} for PBDEs [39]. They were lower than those reported by Focant et al. [4] of 0.5–10 ng mL^{-1} for PBDEs/PCBs, from 0.03 to 0.3 ng mL^{-1} for PCBs and from 0.07 to 1.3 ng mL^{-1} for PBDEs [28]. The linearity of the method was examined by plotting the concentration of PBDEs and PCBs found in the spiked samples against the concentration added to the quality control samples. The native concentration of PBDEs/PCBs in the unspiked sheep serum (used as quality control samples; most exhibited concentrations below the LOD) was calculated, and this value was subtracted from the concentrations found in the spiked samples. Both the results from the initial validation and the second round when investigating the intermediate precision were incorporated when evaluating the linearity. As can be seen from the regression results presented in Table 1, the correlation coefficient was >0.99 for all compounds, and the variation interval of the slopes were narrow (between 0.58 and 1.29). In addition, the chromatograms of real serum samples by the proposed method are shown in Fig. 1.

To further validate the method, a series of recovery tests (using Strata-X SPE cartridge, DCM as SPE eluting solvent, 2 g acid-silica/silica for clean-up and iso-hexane as clean-up eluting solvent, formic acid as denaturant, 1 mL sheep serum) were conducted by spiking sheep serum samples with different concentrations of the standard mixture. For spiked serum samples, the recovery rates were 82–166% for all PCB compounds at spiking rates of 0.02–30 ng g^{-1}, 66–148% for PBDEs 28, 47, 99, 100, 153 and 154 at spiking rates of 0.05–30 ng g^{-1}, and 43–89% for PBDE 183 at spiking rates of 0.10–30 ng g^{-1} (Table 2). The test variability (RSD), based on triplicate analyses for each compound, ranged between 2% and 11%.

The results demonstrated that the PBDEs can be extracted and determined from serum samples by the proposed method, with good accuracy and precision. The combined SPE and GC–MS method developed therefore can be applied to serum samples containing the target PCBs and PBDEs at concentrations as low as pg g^{-1} level.

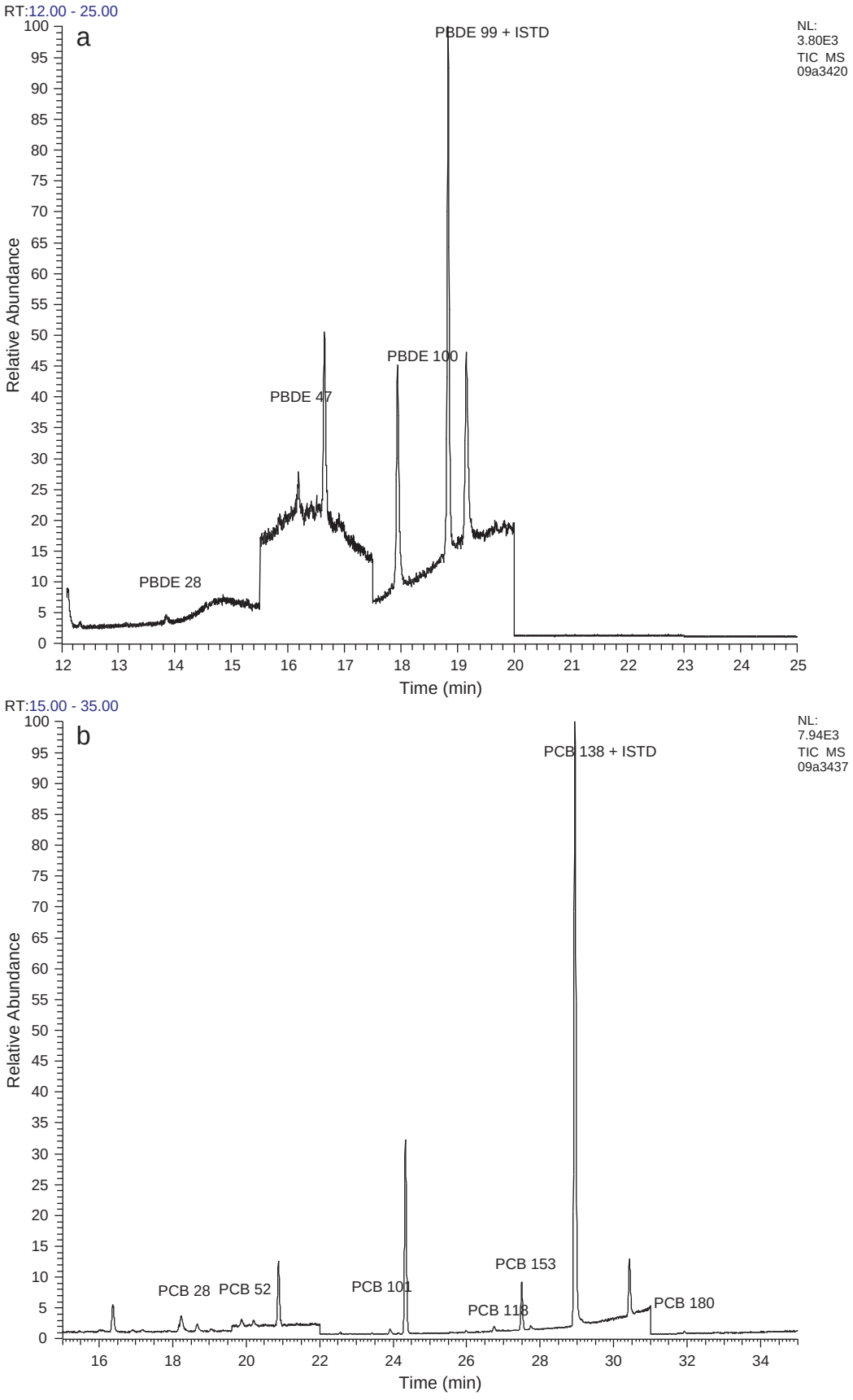

Fig. 1. PBDE and PCB chromatograms obtained from real serum samples (a) PBDEs; (b) PCBs.

After validation, the method was applied to the analysis of target PBDEs/PCBs in the sheep serum samples from Hartwood, Lanarkshire, UK. As shown in Table 3, concentrations of most of the target compounds were low and some were below the limits of detection (LOD). PBDE 47 (0.09–0.94 ng g^{-1}, mean: 0.35 ng g^{-1}), PCB 28 (0.11–0.27 ng g^{-1}, mean: 0.20 ng g^{-1}) and PCB 52 (0.02–0.09 ng g^{-1}, mean: 0.06 ng g^{-1}) were detected in all of these samples. Since PBDE/PCB concentrations in sheep serum have not been reported, we could only compare our data with the concentration in human serum to assess the level of serum contamination. As in human serum, PBDE 47 was identified as the dominant congener, in terms of concentration [50–53]. The mean PBDE concentration was over 20 times higher than those reported in human serum samples collected in Madrid, Spain (PBDE 47: ND–61 pg mL^{-1}, mean: 15 pg mL^{-1}) but PCB 118 (<LOD–0.21 ng g^{-1}, mean: 0.06 ng g^{-1}) was much lower than that reported by Ramos et al. [28] (PCB 118: 20–5920 pg mL^{-1}, mean: 410 pg mL^{-1}). The PBDE 47 concentration was 1–2 orders of magnitude higher than that found in Belgium [39]. The contamination level of PCB 118 (3.81–69.9 ng g^{-1}, mean: 13.1 ng g^{-1}) and PBDE 47 (0.81–1780 ng g^{-1}, mean: 39.0 ng g^{-1}) in Canadian women's blood samples was much higher than those of our results although the former were expressed in terms of lipid weight [49].

4. Conclusion

A method based on SPE and GC–MS for the determination of sheep serum concentrations of various brominated and chlorinated congeners was optimized. A SPE cartridge of Strata-X and DCM were identified as optimal for the sample extraction. 2 g of acid-silica/silica and iso-hexane were used as clean-up and clean-up-eluting solvent, respectively. The denaturation by formic acid resulted in higher recoveries and was preferred. The optimized method has been successfully applied to the analysis of sheep serum samples.

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