Advances in Sample Preparation 7 (2023) 100081

Contents lists available at ScienceDirect

Advances in Sample Preparation

journal homepage: www.elsevier.com/locate/sampre

Green fat removal in the analysis of polychlorinated biphenyls in biotic samples

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ARTICLE INFO

Keywords:
Green sample preparation
Deep eutectic solvents
Fat removal
Polychlorinated biphenyls
Biotic samples
Food analysis

ABSTRACT

A green analytical method based on the use of a deep eutectic solvent (DES) has been proposed for fat removal in the analysis of polychlorinated biphenyl (PCBs) in biotic samples. Using priority PCB congeners as model compounds, a novel sample preparation approach fully avoiding the use of mineral acids has been developed. Once optimized, the procedure allowed fat removal to be accomplished by 30min treatment with [chlorine chloride]:[oxalic acid dihydrated] 1:1 molar ratio at 60°C, with 3s vortexing every 5min to facilitate the homogenization of the slurry. Then, the target PCBs were recovered from the slurry by 20min extraction with 4mL of *n*-hexane. This DES-based method was combined with gas chromatography-quadrupole mass spectrometry (GC-qMS) for final PCB determination. The complete analytical method provided satisfactory recoveries of the target compounds (above 87% for all analytes), using as a small amount of sample as 0.150g. The repeatability of the complete procedure, expressed as relative standard deviation, was less than 5%, and the intermediate precision better than 14%. Final validation of the proposed methodology included the satisfactory comparison of the concentrations calculated for the endogenous PCBs in selected fatty foodstuffs with lipid contents under 18% (w/w fresh weight, fw) with those found when the same samples were analyzed by a more conventional and accepted reference procedure. The limits of detection were in all cases lower than 30 pg/g fw (as calculated for real samples), demonstrating the feasibility of the proposed procedure for accurate determination of the target compounds in biotic samples.

Introduction

Persistent organic pollutants (POPs) are a set of well-known contaminants whose use and production are regulated by the Stockholm Convention because of their toxicity, persistence in the environment once delivered, capability for bio-accumulation and bio-magnification through food webs and capacity for long-range transportation. Due to their hazardous nature, current legislations set maximum residue levels allowed for these pollutants in foodstuffs [1], in particular in fat-containing products, which should be regularly analyzed to ensure consumers' protection.

Current sample preparation methods in use for the determination of organohalogenated legacy POPs in fatty foodstuffs are typically large-scale, highly manipulative procedures involving several sample treatments. The first one consists of the exhaustive extraction of the lipid fraction, in which these analytes are dissolved. This is followed by other treatments allowing fat elimination and the isolation of the target analytes from other co-extracted components before final instrumental determination of the investigated compounds using gas chromatography-based techniques combined with mass spectrometry as detection system (GC–MS) [2]. The efforts carried out during the last decades to green these multi-step procedures have focused on the miniaturization and integration of the several sample treatments [3,4], and on their speed-up by using miniaturized enhanced extraction techniques, such as pressurized liquid extraction [5] or ultrasound-assisted extraction [6]. These alternative, miniaturized approaches have effectively contributed to reduce the amounts of reagents and sample used, the generated wastes, the energy consumption, and have reduced the analyst exposition to hazardous chemicals, while increasing sample throughput. Despite these positive features, fat removal remains as a difficult to green step in these approaches. The reason is that the selective separation between lipids and the target pollutants can only be achieved either by chromatographic fractionation of these two groups of compounds (e.g., by gel permeation chromatography, GPC) or by denaturalization of the former. The latter has been typically carried out by acid digestion due to the high efficiency of this strategy and the chemical stability of POPs. One of the most widely used treatments for acid digestion of lipids in POP analyses consisted of the treatment of the concentrated lipidic extract dissolved on a small volume of a non-polar VOS (e.g., *n*-hexane) with sulfuric acid, which can be directly added to the extract or immobilized on a silica multilayer column [2]. This later approach is cleaner, generates fewer

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https://doi.org/10.1016/j.sampre.2023.100081
Received 19 May 2023; Received in revised form 26 June 2023; Accepted 30 June 2023

wastes than the liquid-liquid extraction-based (LLE) procedure, and prevents from the analyte losses associated with the formation of emulsions. In fact, this is the procedure incorporated in the column-based systems commercialized at present for automatic POP sample treatment [7,8], that is a nowadays widely accepted analytical approach for this type of determination, despite the use of large amounts of reagents (i.e., sulfuric acid, silica and volatile organic solvents, VOSs) for the quantitative elution of the target compounds from the column.

Deep eutectic solvents (DESs) were introduced some two decades ago as custom-made solvents [9] whose physicochemical properties can easily be modified by appropriate mixing of a hydrogen-bond acceptor (HBA) with a hydrogen-bond donor (HBD) at a certain molar ratio and temperature. DESs were described as essentially biodegradable solvents exhibiting appropriate thermal and chemical stability and that could easily be prepared from bulk materials without generating residues or by-products. In addition, DESs exhibited low vapor pressure and flammability, which made then to be rapidly accepted in a variety of application areas [10]. In the analytical field, DESs are considered green solvents and their tunable nature made them particularly attractive as extraction media [11,12] mostly for liquid matrices and in combination with miniaturized techniques [13,14].

Choline chloride (ChCl) has been widely used in the synthesis of DESs [10,12,15]. ChCl combination with nontoxic HBDs, such as carboxylic acids, urea, or polyols, results in eco-friendly, inexpensive and non-flammable, pure eutectic mixtures. In these DESs, the chlorine ion (Cl^-) in ChCl is responsible for forming a strong hydrogen bond with the HBD moiety, so resulting in the formation of the liquid [16]. DESs obtained by the combination of ChCl with oxalic acid (Ox) or urea (Ur) have found application in a number of studies [10]. Interestingly, these ChCl-based DESs have been demonstrated to be a valuable alternative to acids to dissolve macromolecules in the extraction of both, metals and organic compounds [10,17]. However, despite the general satisfactory performance reported in these studies, the use of [ChCl]-based mixtures for the removal of matrix components of solid samples and for the extraction of trace organic pollutants are rare in the literature. In an early study, Helalat-Nezhad et al. [18] reported yields in the 72–110% range with precisions better than 13% for eight polycyclic aromatic hydrocarbons (PAHs) spiked to a marine fish after sample treatment with [ChCl]:[Ox] (1:2M ratio) at 55 °C for 30min. More recently, Noori and Ghanemi [19] found equally satisfactory recoveries (above 95%) when warm [ChCl]:[Ur] and [ChCl]:[Ox] (1:2M ratio in both cases) were used as extraction media for more polar pollutants (i.e., bisphenol A and 4-nonylphenol) from spiked fish muscle, although the later DES was finally preferred because if provided slightly better repeatability. It should be mentioned that, due to the complexity of the investigated matrices, in both studies, further purification of the obtained extracts was mandatory before instrumental determination of the analytes.

The present study evaluates the feasibility of different [ChCl]:[O*x*]- and [ChCl]:[O*x dihydrated*]- ($Ox{\cdot}2H_2O$) based DESs for lipid removal in the treatment of biotic foodstuffs as a green and miniaturized alternative to acidic digestion with strong acids or large-solvent consuming fractionation processes like GPC. The DES allowing the most efficient fat elimination was practically evaluated for the analysis of PCBs in a fortified pork meat sample. The optimized methodology was applied to the determination of the target compounds in foodstuffs with different fat contents and its performance compared with that found using a one-step miniaturized reference sample treatment procedure involving acid digestion [3].

Materials and methods

Reagents and materials

Chlorine chloride (ChCl) 98%, oxalic acid (Ox) 99%, oxalic acid dihydrate ($Ox{\cdot}2H_2O$) 99%, sulfuric acid (95–97%) and silica gel 60 (0.063–0.200mm) were acquired from Merck (Darmstadt, Germany). Isooctane (Chromasolve^{TM} for pesticide residue analysis) was from Honeywell Riedel-de Haën (Hessen, Germany), *n*-hexane (for organic residue analysis) and granular anhydrous sodium sulfate (12–60 mesh) were from J.T. Baker (Deventer, Netherlands). Sea sand was provided by Panreac Quimica SA (Barcelona, Spain).

The PCB Mix-2, containing PCBs isomers No. 18, 28, 52, 101, 138, 153 and 180 dissolved in cyclohexane at a concentration level of 10μg/mL, was provided by Dr. Ehrenstorfer (Augsburg, Germany). (Note: although PCB 18 was included in this commercial mixture, it was excluded from the study because this congener is not part of the six indicator PCBs, ICES-6, considered in current EU regulation [1]). This pure standard was used to prepare a working solution containing the seven PCBs at a concentration of 100 pg/μL of each analyte in isooctane. This working solution was used for further dilution and, when applicable, fortification of the sample used for method development and optimization. Isotopically labeled $^{13}C_{12}$-PCBs-70, –111, –138 and –170 (WP-ISS mix, in nonane) were acquired as a mixture from Wellington Laboratories (Guelph, Ontario, Canada) and used to prepare the spiking solution employed as internal standard in this study (50 pg/μL of each analyte in isooctane). All stock solutions were protected from light and conserved in a fridge at 4 °C until use.

Solid-phase extraction (SPE) glass cartridges (8mL) were obtained from J.T. Baker and Pyrex culture tubes (15 and 18mL) were from SciLabware Limited (Stoke on Trent, UK).

A sample of pork meat muscle (ca. 250g) was acquired in a supermarket in Madrid (Spain) and used for method development. This sample was selected because of its high fat content. The meat sample was sliced, homogenized and freeze-dried at the laboratory. The water content in the sample was gravimetrically determined to be 62.5%. The meat lipid content was determined by extraction of 0.500g of the freeze-dried sample packed in between glass wood plugs in an SPE cartridge with 100mL of a 1:1 (v/v) chloroform:methanol mixture [20]. The lipid content was determined gravimetrically after solvent elimination and determined to be 18% (w/w fresh weight, fw).

A 50g portion of the freeze-dried meat was dispersed in a glass Petri dish, soaked in acetone, and homogenized by manual mixing with a laboratory spoon in a fume hood for 5min. The resulting slurry was then thoroughly mixed with 5mL of an acetone solution containing the appropriate volume of the PCB working solution to yield a final concentration of 10ng/g sample. The Petry dish was covered with a pierced aluminum foil and the solvent was allowed to evaporate overnight. Once dried, the fortified meat sample was allowed to stabilize for one month at room temperature and protected from light before use.

Three non-contaminated food samples, obtained through participation in international interlaboratory exercises organized by Folkehelsa (Oslo, Norway): egg yolk and tuna filet from the 2002 exercise (consensus lipid contents, 25.8% and 2.1%, respectively), and chicken muscle from the 2004 exercise (consensus lipid content, 6.4%), were used for final validation of the developed methodology.

Solvent selection and sample treatment

Four DESs based on ChCl combined with either Ox or $Ox{\cdot}2H_2O$ at two M ratios, 1:1 and 1:2, were evaluated as alternatives to sulfuric acid for fat removal. DESs were prepared by mixing of the two solid components selected in each case under constant rotation in a flask submerged in a water bath kept at 60 °C (± 1°C) until a homogenous, colorless liquid was formed [18]. The prepared solvents were conserved under dried, room temperature conditions and preserved from light until use. Selection among the four assayed DESs was based on their feasibility for practical operation and effectivity for fat elimination. Once the most appropriate DES was selected, the different experimental parameters affecting the efficiency of lipid removal were optimized following a one-variable-at-a-time strategy. These included the sample:DES ratio, the procedure for homogenization of the sample:DES mixture [i.e., manual shaking and vortex (Vortex 4, IKA, Staufen, Germany)], the mixing temperature

and the mixing time. PCBs were back extracted from the obtained slurry by LLE with *n*-hexane. Experimental conditions for this treatment, i.e. volume of hexane, homogenization procedure [i.e., vortex and orbital shaking (AN-2, SBS Instruments, Badalona, Spain)] and number of extraction cycles, were optimized using a one-variable-at-a-time approach. The *n*-hexane phase was separated by centrifugation (NEYA 8 centrifuge, Remi Electrotechnik LTD, Vasa, India) at 3500rpm for 10min. Once optimized, the developed methodology consisted of the spiking of 150mg of the sample with the appropriate volume of the internal standard mixture, followed by its thorough mixing with 2.5g of the [ChCl]:[Ox·2H_2O] 1:1 at 60 ˚C for 30min with additional vortexing of 3s every 5min. Then, PCBs were extracted by LLE of the resulting digested slurry with 4mL of *n*-hexane using an orbital shaker. When required (i.e., during method development and for the analysis of egg yolk), the removal of possible fat traces was accomplished by percolation of the concentrated *n*-hexane extract through a miniaturized multilayer silica column using *n*-hexane as eluent [3]. The fat-free *n*-hexane extract was concentrated under a gentle nitrogen current (Reactic-Therm heating module; Thermo Fisher Scientific, Dreieich, Germany) and reconstituted in 50 μL of isooctane for instrumental analysis.

Otherwise specified, all experiments were carried out in triplicate.

Method validation

The optimized methodology was characterized in terms of accuracy, repeatability and intermediate precision. Accuracy was evaluated by determination of the extracted analytes compared to those spiked to the fortified meat sample. Recoveries were calculated after subtraction of the endogenous values and, when applicable, levels found in the corresponding procedure blank. Repeatability was evaluated as the relative standard deviation (RSD,%) calculated from three separated analyses of the fortified meat sample within the same day. The intermediate precision was evaluated as the RSD values calculated from four separated analyses of the fortified meat sample carried out in different days over a two-weeks period. The feasibility of the proposed DES-based methodology for the analysis of foodstuffs with different fat contents was evaluated through the determination of the target compounds in the three non-contaminated samples indicated in Section 2.1. Final method validation included also the comparison of the results obtained using the optimized method with those found when analyzing the same matrices using a reference treatment procedure involving fat acid digestion. This reference procedure consisted of a miniaturized, generic method allowing the one-step extraction and purification of POPs from biotic matrices [3,21]. In brief, 150mg of the investigated sample was subjected to matrix solid-phase dispersion (MSPD) with silica modified with sulfuric acid (0.3g) and anhydrous sulfate (0.3g). The resulting mixture was then packed in an 8-mL SPE cartridge on top of a multilayer column containing neutral silica (0.5g) and acidic silica (0.5g). The analytes were extracted from the column by two 10min static extraction with *n*-hexane followed by a third dynamic extraction step with the same solvent. Total *n*-hexane consumption was 5mL. Eluates were jointly collected in a Pyrex tube, concentrated under a gentle nitrogen current into chromatographic micro-vials and reconstituted in 50 μL of isooctane for instrumental analysis.

Instrumental analysis

Determination of the investigated PCBs in the final extracts was performed by GC-qMS using a 6890N GC equipped with a 5975C q-MS (Agilent, Palo Alto, CA, USA). Samples were injected in the hot splitless mode (1μL; 275 ˚C; splitless time, 2.0min) in an HP-5 fused silica capillary column (30*m*×0.25mm, 0.25μm film thickness; SGE, Melbourne, Australia). The GC oven was programmed from 70˚C (maintained for 2.0min) to 235˚C at 10˚C/min, then to 270 ˚C at 5 ˚C/min and kept for 5min, and then to 295 ˚C at 20 ˚C/min. This final temperature was maintained for 15min. Helium was used as carrier gas at a constant flow of 0.7mL/min. The transfer line, the source and the quadrupole were kept at 260˚C, 250˚C and 150˚C, respectively. The system was operated in the electron ionization (EI) mode at 70eV and data were acquired in the selective ion monitoring (SIM) mode. Analytes were considered positively detected when the two *m/z* ions selected for each PCB were simultaneously detected at the corresponding retention time and the ratios between them were properly conserved (i.e., ± 25% of the theoretic value determined from pure standards; see Supplementary Information, SI, Table S.1). Native PCBs were quantified using the isotopic dilution procedure, using $^{13}C_{12}$-PCBs as internal standards and the corresponding six-point calibration lines for each analyte (evaluated calibration range, 2–200 pg/μL).

The egg yolk sample was used to determine the limits of detection (LODs) and the limits of quantitation (LOQs) of the complete proposed methodology. LODs were calculated as the concentrations yielding a signal-to-noise (S/N) of three for the quantitation *m/z* value of the corresponding PCB. For LOQs, an S/N ratio of ten was considered.

Quality control and quality assurance

Labeled PCBs with different degree of chloro-substitution (i.e., $^{13}C_{12}$-PCB-70, −111, −138 and −170) were used to control the efficiency of the complete sample preparation procedure proposed at the different stages of the optimization process and during samples analyses. This standard mixture, dissolved in isooctane, was added to the freeze-dried sample (for the DES-based method) and to the dispersed sample (for the MSPD-based method) before treatment [22,23]. The mixture was allowed to stand for 30min before treatment.The fortified meat sample was used in all instances during method development. Analyses of the non-fortified meat were also carried out and, when detected, the levels of the native congeners were subtracted to determine the efficiency of the proposed methodology. Procedural blanks were prepared following the same procedure as for meat but without sample and routinely analyzed every three samples set throughout the entire method optimization and during sample analyses. When applicable, detected levels of the target compounds in the procedural blanks were also subtracted.

Results and discussion

Solvent selection and optimization of the sample preparation procedure

On the basis of previous experience of the group concerning the minimum amount of sample required for accurate determination of priority PCBs in foodstuffs using GC-qMS [3], 150mg of freeze-dried sample were used in this study for method development and optimization. Then, the maximum sample:DES ratio (i.e., g of sample:g of DES) necessary for appropriate sample wetting and easy handling of the obtained slurry was experimentally determined. A sample:DES ratio of 1:10 (corresponding to a 1:21 ratio in a fat base) was insufficient to ensure proper mixing, and consequently proper interaction, between the sample and the DES. Increasing this ratio to 1:13 (1:28 in a fat basis and equivalent to 2.0g of DES) looked to solve the problem and allowed to achieve a satisfactory sample wetting. However, because the goal of this step was to achieve an extensive fat removal, a final ratio of 1:17 (1:35 in a fat basis and corresponding to 2.5g of DES) was adopted for subsequent experiments. This volume of DES was sufficiently large compared to that of the investigated meat to allow complete fat removal (despite its relatively high lipid content, 18%), but small enough to keep the procedure in a miniaturized format minimizing sample and DES consumption, and waste generation. Therefore, these experimental conditions, 150mg of sample and a 1:17 sample:DES ratio (i.e., 2.5g of DES), were selected for further method development. On the other hand, these preliminary experiments evidenced that the relatively high viscosity of the synthesized DESs made the use of a mechanical shaking procedure highly advisable to ensure the homogenous dispersion of meat into the DES. Thereby,

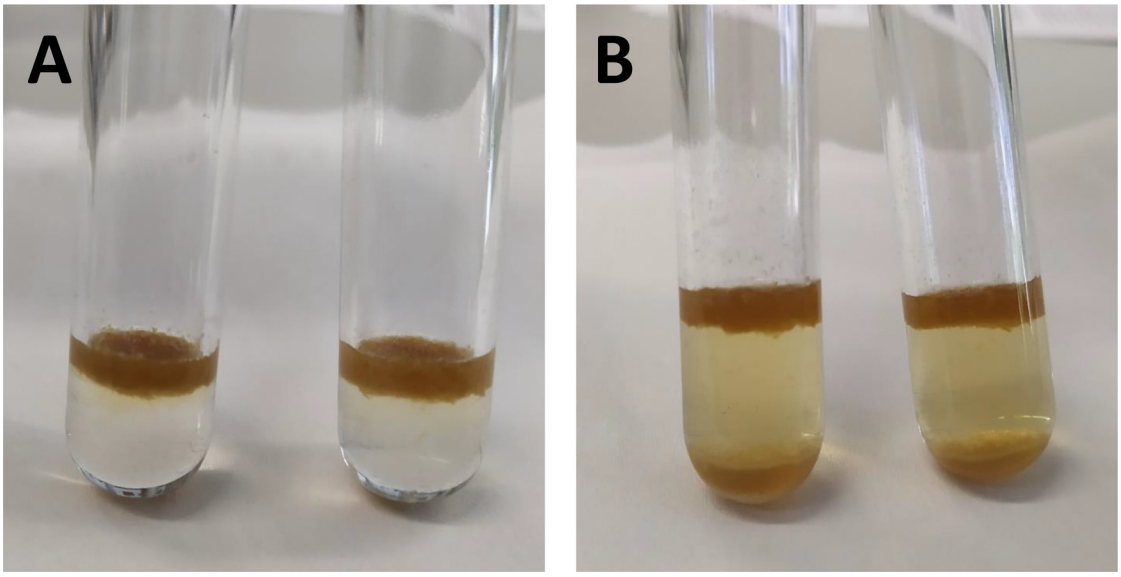

Fig. 1. Appearance of the slurry obtained after treatment of the meat sample (150mg) with 2.5g of (A) [ChCl]:[Ox] 1:1 and (B) [ChCl]:[Ox·2H_2O] 1:1 by heating at 60 ˚C for 30min with additional 3s vortexing every 5min.

in subsequent experiments, vortexing was applied to the mixture to improve the contact between both phases at this step.

The physical state of the synthesized DESs and their viscosity at room temperature were key parameters determining the experimental conditions to be applied for sample treatment. Among the evaluated DESs, [ChCl]:[Ox] 1:1 and [ChCl]:[Ox·2H_2O] 1:1 remained liquid at room temperature. Meanwhile, their equivalent counterparts prepared at a 1:2M ratio were semi-solid and solid, respectively, which made impossible their direct handling at 25˚C (Fig. S.1 in SI). Heating of the solvents before use at 60 ˚C, a temperature intermediate among those previously reported [18,19], melt them and reduced their viscosity. This approach facilitated the handling of [ChCl]:[Ox] 1:2, but not of [ChCl]:[Ox·2H_2O] 1:2, that continued precipitating during pipetting. In consequence, [ChCl]:[Ox·2H_2O] 1:2 was not further considered for method optimization and a temperature of 60 ˚C was adopted for subsequent experiments. In the case of [ChCl]:[Ox] 1:1 and [ChCl]:[Ox·2H_2O] 1:1, it was observed that working at such a temperature simultaneously contributed to facilitate the homogenous interaction between the meat and the DES, while (apparently) improving the efficiency of the fat removal process. On the other hand, in the case of [ChCl]:[Ox] 1:2, after a 30min treatment, and despite the incorporation of an additional vortexing step (3s) every 5min in an attempt to improve the meat-DES interaction, the mixing between both phases was considered insufficient for proper and reproducible sample treatment compared to that achieved with the other assayed DESs (see Fig. S.2 in the SI for a typical example). This fact made [ChCl]:[Ox] 1:2 to be excluded from further evaluation. However, this 3s vortexing step was considered to facilitate the homogeneous contact between the sample and the DES and was, consequently, incorporated to the sample treatment procedure.

The progress of the fat removal process under proposed conditions (i.e., 60 ˚C with additional 3s vortexing every 5min) was then visually evaluated for the two preselected DESs, [ChCl]:[Ox] 1:1 and [ChCl]:[Ox·2H_2O] 1:1. As illustrated by Fig. 1, after 30min of interaction, [ChCl]:[Ox·2H_2O] 1:1 provided a more exhaustive elimination of fat than that reached using [ChCl]:[Ox] 1:1. Therefore, the former DES was selected for further evaluation and method development. It should be noted that no significant progress in fat removal became apparent when the interaction time was extended up to 1h using this DES. Consequently, the defatting time was set at 30min.

The efficiency of fat removal step under the experimental condition proposed, i.e. 30min shaking of 150mg of sample with 2.5g of [ChCl]:[Ox·2H_2O] 1:1 by 3s vortexing every 5min, was evaluated by PCB recovery from the slurry resulting from this treatment. Due to the hydrophilic nature of the DES used in this first treatment, a non-polar solvent with a well-recognised selectivity for the target compounds, *n*-hexane, was selected for the LLE of PCBs from this extract. *n*-Hexane is a toxic VOS and, in consequence, its selection as LLE solvent is far from ideal within the context of green analytical chemistry. However, its affinity for lipids allowed a simultaneous evaluation of the efficiency of the defatting process. In addition, its use in this step prevented from solvent exchange before purification of the obtained extracts in the silica multilayer column used to remove any possible coextracted traces of fat, so avoiding GC damage during method development. The low viscosity of *n*-hexane was also considered an advantageous feature in view of the relatively viscous nature of the DES-based slurry. Following the implications of these considerations, in the context of this study, *n*-hexane was assumed as an acceptable choice in order to determine the efficiency of [ChCl]:[Ox·2H_2O] 1:1 for fat removal.

Homogeneous mixing of [ChCl]:[Ox·2H_2O] 1:1 and *n*-hexane by manual shaking was possible, but somehow hard to achieve due to the viscosity of the slurry from the defatting step. Thereby, orbital shaking (20min, 15rpm) was adopted as shaking procedure. In all instances, the separation between the DES-containing and the *n*-hexane phases was speeded up by 10min centrifugation at 3500rpm. The upper non-polar phase was then easily separated by pipetting.

Next, the volume of *n*-hexane required for LLE of PCBs from the previous slurry was optimized. Assayed volumes were 4 and 5mL and, in both sets of experiments, two LLE cycles (fractions F1 and F2, respectively) were performed to determine the optimum extraction conditions. Results obtained in this part of the study were compared on the bases of the recoveries calculated for the $^{13}C_{12}$-PCBs spiked to the sample before treatment. Data summarized in Table 1 evidenced that quantitative recoveries were obtained with the two assayed volumes, although those found when using 4mL of *n*-hexane were slightly higher than those provided by LLE with 5mL (in the range 91–103% and 87–97%, respectively). In other words, 4mL of *n*-hexane looked to suffice for the quantitative and, repetitive (RSDs, below 12%) extraction of these PCBs from the fat slurry. Consequently, 4mL of *n*-hexane were used in subsequent experiments to minimize the use of this toxic VOS.

Then, the efficiency of the orbital shaking was compared with that of vortexing (1min, 1300rpm) during this LLE process. In addition, in these experiments, the possibility of reducing even more the total vol-

Table 1
Recoveries and RSDs (in parenthesis,%; *n*=3) calculated for the spiked $^{13}C_{12}$-PCBs after LLE with 4mL and with 5mL of *n*-hexane from the slurry obtained by meat treatment with [ChCl]:[Ox·2H_2O] 1:1.

Recovery (RSD,%)	*n*-Hexane volume - 4 mL			*n*-Hexane volume - 5 mL		
LLE cycle	F1	F2	Total	F1	F2	Total
$^{13}C_{12}$-PCB 70	82 (12)	8 (10)	91	76 (5)	12 (32)	87
$^{13}C_{12}$-PCB 111	85 (11)	11 (14)	96	80 (5)	14 (30)	94
$^{13}C_{12}$-PCB 138	86 (12)	11 (12)	97	80 (5)	14 (32)	95
$^{13}C_{12}$-PCB 170	91 (11)	11 (12)	103	83 (5)	14 (35)	97

Table 2
Recoveries (%) and corresponding RSD values (as%, in parenthesis; *n*=3) calculated for the selected PCBs by the analysis of the fortified meat sample using the proposed methodology for fat removal with [ChCl]:[Ox·2H_2O] 1:1 followed by three LLE cycles of the target compounds with 2mL of *n*-hexane using either vortexing or orbital shaking.

Shaking procedure	Orbital			Vortex		
LLE cycle	F1	F2	F3	F1	F2	F3
$^{13}C_{12}$-PCB 70	86 (0.3)	12 (2)	2 (10)	85 (3)	11 (5)	4 (18)
$^{13}C_{12}$-PCB 111	85 (0.6)	13 (4)	2 (11)	87 (2)	11 (4)	2 (12)
$^{13}C_{12}$-PCB 138	86 (0.5)	12 (3)	2 (6)	86 (2)	12 (4)	3 (16)
$^{13}C_{12}$-PCB 170	86 (0.4)	12 (4)	2 (14)	86 (2)	12 (3)	2 (14)
PCB 28	87 (0.5)	11 (2)	2 (10)	86 (5)	10 (2)	4 (9)
PCB 52	86 (0.4)	12 (3)	3 (5)	86 (2)	12 (3)	2 (11)
PCB 101	86 (0.1)	12 (0.4)	3 (4)	87 (2)	12 (3)	1 (11)
PCB 153	86 (0.4)	12 (3)	2 (10)	86 (1)	12 (2)	2 (16)
PCB 138	86 (0.3)	12 (2)	2 (12)	87 (2)	12 (4)	2 (14)
PCB 180	85 (0.8)	12 (5)	3 (14)	85 (2)	13 (5)	2 (17)

ume of *n*-hexane used for sample preparation was simultaneously evaluated. Table 2 summarizes the absolute recoveries calculated in three sequential LLE cycles with 2mL of *n*-hexane with the two assayed shaking procedures for both, the native PCB congeners 28, 52, 101, 153, 138 and 180 present in the fortified meat and the labeled PCB spiked to the meat just before sample treatment. It should be noted that in this table absolute recoveries (i.e., not corrected by the internal standards) are reported. Essentially similar results were obtained for both groups of compounds (i.e., native and labeled) in the three LLE cycles as demonstrated by the overlapping recovery ranges of 85–87% obtained in the first LLE cycle (F1), 10–13% in the second LLE cycle (F2) and below 4% in the third LLE cycle (F3). Satisfactory repeatabilities were also obtained with both shaking procedures. Nevertheless, lower RSDs were systematically observed in the case of the orbital shaking (e.g., 0.1–0.8% vs 1–3% in F1 and 0.4–5% vs 2–5% in F2 for the orbital and vortex shaking, respectively). Furthermore, this shaking procedure allowed to increase the sample throughput (i.e., up to 12 samples can be simultaneously processed). In consequence, orbital shaking was preferred for the following studies. In addition, in view of the slightly higher recoveries obtained when applying this shaking procedure in combination with a single LLE cycle with 4mL of *n*-hexane (Table 1), compared to those found using a single 2mL cycle (Table 2), the former conditions (i.e., a single LLE with 4mL of *n*-hexane) was adopted for subsequent studies.

Table 3 summarized the corrected recoveries (i.e., corrected by internal standard recoveries) and repeatability and intermediate precision values obtained when analyzing the priority PCB congeners in the fortified meat sample applying the newly proposed, green and miniaturized approach for fat removal. Quantitative recoveries (in the range 87–129%) and satisfactory repeatability values (RSDs lower than 5%) were obtained for all investigated analytes. Except for the slight deviations detected for the recoveries of PCBs 28 and 52, these values laid within the recovery range of 60–120% set in current legislation for this type of analysis [24]. The repeatability values were also below the maximum of 20% established in these legislations. The slightly higher recoveries obtained for PCBs 28 and 52 are most probably due to coelution with chromatographic interferences that can be circumvented using either a

Table 3
Corrected recoveries (%) and repeatability (*n*=3) and intermediate precision (*n*=4) values (as RSDs,%) calculated for the selected PCBs in the fortified meat sample using the finally proposed conditions for green fat removal with [ChCl]:[Ox·2H_2O] 1:1 followed by LLE of the analytes with 4mL of *n*-hexane.

PCB congener	Repeatability		Intermediate precision	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
PCB 28	129	3	136	7
PCB 52	124	3	129	6
PCB 101	87	3	100	14
PCB 153	104	2	112	7
PCB 138	97	3	102	6
PCB 180	104	5	110	7

longer GC column (i.e., 60m instead 30m) or an MS system providing improved selectivity, such as tandem MS or high-resolution MS, as most frequently used for this type of determinations [24].

The recovery and repeatability values determined for the method developed in this study were also in the range of those reported in the literature (Table S.2 in SI) for other large-scale procedures involving the well-establish treatment with sulfuric acid [8]. In particular, our results were similar to those obtained by applying the miniaturized one-step sample preparation procedure thoroughly validated in our working group and used as reference method in the present study [3]. It is worth mentioning that our results were also similar [19] or significantly better [18] to those reported by other authors using ChCl-based DESs for fat removal of foodstuffs, although for different organic pollutants and at higher spiking levels. Finally, the results of the repeatability study were consistent with those of the intermediate precision evaluation, which showed only slightly higher RSDs (in the 6–14% range), but far below the 25% set as the maximum in current legislation [24]. These satisfactory results were considered an evidence of the performance and practicality of the proposed approach for the intended determination. Thereby, the final proposed methodology consisted of the treatment of 150mg of freeze-dried sample with 2.5g of [ChCl]:[Ox·2H_2O] 1:1 at 60 °C for 30min with 3s vortexing every 5min, followed by LLE of the resulting slurry with 4mL of *n*-hexane with orbital shaking for 20min at 15rpm; and final phase separation by centrifugation for 10min at 3500rpm.

Fig. 2 shows the typical chromatograms obtained for the fortified meat sample and its corresponding procedure blank after treatment using the optimized methodology. As can be seen, no significant interference or background increase was introduced by the proposed method and the endogenous targeted PCB congeners could be accurately determined.

Method validation

Final method validation was accomplished by comparison of the concentrations calculated for the endogenous priority PCBs in three foodstuffs with different fat content (i.e., egg yolk, tuna filet and chicken muscle) using the optimized DES-based methodology with those found by analyzing the same samples using the miniaturized reference method (MSPD) [3]. Results of this part of the study are summarized in Table 4, where the LODs, as calculated for the egg yolk, are also included. For samples with fat contents lower than the studies pork meat (i.e., tuna fillet, 2.1% and chicken muscle, 6.4%), a satisfactory agreement was observed between the two sets of results for all target congeners (recoveries in the 82–116% range, using the MSPD concentrations as reference values). The only exception to this general good behavior was observed for PCB 138 in tuna fillet. However, it should be noted that this congener was detected at a very low concentration, which made small differences among the levels calculated with both methods (0.06ng/g fw with the DES-based method vs 0.04ng/g fw with MSPD) to result in a large difference when expressed as recovery. It should also be highlighted that, for

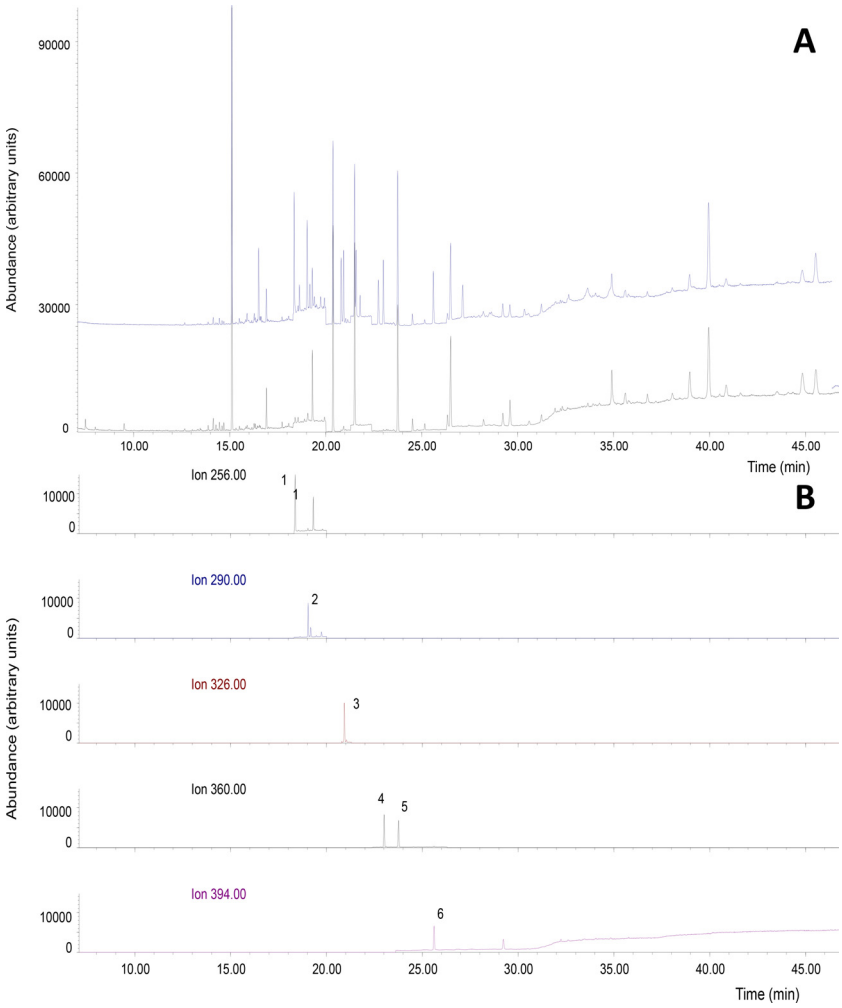

Fig. 2. (A) Total ion current chromatograms obtained for the fortified pork meat (upper blue trace) and the procedure blank (lower black trace) after treatment with the optimised DES-based methodology. Both chromatograms have been adjusted to the same scale. (B) Extracted ion chromatograms obtained for the same pork meat sample. Peak numbering: (1) PCB 28, (1) PCB 52, (3) PCB 101, (4) PCB 153, (5) PCB 138, and (6) PCB 180.

Table 4
LODs (ng/g fw) as calculated for the egg sample prepared using the optimized DES-based method and the reference procedure (MSPD), and comparison of mean concentrations (ng/g fw; *n*=3) obtained when applying these methodologies to the analysis of the selected PCBs in three non-contaminated foodstuffs with different fat contents. Recoveries (%) were calculated using the MSPD concentrations as reference values.

Sample	Egg yolk					Tuna fillet			Chicken muscle		
PCB congener	LOD_{DES}	LOD_{MSPD}	DES	MSPD	Recovery	DES	MSPD	Recovery	DES	MSPD	Recovery
PCB 28	0.01	0.15	0.11	*0.16*	*67* [a]	0.05	0.05	101	0.67	0.58	116
PCB 52	0.02	0.09	0.20	0.30	67	0.16	0.14	116	1.23	1.27	97
PCB 101	0.01	0.11	0.12	*0.29*	*40* [a]	0.21	0.25	82	0.88	0.77	114
PCB 153	0.02	0.08	0.49	1.15	42	0.07	0.08	90	1.53	1.53	100
PCB 138	0.02	0.10	0.31	0.82	38	0.06	0.04	146	1.33	1.14	116
PCB 180	0.03	0.09	0.18	0.50	36	–	0.03	–	0.79	0.86	92

[a] Recoveries in italic letter should be considered as merely indicative because the concentration determined using the reference method laid between the LOD and the LOQ values.

tuna and chicken samples, very clean *n*-hexane extracts were obtained, making further purification of the *n*-hexane extracts unnecessary (Fig. S.3 in SI).

On the contrary, systematically lower PCB recoveries were found for the sample with the highest fat content investigated (egg yolk, 25.8%) using the DES-based method. This finding could indicate that, while the methodology proposed in this study is suitable for the analysis of biotic matrices containing up to 18% of fat fw, its application to samples with a higher fat content could require the use of either larger amounts of DES to ensure complete fat elimination and/or of *n*-hexane for analyte back extraction for the DES-containing slurry. In any case, as illustrated by Fig. S.4 in SI, the proposed methodology provided clean chromatograms with, in general, similar or lower background levels than those obtained with the MSPD procedure, even for this particularly fatty matrix.

As a further illustration of the performance of the proposed methodology, Table 4 summarizes the LODs calculated for the egg yolk sample, which corresponded to the most adverse scenario and complex matrix investigated in this study. Even under these circumstances, and using a qMS as detection system, the calculated LODs were lower than 0.03ng/g fw for all priority PCBs, so demonstrating the general feasibility of the developed method for this type of food analysis.

Conclusions

A new procedure based on sample treatment with [ChCl]:[Ox·2H_2O] 1:1 has been optimized for green fat removal of lipid-rich foodstuffs and applied to the determination of the priority PCBs. The methodology represents an eco-friendly alternative to the conventional sample treat-

ments involving either liquid or immobilized on silica sulfuric acid in use for this type of determinations. Once optimized, the proposed method allowed complete and reproducible fat removal from samples with lipid contents of up to 18% w/w fw in 30min with minimal waste generation. Extraction of the target PCBs from the obtained slurry was accomplished by 20min orbital shaking with only 4mL of *n*-hexane. Although replacement of this toxic VOS is highly desirable in order to approach the principles of green sample preparation, it should be highlighted that this small volume sharply contrasted with the several hundreds of mL required by conventional large-scale treatment procedures in use for this type of analysis. The total time required for sample preparation (ca. 60min) is also shorter than that necessary when using these procedures, although it is longer than those reported for other miniaturized approaches described in the literature also involving an acidic digestion of the investigated sample. Thereby, the method presented in this study is considered a valuable analytical alternative that contributes to green conventional protocols in use in this research field, as it is the first one fully avoiding the use of sulfuric acid for fat removal, while maintaining the analytical quality standards required for this type of food analysis.

Consent to participate

All authors consented to participate in the article preparation.

Consent for publication

All authors give their consent for publication of the article.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

J. Bintanel-Cenis: Methodology, Validation, Data curation, Formal analysis. **L. Herrero:** Methodology, Validation. **B. Gómara:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition. **L. Ramos:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Acknowledgment

This work was funded by project PID2019–106405GB-I00 financed by MCIN/ AEI/10.13039/501100011033. Authors thank the Comunidad of Madrid and European funding from FSE and FEDER programs for financial support (project S2018/BAA-4393, AVANSECAL-II-CM). This article is based upon work from the Sample Preparation Study Group and Network supported by the Division of Analytical Chemistry of the European Chemical Society.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2023.100081.

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