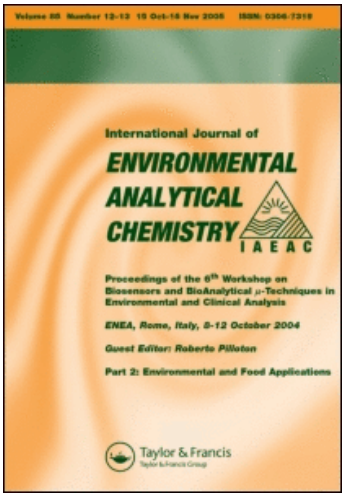

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Comparative Study of Methodologies for the Analysis of Organochlorinated Compounds in Powdered Full-Fat Milk. I. PCBs

Lourdes Ramos[a]; Luis M. Hernandez[a]; Maria J. Gonzalez[a]
[a] Department of Instrumental Analysis and Environmental Chemistry, Organic Chemistry Institute, Madrid, Spain

To cite this Article Ramos, Lourdes , Hernandez, Luis M. and Gonzalez, Maria J.(1998) 'Comparative Study of Methodologies for the Analysis of Organochlorinated Compounds in Powdered Full-Fat Milk. I. PCBs', International Journal of Environmental Analytical Chemistry, 71: 2, 119 — 136
To link to this Article: DOI: 10.1080/03067319808032622
URL: http://dx.doi.org/10.1080/03067319808032622

Intern. J. Environ. Anal. Chem., Vol. 71(2), pp. 119-136
Reprints available directly from the publisher
Photocopying permitted by license only

COMPARATIVE STUDY OF METHODOLOGIES FOR THE ANALYSIS OF ORGANOCHLORINATED COMPOUNDS IN POWDERED FULL-FAT MILK. I. PCBs

LOURDES RAMOS, LUIS M. HERNANDEZ and MARIA J. GONZALEZ*

Department of Instrumental Analysis and Environmental Chemistry, Organic Chemistry Institute, CSIC, Juan de la Cierva, 3, 28006 Madrid, Spain

(Received 20 May 1997; In final form 12 November 1997)

The efficiency of various methods of extraction used for the determination of lipids and PCB levels of powdered full-fat milk is compared using identical milk samples and the same clean-up and quantification procedures.

The solid-liquid procedure provides the best extraction and is the easiest to use and standarize. Silica gel from Bio-Rad and 400 ml of acetone:hexane (1:1, v:v) gives the greatest efficiency for the recovery of the PCBs. The coefficient of variation associated with the determination of PCBs and the lipids extracted from powdered milk using this methodology are below 7.3% and 1.8%, respectively, lower than those found using other extraction methods.

These results show that it is not necessary to extract total lipids in order to determine PCB content. Extraction of apolar lipids alone can yield close to 100% of PCB content, and this approach simplifies the following clean-up steps.

Keywords: PCBs; milk; analysis; extraction

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of ubiquitous environmental pollutants. There are 209 possible PCBs, but in recent years attention has been centred especially on non-ortho, mono-ortho and di-ortho-chlorine-substituted congeners, which show the same type of toxicity as polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs)[1]. The lipophility and persistence of these PCBs has made it possible to find measurable levels of these contaminants nowadays in biological tissues and fatty foods[2–5]. Among these

* Corresponding author. Fax: +34–1–5644853. E-mail: mariche@fresno.CSIC.es.

latter matrices, dairy products and milk in particular have received special attention due to their widespread consumption by the population at large[6].

Data concerning this subject may be found in the literature but a wide variety of analytical techniques have been used to determine their levels, usually based on exhaustive extraction of the lipid fraction from the matrix, followed by a multi-step clean-up procedure[7]. The lack of reference material for this kind of pollutants makes it necessary to validate proposed methodologies by using standard solutions or, in the best case, a fortified matrix, which are based on the assumption that spiked compounds and those from samples have the same behaviour during the extraction step[8]. Even when an incubation step is introduced, it is obvious that spiked molecules are not so strongly retained on the matrix as endogenous ones, making spiked ones easier to extract. The extent of this phenomena is difficult to determine but its consequence is an error in the levels calculated for endogenous compounds.

Another very widespread assumption concerning the analysis of lipophilic pollutants such as PCBs is that the greater the degree of recovery of fat from matrix, the greater the degree of recovery of these compounds. This assumption has been examined in this study.

In this paper, we present the results obtained when different PCB extraction methodologies were applied to commercial powdered full-fat milk. All the extraction methods tested had previously shown good recoveries for the 14 individual coplanar congeners in procedure blanks. Once the different extraction methods were completed, the clean-up and quantification steps used were the same in each case. The comparison of the different extraction methodologies used was done according the endogenous PCB congener levels determined for each one of them. Accuracy, precision and effectiveness of the better extraction protocols were evaluated using statistical methods.

MATERIALS AND METHODS

Sampling

Powdered full-fat milk was purchased from a supermarket. According the manufacturer its percentage composition was 26.0% (w:w) fat, 25.5% (w:w) protein and 39.0% (w:w) carbohydrate.

Materials

All solvents used were pro analysis or pesticide residue quality and they were purchased from Merck and Promochem (Germany). Silica gel 60 (Merk), silicic acid Bio-Sil A (Bio-Rad Lab., Richmond, California., USA) and Florisil (Flori-

din Co., Berkeley Springs, West Virginia, USA) were used as adsorbents. Other chemicals used were analysis grade anhydrous sodium sulphate (Panreac, Spain), anhydrous granulated sodium sulphate (J.T. Baker, Holland), and sodium oxalate, ammonia solution 25% and sulphuric acid 95–97% (all pro analysis quality, Merck).

The 14 individual PCB congeners studied were selected for their toxicity and relative abundance in environmental samples in accordance with WHO criteria[1]. All individual isomers were purchased from Ehrenstorfer (Germany). A working stock solution was prepared from individual coplanar PCB standards (PCBs 77, 101, 105, 118, 126, 138, 151, 153, 156, 167, 169, 170, 180 and 194[9]) with concentrations between 0,51 and 0,12 ng/µl in hexane (Table I). Two individual PCBs (No. 12, 3,4-dichlorobiphenyl and No. 209, 2,2′,3,3′,4,4′,5,5′,6,6′-deca- chlorobiphenyl) were used as internal standards in the quantification step by HRGC.

Sample preparation

The powdered full-fat milk was used in powder form in all experiments. Only when required by the extraction procedure, i.e. liquid-liquid extraction, was milk reconstituted by adding 150 ml of Milli-Q water to a 20g milk-powder sample.

The powdered milk was never spiked, except when the possibility of introducing an incubation step was studied. In this case the reconstituted milk was spiked with 10 µl of the stock solution containing the 14 coplanar PCBs. The sample was then incubated in a heater at 45°C for 90 min and the milk was freeze dried. The possibility of losses from the standards spiked during freeze drying was examined. Freeze dried milk was stored at 4°C until use.

Sample extraction

Milk samples were extracted by the following methods.

Liquid-liquid extraction

Three different methods based on liquid-liquid extraction were evaluated. In each of these methodologies 7g of the powdered milk was reconstituted and then extracted in a separating funnel according to one of the following procedures:

a. Sample was mixed with methanol and extracted with chloroform. After separation from the organic layer the aqueous layer was re-extracted twice with chloroform. Different organic phases were combined and dried with anhydrous sodium sulphate[10].

b. Sodium oxalate and methanol were added to the liquid milk. Subsequently extraction was performed using diethyl ether and then with petroleum ether. After separation from the organic layer the aqueous layer was extracted again using a 1:1 (v:v) diethyl ether:petroleum ether mixture. The ether fractions were combined, concentrated and then washed twice with an aqueous NaCl solution. Water residues were eliminated from the ether phase with anhydrous sodium sulphate[11,12].

c. A 50 ml milk sample (7g) was mixed with 7,5 ml of ammonia. After addition of 50 ml of ethanol and 125 ml of petroleum ether the separating funnel was gently shaken for 1 min. Then, another 125 ml of petroleum ether was added (shaking time 30 seconds). When the organic layer was separated, the aqueous solution was re-extracted. Different organic phases were combined and dried with anhydrous sodium sulphate[13].

Soxhlet extraction

Two different methods based on Soxhlet extraction were evaluated. In both cases, the volume of the solvent mixture was 200 ml.

a. When a 1:1 (v:v) methanol:chloroform mixture was used as solvent, 10g of powdered milk was dried and ground with 4g of sodium sulphate powder. The mixture was extracted for 6h.

b. When a 1:1 (v:v) acetone:hexane mixture was used as solvent, 15g of the powdered milk was mixed with 5g silica gel and 5g sodium sulphate powder. The mixture was extracted for 16h.

Solid-liquid column extraction

A 20g powdered milk sample was mixed with 42g of 1:1 (w:w) silica-gel:anhydrous powdered sodium sulphate. The mixture was ground and loaded into a column. Initial extraction was carried out with 250 ml of a 1:1 (v:v) acetone:hexane mixture.

To study the influence of the silica-gel characteristics on the extraction process, two different commercial silicas were evaluated. The influence of their activation level on the effectiveness of the extraction was also checked.

Lipid determination

Organic solvents produced by each of the extraction methods were evaporated and the total lipid quantity extracted by each technique was determined gravimetrically.

Clean-up protocols

Fat extracts from the different extraction procedures evaluated were submitted to the same clean-up protocol ensuring that the differences in the results were directly related to the specificities of the extraction methods applied.

Two different lipid removal methods were initially examined: i) direct addition of concentrated sulphuric acid to the fat extracts dissolved in hexane[11,14] and ii) elution of the extract through a glass multilayer column containing silica-gel, acidic silica (with 44% concentrated sulphuric acid) and a top layer of granulated anhydrous sodium sulphate[14,15]. The column was eluted with hexane.

A column containing 6g activated Florisil (450°C, 24h)[16] was used for the final clean-up of the extracts. Hexane and dichloromethane were used as eluents.

The PCB fraction was analysed by a high resolution gas chromatograph (HP 5890 Series II) equipped with an electron capture detector and confirmed by HRGC (HP 5890 Series II) coupled to a low resolution mass spectrometer in selected ion monitoring (SIM) mode (HP 5971 A).

HRGC-ECD analysis

The residue containing the PCBs was taken up in a working solution containing PCBs 12 and 209. These individual congeners were added just before chromatographic injection in order to correct injection errors and detector fluctuations. A 0.5 µl aliquot of this solution was injected in the splitless mode (0.60 min) in a 60 m × 0.25 mm i.d. DB-5 fused-silica capillary column with 0.25 µm film thickness (J&W Scientific). The column temperature was programmed from 60°C (1min) to 185°C at 30°C/min and maintained for 3 min, then to 234°C (65.5 min) at 1.9°C/min, and finally to 250°C (30 min) at 4°C/min. The injector and detector temperatures were 280°C and 300°C, respectively. Nitrogen was used as carried gas at a flow rate of about 22 psi.

Chromatographic data were acquired by the System Gold acquisition data system. The identification of the individual PCB congeners was based on the comparison of their retention times with respect to PCB 209 with those of the stock solution mixture. The quantification of the PCB levels in the extracts from the different experiments was based on the individual peak areas and the response factors of the individual congeners with respect to PCB 209.

HRGC-LRMS confirmation

A 0.8 µl aliquot of the PCB fraction was injected in the splitless mode (0.60 min) at 260°C in a 20 m × 0.25 mm i.d. OV-1 capillary column with 0.25 µm film

thickness. The column temperature was programmed from 100°C (1 min) to 130°C at 50°C/min, then to 190°C (2 min) at 4°C/min, and finally to 230°C at 2°C/min. Helium was used as carried gas at a flow rate of about 5 psi. The eluent from the column was transferred to a quadupole mass spectrometer. Electron impact ionization was performed with an electron energy of 70 eV. The ion source and the interphase temperatures were 280°C. The detector was switched to SIM mode. The characteristic ions monitored, the theoretical ion intensity and the confidential limits were as given in a previous paper[3].

RESULTS AND DISCUSSION

Preliminary studies

For testing the precision of the HRGC-ECD response four replicates of a calibration solution containing the 14 individual PCBs studied, along with the internal standard PCBs 12 and 209 were analysed over a period of a month (Table I). A good maintenance in time of both the absolute retention times (t_R, CV $\leq$ 0.2%) and these retention times related to PCB 209 (t_{Rrel}, CV $\leq$ 0.05%) was found for the PCB congeners investigated. A good precision for their quantification was also found. In all cases, the SD and CV were lower than 0.03 and 5.2%, respectively. These values remained steady over time.

Recovery studies were undertaken to check the different steps in the clean-up procedure used. Similar results were obtained when 10 µl of the standard PCB solution in hexane was cleaned-up by direct addition of sulphuric acid, or when a 10 µl of the same standard was eluted through the acidic silica column, thus, this latter treatment was preferred. Table I summarizes the results concerning the precision of this fat removal method calculated from results of three independent analyses. Satisfactory recoveries of the standards (98% as mean) and good reproducibility (CV in the 1.9–8.0% range) were found. In fact, the mean values were not statistically different ($p<0.05$) from the standard PCBs loaded on the column. According with these results and those previously published for the Florisil column[16] it was possible concluded that no significant variability was introduced by the clean-up procedure proposed.

TABLE I PCB structure, IUPAC No. and their concentration (ng/μl) in the stock working solution. Precision of the retention time (t_R), retention time related to PCB 209 (t_{Rrel}), HRGC-ECD and clean-up steps

PCB Structure	*IUPAC No.*	*tr CV,%*	*tr_{rel} CV,%*	*Stock Sol.*	*Precision HRGC-ECD (n=4)*			*Precision SiO_2-H_2SO_4 (n=3)*			
				(ng/μl)	*X**	*SD*	*CV,%*	*X*	*SD*	*CV,%*	*Rec.†,%*
33'44'-TeCB	77	0.02	0.06	0.51	0.51	0.03	4.4	0.52	0.04	5.5	102
22'455'-PeCB	101	0.02	0.004	0.45	0.48	0.03	5.2	0.47	0.05	7.5	104
233'44'-PeCB	105	0.02	0.004	0.38	0.37	0.01	2.0	0.31	0.01	3.1	82
23'44'5'-PeCB	118	0.02	0.05	0.49	0.51	0.01	1.2	0.49	0.01	1.6	100
33'44'5-PeCB	126	0.02	0.03	0.50	0.51	0.03	3.8	0.52	0.05	6.9	104
22'344'5-HxCB	138	0.02	0.03	0.48	0.49	0.02	2.4	0.47	0.03	4.1	98
22'355'6-HxCB	151	0.02	0.03	0.16	0.17	0.01	4.8	0.16	0.02	8.0	100
22'44'55'-HxCB	153	0.02	0.02	0.45	0.44	0.01	1.8	0.42	0.04	5.9	93
233'44'5-HxCB	156	0.01	0.01	0.44	0.43	0.005	0.8	0.45	0.01	1.9	102
23'44'55'-HxCB	167	0.01	0.01	0.48	0.47	0.005	0.7	0.48	0.04	6.5	100
33'44'55'-HxCB	169	0.009	0.02	0.50	0.50	0.02	3.1	0.50	0.04	6.0	100
22'33'44'5-HpC	170	0.01	0.03	0.3	0.23	0.007	2.2	0.23	0.02	5.3	76
22'344'55'-HpC	180	0.005	0.02	0.47	0.47	0.02	3.6	0.48	0.05	6.8	102
22'33'44'55'-OCB	194	0.001	0.02	0.12	0.11	0.003	2.2	0.13	0.008	4.1	108

*X= Average of n independent determinations.
†Rec.= Recoveries.

Extraction

Protocols involving liquid-liquid extraction were performed. In all three cases good recoveries of the spiked compounds were found and the lipid contents determined from the milk compared favourably. The methanol:chloroform mixture (the classical Bligh and Dyer mixture[10]) had been widely recognized as the best alternative available at the time for the total lipid determination[17,18]. Nevertheless, as no appreciable difference was found in the preliminary results, the method using NH_3:ethanol:petroleum ether was preferred to those using chloroform or diethyl ether:petroleum ether as solvents. Whereas in the former the separation of the organic and aqueous layer was spontaneous and rapid (just a few minutes), in the latter this occurred after a long time period (one day) or by centrifugation of small fractions of the mixture for a minimum of 20 min at 3000–3500 rpm. Thus, method C (Official method for lipid content determination in milk[13]) was chosen for the rest of the study to compare with those methods involving Soxhlet or solid-liquid extractions.

Lipid determination

The lipid content extracted was not only highly dependent on the solvent or mixture of the solvents used, but also on the operational extraction conditions (Table II). On the basis of the 26.0% (w:w) of fat content reported by the manufacturer for the sample being studied, the Official method (liquid-liquid extraction) would be adequate for lipid determination (24.8%). The slight difference between both percentages is probably related to the commonly encountered problems in liquid-liquid extraction, such as the formation of separating foam, the saturation of the aqueous phase with the organic solvent, or the droplets of organic solvent which usually remain in aqueous phase[17].

The difference between this fat percentage (24.8%) and that quantified by methanol:chloroform Soxhlet extraction (30.7%) was due to the different extraction ability of the solvent mixtures used. The methanol: chloroform mixture is the best available for total lipid determination (neutral or apolar lipids plus polar or phospo-lipids)[17,18]. Probably, the ethanol:petroleum ether mixture was not able to extract the polar lipids. The differing efficiency of both mixtures for the lipid extraction was not evident from results in liquid-liquid extractions. The difficulties in the separation of both the aqueous and the chloroform phases, the high number of manipulations involved and the substantial amount of variance associated with this methodology are all possible reasons.

TABLE II Total lipid extracted by different methods assayed

Extraction	*Liq.-liq.*	*Soxhlet*		*Solid-liquid*		
Solvent Mixture	*EtOH/Eth. Pet.*	*MeOH/ Chlorof.*	*Acet./Hex.*	*Acet./Hex. (BioRad)*	*Acet./Hex. (Merck)*	*Acet./Hex. (Merck, 200°12h)*
g sample	7.00	10.00	15.00	20.00	20.00	20.00
Replicates (n)	3	4	4	3	3	3
X^* (g)	1.74	3.07	2.73	3.91	3.62	2.43
s.d.	0.21	0.07	0.11	0.08	0.12	0.14
VC, %	8.5	2.0	3.4	1.8	2.8	5.1
% fat (w:w)	24.8	30.7	18.2	19.5	18.1	12.1

*X= Average of n independent determinations.

The acetone:hexane (1:1, v:v) mixture essentially extracted the neutral lipid fraction. Nevertheless, the comparison of the percentages found in the different experiments carried out using this mixture (Table II) showed the high degree of dependence of these values on operational conditions (18.2% by Soxhlet extraction and in the 12.1–19.5% range by solid-liquid extraction depending on the characteristics and activity of the silica).

In solid-liquid extractions the best lipid recoveries and degrees of accuracy were found when the chosen volume of solvents was eluted through the column at a rate of about 3 ml/min. Under these conditions the role of the silica in the extraction process was studied. The fat percentage found with silica from Merck was lower (18.1%) than that found with silica from Bio-Rad (19.5%) and it decreased when the activity of the adsorbent increased (12.1%, when silica Merck was activated at 130°C for 12 h). The quantity of lipid extracted in the experiment using silica from Bio-Rad compared favourably to that found when any adsorbent was used. Nevertheless, in the former case the elution of the solvent through the column was easier and more uniform.

The lipid content found with Soxhlet extraction is highly dependent on the temperature and the time of the extraction[17]. However, it is not easy to explain the differences found in this study between the fat percentages obtained from the Soxhlet (18.2%) and the solid-liquid (19.5%) extractions using the acetone:hexane mixture.

In general, a satisfactory reproducibility was found for all methods. The higher variance was obtained from liquid-liquid extraction (SD= 0.21 and CV= 8.5%). As might be expected, the SD for total lipid determination was lower (0.07) than those found when a partial extraction of the lipids was carried out. The more difficult control of the operational condition on Soxhlet extraction justifies its greater SD (0.11) as compared to that of its equivalent solid-liquid extraction (SD= 0.08).

PCB analysis

After quantification of the extracted fat, lipid fractions were numbered randomly and selected for clean-up and quantification in a random order. Twenty samples were involved: three replicates of the 5 extraction methodologies (1 for liquid-liquid extraction, 2 different Soxhlet methods and 2 solid-liquid procedures); and 5 procedure blanks, one corresponding to each protocol.

The analysis of the native PCB levels in the different lipid extracts showed that the method that provided the highest fat recoveries was not the same method as that which provided the best PCB congener recoveries (Table III). The precision of a given extraction methodology was evaluated by its SD and its CV Values corresponding to HRGC-ECD quantification (Table I) were taken as a reference.

Very different results were obtained by solid-liquid extraction varying according to the properties of the silica employed (Table III). In all cases, the higher PCB values with the lower variability were found when Bio-Rad silica was used. These results seemed to agree with the greater recovery of fat found with this silica. Nevertheless, the differences found for PCB recoveries were greater than was expected from those observed in the fat contents. Following the implications of these results, the silica from Bio-Rad was chosen for the rest of the study.

The PCB levels found using this method were also greater than those found with procedures involving liquid-liquid or Soxhlet extraction (Table III). The lipid content determined by liquid-liquid extraction was greater than that corresponding to the solid-liquid extraction. Nevertheless, the PCB levels found with the former method were between 2–4 times lower than those found with the latter for congeners No. 77, 101, 105, 118, 138, 151 and 167, and between 1.2–1.7 times lower for the rest of the PCBs. These results showed that the general assumption of greater fat recovery from the matrix implying greater recovery of this kind of compound is not completely true. This fact was particularly clear when the results found for the fat content and the PCB levels determined by the Bligh and Dyer mixture were compared with those found by the solid-liquid extraction.

TABLE III PCB levels found by different methods assayed. (Results as average of three independent determinations, ng/g sample)

Extraction	*Liquid-liquid*			*Soxhlet*						*Solid-liquid*					
Solvents	*EtOH/Eth. Petr.*			*MeOH/Chloroform*			*Acetone/Hexane*			*Acet./Hex., Bio-Rad*			*Acet./Hex., Merck*		
PCB No.	*X**	*SD*	*CV,%*	*X*	*SD*	*CV,%*	*X*	*SD*	*CV,%*	*X*	*SD*	*CV,%*	*X*	*SD*	*CV,%*
77	0.62	0.15	17	0.57	0.02	2.4	0.72	0.01	1.1	1.79	0.08	3.2	0.05	0.006	9.3
101	0.77	0.19	17	0.78	0.04	3.3	0.89	0.05	4.2	1.81	0.14	5.3	0.10	0.01	8.2
105	0.05	0.001	1.5	0.04	0.006	11	0.12	0.007	4.1	0.20	0.005	1.6	0.02	0.002	8.5
118	0.28	0.05	13	0.22	0.04	14	0.28	0.01	2.7	0.64	0.004	0.4	0.21	0.02	8.8
126	0.14	0.03	12	0.13	0.005	2.8	0.15	0.003	1.4	0.17	0.01	4.4	0.17	0.02	10
138	0.61	0.14	17	0.68	0.04	4.5	0.75	0.04	3.5	1.22	0.02	1.0	0.24	0.02	10
151	0.17	0.03	12	0.19	0.01	4.3	0.23	0.03	9.9	0.36	0.04	7.1	0.03	0.02	6.7
153	0.71	0.18	18	0.66	0.06	6.5	0.82	0.001	0.1	1.21	0.07	3.9	0.59	0.05	6.3
156	0.01	0.002	10	0.02	0.002	7.9	0.02	0.001	6.4	0.02	0.001	2.1	0.01	0.001	7.6
167	0.15	0.02	9.2	0.30	0.03	8.3	0.31	0.04	9.5	0.33	0.04	9.1	ND	-	-
169	ND	-	-	ND	-	-	ND	-	-	ND	-	-	ND	-	-
170	0.08	0.01	9.8	0.12	0.01	6.6	0.13	0.01	5.8	0.14	0.01	5.6	0.02	0.001	5.9
180	0.35	0.01	2.7	0.47	0.06	8.6	0.42	0.04	6.4	0.45	0.02	3.8	0.23	0.02	5.2
194	0.02	0.002	7.7	0.03	0.004	10	0.03	0.001	2.4	0.03	0.0004	0.8	0.01	0.001	6.8

*X= Average of three independent determinations.

Two groups of PCBs could be differentiated when the results from Soxhlet methods were compared with those obtained using the solid-liquid extraction with Bio-Rad silica (Table III). One group, including congeners No. 126, 156, 167, 169, 170, 180 and 194, showed very similar recoveries in all extraction methods used (including liquid-liquid extraction). In contrast to this group there was another, which included PCBs 77, 101, 105, 118, 138, 151 and 153, that showed greater recoveries in solid-liquid extraction than in methods involving the Soxhlet apparatus. The PCBs of this second group are more volatile than those of the former. Furthermore, the effect is more apparent when the volatility of the solvents increased (i.e., greater for the methanol:chloroform mixture than for the acetone:hexane mixture). Their lower recoveries could be related to loss of the compounds as a result of solvent evaporation occurring during the extraction process due to their higher vapour pressure. These results agree with the results found using other extraction methodologies which imply heating the extraction solvents[19].

In general, the extraction methods which use the acetone:hexane mixture as solvent provided the best recoveries of native PCBs. This mixture was only able to extract neutral lipids, but the levels found for the less volatile PCBs compared favourably with those found when an exhaustive extraction of the fat was carried out (methanol:chloroform mixture). These results suggest the possibility that the PCBs were associated mainly or even exclusively with the neutral or apolar fraction of the milk lipids. None or only a small part of the PCBs would be associated with the polar or phospho-lipids. Therefore, the determination of the PCB levels in milk can be performed by extracting just the most apolar lipids, with the advantage that this approach could greatly simplify the following clean-up process.

Effectiveness of the extraction

The extraction efficiency of the methods which provided the best results, Soxhlet and solid-liquid extraction with the acetone:hexane mixture, was examined. In these experiments unspiked powdered milk was submitted sequentially (three times) to the corresponding extraction methodology using the same amount of solvent in each extraction (200 ml in Soxhlet and 250 ml in solid-liquid extraction). The comparison of the PCB levels found in the fractions extracted sequentially by the solid-liquid method with 250 ml of the mixture and those found with the Soxhlet apparatus showed that the former method was more efficient in the recovery of the PCBs from powdered milk than the latter (Table IV). In general, both the PCB levels and their percentages of the total PCB extracted in the first fraction from solid-liquid method were greater than those of the corresponding fraction from the Soxhlet method. Nevertheless, under these experimental conditions, some PCBs were found even in the third extract at non-negligible levels.

TABLE IV Effectiveness of the extraction methods using the acetone/hexane mixture. (Results as average of three independent determinations, ng/g sample)

Extr.	*Soxhlet (200 ml)*						*Solid-liquid (250 ml)*						*Solid-liquid (400 ml)*					
Extr. No.	*1*		*2*		*3*		*1*		*2*		*3*		*1*		*2*		*3*	
PCB No.	*X**	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*	*X*	*CV,%*
77	0.72	1.1	0.29	20	0.03	16	1.79	3.2	0.26	15	0.21	18	2.21	1.9	0.03	11	ND	-
101	0.89	4.2	0.40	8.0	0.05	19	1.81	5.3	0.27	12	0.22	15	2.20	3.1	0.04	13	ND	-
105	0.12	4.1	0.46	6.5	0.07	15	0.20	1.6	0.05	1.6	0.08	19	0.31	1.4	0.01	12	ND	-
118	0.28	2.7	0.09	14	0.01	18	0.64	0.4	0.04	7.6	0.04	23	0.68	0.3	0.02	13	0.02	14
126	0.15	1.4	0.10	15	0.01	9.8	0.17	4.4	0.09	12	0.06	15	0.27	2.0	0.03	10	0.01	14
138	0.75	3.5	0.34	14	0.03	19	1.22	1.0	0.35	8.8	0.27	1.4	1.62	0.7	0.15	14	0.3	15
151	0.23	9.9	0.12	17	0.01	18	0.36	7.1	0.13	24	0.09	2.9	0.60	4.4	0.03	5.8	ND	-
153	0.82	0.1	033	11	0.04	14	1.21	3.9	0.36	4.1	0.28	2.8	1.70	2.9	0.12	6.0	0.01	14
156	0.02	6.4	0.01	11	ND	-	0.02	2.1	0.01	12	ND	-	0.02	1.6	ND	-	ND	-
167	0.31	9.5	0.13	14	0.02	13	0.33	9.1	0.07	22	0.05	6.3	0.33	7.3	0.04	9.3	0.03	11
169	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-	ND	-
170	0.13	5.8	0.05	5.9	ND	-	0.14	5.6	0.09	4.9	0.07	5.1	0.31	1.3	0.01	18	ND	-
180	0.42	6.4	0.33	8.8	0.04	11	0.45	3.8	0.37	6.8	0.22	1.9	0.98	2.5	ND	-	ND	-
194	0.03	2.4	ND	-	ND	-	0.03	0.8	ND	-	ND	-	0.03	0.4	ND	-	ND	-
g fat	2.73	3.4	0.48	1.7	0.11	4.8	3.91	1.8	0.06	4.4	0.09	2.7	4.38	2.0	ND	-	ND	-
% fat (w:w)	18.2		3.2		0.7		19.5		0.3		0.5		21.9		-		-	

*X= Average of three independent determinations.

TABLE V Precision and accuracy of the solid-liquid methodology at three different levels of concentration. (Resuls as average of three independent determinations)

PCB No.	Level 1						Level 2						Level 3					
	$ng_{Spik.}$	ng_{Found} (Rec.,%)	Accuracy		Precision		$ng_{Spik.}$	ng_{Found} (Rec.,%)	Accuracy		Precision		$ng_{Spik.}$	ng_{Found} (Rec.,%)	Accuracy		Precision	
			$E_{Abs.}$	$E_{Rel.}$	SD	CV (%)			$E_{Abs.}$	$E_{Rel.}$	SD	CV (%)			$E_{Abs.}$	$E_{Rel.}$	SD	CV (%)
77	2.04	1.87(92)	-0.17	-8.3	0.16	8.6	4.59	4.92(107)	0.33	4.92	0.54	11	8.16	7.97(98)	-0.19	-2.3	0.82	10
101	1.79	1.99(111)	0.2	11	0.23	11	4.02	4.51(112)	0.49	12	0.51	11	7.15	7.76(108)	0.61	8.5	0.22	2.8
105	1.50	1.39(93)	-0.11	-7.3	0.08	5.7	3.38	3.11(92)	-0.27	-8.0	0.16	5.3	6.00	5.68(95)	-0.32	-5.3	0.41	7.3
118	1.97	1.95(99)	-0.02	-1.0	0.15	7.6	4.44	4.37(98)	-0.07	-1.6	0.31	7.0	7.89	7.69(97)	-0.20	-2.5	0.70	9.1
126	2.00	1.85(93)	-0.15	-7.5	0.09	4.6	4.50	4.98(111)	0.48	11	0.27	5.4	8.00	8.34(104)	0.34	4.3	0.74	8.9
138	1.92	1.87(97)	-0.05	-2.6	0.17	8.9	4.31	4.11(95)	-0.2	-4.6	0.20	4.8	7.66	7.70(101)	0.04	0.52	0.66	8.5
151	0.65	0.58(89)	-0.07	-11	0.04	7.4	1.46	1.59(109)	0.13	8.9	0.16	9.9	2.59	2.59(100)	0	-	0.16	6.1
153	1.79	1.80(100)	0.01	0.6	0.14	7.8	4.02	4.49(112)	0.47	12	0.28	6.2	7.15	7.62(107)	0.47	6.6	0.82	11
156	1.74	2.03(117)	0.29	17	0.12	6.1	3.92	3.69(94)	-0.23	5.9	0.16	4.2	6.96	6.91(99)	-0.05	-0.42	0.63	9.1
167	1.91	2.28(119)	0.37	19	0.14	6.1	4.30	4.82(112)	0.52	12	0.24	4.9	7.65	7.77(102)	0.12	1.6	0.36	4.7
169	2.00	2.23(111)	0.23	12	0.08	3.7	4.50	4.61(103)	0.11	2.4	0.10	2.1	8.00	7.93(99)	-0.07	-0.88	0.92	12
170	0.93	0.78(84)	-0.15	-16	0.07	9.4	2.10	2.02(96)	-0.08	3.8	0.02	0.8	3.73	3.80(102)	0.07	1.9	0.34	8.9
180	1.89	1.65(87)	-0.24	-13	0.20	12	4.25	4.07(96)	-0.18	4.2	0.04	1.0	7.55	7.24(96)	-0.31	-4.1	0.68	9.4
194	0.46	0.37(80)	-0.09	-20	0.05	12	1.04	1.11(107)	0.07	6.7	0.006	0.55	1.86	1.88(101)	0.02	1.1	0.13	7.0

When the solvent volume of the acetone:hexane mixture in the solid-liquid extraction was increased from 250 to 400 ml, the PCB percentages in the first fraction were above 90%. Extremely low PCB levels were found in the second fraction and levels were negligible or below the threshold of detection in the last fraction. In this experiment the total lipid content extractable from this mixture was extracted in the first fraction. Therefore, this method was chosen as the best methodology for PCB determination in powdered full-fat milk and was used in subsequent studies.

Precision and accuracy studies

The precision of the whole methodology finally proposed (solid-liquid extraction using 400 ml of acetone:hexane mixture and clean-up using the acidic silica and Florisil) was evaluated (Table IV). The CVs associated with the determination of endogenous PCBs were below 4.4%, except for PCB 167 with CV=7.3%. These CVs were lower than those found in the literature for spiked samples with higher PCB levels[20].

A number of experiments were undertaken to evaluate the accuracy of this analytical procedure. The solid matrix residues resulting from the solid-liquid efficiency studies in which any PCB levels were detected in the last extraction were used as matrix blanks. After evaporating the solvent residues from the solid matrix, it was re-loaded in a column, spiked with a mixture containing the 14 individual studied PCB congeners and the solid-liquid extraction was performed as previously described. The accuracy of the whole proposed methodology was checked at three different levels of concentration (0.02–0.10 ng/g sample, 0.05–0.23 ng/g sample and 0.09–0.41 ng/g sample, Table V). The overall recoveries ranged from 95% to 108% at the upper concentration level and from 92% to 112% at the medium level. The relative error was less than 8.5% in the former and less than 12% in the latter. The CVs were less than 12% in both cases. At the lowest concentration level the recoveries ranged from 80% to 119% (0.02–0.23 ng/g sample), CVs were in the 3.7–12% range and the relative error was lower than 20%.

Incubation step

When the addition of spiked labelled standards was necessary before the analysis was conducted, an incubation step was recommended in some methodologies[8]. The efficiency of this incubation step when followed by freeze drying of the samples was examined.

Six independent 20 g powdered milk samples were reconstituted as described in the Sample Preparation section. Three of them were spiked with 10 μl of the

stock working solution containing the 14 individual studied PCB congeners in hexane. Then, all samples were incubated as previously described and freeze dried. The six freeze dried samples were extracted and purified by the proposed methodology. No significant differences ($p<0.05$) were observed between the PCB levels found in the freeze dried unspiked samples and those found in the same powdered milk directly submitted to solid-liquid extraction (Table IV). That is to say, the endogenous PCBs were not affected by the freeze drying process. Nevertheless, very important differences were observed between the spiked PCB levels and those found after the freeze drying process. The latter were calculated as the difference in the PCB levels found in the spiked and the unspiked reconstituted samples. Their recoveries are summarized in Table VI as percentages of PCBs added. The worst recovery (22%) with the upper CV (34%) corresponded to PCB 105. For the rest of the congeners the overall recoveries ranged from 52% to 94% with a mean recovery of around 71 % and a mean CV of 10%. Similar kind of losses has been reported previously for the lower chlorinated PCBs[21].

These results showed that even when an incubation step was introduced in the methodology, differences in behaviour can be seen between spiked and native molecules during the freeze drying of the sample. Therefore, when it was necessary to spike the samples, the compounds were loaded on the head column before the solid-liquid extraction.

TABLE VI Percent recoveries of spiked congeners from spiked freeze dried reconstituted powdered milk samples. (Results as average of three independent determinations)

PCB No.	*Spiked level (ng/g sample)*	*Recovery (%)*	*CV(%)*
77	0.26	87	6.8
101	0.22	114	9.6
105	0.19	22	34
118	0.25	65	18
126	0.25	81	7.6
138	0.24	91	5.5
151	0.08	52	9.7
153	0.23	69	7.8
156	0.22	85	7.7
167	0.24	66	7.6
169	0.25	63	12
170	0.12	85	8.1
180	0.24	80	3.1
194	0.06	56	3.1

CONCLUSIONS

Significant differences in the lipid content of powdered milk were obtained using different extraction methodologies. These results imply that comparison of the lipid contents found during an investigation with those in the literature is not always a good practice when different extraction methods are used.

An alternative would be to report the PCB contents on a fresh weight basis, mainly when comparison among data from different surveys is to be carried out.

Good recoveries obtained with spiked standard solutions do not automatically imply that similar results would be obtained with real samples. Determining the "real" efficiency of the extraction step of the proposed methodology is difficult but, nevertheless, one of the critical aspects of method validation. Spiked samples usually indicate the accuracy and precision of the subsequent analytical steps, but do not necessarily determine the efficiency of the extraction step. As an alternative method, an unspiked sample could be sequentially extracted by the same method in order to optimise the conditions for the maximum removal of the studied compounds. In this paper, the solid-liquid extraction method using silica from Bio-Rad and 400 ml of the acetone:hexane mixture for each 20 g of powdered milk sample was chosen as the best alternative for PCB analysis. The proposed methodology provided the most efficient recovery of the native PCBs from the milk with CV below 7.3%. Good accuracy at the different evaluated concentration levels was found (CV below 12%). In addition, this method provided the lowest variability in the lipid determination (CV=1.8%), in spite of the fact that only the neutral or apolar fraction of the milk lipids was extracted. This more selective extraction of the PCBs simplified the subsequent clean-up steps and showed that these pollutants could be associated mainly or exclusively with apolar milk lipids. From this point of view, the traditional requirement of an exhaustive extraction of the lipids from the sample in this kind of trace organic analysis could be revised.

References

[1] H.G. Ahlborg, G.C. Becking, C.S. Birnbaum, A. Brouwer, H.J.G.M. Derks, M. Feeley, G. Golor, A. Hanberg, J.C. Larsen, A.K.D. Lieh, S.H. Safe, C. Schlatter, F. Waern, M. Younes and E. Yrjänheikki, *Chemosphere*, **28**, 1049–1067 (1994).

[2] D.T. Willians and G.L. Lebel, *Chemosphere*, **22**, 109–128 (1991).

[3] M.J. González, L. Ramos and L.M. Hernández, *Bull. Environ. Contam. Toxicol.*, **54**, 349–356 (1995).

[4] K. Fytianos, G. Vasilikiotis and L. Weil, *Bull. Environ. Contam. Toxicol.*, **34**, 504–508 (1985).

[5] C. de la Riva and A. Anadon, *Bull. Environ. Contam. Toxicol.*, **46**, 527–533 (1991).

[6] J. Mes and W.H. Newsome, *Food Addit. Contam.* **6**, 365–375 (1989).

[7] H. Schimmel, B. Griepink, E.A. Maier, G.N. Kramer, A.H. Roos and L.G.M.T. Tuinstra, *Fresenius J. Anal. Chem.*, **348**, 37–46 (1994).

[8] P. Hess, J. de Boer, W.P. Cofino, P.E.G. Leonards and D.E. Wells, *J. Chromatogr. A*, **703**, 417–465 (1995).
[9] K. Ballschmiter and M. Zell, *Fresenius Z. Anal. Chem.*, **320**, 20–31 (1980).
[10] E.G. Bligh and W.J. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911–917 (1959).
[11] P. Furst, C. Furst and W. Groebel, *Chemosphere*, **20**, 787–792 (1990).
[12] P.W. O'Keefe, M. Meselson and R.W. Baughman, *J. Assoc. Off. Anal. Chem.*, **61**, 621–626 (1978).
[13] AOAC. "Official methods of analysis". Method 989.05, 14th ed. Association of Official Analytical Chemists, Arlington, USA (1990).
[14] B.D. Eitzer, *Chemosphere*, **30**, 1237–1248 (1995).
[15] E. Storr-Hansen and T. Cederberg *Chemosphere*, **24**, 1181–1196 (1992).
[16] L. Ramos, L.M. Hernández and M.J. González, *J. Chromatogr. A*, **759**, 127–137 (1997).
[17] W. Cofino and D.E. Well, Resume of "Quality Assurance of Information in Marine Environmental Monitoring Programme in Europe (QUASIMEME)". Guidelines on quality assurance for marine monitoring, U. K. (1994).
[18] R.G. Jensen, *Chemophere*, **31**, 4197–4205 (1995).
[19] L. Ramos, G.P. Blanch, L. Hernández and M.J. González, *J. Chromatogr. A*, **690**, 243–249 (1995).
[20] P. de Voogt, P. Haglund, L.B. Reutergardh, C. de Wit and F. Waern, *Anal. Chem.*, **66**, 305A-311A (1994).
[21] B. Bush, J.T. Snow and S. Connor, *J. Assoc. Off. Anal. Chem.*, **66**, 248–255 (1983).