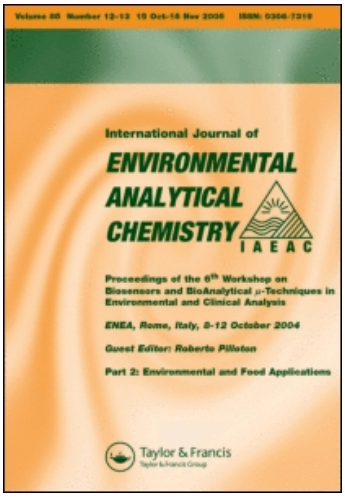

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Janina Lulek[a]; Katarzyna Szyrwińska[a]; Barbara Szafran[a]; Anna Pachcińska[a]; ElżBieta Nowak[a]
[a] Department of Inorganic and Analytical Chemistry, University of Medical Sciences in Poznań, Poznań, Poland

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VALIDATION OF THE METHOD OF SELECTED POLYCHLORINATED BIPHENYLS DETERMINATION IN HUMAN MILK SAMPLES

JANINA LULEK*, KATARZYNA SZYRWIŃSKA, BARBARA SZAFRAN, ANNA PACHCIŃSKA and ELŻBIETA NOWAK

Department of Inorganic and Analytical Chemistry, University of Medical Sciences in Poznań, 6 Grunwaldzka St., 60–780 Poznań, Poland

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A method for determination of trace concentrations of individual PCB congeners in human milk was validated. The analytical procedure included the following steps: acetone : hexane extraction, clean-up of extracts with concentrated sulfuric acid and solid phase extraction (SPE) on Florisil. The identification and quantification of analytes in purified extracts were carried out by high-resolution gas chromatography (HRGC) with electron capture detection (ECD) and/or with low-resolution mass spectrometry (LRMS). Recoveries of 14 PCB congeners from spiked cow milk samples, based on HRGC-ECD were between 87.3 and 93.6%. The precision of analyte determination was established as close to or less than 10%. The detection limits ranged between 0.14 and 0.26 ng/g fat and the quantification limits between 0.57 and 0.86 ng/g fat. The method was linear and characterized by good correlation coefficients (>0.99) for most of the compounds studied. The quality of the method under validation was verified by the analysis of Standard Reference Material (CRM-450) and interlaboratory exercise.

Keywords: PCB; Milk; Quality assurance; CRM-450

INTRODUCTION

Following the initial identification of their presence in Baltic Sea fauna [1], it has become clear that polychlorinated biphenyls (PCBs) are present throughout the environment, including the biosphere, in which they have been shown to exhibit both chronic and acute toxic effects.

Human breast milk, because of its relatively high fat content, is both a major source of bioaccumulated contaminants for nursing infants and a suitable matrix for studies of long-term exposure to persistent organic pollutants, including PCBs [2–4].

In order to accurately determine whether the modest polychlorinated biphenyls (PCBs) exposure experienced with the declining global load involves a real health risk, it is necessary to examine the exposure more accurately and in more detail [5]. Estimates of the dietary intake of lipophilic PCBs by the breast-fed infant may be

*Corresponding author. E-mail: lulekjan@main.amu.edu.pl

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obtained from residue levels in human milk data. It is crucial that data should be as accurate and reliable as possible.

The first and second WHO-coordinated studies of PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in breast milk have revealed problems in the data comparability and validity, especially for PCBs [6,7]. These probably result from the fact that for the last 30 years different and sometimes poorly reliable analytical methods for PCBs' quantification have been used [6–8].

To ensure the quality of the results of exposure and risk assessment, a specific analytical method used for monitoring PCBs should be validated in laboratory experiments.

The aim of this study was to validate a method for determination of selected PCB congeners in human milk in accordance to the requirements of modern analytical chemistry.

EXPERIMENTAL

Materials

On the basis of their reported abundance [2–4] and toxicity, the following congeners (IUPAC Nos) 28, 74, 101, 105, 114, 118, 128, 138, 149, 153, 156, 170, 180 and 187 were targeted for validation of the method of determining specified PCBs in human milk. The individual chlorobiphenyls mentioned, as well as PCBs 30 and 209 (internal standards) were purchased from Dr Ehrenstorfer Laboratories (Augsburg, Germany). The different working standard solutions were prepared by adding the appropriate weights of primary standards to isooctane (Baker® ultra-resi analysed quality) or to acetone (Baker® ultra-resi analysed quality). All glassware and equipment used for the preparation of standard solutions and samples were thoroughly cleaned before use.

The concentrated H_2SO_4 Merck®, *n*-hexane Mallinckrodt nanograde®, granulated sodium sulfate anhydrous "Baker ultra-resi analysed" and Florisil® (for residue analysis) "Baker analysed" were used. Florisil was activated at 650°C for 4 h, stored in a desiccator and kept at 130°C for 2 h before use.

The samples of cow milk used for validation of the analytical procedure came from an individual Polish small farm. A certified standard of powdered milk (CRM-450) was supplied by EEC Community Bureau of Reference (BCR). Seven samples of human milk from the Wielkopolska region in Poland were collected in 2001.

Analytical Procedure

Lipid Determination

The lipid content in cow milk and human milk samples was determined according to the method of Hong *et al.* [9]. 5 g of milk sample was weighed in a 50-mL centrifuge tube. 10 mL of ethanol and 9 mL of hexane were added to the centrifuge tube, shaken vigorously for 1 min and centrifuged at 4000 rpm for 10 min. The upper layer was transferred to an Erlenmeyer flask containing anhydrous Na_2SO_4. The aqueous residue was extracted twice with 7 mL hexane. The combined extracts were used for a gravimetric lipid determination.

PCBs Determination

Congener-specific analysis was performed by the modified method of Galceran *et al.* [10].

The homogenized unspiked or spiked milk sample (about 20.0 g) was placed in a mortar and then 12 g of Florisil and 130 g of anhydrous Na_2SO_4 were added. The mixture was mixed to yield a dry powder and placed on a column previously filled with a small plug of glass-wool. After elution with 100 mL of *n*-hexane–acetone (2 : 1, v/v), the eluate was concentrated to less than 3 mL and cleaned-up twice with concentrated H_2SO_4. The combined extract was concentrated to about 0.5 mL and quantitatively transferred to a Florisil SPE cartridge (500 mg), previously activated with 10 mL hexane. The PCB congeners were eluted with 5 mL hexane. The internal standards (PCB 30 and 209) were added to the concentrated extracts before analysis by gas chromatography.

The PCB identification and quantification were performed by HRGC/ECD (high-resolution gas chromatography with electron capture detection). A Shimadzu GC-14A gas chromatograph equipped with a ^{63}Ni electron capture detector and split/splitless injector was used. Chromatographic separation of the examined PCB congeners was carried out on a 60-m $RT_x^{®}$-5, Restek Corporation (0.25-mm i.d.; film thickness 0.25 μm) fused-silica capillary column (5% diphenyl polysiloxane, 95% dimethyl polysiloxane). The detector temperature was 300°C with nitrogen as make-up gas at a flow rate of 48.0 mL/min. The temperature program of the column was 2 min at 125°C; 7.5°C/min up to 190°C and 2°C/min up to 280°C, holding for 15 min. The chromatographic data were recorded on a Chrompack integrator.

Each congener was identified by a comparison of the relative retention times (RRT_{30}) of the peaks from calibration standards with peaks from cleaned-up extracts of milk. The repeatability of the RRT_{30}, calculated from 18 replicate analyses of a standard mixture of the PCB congeners was between 0.012 and 0.049%. The following elution order of congeners was established: 30 IS, 28, 74, 101, 149, 118, 114, 153, 105, 138, 187, 128, 156, 180, 170 and 209 IS.

The linearity of the ECD response for each congener was determined by plotting calibration graphs of peak height/mass injected *versus* mass injected [11]. The linear range for the PCB congeners was between 20 and 350 ng/mL. The repeatability of the peak heights calculated from five replicate analyses of a standard mixture of the PCB congeners was between 3.82 and 6.51%.

For confirmation of the results, the extracts were analyzed by HRGC/LRMS (high-resolution gas chromatography–low-resolution mass spectrometry) on a Perkin–Elmer AUTOSYSTEM XL connected *via* a direct interface to a Turbomass spectrometer detector. The MS acquisition parameters were: ion source 300°C; electron ionization − 70 eV. Dwell times were set at 0.1 s. Full-scan spectra were run in the electron impact (EI) mode from m/z 100 to 650. In addition, five ions were monitored in EI selected ion recording mode (SIR) for the analysis of the trichlorinated biphenyls through heptachlorinated biphenyls, one ion for each homologue group and one ion for decachlorinated biphenyl (209 IS). The masses of these ions were 255.96, 291.92, 325.88, 359.84, 393.80 and 497.68. The GC oven program for 60 m PE-5MS column (0.25-mm i.d.; film thickness 0.25 μm) was as follows: initial temperature 80°C retained for 0.5 min, increased at a rate of 25°/min to 140°C, than increased at a rate 3°/min to 300°C and retained for 10 min.

The repeatability of the RRT_{209}, calculated from 18 replicate analyses of a standard mixture of the PCB congeners was between 0.012 and 0.037%.

Quantitative measurements of PCB congeners in spiked cow milk, certified milk powder and human milk samples were carried out on the basis of peak heights. The calibrations were performed with bracketing standards calculated to be $\pm 10\%$ of the concentration of each PCB as determined by a preliminary analysis.

Blank experiments were included in each batch of samples to minimize the risk of introduction of any artifacts.

RESULTS AND DISCUSSION

Limit of Detection and Quantification

The limit of detection (signal-to-noise ratio = 3) and quantification limit (signal-to-noise ratio = 10) for the specific compounds depended significantly on the detection method in the HRGC analysis. As results from the data presented in Table I indicate, the method's limit of detection ranged between 0.11 ng/g fat and 0.26 ng/g fat for ECD and between 6.57 ng/g fat and 17.14 ng/g fat for LRMS-SIR. Similarly, quantification limits of congeners are lower for ECD (0.57–0.86 ng/g fat) than for LRMS-SIR detection (21.71–56.86 ng/g fat).

Recovery

Conventional recovery calculation of PCBs from real matrices uses the addition of known quantities of each of the compounds measured at one or more levels of concentration. In our studies the recovery determination of congeners from real milk samples was similar to that of the certification exercises performed by BCR on PCBs [12].

Preliminary analysis of triplicate unspiked cow milk sample was performed using the method examined. The chromatogram of purified milk extract did not contain

TABLE I Detection and determination limits of the method of selected PCB determination in milk (fat content = 3.5%)

PCB	*Limit of detection* (ng/g fat)		*Limit of determination* (ng/g fat)	
	LRMS-SIR	*ECD*	*LRMS-SIR*	*ECD*
28	6.86	0.26	25.43	0.86
74	9.14	0.26	31.71	0.86
101	6.57	0.26	22.57	0.86
149	9.14	0.26	30.57	0.86
118	6.57	0.20	24.29	0.57
114	6.57	0.14	21.71	0.57
153	14.29	0.26	47.43	0.86
105	8.29	0.20	29.43	0.57
138	17.14	0.23	30.57	0.86
187	13.71	0.23	48.86	0.86
128	15.71	0.17	51.71	0.57
156	17.14	0.26	56.86	0.86
180	15.43	0.20	50.29	0.57
170	15.43	0.11	50.29	0.57

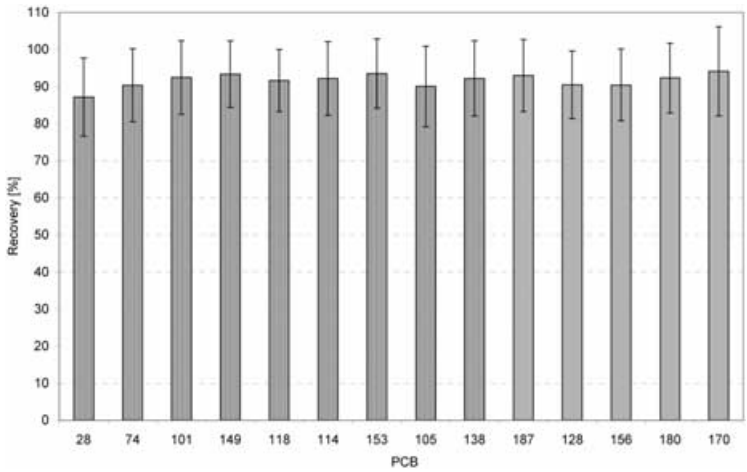

FIGURE 1 Recovery of selected PCB congeners from spiked milk cow samples (mean ± RSD, $n = 12$).

the peaks whose RRT_{209} corresponded to the congeners presented in the standard solution of PCBs. Then the cow milk samples were spiked with the PCB standards at four different levels between 27 ng/g fat and 3000 ng/g fat in triplicate for each of the congeners.

As shown in Fig. 1, the mean recovery of all PCBs from spiked milk samples has been established above 85% and varies from 87.3% for PCB 28 to 93.6% for PCB 153.

The AOAC (American Association of Official Analytical Chemists) requirement [13] concerning the level of analyte recovery at different concentrations (mean recovery of analyte on ppb level, 80–110%) for all examined congeners has been satisfied. In all cases the relative standard deviations (RSDs) are close to or less than 10%.

These recovery results were comparable to those reported by other authors for the same matrix and same analyte concentrations [14,15].

Linearity

The linear dependence between PCB concentration determined in spiked milk samples and the quantities of congeners added to the sample was examined.

The parameters of the calibration equation and the correlation coefficients for all congeners studied were calculated. As results from the data presented in Table II indicate, the linear regression is characterized by good correlation coefficients (> 0.99) for all analytes, except PCBs 105 and 156.

Accuracy and Precision

As a check of the accuracy and precision of the method, the standard reference material CRM-450 – milk powder was analysed five times. The typical HRGC-ECD chromatogram of cleaned-up extract of reference milk sample is shown in Fig. 2.

The quantitative results for the replicate analyses of milk samples are presented in Table III, together with the certified concentration for each congener.

TABLE II Calibration equations of the method of selected PCB determination in milk

PCB	*Calibration equation*	*r*
28	$Y = 0.905 \cdot x - 3.989$	0.9952
74	$Y = 0.922 \cdot x + 0.901$	0.9939
101	$Y = 0.936 \cdot x + 0.868$	0.9930
149	$Y = 0.930 \cdot x - 6.804$	0.9934
118	$Y = 0.930 \cdot x + 3.789$	0.9926
114	$Y = 0.945 \cdot x + 5.002$	0.9906
153	$Y = 0.938 \cdot x + 14.841$	0.9921
105	$Y = 0.906 \cdot x + 6.737$	0.9878
138	$Y = 0.951 \cdot x + 2.542$	0.9924
187	$Y = 0.946 \cdot x + 2.438$	0.9921
128	$Y = 0.921 \cdot x + 0.460$	0.9919
156	$Y = 0.943 \cdot x - 7.980$	0.9897
180	$Y = 0.947 \cdot x - 1.730$	0.9932
170	$Y = 0.923 \cdot x + 12.000$	0.9905

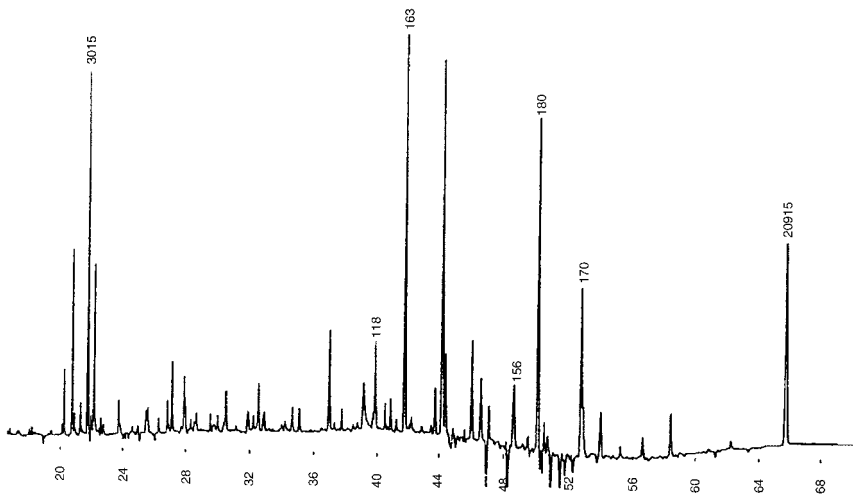

FIGURE 2 HRGC-ECD chromatogram of the extract of certified powder milk CRM-450.

TABLE III The results from five replicate analyses of a milk powder, CRM-450

PCB	*Concentration certified* $\pm \mu^*$ (μg/kg *milk*)	*Concentration determined* (μg/kg *milk*)						*Recovery* (%)
		I	**II**	**III**	**IV**	**V**	**Mean** $\pm$ **SD**	
118	3.29 ± 0.4	2.57	2.96	2.52	3.20	2.79	2.81 ± 0.28	85.3
153	18.95 ± 0.7	15.28	16.49	14.55	17.78	16.59	16.1 ± 1.25	85.2
156	1.62 ± 0.2	1.63	1.86	1.57	1.59	1.44	1.6 ± 0.15	99.9
170	4.8 ± 0.6	3.66	3.90	3.71	4.61	4.19	4.0 ± 0.39	83.6
180	11.09 ± 0.7	11.43	10.16	10.49	10.32	9.44	10.4 ± 0.72	93.5

μ^* = uncertainty expressed as μg/kg [15].

The results obtained are characterized by good accuracy and precision. Similarly to the results presented above, the recoveries of certified congeners are higher than 80% and for all analytes STD did not exceed 10%.

It is worth noting that the concentrations (not certified) of congeners PCB 28, 101, 105, 128, 138 and 149 determined in milk CRM-450 by the analytical procedure studied were comparable to those presented in the Certification Report [15]. Similarly to BCR results, the highest RSDs for triplicate analysis were obtained for PCB 105 (11.5%), PCB 28 (13.8%) and PCB 101 (11.3%).

Quality Control

The quality of the method examined was verified in an interlaboratory exercise. Analyses of seven Polish breast milk samples were performed simultaneously in our laboratory and at the Institute of Applied Environmental Research at the Stockholm University. Figure 3 presents a comparison of the concentrations of selected PCB congeners (118, 138, 153 and 180) determined with different analytical methods in both laboratories.

The results were compared on the basis of statistical criteria. Student's paired t-test was employed to check if the concentrations of the congeners examined determined at both laboratories are significantly different. The calculated t values were in each case smaller than the critical value t_{crit} read from tables for a specified degree of freedom ($f = n - 1$) and 95% confidence level. The slight differences in the concentration levels presented in Fig. 3 are probably due to random errors.

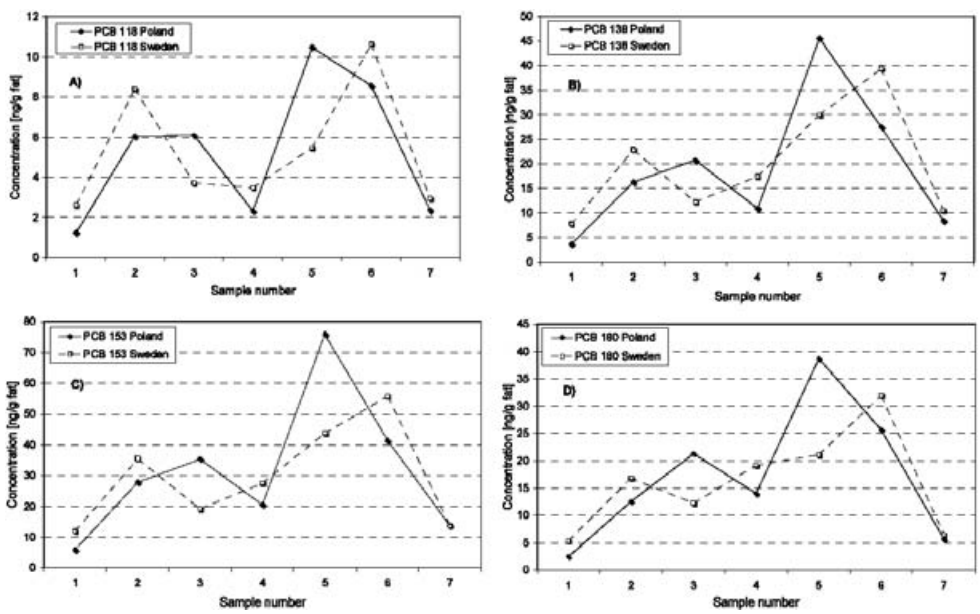

FIGURE 3 Interlaboratory comparison of the results of PCB determination in human milk samples: (A) PCB 118; (B) PCB 138; (C) PCB 153; (D) PCB 180.

CONCLUSION

As follows from the precision, accuracy, linearity, detection and quantification limits the analytical procedure studied has been proved to be suitable for determination of individual PCB congeners in human milk samples.

Taking into consideration the PCB concentrations recently detected in human milk from different regions of the world, the validated method should be useful for the accurate and reliable study of assessment of breast-fed infants' exposure to PCBs.

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