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Determination of polychlorinated biphenyl congeners in human milk by gas chromatography–negative chemical ionization mass spectrometry after sample clean-up by solid-phase extraction

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Abstract

This paper describes a simple and efficient procedure for measuring 25 congeners of polychlorinated biphenyls in human milk. The limit of quantitation was 0.1 ng/ml for five less chlorinated congeners (PCB 70, 74, 87, 99,101), and 0.01 ng/ml for the remaining 20 congeners (PCB 77, 105, 118, 126, 128, 138, 151, 153, 156, 169, 170, 180, 183, 187, 191, 194, 205, 206, 208 and 209). Solid phase extraction technology was applied to extract the analytes from the matrix and to remove lipids. Three columns were used sequentially, and they were a Bond Elut C_{18}, a Sep-Pak Plus NH2 and a Bond Elut PCB cartridge. The instrumental method was gas chromatography–mass spectrometry with negative chemical ionization, and selected ion monitoring mode was used.
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1. Introduction

Polychlorinated biphenyls (PCB) are ubiquitous environmental contaminants as a result of their resistance to chemical transformation. They are known to cause a number of adverse health effects both in humans and wildlife. Their presence in human milk is of major concern, as it is the primary source of exposure in infants. It has been shown that the body burden of a mother decreases during the lactation period, and this indicates that breastfeeding is a good way of getting rid of the contaminants. However, it is the infant that is on the receiving end of these chemicals [1]. It is an important public health issue to know the distribution of toxic chemicals, including PCB, in breast milk. Some PCB congeners such as the coplanar ones: PCB 77, PCB 126 and PCB 169, can cause toxic effects even at very low concentrations. Therefore, it is essential to be able to detect these compounds down to sub-nanogram per milliliter level.

Many analytical procedures had been described, and the instrumental technique was invariably gas chromatography (GC) mostly with electron capture detection because of its excellent sensitivity. Recently, there was a shift to GC–mass spectrometry (MS) because of enhanced selectivity [2]. The sensitivity of the technique is enhanced with the use of selected ion monitoring (SIM). We have developed a procedure that utilizes GC–MS with negative chemical ionization (NCI). This procedure takes advantage of the electron capturing properties of PCB molecules, and the selectivity of mass spectrometric analysis.

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Cochran and Frame [3] have recently reviewed this subject. In NCI-MS, a buffer gas at higher pressure is introduced in the ion source. The electron beam in the mass spectrometer collides with the buffer gas molecules, and thermal electrons with much lower energy are produced. PCB molecules, being highly chlorinated, have great ability in capturing the thermal electrons to form negative ions.

Because of the high contents of fat in milk, a major challenge in the analysis is sample clean-up. If the lipids are not removed in the sample preparation process, these non-volatile compounds will be injected into the GC–MS system, resulting in lower sensitivity, shorter column life and more frequent instrument maintenance. Traditionally PCBs were extracted from milk by solvent extraction. Some authors would freeze-dry the milk before extraction [4], others would initially separate the fat by centrifugation [5], and still others would perform extraction on liquid milk [6]. The co-extracted lipids may be digested with concentrated sulfuric acid [4]. Additional sample clean-up was in general necessary, especially when there was no acid digestion step. Sample clean-up was mostly by column chromatography, using alumina [4], Florisil [5,6], and silica gel [7]. Clean-up by high-performance liquid chromatography was also used [8]. One drawback of solvent extraction is the large amount of solvent that has to be used. Column chromatographic clean-up is cumbersome and labor intensive. Solid phase extraction (SPE) is a newer technique used in the isolation of chemical compounds. A procedure was described where PCBs were extracted from milk using an C_{18} SPE column, and the extract was analyzed by GC without further purification [9]. Good sensitivity and recovery were reported. Our experience was that lipids were not removed by this one SPE step. We will describe a procedure whereby SPE technology is used for both extraction and sample clean-up. This procedure requires only 2 ml of milk sample, and can detect down to 0.01 ng/ml of most of the 25 congeners targeted in this procedure.

2. Experimental

2.1. Materials

The GC–MS system was a Hewlett-Packard (Palo Alto, CA) 6890 gas chromatograph linked to a HP 5973 mass spectral detector (MSD). We used a DB-5MS, 5% phenylmethylpolysiloxane GC column (J & W Scientific, Folsom, CA), 30 m×0.25 mm I.D.; film thickness was 0.25 μm. The carrier gas was helium (Air Liquide, Montreal, Quebec). The buffer gas for chemical ionization was methane (Matheson, Edmonton, Alta).

A RapidTrace SPE Workstation (Zymark, Hopkinton, MA), and a Visiprep SPE Vacuum Manifold (Supelco, Oakville, ON) were used for solid-phase extraction. Other minor equipment used were a B-220 Sonicator (Branson, Shelton, CT) and Reacti-Therm Heating Modules (Pierce, Rockford, IL).

PCB standards were purchased from Cambridge Isotope Laboratories (Andover, MA) and Radian (Austin, TX). Solvents and chemicals were of the best available grade from various commercial sources.

Bond Elut C_{18} (6 cc/500 mg) and Bond Elut PCB SPE (3 cc/1 g) cartridges were from Varian (Harbor City, CA) and Sep-Pak Plus NH2 SPE (360 mg) cartridges were from Waters (Milford, MA).

2.2. Validation samples

A total of 25 PCB congeners were analyzed in this procedure. Some of them had lower sensitivities and were segregated in group I: PCB 70, 74, 87, 99 and 101. The congeners in group II were PCB 77, 105, 118, 126, 128, 138, 151, 153, 156, 169, 170, 180, 183, 187, 191, 194, 205, 206, 208 and 209. Homogenized cow milk (3.25% fat) was fortified with the appropriate amounts of PCB congeners in methanolic solutions in the preparation of validation samples.

2.2.1. Linearity studies

A milk sample was fortified with 10 ng/ml of group 1 PCBs and 5 ng/ml of group 2 PCBs. This sample was serially diluted with blank milk down to 0.01 and 0.005 ng/ml, respectively, of the two groups of PCBs.

2.2.2. Precision studies

A milk sample was fortified with 1.0 ng/ml of group 1 PCBs and 0.1 ng/ml of group 2 PCBs, and this experiment was performed with six replicates.

2.2.3. Recovery studies

Absolute recovery of PCBs in this procedure was investigated with a milk sample fortified to contain 1.0 ng/ml of group 1 PCBs and 0.1 ng/ml of group 2 PCBs. This was done in triplicates.

2.3. Calibration standards

In quantitative analysis, the PCB concentration in a human milk sample is calculated against a calibration curve constructed via the analysis of five calibration standards. The calibration standards are prepared by fortifying homogenized cow milk with the PCB congener to the following concentrations: for group 1, they are 0, 0.1, 0.5, 1.0 and 2.0 ng/ml, and for group 2, they are 0, 0.01, 0.05, 0.1 and 1.0 ng/ml. They are extracted along with the human milk samples. The response ratio of the PCB congener to the internal standard is plotted against the congener concentration to construct the calibration curve. Three internal standards are used: PCB 114, PCB 189 and PCB 202. These are selected as they are not normally present in human milk.

2.4. Extraction procedure

Prior to aliquoting, the stored milk (−20°C) was brought to room temperature and homogenized by vortexing vigorously for 5 min. To 2 ml of milk sample 10 μl of the internal working standard solution (500 ng/ml each of three congeners in methanol) were added. A 2-ml sample of glacial acetic acid was added to the milk, then 2 ml of methanol. This mixture was vortexed briefly and then sonicated for 30 min. It was then applied at ~1 ml/min flow-rate to a Bond Elut C_{18} cartridge that had been pre-washed with hexane and conditioned with 5 ml each of methanol and deionized water in a Visiprep SPE Vacuum Manifold. The cartridge was then washed with 5 ml of water, and maximum vacuum was applied to dry the cartridge. It was then coupled to a Sep-Pak Plus NH2 cartridge that had been pre-washed with hexane. To elute the PCBs, 6 ml of hexane were applied at a flow-rate of ~0.5 ml/min to the coupled columns. The hexane eluent was then applied at a flow-rate of ~0.5 ml/min to a Bond Elut PCB cartridge, which was pre-washed with hexane in a RapidTrace SPE Workstation. The PCB cartridge was further rinsed with another 3 ml of hexane. The hexane fractions were pooled, and concentrated to ~3 ml under a stream of nitrogen at 40°C. Then 500 μl of isooctane were added. This mixture was further concentrated down to ~100 μl, and 2 μl were injected into the GC–MSD.

2.5. Chromatography and mass spectrometry

The GC was run in the splitless injection mode with a purge time of 1.0 min. The injection port temperature was set at 250°C. The temperature program of the GC oven is listed in Table 1. The linear velocity of the helium carrier gas was 46 cm/s at 90°C.

The temperature of the transfer line to the MSD was 280°C. The MSD was operated under negative chemical ionization mode using methane as the buffer gas. The flow of methane was set at 2.0 ml/min as recommended by the manufacturer, re-

Table 1
Temperature program of gas chromatographic oven

	Temperature (°C)	Rate (°C/min)	Time (min)	Hold time (min)
Initial	90			2
Program (1)		25		
Intermediate (1)	150		2.4	0
Program (2)		3		
Intermediate (2)	200		16.7	0
Program (3)		8		
Intermediate (3)	280		10.0	0
Program (4)		20		
Final	300		1.0	9

Total time = 41.1 min

sulting in a pressure of 2×10^{-4} Torr in the ion source. The ion source was maintained at a temperature of 150°C and the mass analyzer was at 106°C.

Selected ion monitoring was used in the quantitative analysis of PCB congeners in milk. The base ion of each PCB congener was selected as the quantifying ion to be used for the calculation of concentrations. Two other prominent ions were chosen as qualifying ions. The ion abundance ratio of each qualifying ion against the quantifying ion should fall within 20% of the same ratio in calibration standards for positive identification. Because of the nature of chemical ionization, these ions usually belong to the molecular cluster. The ions used in the monitoring of the various PCBs are listed in Table 2.

For comparison of sensitivity against electron ionization mass spectrometry, the MSD was operated in the full scan mode. Six PCB congeners, PCB 77, 126, 138, 153, 169 and 180 were analyzed with 80 pg of the neat standard injected into the GC–MSD system. The root mean square signal-to-noise ratios of the quantifying ions were calculated. The same analysis was performed in a GC–MSD using electron ionization in the full scan mode under identical GC conditions.

3. Results and discussion

Negative chemical ionization is a technique that is very useful for the analysis of PCBs. The essence of this technique is ionization through the capturing of a low energy electron by the analyte molecule. Compounds that contain electronegative atoms, such as the highly chlorinated PCBs, are very sensitive towards this mode of detection. While the positions of the chlorine atoms could make some difference, our results demonstrated that sensitivity in general

Table 2
Ions monitored in the analysis of PCB congeners

PCB congener	Retention time (min)	Quantifying ion (m/z)	Qualifying ion #1 (m/z)	Qualifying ion #2 (m/z)
74	20.92	292	290	294
70	21.20	292	290	294
101	22.52	326	324	328
99	22.71	326	324	328
87	23.65	326	324	328
77	24.11	292	290	294
151	24.47	360	362	358
118	25.09	326	324	328
114 (I.S.)	25.42	326	324	328
153	25.87	360	358	362
105	25.96	326	324	328
138	26.74	360	362	358
126	27.08	326	324	328
187	27.22	394	396	392
183	27.39	394	396	392
128	27.55	360	362	358
202 (I.S.)	28.15	430	428	432
156	28.31	360	358	362
180	28.79	394	396	360
191	28.91	394	396	360
169	29.39	360	358	362
170	29.54	394	396	392
189 (I.S.)	30.34	394	392	396
208	30.61	464	462	430
194	31.20	430	428	394
205	31.29	430	428	432
206	31.97	464	462	430
209	32.58	498	464	462

increased with chlorination of the analyte. Fig. 1 shows the extracted ion chromatograms of six PCB congeners obtained in full scan analysis. The top panel represents the results of NCI analysis. The base ions of the PCBs were used to monitor the compounds: *m/z* 292, 360, 360, 326, 394 and 360, respectively, for PCB 77, 153, 138, 126, 180 and 169. These are the ions that belong to the molecular clusters of the different congeners. The lower panel represents the same in an EI analysis. It is evident that the responses were much stronger in NCI analysis. The more telling parameter was the signal-to-noise (S/N) ratio as tabulated in Table 3. There were significant increases in S/N ratio from EI analysis to NCI analysis. For PCB 77, a tetra-chlorinated compound, the increase in S/N ratio was seven times, while that for PCB 180, a hepta-chlorinated compound, was 110 times.

Human milk is a complex liquid and may contain between 3 and 5% of lipids [10]. The lipids are almost exclusively triglycerides (TG, 98%). There are small amounts of phospholipids (0.8%) and cholesterol (0.5%). The major fatty acids in TG are myristic acid (9.4%), palmitic acid (27.0%) and oleic acid (34.2%). Cow milk is similar to human milk in that TG also constitutes 98% of the lipids, with the same major fatty acids: myristic acid (11.2%), palmitic acid (23.9%) and oleic acid (24.0%) [11]. The major difference is that cow milk has some shorter chain fatty acids, for example butyric acid (11.8%). This justified the use of cow milk, other than for practical reasons, as the matrix for method development and preparation of calibration standards. A major challenge in the gas chromatographic analysis of PCBs is the removal of lipids in the final extract. Lipids cause poor chromatography and consequently higher detection limits. Furthermore, lipids have deleterious effects on the performance of the chromatographic column and reduce column life drastically. They will also deposit on the ion source of the mass spectrometer, resulting in the necessity of source cleaning. Therefore for efficiency and economics, it is imperative to have a thorough sample clean-up prior to instrumental analysis. Furthermore, the milk may contain 5% of lipids, or 50 mg per ml, and this well illustrates the analytical problem at hand when we need to measure down to a fraction of ng per ml of a PCB congener. While most of the milk lipids are TGs, they may be hydrolyzed enzymatically into free fatty acids on storage [10]. Therefore the clean-up strategies should include the removal of free fatty acids, TGs, diglycerides, monoglycerides and glycerol. We employed a multi-step solid-phase extraction (SPE) procedure. Prior to extraction, the milk protein was precipitated with acetic acid, and the fat globules were disturbed by the addition of methanol. A Bond Elut C_{18} column was then used to isolate the PCB compounds from the milk matrix. Hexane was then used to elute the analytes. The eluate was then passed through a Sep-Pak NH2 column, which will remove the free fatty acids. Further clean-up was effected by using a Bond Elut PCB column. This column is a multiphase SPE column with a strong cation exchange bed and a silica bed. This was designed to extract PCBs from oil or organic matrices. After this three-step SPE process, we obtained a clean extract, with no detectable lipids, ready for injection into the GC–MS system. No additional maintenance of the GC–MS system was required after analyzing over 200 human milk samples, and this also demonstrated that the extract was clean.

The precision of this procedure is listed in Table 4. The concentrations of the group 1 PCB congeners in the milk sample were 1.0 ng/ml and those of group 2 PCB congeners were 0.1 ng/ml. The mean coefficients of variation ($n=6$) ranged from 2 to 7%.

The absolute recovery of PCB congeners in this procedure is listed in Table 5. The validation sample was cow milk fortified with PCB congeners to concentrations of 1.0 and 0.1 ng/ml, respectively, for group 1 and group 2 analytes. In the determination of absolute recovery, the validation sample was extracted according to the described procedure without internal standards, which were added to the final extract. The results were compared to an analysis of unextracted PCB standards at the same concentrations. Most recovery rates were ~100%.

For group 1 PCBs, linearity ranged from 10 down to 0.1 ng/ml, while that for group 2 PCBs was from 5 down to 0.01 ng/ml. The lower concentrations were the limits of quantitation.

Fig. 2 shows the ion chromatograms of nine PCB congeners in a human milk sample with a fat content of 3.9%. Their concentrations in ng/ml were 0.36 (PCB 74), 0.17 (PCB 87), 0.20 (PCB 99), 0.14

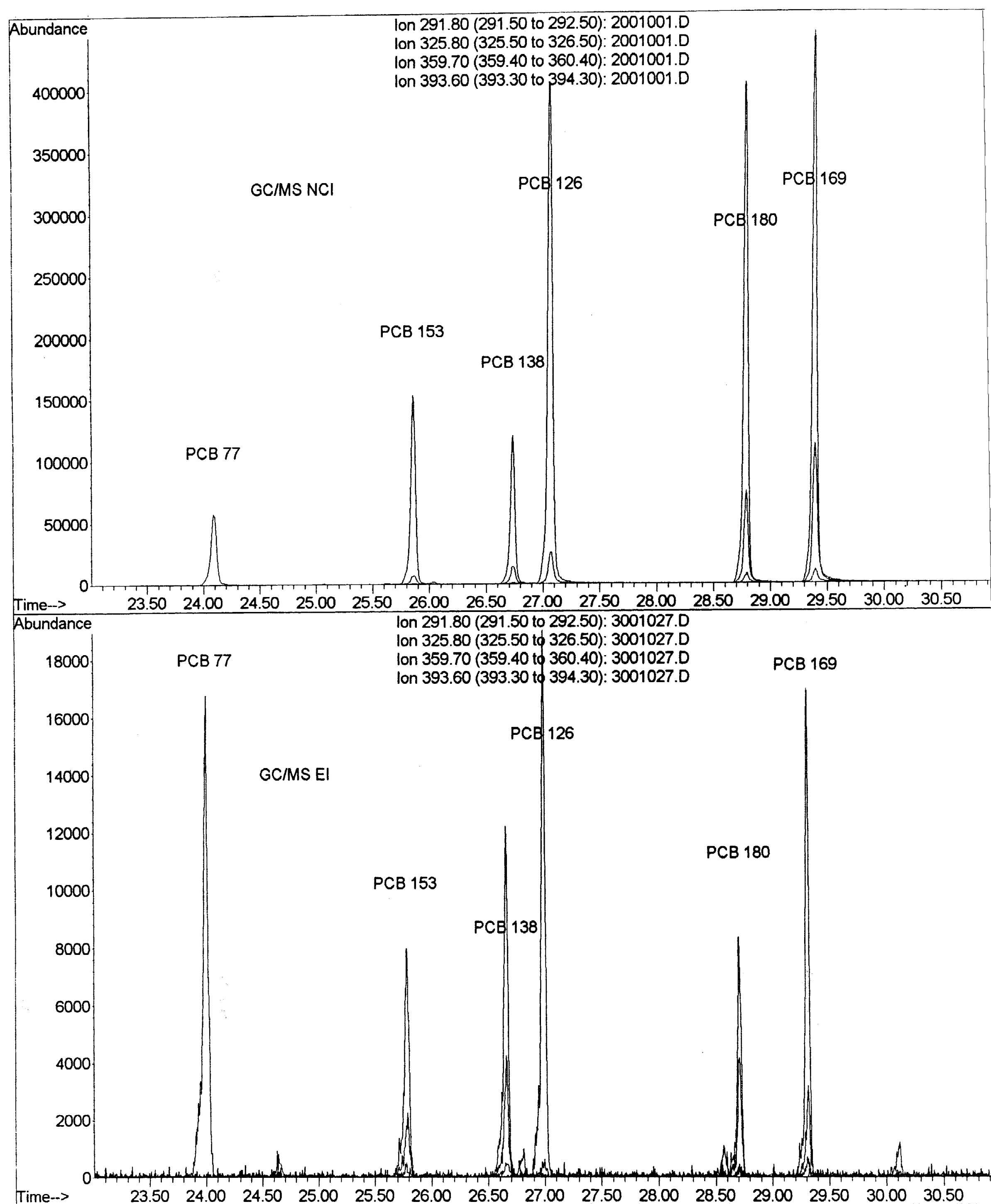

Fig. 1. Comparison of extracted ion chromatograms of six PCB congeners (80 pg each on column) in the negative chemical ionization (top panel) and electron ionization (bottom panel). The ions monitored were m/z 292 for PCB 77, m/z 360 for PCB 153, 138 and 169, m/z 326 for PCB 126 and m/z 394 for PCB 180.

Table 3
Comparison of root mean square signal-to-noise (S/N) ratios between negative chemical ionization and electron ionization

PCB congener	Chlorination	S/N (NCI)	S/N (EI)	Factor
77	4	1947	263	7.4
126	5	17 789	196	90.7
138	6	4468	164	27.2
153	6	5684	107	53.1
169	6	16 478	224	73.6
180	7	17 733	161	108.1

Table 4
Precision of PCB concentration measurements

PCB congener	Coefficient of variation	PCB congener	Coefficient of variation
70	3.32	156	4.87
74	2.26	169	4.60
77	5.85	170	6.17
87	4.03	177	4.01
99	2.45	180	5.11
101	2.09	183	5.78
105	2.97	187	5.16
118	2.19	191	5.85
126	3.00	194	3.89
128	5.62	205	5.09
138	5.69	206	3.84
151	6.58	208	3.19
153	4.76	209	2.95

(PCB 118), 0.18 (PCB 138), 0.31 (PCB 153), 0.06 (PCB 170), 0.12 (PCB 180) and 0.02 (PCB 194). The first eight congeners had the highest concentrations in this sample, and PCB 194 was included to demonstrate the sensitivity of this procedure. Our results also showed significant drop-off of sensitivity in the group 1 congeners. This is the characteristic of negative chemical ionization that sensitivity increases with chlorination. The ion chromatograms were clean and did not show any interference that may cause uncertainty in identification by retention time. Furthermore, increased confidence was achieved in the identity of the PCB congener by monitoring three ions of one analyte, and requiring the qualifying ion ratios be within 20% of the same in the calibration standards. On the other hand, this technique offers less selectivity as there is limited fragmentation in chemical ionization. Consequently gas chromatographic separation is of paramount importance. Cochran and Frame [3] pointed out that no one GC column is capable of separating all PCB congeners that exist in Aroclors, or those that are regulated and/or those that have some toxic effect on humans or other biota. Of greater concern is that there is no contribution to the toxic coplanar PCBs: 77, 126 and 169 from other congeners. There is a potential interference of PCB 77, a tetrachlorobiphenyl, by PCB 110, a pentachlorobiphenyl, with the GC column (DB-5 MS) we used [3]. The ions used to monitor PCB 77 are those from the molecular cluster and are minor fragmentation ions of PCB 110. Our results showed that the contribution of PCB 110 to PCB 77 in the analysis was ~1%. In other words, it will take a significant concentration of PCB 110 to cause a false positive of PCB 77.

Table 5
Mean absolute recovery of PCB congeners

PCB congener	Recovery	PCB congener	Recovery
70	117	156	104
74	109	169	100
77	109	170	106
87	120	177	104
99	114	180	104
101	115	183	103
105	106	187	104
118	111	191	104
126	106	194	103
128	124	205	102
138	129	206	101
151	128	208	97
153	112	209	101

In our protocol, we would perform duplicate analysis on selected samples in each batch of samples. Table 6 shows the reproducibility of this procedure.

We have demonstrated that we have a very sensitive procedure for PCB analysis in human milk, at least for the higher chlorinated congeners. Productivity is improved through the use of SPE technology. Multiple samples can be processed simultaneously in the Bond Elut C_{18} extraction and Sep-Pak NH2 clean-up using the Visiprep SPE Vacuum Manifold. The Bond Elut PCB clean-up is sequential, but is automated with the RapidTrace SPE Workstation, so that unattended operation can be carried out. Use of solvents is also minimal: 9 ml of hexane for elution per milk sample.

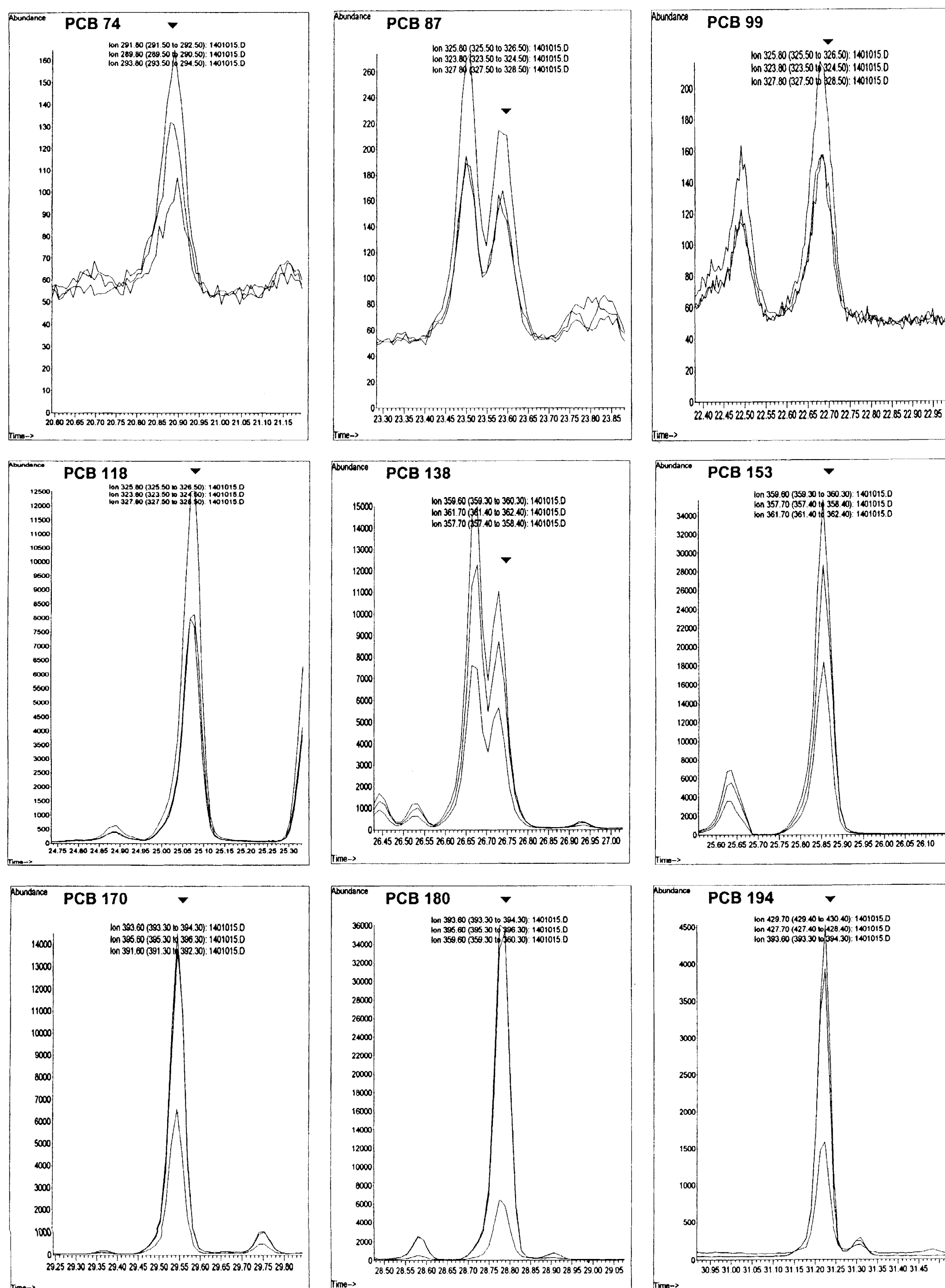

Fig. 2. SIM ion chromatograms of nine PCB congeners obtained from a human milk sample. Concentrations of these congeners are described in the text. ▼ Identifies the chromatographic peak of the PCB congener.

Table 6
Reproducibility of PCB congeners quantitation in duplicate analysis

PCB congener	Concentration (ng/ml)	
	Duplicate sample #1	Duplicate sample #2
70	0.00	0.00
74	0.27	0.25
77	0.00	0.00
87	0.00	0.00
99	0.10	0.10
101	0.00	0.00
105	0.01	0.01
118	0.07	0.07
126	0.00	0.00
128	0.00	0.00
138	0.12	0.11
151	0.00	0.00
153	0.25	0.25
156	0.03	0.03
169	0.00	0.00
170	0.06	0.06
180	0.13	0.13
183	0.02	0.02
187	0.03	0.03
191	0.00	0.00
194	0.03	0.02
205	0.00	0.00
206	0.00	0.00
208	0.00	0.00
209	0.00	0.00

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