Letter to the Editor

Optimization of Gas Chromatography-electron Ionization-tandem Mass Spectrometry for Determining Toxic Non-*ortho* Polychlorinated Biphenyls in Breast Milk*

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One of the most commonly used non-invasive methods for assessing human exposure to pollution is the analysis of human milk. Human milk analyses help estimate the exposure of infants[1]. This is why breast milk is receives scientific interest, and various methods for determining different pollutants from the environment are being developed[2,3]; much attention is given to polychlorinated biphenyl (PCB) analysis[4]. The majority of published studies used gas chromatography-high resolution magnetic sector mass spectrometry instruments[5], but high costs and complexity of data processing limits its usage in routine analyses. Recent advances in gas chromatography-triple quadrupole mass spectrometry technology have allowed for high sensitivity and selectivity.

The aim of this study is to optimize conditions for PCB determination in the lowest concentration range and achieve the best sensitivity possible using gas chromatography–electron ionization–tandem mass spectrometry (GC-EI/MS/MS) in the programmable temperature vaporizer-large volume injection mode (PTV-LVI) to quantify non-*ortho* PCBs (PCB-77, PCB-81, PCB-126, and PCB-169) in breast milk samples.

Breast milk samples were collected from 46 mothers living in Zadar (Croatia) according to the Ethical Permissions of the Zadar County Health Centre Ethics Committee (01-745/2011 and 01-405/2014). The mothers were between 19 and 41 years of age; 23 were primiparae (first child delivery) and 23 secundiparae (second child delivery).

Details published previously[6].

The analyses were performed by GC-EI/MS/MS using an Agilent 7890 B gas chromatograph equipped with a PAL RTC 120 autosampler and coupled to an Agilent 7000 C tandem mass spectrometer operating in EI mode.

The GC separation was performed using a fused silica HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). The oven temperature was programmed as follows: 60 °C (2.54 min), 45 °C/min to 200 °C, and 8 °C/min to 285 °C (2 min). Helium was used as a carrier gas at a flow rate of 1.2 mL/min. The mass selective detection (MSD) transfer line and ion source were set to 340 °C. An Agilent Multimode Inlet (MMI) was operated as a programmable temperature vaporizer in solvent vent mode under helium flow at 60 mL/min. The injection volume was 3 µL. The MMI temperature program was as follows: 60 °C held for 0.04 min (at which point the vent was closed), ramped to 400 °C at 600 °C/min, and held at 400 °C for the rest of the GC run. This allowed for fast and efficient transfer of the analytes to the GC column (Table 1).

Descriptive statistics and data processing were conducted using STATISTICA 8.0 software (StatSoft, Inc., Tulsa, OK, USA). The Mann-Whitney *U* test was applied to determine significant differences ($P < 0.05$) between primiparae and secundiparae. Kruskal-Wallis ANOVA was used to compare levels between different age groups of mothers (19–24, 25–30, > 31).

The identification and quantitation of non-*ortho*

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PCBs is complicated by the serious interference caused by other congeners. For isomeric compounds or compounds with similar structures (e.g., PCB-81 and PCB-77), chromatographic separation is important because they have the same precursor and product ions. The GC oven program was optimized with the objective of attaining chromatographic separation by focusing on co-eluting pairs, such as PCB-81 and PCB-77. However, the sensitivity was limited, and injection of 1 µL standard solution *via* splitless injection was not enough to achieve lower quantification limits up to the expected values of PCBs in the breast milk samples. The samples were introduced through a PTV-LVI in the solvent vent mode. Larger injection volumes were preferable to increase the overall sensitivity of the method.

The increase in sample injection volume to 3 µL resulted in a higher sensitivity and sharper peaks for less sensitive compounds like PCB-126 and PCB-169. The PTV injection helped to reduce peak widths (at the base) by three times for PCB-126 and up to four times for PCB-169.

To design the MS/MS quantification method, individual injections of each target compound in full-scan mode (scan range, *m/z* 50–500) were completed to obtain their retention times and select the optimal precursor ions. After obtaining the full scan spectra, the two most intense precursor ions with the highest *m/z* for each analyte were selected. One of the selected precursor ions was also the molecular peak (*m/z* 290 for PCB-77 and PCB-81, *m/z* 324 for PCB-126), except for PCB-169 whose molecular peak was third in intensity. A product ion scan was performed using different collision energies (CE), between 10 eV and 40 eV, to determine the most selective product ions. The common characteristic for all PCBs was that the two most intense transitions corresponded to a loss of Cl_2 (M-70).

For those analytes that had the same multiple reaction monitoring (MRM) ion transitions (same homologue group chemicals as with PCB congeners −77 and −81), achieving sufficient chromatographic separation was essential for their reliable identification and quantification. Figure 1 provides the total ion current (TIC) chromatogram of the PCBs obtained for the mid-level calibration standard and extracted ion current (EIC) chromatograms of the PCBs in the real sample using the developed procedure.

To obtain low detection limits and well-shaped chromatographic peaks, the dwell time parameter was also optimized to provide at least 10 points/peak. This parameter was modified to between 10 ms and 500 ms (Table 1).

The linearity of the detector response was studied by injecting reference standard solutions in triplicate in the range of 0.001–0.100 ng/mL. The values of the regression coefficient were higher than 0.99 for all compounds over the whole test range with residuals lower than 20%. The estimated detection limits were in the low pg/mL range, from 0.35 pg/mL to 1.14 pg/mL across the target compounds, while quantification limits varied from 1.15 pg/mL to 3.80 pg/mL. The calibration drift check had an average repeatability of 7.2%.

The following conditions are required to confirm the detection of non-*ortho* PCBs in breast milk samples using the method optimized in this study:

Table 1. MS/MS settings

Time segment	Segment start time (min)	RT (min)	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Q/q	Dwell time (ms)	Collision energy (eV)	*Q/q* ratio*
1	5.00	10.971	PCB-81	291.9	222.0	q	150	28	30.83 (16%)
				289.9	220.1	Q	150	28	
		11.142	PCB-77	292.0	222.1	q	150	28	30.05 (13%)
				289.9	220.1	Q	150	28	
2	11.50	12.692	PCB-126	325.9	255.8	q	300	28	91.26 (12%)
				324.0	253.9	Q	300	28	
3	13.00	14.239	PCB-169	359.9	289.9	Q	300	28	103.17 (8%)
				357.8	287.8	q	300	28	

Note. * Average value calculated from 15 injections of standard solutions (five concentrations levels with three replicates each), and the risk specific dose (RSD) is in parenthesis. PCB, polychlorinated biphenyl.

retention times for the selected MRM transitions may deviate by ± 0.1 s with $S/N > 3$, and the Q/q ratio may vary by 15% of the theoretical value. The experimental Q/q ratio for each PCB that could be detected [> limit of detection (LOD)] in breast milk samples was compared with the theoretical value of the Q/q ratio of the standard solutions to confirm the identity of each PCB in the sample. For all PCBs, the RSD value was lower than 15%, except for PCB-81 (16%), which is likely due to its low sensitivity. These results showed that the Q/q ratios for the analyzed compounds meet the requirements for the positive identification of non-*ortho* PCBs in breast milk samples. Slight variations in the Q/q ratio values and retention times were found in the samples analyzed in this study for all PCBs.

After observing the chromatograms for real samples and comparing them with the chromatogram of the calibration standard, a shift in retention time was observed: 0.005 min for PCB 81, 0.028 min for PCB 77, 0.021 min for PCB 126, and 0.027 min for PCB 169. Shifts in retention time were less than 3%; therefore, the confirmation was considered to be sufficiently accurate. The compounds that did not meet the confirmation criteria were considered 'non-detected', i.e., below the LOD. The method recovery and reproducibility ranged from 66% to 89%, and the RSD ranged from 4% to 7%.

Maternal age and parity are the most frequently appraised since their increase has been reported to have the opposite effect on persistent organic pollutants (POPs) in a mother's body. The concentrations of dioxin-like PCBs including PCB-77, PCB-126, and PCB-169 have been expected to increase in the body fat of elder mothers, which is probably because younger mothers exhibit a shorter lifetime exposure to the pollutants[7]. Some authors have reported the absence of significant associations between maternal age and PCB levels in breast milk, mainly due to the limited number of observations[8]. Conversely, child delivery is considered to reduce the PCB body burden since a long breast-feeding period decreases congener levels by 5%–25%[9], making lactation the main path for pollutant excretion from the body. As reported in a comprehensive literature review[5], the breast milk of multiparous mothers is assumed to contain significantly lower levels of contaminants compared to that of primiparous mothers. In this study, no significant differences in the levels of targeted non-*ortho* PCBs were observed either between the mother age groups or mothers with first or second child delivery (Figure 2). In conclusion, this finding is a result of limited data size (n = 46) that can be further re-verified using extensive datasets and modeling methodologies to reveal the statistically interrelated and possibly non-linear dynamics of PCBs in breast milk[10].

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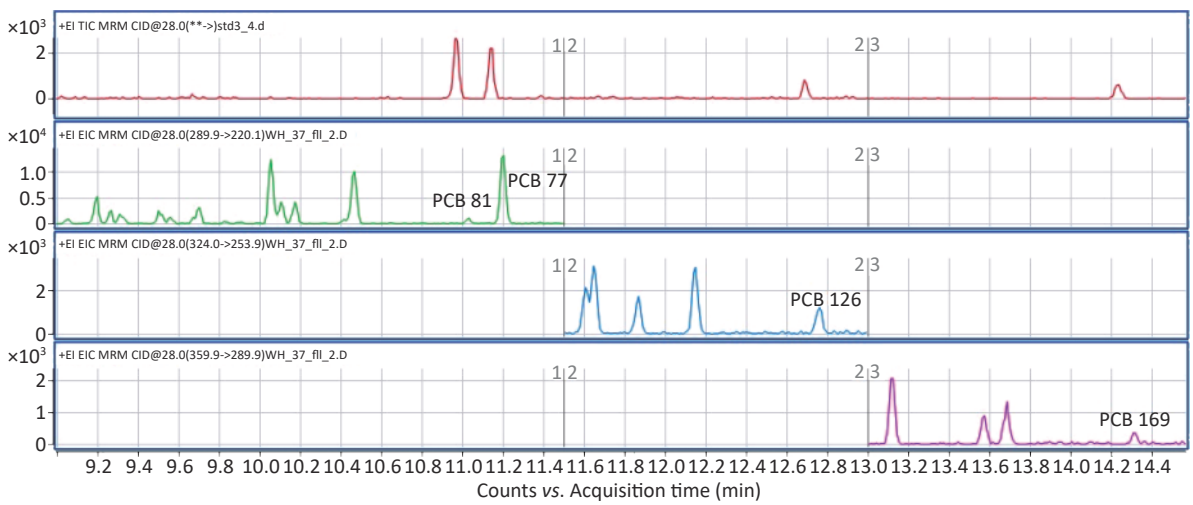

Figure 1. Total ion current (TIC) chromatogram of the PCBs obtained for mid-level calibration standard, and extracted ion current (EIC) chromatograms of the PCBs in the sample. PCB, polychlorinated biphenyl.

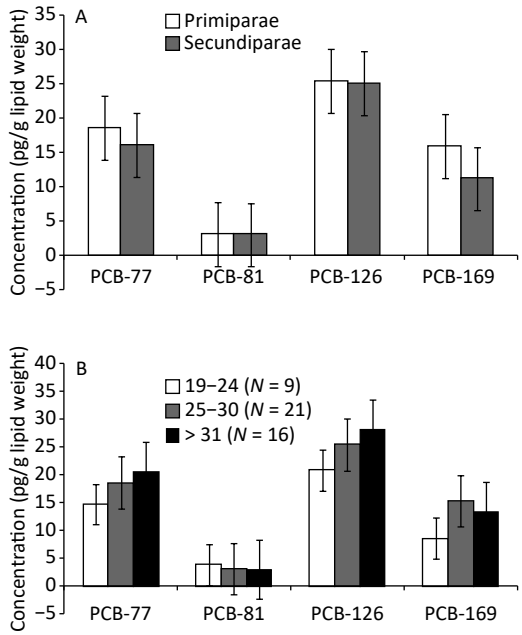

Figure 2. Median concentrations of non-*ortho* PCBs in breast milk samples collected from the Zadar area in 2014 and classified according to (A) parity and (B) mother age groups; as shown by the Mann-Whitney *U* test and Kruskal-Wallis ANOVA, no significant differences were observed between groups.

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