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Stir bar sorptive extraction–thermal desorption–capillary gas chromatography–mass spectrometry applied to the analysis of polychlorinated biphenyls in human sperm

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Abstract

Stir bar sorptive extraction (SBSE) on polydimethylsiloxane (PDMS) was applied to the enrichment of polychlorinated biphenyls (PCBs) from human sperm. The seven Ballschmiter PCBs were used as model compounds. The extracted PCBs were then thermally desorbed from the stir bar and analysed on-line by capillary gas chromatography (CGC) with mass spectrometric detection (MS). Method development started with the analysis of PCBs spiked in water. Methanol had to be added to the samples in order to reduce the influence of glass adsorption on recovery and reproducibility. Recoveries in water for all PCBs varied around 50–60% and were limited for low molecular mass (MM) PCBs by polarity changes in the sample due to methanol addition and for high MM PCBs by non-equilibrium conditions. Matrix suppression by the lipophilic medium lowered the recoveries in the sperm samples proportional with PCB polarity. The method was validated and although limits of detection (LOD) for the individual congeners were in the sub-ppt level (<pg/ml), the limit of quantification (LOQ) was set at 10 ppt (10 pg/ml).

Keywords: Stir bar sorptive extraction; Polychlorinated biphenyls

1. Introduction

In the last decade, scientific concern and public awareness concerning potential effects of environmental exposure to chemical substances that interact with the endocrine system were growing steadily. The majority of endocrine disruptors, also defined as xeno-estrogens, have been linked to hormone-related cancers and developmental abnormalities in both humans and animals [1]. Lately, considerable attention has been focused on reproductive problems, especially on the observed decrease in human sperm quality, which seems to be linked with exposure to these chemicals [2,3]. In this respect the ubiquitous PCBs, a class of synthetic organic chemicals are of concern [4]. The unawareness of their toxicity from the 1950s to the 1970s and their high degree of stability resulted in their omnipresence in our environment and food chain. The latter is the prime source for human exposure and, due to their lipo-

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philic character, of accumulation in the body. Several studies have shown trace levels of PCBs in e.g. the human brain [5], blood [6], adipose tissue [7] and breast milk [8,9]. Since PCBs have been associated with a decline in semen quality [10], our interest was focused on the development of a method for ultra-trace analysis of PCBs in human sperm.

Analysis of PCBs is routinely performed by liquid–liquid extraction [11], solid-phase extraction [12], microwave extraction [13], solid-phase microextraction [14] or supercritical fluid extraction [15] followed by either capillary gas chromatography (CGC) [16] or liquid chromatography (HPLC) [17]. Due to its superior separation efficiency CGC is the method of choice. Moreover, specific detection systems, such as electron capture detection (ECD) and mass spectrometry (MS) significantly decrease detection limits [18]. The susceptibility, however, of the ECD response towards co-eluting interferences renders mass spectrometry in the selected ion monitoring (SIM) mode the preferred detection technique.

Recently, a novel approach for sample enrichment in aqueous solutions namely stir bar sorptive extraction (SBSE), was developed [19]. In SBSE a glass-lined magnetic bar is covered with a thick layer of polydimethylsiloxane (PDMS). By magnetically stirring the bar in the sample solution the components are enriched in the PDMS phase. After this concentration step, analytes are thermally desorbed (TD) from the stir bar on-line with CGC–MS. Major advantages of this technique are ease-of-use, improved sensitivity, high accuracy of analysis and reduced risks of contamination compared with other sample preparation techniques.

The aim of this study was to evaluate the possibilities of SBSE–TD–CGC–MS for determination of PCBs in human sperm at the ppt level. The recovery of PCBs from water and a sperm sample spiked with the seven ''Ballschmiter PCBs'' [20] is compared.

2. Experimental

2.1. Chemicals

A PCB standard mixture (10 mg/l in isooctane) of the seven Ballschmiter congeners was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Compounds are depicted in Table 1. Octachloronaphthalene (10 mg/l in dichloromethane) was used as internal standard (IS) and was kindly supplied by the Research Institute for Chromatography (RIC, Kortrijk, Belgium). Methanol (HPLC grade) was purchased from Romil (Merelbeke, Belgium). Milli-Q water was prepared by purification and deionization of tap water in a Milli-Q Plus water system (Millipore, Bedfore, MA, USA).

2.2. Sample preparation: stir bar sorptive extraction

Samples of human sperm, approximately 3 ml each, were collected from fifteen randomly selected donors at the Ghent University Hospital, and pooled to form a general sperm mix for method development of xeno-estrogen determination. During this study it was proven that the PCB concentration was far below 10 ppt which is the LOQ of the presented method. Due to their lipophilic character, PCBs were expected to accumulate inside the membrane of the spermatozoa. In order to break the membrane and

Table 1
The seven ''Ballschmiter'' PCBs and the internal standard with their respective $K_{o/w}$ values and ions used in selected ion monitoring

PCB	Structure	$K_{o/w}$	SIM ions
28	2,4,4′-trichlorobiphenyl	4.0×10^{5}	256/258
52	2,2′,5,5′-tetrachlorobiphenyl	3.0×10^{6}	290/292
101	2,2′,4,5,5′-pentachlorobiphenyl	2.5×10^{6}	326/328
118	2,3′,4,4′,5-pentachlorobiphenyl	2.0×10^{6}	326/328
138	2,2′,3,4,4′,5′-hexachlorobiphenyl	5.0×10^{6}	360/362
153	2,2′,4,4′,5,5′-hexachlorobiphenyl	8.0×10^{6}	360/362
180	2,2′,3,4,4′,5,5′-heptachlorobiphenyl	1.6×10^{7}	394/396
I.S.	Octachloronaphtalene	2.3×10^{7}	404/406

release the target components, the sperm mix was ultrasonically treated for 3 min at 23 kHz.

Analyses were carried out on diluted sperm mixtures (1 ml of sperm in 9 ml of water/methanol 1:1 by volume). Dilutions were made directly in 20 ml headspace vials, which were capped during stirring. The stir bar (Twister™) was purchased from Gerstel (Mülheim a/d Ruhr, Germany) and was coated with a PDMS layer of 50 mg. The stir bar was conditioned overnight at 325°C under a helium stream. Enrichment was carried out by stirring the twister for 45 min at 1000 rpm in the diluted sperm mixture. When completed, the stir bar was removed from the solution by a forceps, washed with water and dried with tissue paper. Subsequently, the stir bar was placed inside an empty conditioned glass tube (187 mm L×4 mm I.D.) for desorption. Stir bars were re-used without additional cleaning and conditioning.

2.3. Thermal desorption–capillary gas chromatography–mass spectrometry (TD–CGC–MS)

The stir bar was thermally desorbed using a Gerstel TDS-2 thermodesorption system. This system basically consists of two programmable temperature vaporization (PTV) injectors placed in series. The first injector is the actual thermodesorption (TD) unit in which the glass desorption tube is placed. The second PTV is a Gerstel CIS-4 injector for cryofocusing and fast injection of desorbed analytes onto the capillary column. Details of the system were given elsewhere [21].

In order to achieve complete desorption of the PCBs from the stir bar, the TD-unit was heated from 20 to 325°C at 60°C/min, with an initial and final time of 1 and 10 min, respectively. Compounds were swept towards the CIS-4 at 100 ml/min and trapped at −150°C in an empty baffled liner. When desorption was completed the CIS-4 was heated from −150°C at 12°C/s to 350°C, which was held for 5 min. The CIS-4 temperature program started after an additional 30 s at −150°C to allow full flow stabilization inside the PTV-liner after closure of the split valve. The total splitless time was 2.5 min. The TDS-2 was mounted onto a HP 6890 GC/5973 MSD (Agilent Technologies, Little Falls, DE, USA). The MSD was operated in the electron impact mode (70 eV), generating full scan spectra between 50 and 550 amu at 3 scans/s for PCB peak assignment. The system was also equipped with a Wiley database for spectral identification. For the actual experiments the MSD was used in the time-scheduled SIM mode using two ions per congener (Table 1). An HP-5MS capillary column (30 m L×250 μm I.D., 0.25 μm d_f) was used during the experiments. Helium was the carrier gas at 35 cm/s. The oven was programmed from 40°C (2.5 min) at 25°C/min to 150°C and then to 280°C (3 min) at 10°C/min.

2.4. Data processing

Limit of detection (LOD) was defined as the concentration derived from a signal three times the noise level, while for the limit of quantitation (LOQ) normally defined as ten times the background level, sample and system contamination had to be taken into account. Weighted linear regression was performed in an effort to account for data heteroscedasticy. Calibration graphs were constructed based on the responses from standard solutions (10 ml) containing the target PCBs from 0.01 up to 100 ng/ml sample (0.01, 0.1, 1, 10 and 100 ng/ml). Each calibrator sample was spiked with the I.S. (1 ng/ml), to obtain a peak area ratio of approximately 1 (analyte/I.S.) in the centre of each calibration curve. All samples were analysed five times in replicate. Recoveries were determined by comparing detector responses of the analysis of the 1 ppb solution with those obtained by spiking 1 μl of the stock PCB solution (10 mg/l) on a small plug of glass wool, placed inside an empty desorption tube. Method precision was evaluated at the 10 ppt level using both within- and between-day repeatability (calculated as %RSD on five replicates).

3. Results and discussion

Initial experiments were performed on aqueous samples spiked with PCBs to determine recoveries as function of extraction time. Curves describing this relationship are characterized by a steady increase and reach a maximum when the system is fully equilibrated. Once equilibrated, longer enrichment times show no improved recoveries. This equilibra-

tion time is often very long, especially for higher molecular mass (MM) components. Equilibrium curves were constructed with stirring times from 15 to 90 min, with 15 min intervals on 10 ml water samples spiked with 1 ppb of the PCB test mixture and the I.S. The stir bar was rotated at 1000 rpm. Analyses were characterized by very poor recoveries of less than 10% for the higher MM PCBs combined with repeatabilities in the range of 30–60% RSD for all compounds. Previous studies have shown that solutes with an octanol/water partitioning coefficient ($K_{O/W}$) larger than 500 can be extracted quantitatively into the PDMS [19]. Since the $K_{O/W}$'s of the Ballschmiter PCBs and of the I.S. (Table 1) well exceed 500, exhaustive extraction of these apolar solutes from the aqueous solution is (theoretically) possible. The relationship between recovery and the compounds' polarity suggested glass adsorption as the main reason for these unsatisfactory results. Reducing the polarity of the solution by adding small amounts of organic solvent, is an effective way to minimise this effect, which is particularly pronounced in the analysis of apolar compounds in water. In agreement with the analysis of PAHs, different percentages of methanol were added to the sample solution [19].

Samples were diluted in methanol/water mixtures with methanol percentages of 10, 15, 20, 25 and 50%. Also pure methanol was evaluated. All samples were stirred for 45 min. Starting from water, recoveries increased gradually with increasing methanol percentages to reach a maximum depending on PCB polarity. Higher methanol percentages resulted in a recovery descend for each specific congener. When spiked in pure methanol, recoveries for all PCBs were less than 5%. In Fig. 1 recovery variations as function of methanol percentage are given for PCB 28 (3 chlorines) and PCB 180 (7 chlorines).

PCBs 28 and 180 showed maximum recoveries at 25 and 50% methanol addition, respectively. Other PCBs exhibited similar curves at intermediate methanol percentages. Maximum recovery values varied around 50–60% for each component. Not only reduction of glass adsorption but also repeatability was positively affected by methanol addition. Repeatability is expressed as %RSD for 5 replicate analyses and is denoted in Fig. 1 by means of error bars. In the 50% methanol mixture %RSDs decreased substantially to values below 7% for both within- and between-day replicates. All further experiments were performed using the 50% methanol mixture. The small loss in recovery for the lower MM PCBs at this level of methanol was considered negligible compared with the significant gain in repeatability. Recoveries, however, do not solely depend on the extent of glass adsorption, also sampling time compared to the equilibrium time is essential. Therefore, equilibration curves in 50% methanol were determined. After 180 min of stirring, recoveries remained constant (equilibrium) and had increased compared to the 45 min sampling time (Table 2).

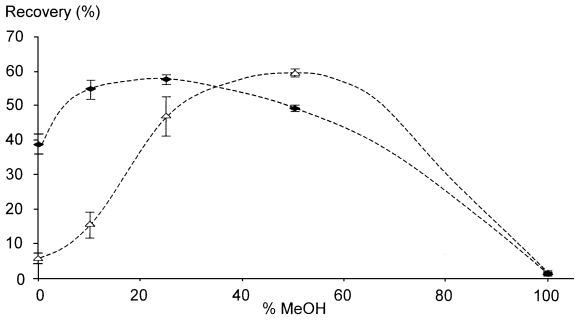

Fig. 1. Influence of MeOH on recovery and repeatability of PCBs 28 (♦) and 180 (△) at 1 ng/ml, stirred during 45 min at 1000 rpm.

A clear difference in equilibrium recoveries for the different PCBs is observed. PCB 180 was characterized by a nearly complete extraction (recovery 91%) compared with direct injection. Other PCBs, on the contrary, were characterized by much lower equilibrium recoveries, which are directly proportional with their MM. This decrease in recovery is obviously

Table 2
Recoveries of PCBs from a 10 ml sample at 1 ppb as a function of stirring time in SBSE

PCB	15 min	30 min	45 min	60 min	180 min
28	23	37	49	54	54
52	21	36	49	54	56
101	23	41	57	62	71
118	24	43	59	66	76
138	24	44	60	66	81
153	24	44	59	67	82
180	23	44	62	67	91

caused by the influence of methanol on the polarity of the sample, reducing the equilibrium constant between sample and PDMS phase, i.e. the affinity of the target compounds towards the stir bar stationary phase. This effect is more expressed for the lower MM PCBs, which are already characterized by a lower initial PDMS/water equilibrium constant ($K_{PDMS/W}$). In practice, full equilibration is not essential for accurate quantification and therefore in analogy to non-equilibrium solid-phase microextraction (SPME) [22] calibration curves were constructed using an enrichment time of 45 min. It should be noted that the use of 13C-labelled PCB congeners as internal standards instead of octachloronaphthalene would simplify quantitation especially for the low MM PCBs.

The method was then applied to the sperm sample. As for water, 1 ml of sperm, spiked with internal standard and the appropriate amounts of PCBs, was diluted in 9 ml methanol–water (1:1 by volume). A time-scheduled SIM chromatogram of the sperm sample spiked with 10 ppt level (A) and at the 1 ppt level (B) of the PCB mixture are presented in Fig. 2.

Although it can be deduced from Fig. 2B-PCB 180 that the LOD is as low as 0.1 ppt, the LOQ cannot be set at 0.33 ppt. Comparing Fig. 2A and B, the abundances should be in a ratio 10 to 1 which is not the case e.g. PCB 52 and 180 ratios are 4 and 3.2, respectively. This means that background levels in the low ppt level are present although it should be confirmed by high resolution mass spectroscopy (HRMS) if these signals are really PCBs. In principle, the background can originate from the sperm sample or from the analytical procedure. Whatever tried out to reduce cross-contamination at that level we could not reduce the background values even for blank water samples which indicates that the total analytical procedure seems to be the main source of the cross contamination. This phenomenon has often been observed in our laboratory for ultra-trace analysis of hydrophobic solutes like PCBs, polyaromatic hydrocarbons (PAHs) and organochloro pesticides (OCPs) made. The LOQ was therefore set at 10 ppt for the presented experimental conditions and calibration graphs were made. The LOQ value eventually can be reduced using larger sperm quantities. SBSE indeed has been applied to 200 ml water samples [19] which corresponds to 20 ml sperm with the dilution factor applied. The calibration curves were linear in the selected range (0.01–100 ng/ml sample) with correlation coefficients (r) higher than 0.999. The regression equations and corresponding correlation coefficients for all seven PCBs are given in Table 3. The within-day and between-day reproducibility at 10 ppt were below 7%.

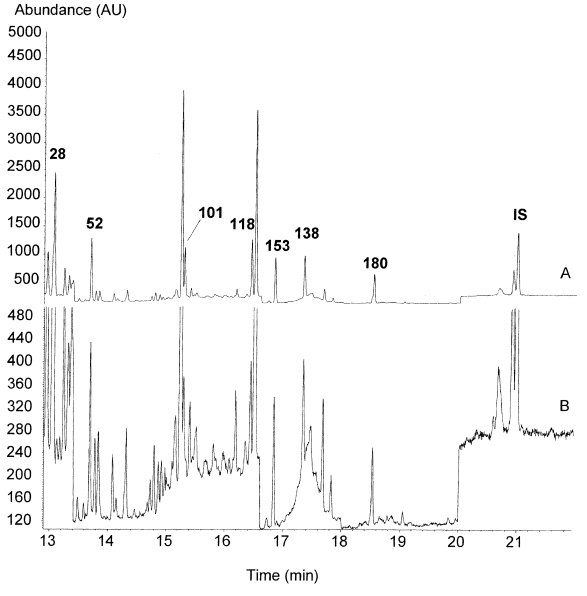

Fig. 2. Time-scheduled SIM chromatogram of 7 PCBs spiked in human sperm at 10 ppt (A) and 1 ppt (B). Two ions per compound were monitored (see Table 1).

As expected, an extra adsorptive contribution caused by the presence of cellular material was observed. Recoveries compared to the water samples dropped to 30–40% (Table 3). Linearity and repeatability, however, were not affected. Matrix suppression was proportional with the hydrophobicity of the PCBs. PCBs with higher $K_{O/W}$ values, were lost relatively more, i.e. the percentage of response suppression by the matrix for PCB 28 is much less pronounced than for PCB 180. This phenomenon is explained by the increasing affinity of the analytes for lipophilic substances in the sperm matrix.

The SBSE–TD–CGC–MS method is presently applied to the analysis of PCBs in different individual sperm samples in order to establish if the hypothetic relation semen quality–PCB contamination can be experimentally verified. During this study other solutes with estrogenic activity were detected

Table 3
Linearity of sampling and analysis of PCBs in the range of 10 ppt to 100 ppb in sperm[a]

PCB	Equation	R^2	RSD (%)	Recovery (%) water	Recovery (%) sperm
28	$y = 10.07x + 0.136$	0.99942	5.6	48	37
52	$y = 6.06x + 0.153$	0.99945	3.3	48	38
101	$y = 5.34x + 0.007$	0.99974	6.6	55	40
118	$y = 6.50x + 0.050$	0.99981	3.9	58	38
138	$y = 4.48x + 0.091$	0.99998	3.7	59	37
153	$y = 4.78x + 0.117$	0.99989	3.9	59	36
180	$y = 3.26x + 0.082$	0.99999	3.2	61	30

[a] Repeatability (%) in sperm at the 10 ppt level and recovery (%) in water and sperm at the 10 ppt level.

in the sperm pool and this is the subject of present research.

4. Conclusion

Sorptive extraction using stir bars coated with PDMS (SBSE) followed by thermal desorption–capillary GC–MS was evaluated for the analysis of the Ballschmiter PCBs in water and human sperm. Methanol was added to the sample solutions to minimize the effect of glass adsorption on recovery and repeatability. The developed method was highly repeatable and sensitive with %RSDs below 7% and LOD in the low ppq region. However, because of cross-contamination the LOQ was set at 10 ppt. For lower LOQs, larger sperm amounts or the application of HRMS should be considered.

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