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Application of solid phase micro extraction for the rapid analysis of chlorinated organics in breast milk

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Abstract The method presented here allows the monitoring of persistent organochlorine compounds in breast milk using the solid phase microextraction technique (SPME) and gas chromatography with electron capture detection (GC-ECD). It describes the determination of hexachlorobenzene (HCB), α-, β-, and γ-hexachlorocyclohexane (HCH), dichlorodiphenyltrichloroethane (DDT) and its derivatives, and some important congeners of the polychlorinated biphenyls (PCB). Also included are more polar substances such as tri-, tetra- and penta-chlorophenols, which can be analyzed simultaneously with the afore-mentioned less polar compounds without the need of a derivatization for the determination of the phenolic compounds. The reproducibility of the results is very good down to the lower µg/L-region. The method is very fast and of low cost compared to the classic extraction and determination procedures.

Introduction

The common trace analysis of pesticides and related compounds still relies upon time-consuming and costly procedures, especially from complex matrices such as body fluids or adipose tissue. These procedures usually include various steps of sample pretreatment, extraction and separation before the final quantitative estimation. Very popular among pesticides were and still are some organochlorine compounds, such as chlorobenzenes, chlorophenols and DDT which, on one hand are very effective, but on the other toxic to humans, too, and apart from that rather persistent with a tendency to accumulate in organisms. Therefore, it is very important to have a powerful and fast analytical method to gain a broad knowledge about the occurrence and concentrations of a number of important pesticides in the environment and/or organisms ("Biomonitoring").

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We had already developed a method for the determination of chlorinated organics in whole blood [1] using the new solid phase micro extraction technique [2] combined with GC/ECD determination. This proved to be a fast and low-cost alternative to the established sample pretreatment techniques that are commonly used. These include solvent extraction (liquid-liquid extraction, LLE) or separation on solid materials, such as silica gel (solid phase extraction, SPE) [3–6]. So the idea of using SPME sampling for the determination of pesticides and related compounds in breast milk seemed attractive.

We succeeded in developing an SPME method for an easy separation of the same spectrum of chlorinated organics as described in [Röhrig L, Püttmann M, Meisch HU (1998) Fresenius J Anal Chem 361: 192–196] from breast milk samples, followed by GC with ECD detection.

Experimental

Sample pretreatment

Spiking procedure. The milk samples were collected from apparently healthy women, who had been working at the laboratories of the Universität des Saarlandes before their pregnancies, by using manual or automatic pumps. As soon as possible after collection, the milk was frozen at –20 °C. The samples were only allowed to thaw for spiking and analyzing procedures. The milk samples were spiked to known concentrations of organochlorine compounds following a method developed by Waliszewski and Szymczynski [8], which we had already used for the determination of chlorinated organics in blood [1]. To get a concentration of 10 µg/L of every substance, a corresponding volume of standard solution was pipetted to a 25 mL head space vial and the solvent was vaporized in a nitrogen stream. 20 mL of the individual milk sample were added, the vial sealed and shaken thoroughly. The sample was cooled at about 4 °C for 24 h (to enable the active biological milk compounds to bind to the added organochlorine compounds) before being analyzed and was shaken thoroughly from time to time. Samples of different concentrations were obtained by diluting the spiked milk with unspiked milk from the same donator. All dilutions were analyzed after 24 h in the refrigerator.

SPME procedure. The SPME procedure follows the method we used for the biomonitoring of blood [1] with slight variations. To 0.5 mL of a milk sample, 0.5 mL of perchloric acid (1 M) were added

Table 1a Materials and chemicals

Milk:	Individual samples of breast milk collected from 4th to 15th feeding week
Standard solutions:	Solutions of 2,4,6- and 2,4,5-trichlorophenol, 2,3,4,6-, 2,3,5,6- and 2,3,4,5-tetrachlorophenol, pentachlorophenol, 2,4,6-tribromophenol, α-HCH, β-HCH, γ-HCH, hexachlorobenzene, o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD, o,p′-DDT, p,p′-DDT, PCB 28, 52, 101, 153, 138, 180 (c = 10 ng/μL each) in cyclohexane (Dr. Ehrenstorfer)
Other chemicals:	Perchloric acid 30% Suprapur (Merck), diluted to c($HClO_4$) ≅ 1 mol/L, sodium sulfate anhydrous, p.A. (Merck)
SPME equipment: (Supelco)	SPME holder for manual sampling / SPME fiber assembly 85 μm polyacrylate coating for polar semivolatiles SPME sampling stand with heated magnet stirring device
Sample vials:	4 mL vials with screw caps and septa (CS GmbH, D-Langerwehe)

Table 1b Gas chromatography

GC apparatus:	Shimadzu GC-14 A with ECD and split/splitless injector
Integration device:	Shimadzu CR-4 A / thermal printer
Capillary column:	SE-54, 50 m × 0.32 mm ID, d_f 0.35 (Macherey-Nagel)
Carrier gas:	hydrogen (10^5 Pa)
Split:	split closed for 5 min, split ratio: ~30:1
Injector:	temperature 280 °C, split insert liner (4 mm inner diameter)
Detector:	ECD, temperature 310 °C
Column oven:	60 °C (1 min) ⇒ 200 °C (10 °C/min, holding time 0 min) ⇒ 240 °C (20 °C/min, holding time 11 min) ⇒ 300 °C (10 °C/min, holding time 10 min)

to a 4 mL vial. Also added were 0.15 g sodium sulfate to speed up the transition of the analytes from the liquid to the gas phase. The vial was sealed with a screw cap and septum (PTFE coated butyl-rubber). A Vortex shaker was used for homogenization. The vial was placed into a heating block at a temperature of 100 °C. The SPME fiber (85 μm polyacrylate) was exposed to the head space of the sample for 40 min while stirring vigorously with a magnetic stirring bar. The fiber was inserted into the GC injector (280 °C) for 10 min to enable thermal desorption of the analytes from the SPME fiber.

Results and discussion

The solid phase micro extraction technique

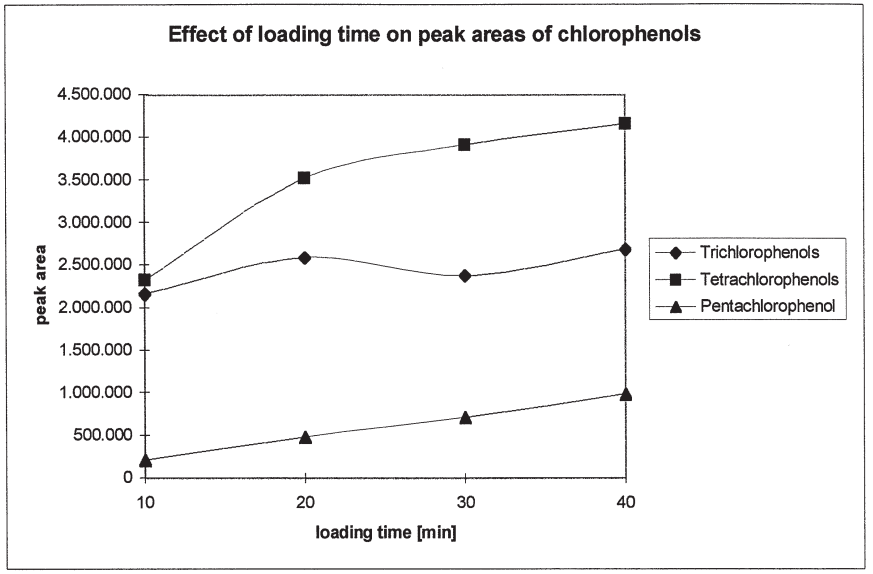
Choice of the convenient SPME fiber. In our earlier experiments on SPME sampling of the same range of organochlorine compounds from blood [1], various SPME fiber coatings were tested, but best results were obtained with a 85 μm polyacrylate coated fiber. For sampling from milk, also, the use of this fiber coating allowed the best determination of a wide variety of chlorinated organics in a single sampling step, compared to other tested fiber coatings (7 μm polydimethylsiloxane (PDMS) and 100 μm PDMS). The latter gave unsatisfactory results with respect to a simultaneous sampling of all relevant organochlorine compounds.

Conditioning of new SPME fibers. Before first use, SPME fibers had to be heated in the GC injection port for at least 2 h, because a rather high noise signal during the first GC runs had been observed resulting from residues of the manufacturing process of the fibers, especially from glue that fixes the fiber in its mounting. By heating the SPME fiber in the injection port at 280 °C for three complete GC runs, this problem can be eliminated. The resulting chromatograms from these conditioning runs allow a direct control of the quality of the fiber.

Direct immersion (DI) – vs. head space (HS) – technique. We chose a head space technique for the SPME sampling from milk, also, as described in [1]. Milk contains a lot of native compounds (especially fats and proteins) that are likely to be adsorbed on the fiber in a DI-SPME sampling besides the relevant analytes. Apart from peak interfering problems in the resulting chromatogram, this may also drastically shorten the lifetime of the fiber. In the HS-SPME process selected, the sample is heated in a sealed vial at 100 °C while the SPME fiber is exposed to the head space. This avoids interferences of the analysis by other milk compounds and the fiber can be used for at least 50 to 100 runs with good reproducibility. The high temperature provides a good retrieval even of less volatile substances (like some of the PCB congeners). Higher temperatures than 100 °C can cause problems with the sealing of the sample vials.

During analysis, the vials have to be sealed tightly. Therefore, the seals of the vials were partially pierced with an injection needle before heating the sample to 100 °C. Otherwise, a vial containing a heated sample can leak when pierced by the SPME fiber, because the fiber guiding needle is not sharp and has a rather high diameter compared to usual syringe needles.

Ion strength of the solution to be analyzed. As the equilibrium between molecules in a solution and the head space area depends strongly on the ion strength in the solution, the addition of salt up to a saturation can speed up the transition of dissolved non-electrolytes from solution into the


Effect of loading time on peak areas of chlorophenols
peak area
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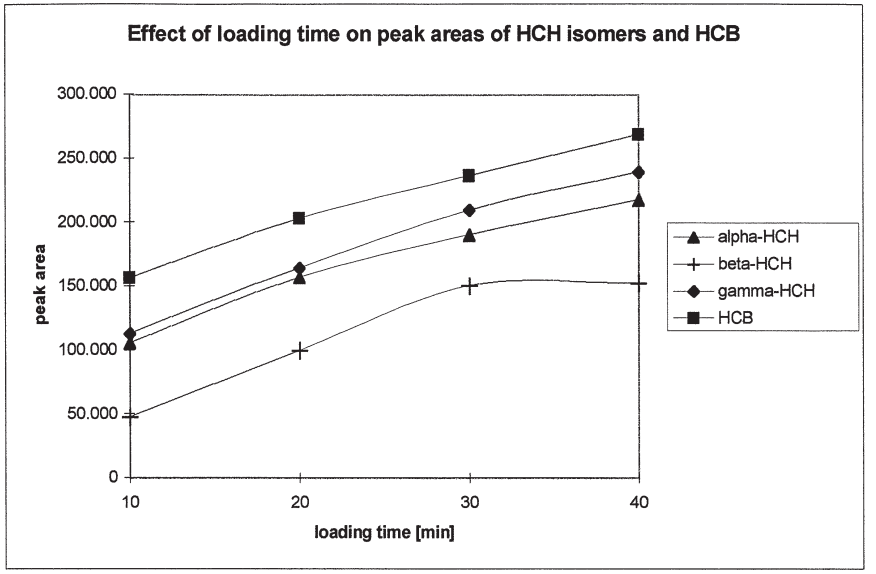
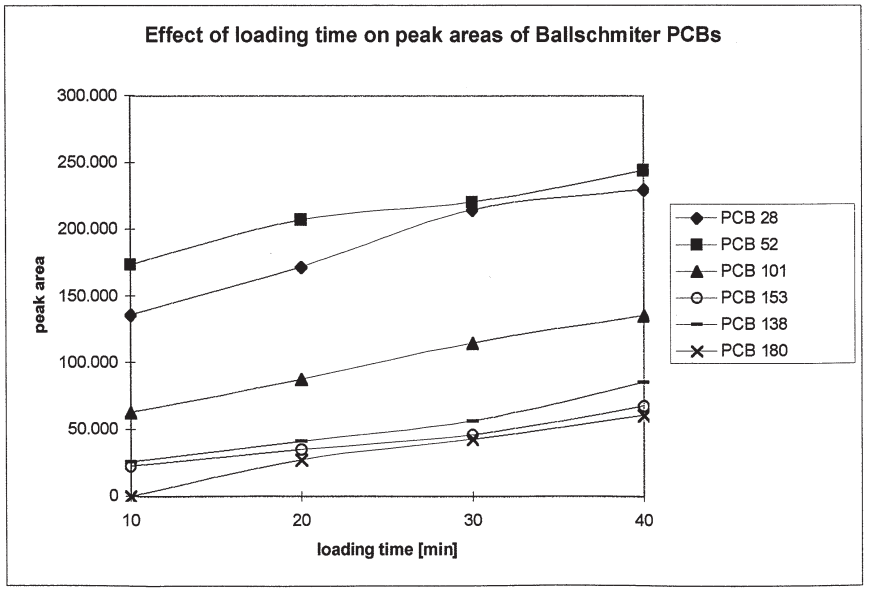
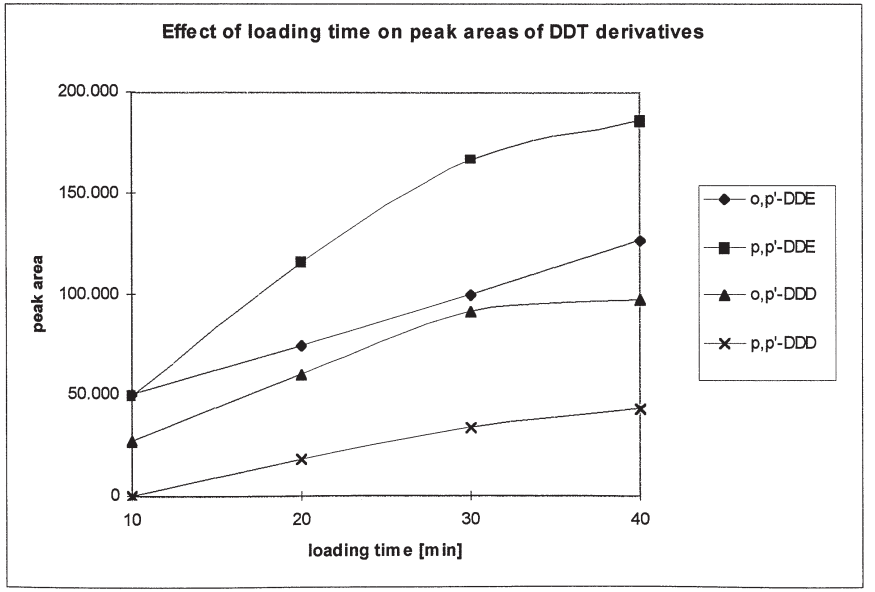
4.000.000
3.500.000
3.000.000
2.500.000
2.000.000
1.500.000
1.000.000
500.000
0
10
20
30
40
loading time [min]
Trichlorophenols
Tetrachlorophenols
Pentachlorophenol


Effect of loading time on peak areas of HCH isomers and HCB
peak area
300.000
250.000
200.000
150.000
100.000
50.000
0
10
20
30
40
loading time [min]
alpha-HCH
beta-HCH
gamma-HCH
HCB


Effect of loading time on peak areas of Ballschmiter PCBs
peak area
300.000
250.000
200.000
150.000
100.000
50.000
0
10
20
30
40
loading time [min]
PCB 28
PCB 52
PCB 101
PCB 153
PCB 138
PCB 180


Effect of loading time on peak areas of DDT derivatives
peak area
200.000
150.000
100.000
50.000
0
10
20
30
40
loading time [min]
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p,p'-DDE
o,p'-DDD
p,p'-DDD

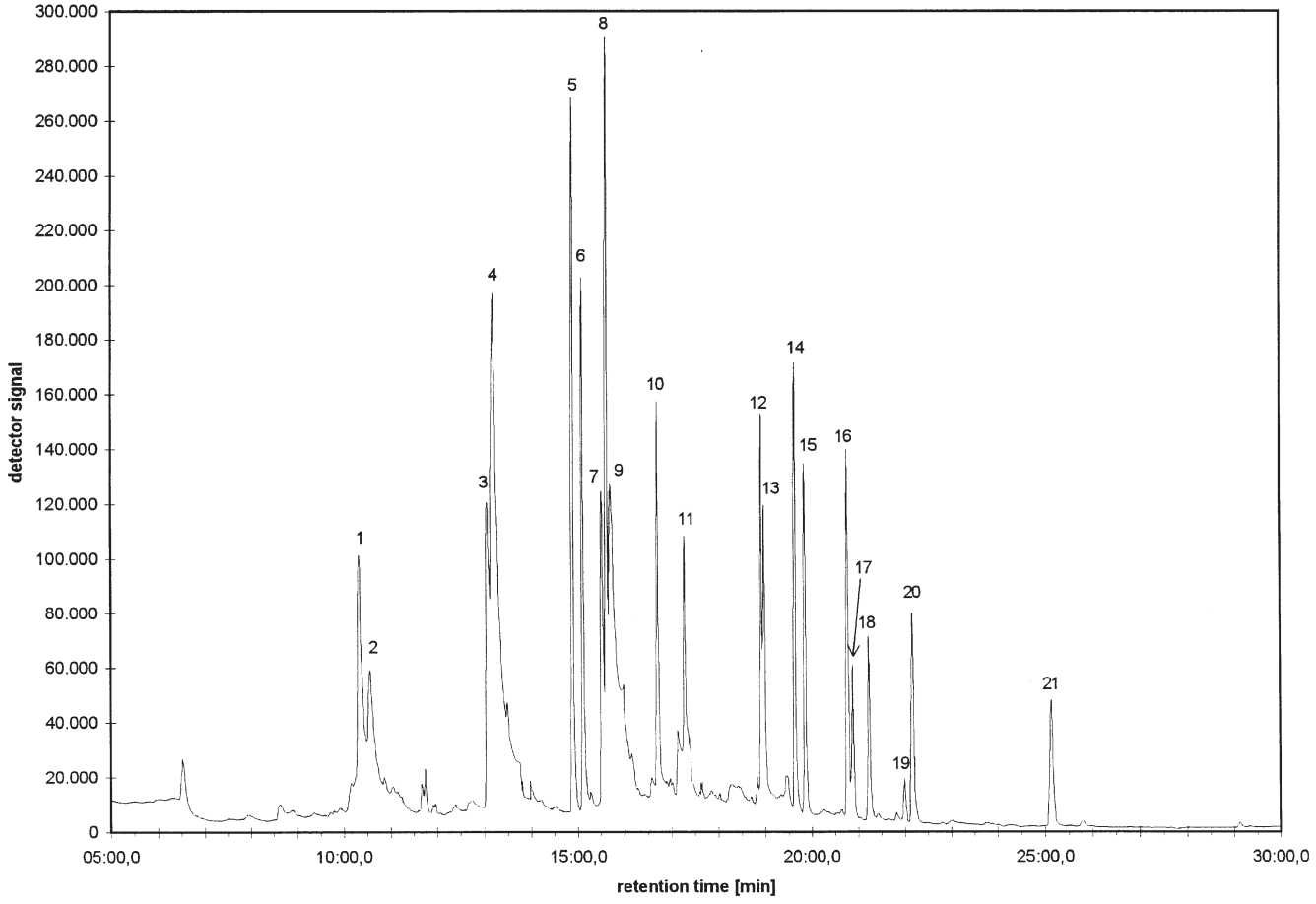

Fig. 2 Chromatogram of a breast milk sample, spiked to 10 µg/L per substance; chlorophenols, β-HCH and p,p′-DDT each 20 µg/L (peak identification see Table 2, analytical conditions see *Gas chromatography*)

head space [9]. This so-called salt-out effect is used in many SPME applications [10–13] In preceding experiments, different salts (NaCl, Na_2SO_4, $MgSO_4$) as well as different amounts of salt were tested. Best results were obtained with the addition of 0.15 g of sodium sulfate to 2 mL of the prepared milk solution. A saturation of this solution with $NaSO_4$ (about 0.36 g) showed no better results.

Loading time. The time while the analytes are adsorbed on the fiber, *i.e.* the time, while the SPME fiber is exposed to the sample or its head space, is another important parameter for SPME sampling. It has to be long enough to enable an equilibrium between the concentration of the analytes in the sample or the head space and their concentration on the fiber itself. During this loading time, the sample should be moved continuously, best by vigorous stirring in order to speed up the phase transition of the analytes from the liquid to the gas phase [14, 15]. Milk samples of known concentrations of chlorinated compounds were prepared as described previously and treated by SPME. Each sample was analyzed twice and the results were averaged. The loading time was varied between 10 and 40 min. Fig. 1 shows the results. As saturation is reached after 30–40 min, a 40 min sampling time was chosen for further studies. This includes the time for heating the sample to 100 °C (< 1 min).

A 10 min desorption time was chosen which ensured a complete desorption of the analytes together with a substantial cleaning of the fiber.

◀ **Fig. 1** Effect of extraction time on peak areas of chlorophenols; HCH isomers and HCB; Ballschmiter PCBs and DDT derivatives on an SPME fiber from breast milk (analytical conditions see *Gas Chromatography*)

Gas chromatography

Analytical method. As already stated in [1], there is no need for a preceding derivatization step for the determination of chlorophenols in SPME/GC-ECD, because of the solvent-free technique. The possible need for a derivatizing step (*i.e.* due to the polar chlorophenols) mainly depends on the nature of the analyte and the analyzing technique, but not on the matrix from which the analytes are extracted [16]. We found that the chlorinated phenols can be determined without any problems from breast milk by SPME/GC, without derivatization as there is no solvent in the injection port and the thermal desorption of the analytes is being performed directly from the SPME fiber.

Table 2 Calibration values, detection and determination limits (LOD, LODmn) and retention times [min] of organochlorine compounds in breast milk determined by SPME – GC/ECD (analytical conditions see *Gas chromatography*)

ID-No	Compound	Calibration curve equation	Linearity	LOD [µg/L]	LODmn [µg/L]	Retention time [min]
1, 2	Trichlorophenols	y = 44797x + 5923.7	R^2 = 0.9383	0.56	2.12	10.458/10.711
3, 4	Tetrachlorophenols	y = 122985x – 27279	R^2 = 0.9649	1.01	3.83	13.274
9	Pentachlorophenol	y = 65191x – 44539	R^2 = 0.9476	0.98	3.71	15.777
5	α-HCH	y = 14061x + 14715	R^2 = 0.9781	0.31	1.17	14.900
7	β-HCH	y = 17215x + 273	R^2 = 0.9952	0.70	2.65	15.511
8	γ-HCH	y = 24830x + 46828	R^2 = 0.9470	3.41	12.92	15.618
6	HCB	y = 17036x + 3282	R^2 = 0.9807	0.73	2.77	15.125
10	PCB 28	y = 20771x + 7491	R^2 = 0.9934	0.41	1.55	16.712
11	PCB 52	y = 20954x + 15736	R^2 = 0.9942	0.93	3.52	17.132
13	PCB 101	y = 9464x + 19709	R^2 = 0.9721	2.45	9.28	18.972
20	PCB 138	y = 7599x + 2325	R^2 = 0.9737	1.15	4.36	22.158
18	PCB 153	y = 6316x + 3525	R^2 = 0.9810	1.97	7.47	21.217
21	PCB 180	y = 6668x + 1970	R^2 = 0.9478	0.06	0.23	25.153
12	o,p′-DDE	y = 13080x + 5054	R^2 = 0.9969	1.36	5.15	18.910
14	p,p′-DDE	y = 23893x + 8409	R^2 = 0.9984	1.92	7.28	19.627
15	o,p′-DDD	y = 11774x + 7266	R^2 = 0.9878	1.85	7.01	19.843
16	p,p′-DDD	y = 9748x + 4217	R^2 = 0.9814	1.62	6.14	20.760
17	o,p′-DDT	y = 4013x + 2868	R^2 = 0.9682	2.79	10.57	20.872
19	p,p′-DDT	y = 1376x + 5289	R^2 = 0.9138	0.80	3.03	21.978

The organochlorine compounds were finally detected by gas chromatography with electron capture detection (GC-ECD). A typical gas chromatogram with peak indentification from SPME sampling of the afore-mentioned chlorinated organics from breast milk can be seen in Fig. 2.

Calibration by external standard. In the GC determination of the PCB congeners, DDT-, and HCH-isomers, calibration can be performed with decachlorobiphenyl (PCB 209) as internal standard (IS), but this compound has a very high boiling point (> 300 °C) and so it cannot be analyzed properly with our head space method. For the analysis of the phenolic compounds (tri-, tetra- und pentachlorophenols), 2,4,6-tribromophenol could be used as IS. The external standard method, which had been used successfully for blood [1], was also used for the milk experiments.

Reproducibility experiments. Linearity experiments were carried out with samples of breast milk, spiked with each of the chlorinated compounds mentioned before in a concentration range from 0.25 µg/L to 10 µg/L for each compound. The results from five different experiments were used to calculate the calibration curve equations and the regression coefficients (Table 2).

The experiments gave a very good linearity in the examined concentration range for almost all compounds. Some substances, however, such as PCP or PCB 101, showed just adequate correlation. The reason is a partial interference of these peaks with signals from other compounds from the milk samples that were not identified here. So a correct integration of these peaks is difficult. Problems like this could be eliminated by doing a post-run calibration of each peak or an additional cleaning step to remove these substances. Both actions, however, would have increased the analysis time considerably and so we decided to accept an approximate calibration for a few compounds in favor of the rapidity of the method.

Limits of detection and determination. The limit of detection (LOD) and limit of determination (LODnm), respectively, of a method of analysis is defined as the smallest amount or concentration of a compound which differs qualitatively (LOD) or quantitatively (LODmn) with a given probability from those given at concentration zero [17].

The limits of detection and determination can be ascertained from the calibration curves by using data from tabular forms [18]. The calculated limits of detection and determination from these experiments are also included in Table 2.

Apart from blood, breast milk is a body fluid which is used often for the monitoring of hazardous substances. As the compositions of blood and milk are rather different concerning the protein, carbohydrate, and fat content [19], our SPME method developed earlier [1] had to be adapted for milk analysis.

Firstly, with the change of the type of added acid from hydrochloric to perchloric acid for breaking down analyte/matrix composites and the protonization of the phenolic compounds, a much higher recovery of the analytes was obtained, thus increasing the sensitivity of the method. Secondly, the addition of salt to the milk before the SPME seemed to be useful, as the corresponding experiments showed. Best results were obtained with the addition of 0.15 g of sodium sulfate to 2 mL of the prepared milk solution. With the described modifications, the other SPME parameters (loading temperature, loading time, desorption time) did not have to be altered from the blood method, as corresponding experiments proved.

The milk samples examined here came from different phases of breast feeding (4th to 15th feeding week). It is known that the composition of mother's milk does not change much after the 3rd week. Only the yellowish milk from the first days of lactation, so-called colostrum, has rather different protein, fat and carbohydrate contents [20], but no milk from that early phase was analyzed here, mainly because colostrum is more difficult to obtain and the results from its analysis are considered to be less representative.

As we used individual milk samples instead of pooled samples, a more or less higher background signal from different substances (especially PCB congeners) in the unspiked milk samples could be measured. Therefore, every spiked sample was calibrated for the linearity and LOD/LODnm experiments with an unspiked sample from the same donator to eliminate this background, thus avoiding wrong results.

Conclusions

With the adaptions discussed before, the SPME technique allows a very fast and sensitive extraction and determination of persistent organochlorine substances from milk. The linearity of the method is very good in the concentration range from 0.25 μg/L to 10 μg/L for every relevant substance. Furthermore, the limits of detection and determination are low enough for biomonitoring purposes. They can be compared with reference values and reference ranges from the literature. These reference ranges are obtained from as many samples as possible, which were chosen at random and contain the contaminations in biological material that usually appear in samples from a defined geographical area. Therefore, a measuring method suited for biomonitoring has to be at least as sensitive to fall below these reference values. In case of chlorinated organics in breast milk, the reference values are in the range 0.1 to 2 mg/kg milk fat for a single substance. The calculated limits of detection and determination from our method are in almost all cases drastically lower, thus showing not only the suitability for biomonitoring, but also the power and efficiency of SPME for the determination of chlorinated organics in breast milk.

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