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Development of a high-performance liquid chromatography carbon column based method for the fractionation of dioxin-like polychlorinated biphenyls

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Abstract

A method to separate polychlorinated biphenyls (PCBs) by using high-performance liquid chromatography (HPLC) was developed. The HPLC column was packed with Amoco PX-21 activated carbon dispersed on octadecylsilane (ODS). The separation was carried out by gradient elution with *n*-hexane–dichloromethane and toluene in the forward direction followed by reversed elution with toluene. The results show that this HPLC method is useful for the separation of PCBs according to the number of substituted *ortho* chlorine atoms attached to the biphenyl structure. Average recoveries for a number of individual di-*ortho* PCBs, mono-*ortho* PCBs, and non-*ortho* PCBs in three selected elution windows were 97, 92, and 96%, respectively. Clophen A50, a herring sample, and a cod liver oil sample were fractionated on the column and the analytical results are compared with data from the literature. The method presented here is useful for quantitative separations of mono-*ortho* PCBs as well as non-*ortho* PCBs which have been assigned toxic equivalency factors by the World Health Organisation. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Fish; Oils; Food analysis; Polychlorinated biphenyls; Clophen A50

1. Introduction

Recently polychlorinated biphenyls (PCBs) once again were at the centre of interest. In January 1999, 500 000 kg of animal feed contaminated with approximately 50 kg of PCBs and 1 g of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were distributed to farms in Belgium, and to a lesser extent to other nearby countries [1]. Originally observed as a PCDD/Fs problem, it soon became clear that the animal feed was contaminated with PCB oil which contained PCDFs as unwanted by-products [2]. The 2,3,7,8-substituted PCDD/Fs but also non-*ortho* PCBs and mono-*ortho* PCBs contribute to the toxicity in food products.

A summary of the total number of the different *ortho* PCBs for each homologue group, respectively, is listed in Table 1. There are 12 PCBs that have been identified as producing dioxin-like toxicity, viz. non-*ortho* PCB 77, 81, 126, 169 and mono-*ortho* PCB 105, 114, 118, 123, 156, 157, 167, 189 [3–6]. These PCB congeners can adopt a planar configuration and are characterised by four or more chlorine atoms substituted at the *meta* and *para* positions.

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Table 1
PCB nomenclature summary

Homologue	Non-*ortho* PCBs	Mono-*ortho* PCBs	Di-*ortho* PCBs	Tri-*ortho* PCBs	Tetra-*ortho* PCBs
MonoCB	2 (3)	1 (1)			
DiCB	5 (15)	5	2 (10)		
TriCB	5 (37)	11 (28)	7	1 (19)	
TCB	5 (*77*,80)	14 (66)	17 (52)	5	1 (54)
PeCB	2 (*126*)	11 (*105,114,118,123*)	20 (87,97,101,110)	11	2 (104)
HxCB	1 (*169*)	5 (*156,157,167*)	17 (128,138,153)	14	5 (155)
HpCB		1 (*189*)	7 (170,180)	11	5 (188)
OCB			2 (194,205)	5	5 (202)
NCB				1 (206)	2 (208)
DCB					1 (209)
Total	20	48	72	48	21

The total number of different *ortho* isomers for each PCB homologue group are indicated. Tested PCB congeners on the HPLC carbon column are in parentheses. PCB congeners in *italics* are WHO-PCBs.

The toxic non-*ortho* PCBs, mono-*ortho* PCBs, and PCDD/Fs are considered to act through the same mechanism of action and have been assigned toxic equivalency factors (TEFs) by the World Health Organisation (WHO) [4]. The TEF values based on the toxicological potencies relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), are used to calculate a single toxic equivalent concentration (TEQ) in a sample.

More than 34% (72 PCBs) of all PCBs are di-*ortho* substituted PCBs and these congeners are predominant in technical mixtures, e.g. Clophen A50. TEFs of some di-*ortho* PCBs, as suggested earlier by Safe [3] and Ahlborg et al. [6], have been withdrawn depending on insufficient toxicological evidence. Recent data from an international interlaboratory comparison study have shown that the contribution of PCBs to the TEQ can exceed the contribution of the PCDD/Fs [7]. In cows' milk collected in Germany, the concentrations of non- and mono-*ortho* PCBs expressed in TEQs contributed 16 times more to the total TEQ than PCDD/Fs [8].

Individual dioxin-like PCBs are often not detected in a general analysis of PCBs, since co-eluting *ortho* substituted PCBs are present at much higher levels. Isolation and enrichment of non-*ortho* PCBs and mono-*ortho* PCBs are complicated by the presence of these co-extracted poly-*ortho* PCBs (di-*ortho* PCBs, tri-*ortho* PCBs and tetra-*ortho* PCBs) that can interfere in the final analysis by high-resolution gas chromatography and mass spectrometry (HRGC–MS).

The clean-up and analysis of non-*ortho* PCBs and mono-*ortho* PCBs have been critically reviewed in the literature [9–11]. Activated carbon columns are often used in one of the clean-up steps prior to the final analysis. Activated carbon can achieve the separation of planar aromatic compounds, e.g. non-*ortho* PCBs present at pg/g levels in environmental samples, from interfering non-planar aromatic compounds, e.g. poly-*ortho* PCBs and pesticides present at ng/g or μg/g levels. Chromatography using activated carbon separates chlorinated aromatic hydrocarbons on the basis of molecular planarity and to some extent on the degree of chlorination. The separation properties of the activated carbon are based upon interactions between the graphite structure and the diaromatic system (π-electrons) of the biphenyls. These carbon properties can be used to separate PCBs into classes depending on the amount of twisting out of the plane of the biphenyl rings [12–15]. In addition, activated carbon can be used to separate PCDD/Fs, and other structurally similar compounds, e.g. polychlorinated naphthalenes (PCNs), polychlorinated dibenzothiophenes (PCDTs), and alkylated polychlorinated dibenzofurans (R-PCDFs) from non-planar compounds. Historically, different types of stationary phases as well

as different types of supporting materials have been used for the separation of mono-*ortho* PCBs and non-*ortho* PCBs in environmental samples [9–11].

Traditionally, activated carbon is used in open column liquid chromatography [5,9–11,13–28] but in recent years HPLC has received increasing attention because of its higher reproducibility and its potential for automisation [8–12,29–44]. Different HPLC carbon columns but also the 2-(1-pyrenyl-) ethyldimethylsilylated silica column (PYE column) can separate the PCBs into suitable fractions [30–32]. It is important that the HPLC column material is insensitive to matrix effects. In addition, a large active surface area is necessary to avoid changes in retention times caused by an overloaded column or blocked active sites. These considerations are crucial for the choice of column material. Commercially available columns like porous graphitic carbon (PGC) columns [12,33,34,40,41,44] can be used for the PCB separation as well as laboratory-prepared HPLC PX-21 carbon columns [29,39,43].

Early experiments with Amoco PX-21 carbon were conducted during the late 1970s by Stalling et al. [13] and Huckins et al. [14]. This method was refined by Smith et al. [15,16] and used for analyses of biological and sediment samples. A sophisticated clean-up method with two HPLC PX-21 carbon columns containing 10 and 200 mg Amoco PX-21 carbon was developed by Feltz et al. [43]. This method produced four discrete fractions containing poly-*ortho* PCBs, mono-*ortho* PCBs, non-*ortho* PCBs, and PCDD/Fs.

The aim of this work was to develop an automated fractionation method using a gradient of toluene in the forward direction, which makes the quantification of the dioxin-like PCBs possible, and at the same time increases the sample throughput in the laboratory.

The method is a straightforward HPLC carbon column method using only one carbon column with approximately 100 mg Amoco PX-21 activated carbon dispersed on common HPLC supporting materials in order to produce three discrete fractions, a di-*ortho* PCB fraction, a mono-*ortho* PCB fraction, and a non-*ortho* PCB fraction also containing other planar compounds such as PCDD/Fs, PCNs, PCDTs, and R-PCDFs.

Solvent mixtures of *n*-hexane and dichloromethane (DCM) or pure *n*-hexane, together with a gradient of toluene, and a final elution with pure toluene in the reversed direction, enabled the optimisation of the separation of the different classes of PCBs from interfering compounds and a proper quantification of the target compounds.

An important advantage of the HPLC method presented here is that the use of organic solvents was minimised resulting in a healthier laboratory environment for the chemists and a more cost efficient clean-up.

2. Experimental

2.1. Chemicals and materials

All solvents, *n*-hexane, cyclopentane, dichloromethane, and toluene were glass-distilled grade (Burdick and Jackson, Muskegon, MI, USA). Glassware of high quality were machine washed with alkaline detergent and solvent rinsed prior to use.

A PCB window defining mixture for use with DB-5 type GC-columns and a PCB predominant congener mixture were purchased from Wellington Labs., Ont., Canada. The PCB window defining mixture contained PCB 1, 3, 10, 15, 19, 37, 54, 77, 104, 126, 155, 169, 188, 189, 202, 205, 206, 208, and 209 and the PCB predominant congener mixture PCB 1, 15, 28, 52, 118, 153, 180, 194, 206, and 209. The PCB window defining and PCB predominant congener mixtures were used for carbon column retention experiments. Another PCB mixture consisting of target PCBs (PCB 77, 105, 114, 118, 123, 126, 128, 138, 156, 157, 167, 169, 170, 180 and 189) provided by Ted Schwartz (USGS, Columbia Environmental Research Center, Columbia, MO, USA) was also used in retention experiments. The target PCB mixture contained all the non-*ortho* and mono-*ortho* PCB target compounds except PCB 81. A mixture of PCBs containing PCB 77, 126, 169, 105, 118, 123, 156, 157, 101, 110, 138, 153, and 180 was made from individual PCB standards (Cambridge Isotope Labs., CIL, Woburn, MA, USA) and used in recovery experiments. The concentrations of the individual PCBs in this mixture was in the range of 0.9–5.0 ng/μl.

^{13}C-Labelled PCB 77, 80, 101, 118, 126, 153 and

169 (Cambridge Isotope Labs.) were used as internal standards and recovery standards. Unlabelled PCB 110 was purchased from the same supplier in order to find out if the PX-21 carbon column could separate this congener from PCB 77. The total number of individual PCBs used in retention and recovery experiments was 40 (Table 1).

A sample-like PCB fractionation on the column was studied using a technical Clophen A50 mixture (Bayer, Germany). Carbon column fractionations of Clophen A50 were carried out at several occasions throughout the investigations of the carbon columns and used as a quality assurance (QA) sample. By using this mixture, it was possible to investigate the retention behaviour of the PCBs, and if the column deteriorated, over time.

Two different 250×4.5 mm stainless steel HPLC columns (columns 1 and 2) were extensively tested separately during 12 months without any notable pressure changes. Column 1 contained 100 mg Amoco PX-21 activated carbon (2–10 μm, Amoco Research, Chicago, IL, USA), and 2.20 g Lichroprep RP-18 (particle size 15–25 μm, EM Science, USA). This column was provided by Ted Schwartz and filled by Kevin Feltz (USGS, Columbia Environmental Research Center). Column 2, filled at our department, contained 92 mg Amoco PX-21 activated carbon (2–10 μm, Amoco Research) dispersed on 2.01 g Nucleosil RP-18 (particle size 15–25 μm, Macherey–Nagel, Düren, Germany). Both columns were used in the recovery experiments.

The analysis of a herring oil sample, earlier analysed by laboratories in an inter-laboratory study, was used to validate the performance of the carbon column. This herring oil sample was prepared from herring (*Clupea harengus*) collected from the Gulf of Bothnia. A second validation sample, cod liver oil (*Gadus morhua*, Standard reference material SRM 1588), was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The cod liver oil sample had no certified non- and mono-*ortho* PCB concentrations, but the separation of PCBs on the PX-21 carbon column enabled a quantification of certified di-*ortho* PCB concentrations for comparisons.

2.2. HPLC set-up

The HPLC equipment used for the separation of the PCBs is shown in Fig. 1. It consisted of a binary pump system (Model 2150, LKB, Bromma, Sweden) with a controller (Model 2152, LKB), a mixing device (Tee, Valco Instruments, TX, USA), an autosampler AS-4000 (Hitachi, Japan) equipped with a 200- or a 500-μl sample loop, a pre-column filter (A-315, 2 μm porosity, Upchurch Scientific, WA, USA) mounted after the autosampler, a HPLC PX-21 carbon column (column 1 or column 2), and a Gilson FC 204 fraction collector (Gilson Medical Electronics, Middleton, WI, USA). A two-position six-port valve (AC6 W, Valco Instruments) was mounted between the carbon column and the pre-column filter to change the direction of the mobile phase through the carbon column. A two-position air actuator (A60, Valco Instruments), controlled by a digital valve interface (DVI-220, Valco Instruments) provided an automatic valve switching. The solvent reservoirs contained *n*-hexane–DCM or pure *n*-hexane (Fig. 1, solvent A) and toluene (Fig. 1, solvent B). All solvents were degassed with argon and the solvent flow-rate was 4 ml/min. The pressures measured in the forward and the reversed flow directions were 9.5 MPa (~1400 p.s.i.) and 15 MPa (~2200 p.s.i.), respectively.

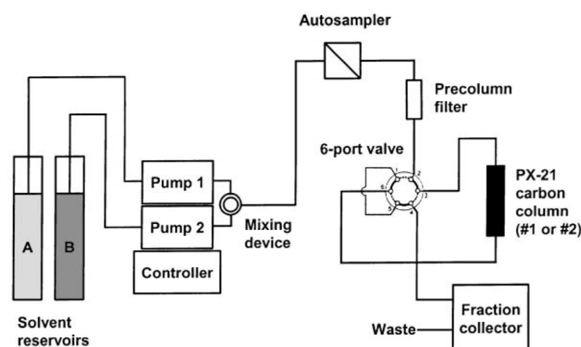

Fig. 1. HPLC equipment. Solvent reservoir 1 contains a mixture of DCM in *n*-hexane or pure *n*-hexane (A) and solvent reservoir 2 contains toluene (B). The solvents are homogenised in a mixing device (tee) directly after the HPLC pumps. The six-port valve controls the direction of the mobile phase through the column. Fractions are collected after the carbon column with a fraction collector. The pre-column filter, mounted after the autosampler, filters the mobile phase and protects the column from particles larger than 2 μm.

Before injection, the column was washed and reconditioned with toluene (20 ml, backflush) and

n-hexane–DCM (20 ml, backflush) followed by *n*-hexane–DCM for 10 min (40 ml) in the forward direction. Injections of extracts (150–300 μl) were executed by the pre-washed auto-sampler using a full injection method. Following sample injection, a linear gradient of solvent B changed the composition of the mobile phase. After 40 min, the six-port valve switched to the second position and 100% B (80–160 ml) backflushed the column. Appropriate fractions (3, 4, 17, or 18 fractions) were distributed by the fraction collector to collection vessels (300 mm×24 mm I.D., 130 ml). A keeper (30 μl) of toluene or tetradecane and a recovery standard (^{13}C-labelled PCB 101) were added to the fractions prior to evaporation and the final GC–MS analysis, respectively.

2.3. GC–MS analysis

Mono-*ortho* PCBs and samples with high non-*ortho* PCB levels were analysed on a high-resolution gas chromatography–low-resolution mass spectrometry (HRGC–LRMS) system (VG 12-250). Environmental samples (herring and cod liver oil) with low non-*ortho* PCB levels were analysed on a high-resolution gas chromatography–high-resolution mass spectrometry (HRGC–HRMS) system (VG 70-250S). Both instruments were operated in electron ionisation (EI) mode using electron energies of 70 and 35 eV, respectively. The HRMS instrument operated at a mass resolution of 8000. The GC systems used for the analyses were Hewlett-Packard 5890. The GC capillary fused-silica columns used were an RTx-5, 60 m×0.32 mm I.D., 0.25-μm film thickness (Restek, Bellefonte, PA, USA) or a DB-5, 60 m×0.25 mm I.D., 0.25-μm film thickness (J&W Scientific, Folsom, CA, USA). Carrier gas was helium and the column head pressures were 28 p.s.i. (~190 kPa) or 18 p.s.i. (~120 kPa), respectively. The GC columns were directly interfaced to the VG 12-250 (LRMS) or the VG 70-250S (HRMS) mass spectrometer (VG Analytical, Manchester, UK). The samples (3 μl) were injected in splitless mode. The ovens were temperature programmed as follows: 80 °C (2 min), 15 °C/min, 200 °C, 4 °C/min, 300 °C (10 min), when the keeper was toluene and 180 °C (2 min), 20 °C/min, 200 °C, 4 °C/min, 300 °C (15 min), when the keeper was tetradecane. Injector, interface, and ion source temperatures were 250, 280, and 250 °C, respectively. The analytes were recorded with full scan (m/z = 100–500) or selected ion recording (SIR), monitoring the two most abundant ions in the molecular ion chlorine distribution cluster for each PCB homologue (m/z 188/190, 222/224, 256/258, 290/292, 326/328, 360/362, 394/396, 428/430, 464/466, and 498/500). For ^{13}C-labelled compounds, m/z 304, 338, and 372 were monitored. Detection limits ($S/N > 3$) were 50 pg (full scan) and 5 pg (SIR) for the low-resolution instrument and 0.1 pg (SIR) for the high-resolution instrument.

2.4. HPLC elution experiments

The elution experiments were divided into three parts: HPLC retention experiments, recovery experiments, and Clophen A50 fractionation. In the HPLC retention experiments, the HPLC conditions were varied to enable a proper fractionation and quantification of the target PCBs. The window defining, predominant, and target PCB mixtures were all fractionated on the HPLC system investigating the retention of the PCBs on the column under these different conditions. The optimal HPLC conditions found were then used in the subsequent experiments.

Studies of the HPLC method's repeatability and reproducibility were carried out in recovery experiments. Within-run and between-run precision of the method were investigated in these experiments using two PX-21 carbon columns (column 1 and column 2). Triplicate injections of 10, 50 and 100 μl each of a specially made recovery mixture were used to explore the separation precision of the HPLC method.

The column's separation efficiency for complex mixtures was investigated in the final elution experiment, the fractionation of Clophen A50. Co-eluting PCBs and other interfering compounds in the established fractions could be detected and identified by the use of this sample-like mixture. Full-scan mass spectrometry (m/z 100–500) recorded PCBs, interferences, and impurities in the obtained fractions. No internal and recovery standards were added to Clophen A50 or the fractions keeping the analyses clean from outer influences. Quantification of PCBs in Clophen A50 was thereby impossible in this experiment.

2.5. Clean-up and fractionation of validation samples

The clean-up of herring and cod liver oil was carried out by means of dialysis with semi-permeable membranes (SPMs) to reduce the bulk of the lipids using cyclopentane [45]. The SPMs were made from a polyethylene dialysis tube, 500 mm length, 26 mm wide and 80 μm thick (EST, St Joseph, MO, USA). The dimensions of the glass funnels used for the dialysis procedure were 500 mm×45 mm I.D., with a PTFE stopcock placed at the bottom. The fish oil was dissolved in cyclopentane and transferred to the SPM, which was mounted inside the dialysis funnel. The dialysis was accomplished by changing the outer cyclopentane (dialysate) solvent after 12, 40 and 64 h. The dialysate fractions were combined and evaporated to a few millilitres. Before dialysis, five ^{13}C-labelled PCBs (PCB 77, 118, 126, 153 and 169) were added to the fish oil extracts as internal standards. The dialysis extract was further cleaned up by elution on a silica column (10% deactivated) with *n*-hexane and fractionated on an HPLC aminopropyl silica column [46]. A fraction from the column containing dicyclic aromatic compounds was introduced to the HPLC PX-21 carbon column. Four fractions were collected and the fractions that contained target compounds were analysed. Two procedure blanks were processed concurrently with samples.

3. Results and discussion

3.1. HPLC retention experiments

The main objective of these experiments was to find an optimal separation of different *ortho*-PCBs, making it possible to quantify the 12 dioxin-like PCB congeners as defined by the WHO (WHO-PCBs). The major goal was consequently to separate the PCBs into a mono-*ortho* PCB fraction and a non-*ortho* PCB fraction free from co-eluting PCBs and other interferences, but a beforehand self-stipulated demand also included a fraction containing di-*ortho* PCBs. The basic idea behind the optimisation was to collect the tetra- and tri-*ortho* PCBs in the first 10-ml fraction and the non-*ortho* PCBs in the final toluene fraction. In addition to that the di-*ortho* PCBs and the mono-*ortho* PCBs should be distributed into the fractions between the first and the final fraction. Traditionally, when fractionating PCBs on carbon, a constant composition of the mobile phase is used. In this work, the composition of the mobile phase is changed by a toluene gradient to be able to achieve the goals of fractionation.

The PCB separation was optimised within a relatively short time (~1.5 h) including fractionation, washing, and reconditioning of the column. This allows fractionation of many samples during one day.

Six consecutive experiments (Exp. 1–Exp. 6) with different solvent systems and HPLC gradient conditions were used for the investigation of the retention behaviour of a broad spectrum of PCBs (Fig. 2, section B, left) on the PX-21 carbon column. The HPLC elution experiment conditions are presented in Table 2. In these experiments, the eluate was divided into 17 or 18 fractions. The first 16 fractions contained 10 ml eluate and the final fractions 80 or 120 ml toluene.

The results obtained by Exp. 1 conditions showed a poor separation efficiency between the tetra-, tri-, and di-*ortho* PCBs. These poly-*ortho* PCBs were mainly collected in the first three fractions. Non-*ortho* PCBs with three or more substituted chlorine atoms were all collected in the final reversed pure toluene fraction. Furthermore, the retention times of mono-*ortho* PCBs were much shorter than the non-*ortho* PCBs and many mono-*ortho* PCBs co-eluted with di-*ortho* PCBs. The initial experiment showed that a more systematic investigation of the separation between the different *ortho*-PCBs was needed.

In Exp. 2, where the toluene gradient was changed from 0–10 to 0–5%, the mono-*ortho* PCBs were more retained on the column resulting in a sufficient separation between these congeners and the poly-*ortho* PCBs. Mostly all the mono-*ortho* PCB retention times were between 10 and 40 min in this experiment. One mono-*ortho* PCB congener however, PCB 189, was quantitatively (50%) found in the final planar fraction. Non-*ortho* PCBs were still collected in the last fraction (17th fraction). The separation of tetra- and tri-*ortho* PCBs from di-*ortho* PCBs was still unsatisfactory. The change in mobile phases (solvent A) in Exp. 3 and Exp. 4 from 10 to

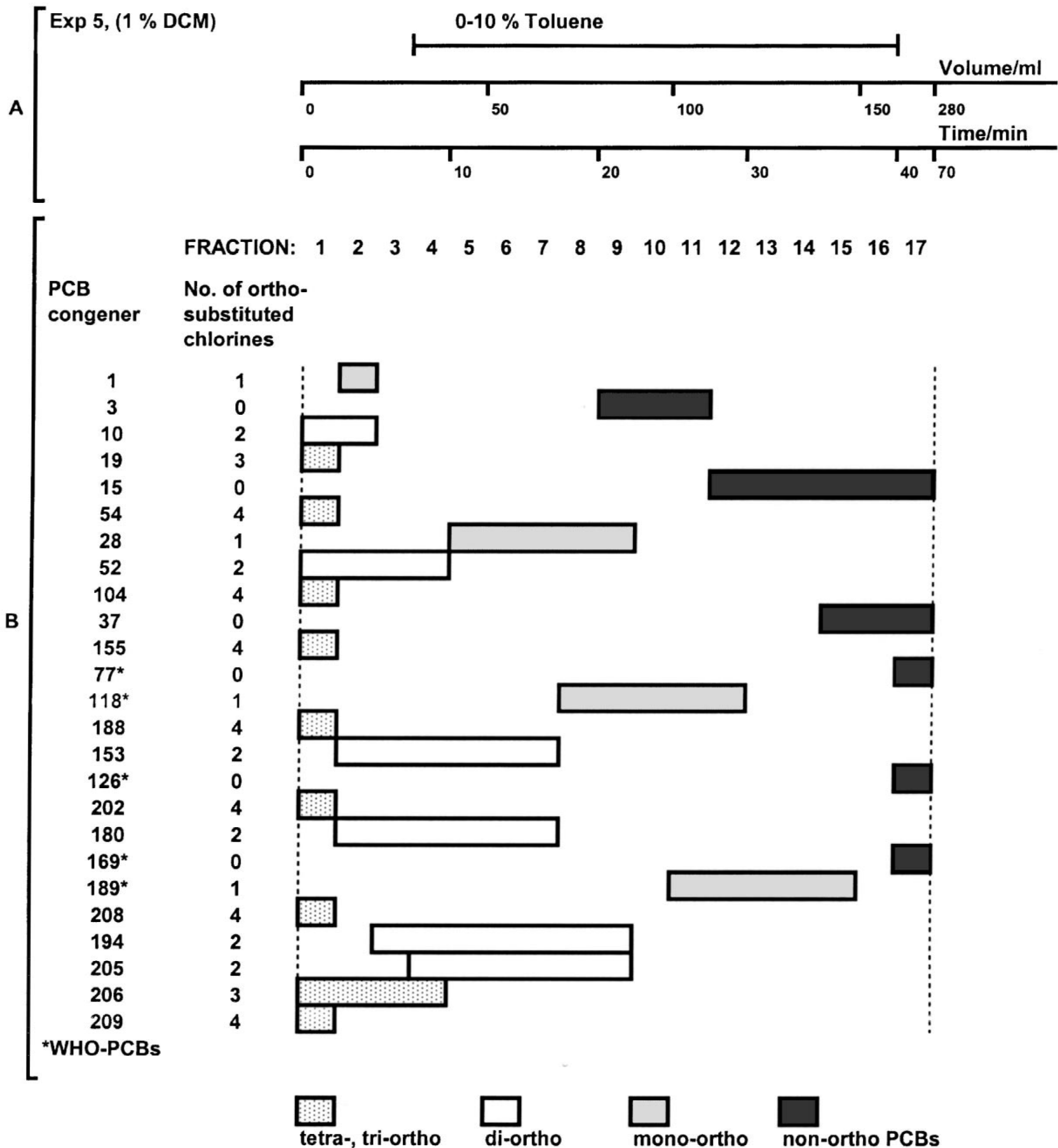

Fig. 2. The elution order of a PCB mixture using the experimental conditions in Exp. 5 (Table 2). A diagram with solvent volume, analysis time and used toluene gradient is shown in section A, top. The eluate was divided into 16 equal fractions (10 ml) and a planar fraction (120 ml). The identified PCBs in the 17 fractions are shown in section B, bottom.

5% or 1% DCM in *n*-hexane separated these PCBs to a higher degree.

In Exp. 4, numerous mono-*ortho* PCBs eluted off the column into the final non-*ortho* PCB fraction (planar fraction). The analyses of PCBs in the 18th fraction (pure toluene, 80 ml, backflush) in these

Table 2
HPLC elution experiment conditions

Exp.	Solvent A	Solvent B	Toluene gradient (%) Time/min (volume/ml)	No. of fractions	Total volume/ml	Total time/min
1	10% DCM in hexane	Toluene	0–10% Toluene 5–40 min (20–160 ml)	17	240	60
2	10% DCM in hexane	Toluene	0–5% Toluene 5–40 min (20–160 ml)	17	240	60
3	5% DCM in hexane	Toluene	0–6% Toluene 7.5–40 min (30–160 ml)	18	320	80
4	1% DCM in hexane	Toluene	0–5% Toluene 7.5–40 min (30–160 ml)	18	320	80
5	1% DCM in hexane	Toluene	0–10% Toluene 7.5–40 min (30–160 ml)	17	280	70
6	Hexane	Toluene	0–10% Toluene 7.5–40 min (30–160 ml)	17	280	70

Flow-rate: 4 ml/min, column 1; DCM, dichloromethane.

experiments resulted in non-detectable levels of mono-*ortho* PCBs and non-*ortho* PCBs. The change in the toluene gradient from 0–5 to 0–10% toluene in Exp. 5 and the use of the same percentage of DCM (1%) as in Exp. 4 resulted in a satisfactory separation between the PCB classes (Fig. 2).

In the last experiment (Exp. 6), where pure *n*-hexane was used as solvent A, some of the higher chlorinated mono-*ortho* PCBs eluted into the planar fraction.

The HPLC conditions in Exp. 5 were considered to be the best of the tested solvent conditions for the separation of the target analytes. The optimised fractionation of the PCBs enabled a precise quantification of the WHO-PCBs and to some extent the di-*ortho* PCBs. The results of the separation of a PCB mixture using Exp. 5 conditions are shown in Fig. 2. The linear toluene gradient (0–10%) was initiated at 7.5 min and finished after 40 min. The total solvent volume used was 280 ml (Fig. 2, section A). The relatively low amounts of organic solvents used were advantageous in comparison to other similar methods [39,43]. Positive identifications of PCB peaks in the fractions above the detection limit are shown in Fig. 2, section B. The tetra- and tri-*ortho* PCBs, di-*ortho* PCBs, mono-*ortho* PCBs, and non-*ortho* PCBs are mainly collected in the 1st (0–10 ml), 2nd to 6th (10–60 ml), 7th to 16th (60–160 ml), and 17th fraction (160–280 ml), respectively. The figure shows that the retention time of the individual PCBs on the PX-21 carbon column depends on the number of chlorine substituents in *ortho* positions (Fig. 2, section B). Fewer *ortho* chlorine atoms substituted to the biphenyl molecule increase the retention time. The non-*ortho* substituted PCBs are more retained on the PX-21 carbon, having a stronger interaction with the activated carbon compared to the *ortho*-substituted congeners. The non-*ortho* PCBs with three or more chlorine atoms (PCB 37, 77, 126, and 169) were all collected in the last planar fraction. In addition to molecular planar structures, the selectivity of PX-21 carbon is governed by the number of electronegative substituents on the biphenyl skeleton [13,14]. Heptachlorobiphenyls, e.g. PCB 189, have thus longer retention times than those of trichlorobiphenyls, e.g. PCB 28 (Fig. 2, section B).

Approximately 1.2 μg each of three selected PCBs (di-*ortho* PCB 153, mono-*ortho* PCB 118, and non-*ortho* PCB 80) were injected onto the carbon column and eluted into 17 fractions using the Exp. 5 conditions. The obtained elution profiles are illustrated in Fig. 3. The calculated percentages of each congener in each fraction were plotted versus the elution volume. The peaks are sharp, and more than 90% of the congeners were collected within 30 ml of eluting solvent, despite the high amount injected. The peak profiles are narrow with some tailing which is typical for chromatography on activated carbon [12]. The investigated congeners are well separated on the column, only minor portions ($<0.1\%$) co-elute. PCB 80 was selected in this experiment due to

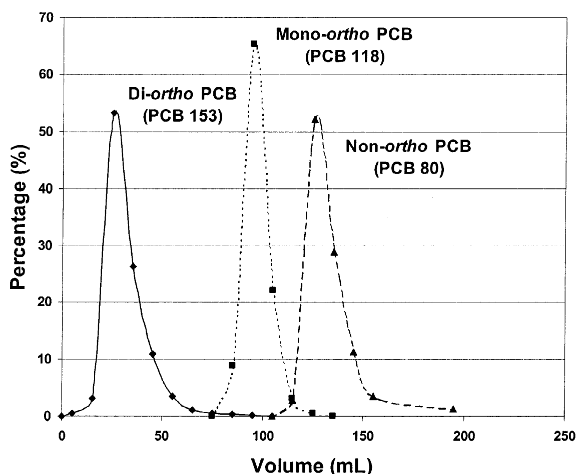

Fig. 3. Elution profile of PCB 153 (di-*ortho* PCB), PCB 118 (mono-*ortho* PCB) and PCB 80 (non-*ortho* PCB) on the PX-21 carbon column using the experimental conditions as in Exp. 5 (Table 2).

the fact that this non-*ortho* PCB congener had been identified in the mono-*ortho* PCB fraction in earlier experiments not presented in this article. This congener is less retained on the carbon column than PCB 77 and PCB 81 demonstrating dissimilar properties among these non-*ortho* PCBs.

3.2. Recovery experiments

Recovery experiments with selected di-*ortho*, mono-*ortho*, and non-*ortho* PCBs were carried out at three different levels and in triplicate. The total-PCB amounts loaded on the carbon column were 0.27 μg (low level), 1.33 μg (medium level), and 2.65 μg (high level). The recovery standard used was ^{13}C-labelled PCB 118 and the amounts of individual PCBs loaded on the column were similar to expected amounts in samples: 9–50 ng (low level), 45–250 ng (medium level), and 90–500 ng (high level).

The higher PCB amounts were at the same time used to investigate the carbon column's loading capacity and if cross-contamination or memory effects were problematic for the HPLC fractionation method. The fractionation on the carbon column was performed using Exp. 5 conditions and the chosen retention windows for tetra- and tri-*ortho*, di-*ortho*, mono-*ortho*, and non-*ortho* PCB fractions were 0–2.5 min (10 ml), 2.5–15 min (50 ml), 15–40 min

Table 3
PCB recoveries in the non-*ortho*, mono-*ortho*, and di-*ortho* PCB fractions using the conditions of Exp. 5 and carbon column 1

PCB congener	Recovery±RSD (%)		
	Low level	Medium level	High level
Non-*ortho* PCBs			
77	99±6	96±4	91±5
126	86±6	99±5	100±4
169	82±13	97±5	118±3
Mono-*ortho* PCBs			
105	88±7	88±4	78±5
118	84±8	88±4	83±3
156	97±7	106±8	94±3
157	98±6	105±5	93±8
Di-*ortho* PCBs			
101	109±7	96±4	85±8
110	94±5	83±6	77±6
138	95±6	110±6	113±10
153	97±4	104±8	100±8
180	92±11	88±10	113±11

Three levels of PCBs were injected; low level (9–50 ng), medium level (45–250 ng), and high level (90–500 ng). The mean recovery values of three fractionation experiments on the carbon column for each level together with relative standard deviations (RSDs) are reported. ^{13}C-Labelled PCB 118 was used as a recovery standard.

(100 ml), and 40–70 min (120 ml), respectively. The recovery experiment results are summarized in Table 3. The chosen elution cutting times for the di-*ortho*, mono-*ortho*, and non-*ortho* PCB fractions resulted in high recoveries and only minor amounts (<3%) of the analysed PCBs were found in wrong fractions.

The determined recoveries were in the range of 77–118%. Average recoveries for di-*ortho*, mono-*ortho,* and non-*ortho* PCBs were 97, 92, and 96%, respectively. The experimental results with recoveries near 100% and low relative standard deviations (RSDs) for the three groups of PCBs showed that the PCB fractionation was accurate and precise (Table 3). The recoveries were similar for high as well as low amounts of injected PCBs. This demonstrated that the carbon column's loading capacity is excellent for this range representing real sample levels. The 100 mg PX-21 carbon, with a large surface area (~200 m^2), has a high capacity and the recovery experiments have shown that this amount of PX-21 carbon is applicable for a wide range of PCB concentrations. Carbon with a lower surface area

(e.g. porous graphitic carbon) has a greater potential to be overloaded causing a reduction of fractionation efficiency [41]. Cross-contamination that originated from the injector or memory effects (irreversible adsorption of PCBs) on the PX-21 carbon column was not observed in these experiments. The reproducibility of the method was tested in these recovery experiments using a second PX-21 carbon column (column 2). Recoveries and RSDs values obtained for this column and column 1 were equivalent.

3.3. Clophen A50 fractionation

Clophen A50 (10 μg) in *n*-hexane was injected onto the HPLC system by the autosampler. The experiment was performed using Exp. 5 conditions and established retention windows. The results of the HRGC–MS analyses of the four fractions (non-*ortho* PCB fraction, mono-*ortho* PCB fraction, di-*ortho* PCB fraction, and tetra and tri-*ortho* PCB fraction) are shown in Figs. 4 and 5. Identification of the PCBs in Clophen A50 was made by comparison with data from the literature [47,48]. Few chromatographic peaks were recorded in the non-*ortho* PCB and mono-*ortho* PCB fractions and the fractions were relatively free from interferences (Fig. 4). As seen in the chromatograms, the target compounds (mono-*ortho* PCB 123, 118, 114, 105, 167, 156, 157, and 189; non-*ortho* PCB 77, 126, and 169) were collected and identified in the expected fractions. Minor portions (<2%) of high level di-*ortho* PCBs (PCB 101, 110, 138, 153, and 163) and high level mono-*ortho* PCBs (PCB 105, and 118) in Clophen A50 were identified in the mono-*ortho* PCB and non-*ortho* PCB fractions, respectively. Critical co-eluting pairs of PCBs on a DB-5 capillary GC column such as PCB 77 and 110 are clearly separated on the carbon column. These results are in agreement with the results obtained for pure standards (Fig. 2).

The analyses of the di-*ortho* PCB and the tetra-, tri-*ortho* PCB fractions resulted in complex chromatograms containing many different PCBs (Fig. 5). The dominant peaks in the analysed di-*ortho* PCB fraction were PCB 101, 110, 153, 138, 170, and 180. As can be seen in the chromatogram, some of the lower chlorinated PCBs (e.g. mono-*ortho* PCB 66, 70, 74, and 76) were collected in a wrong fraction. Minor portions (<5%) of mono-*ortho* PCB 105 and 118 were identified in the di-*ortho* PCB fraction. Another interesting observation was that the separation of mono-*ortho* PCBs from di-*ortho* PCBs and non-*ortho* PCBs was more pronounced compared to the separation of di-*ortho* PCBs from tetra- and tri-*ortho* PCBs and mono-*ortho* PCBs, cf. Figs. 4 and 5. There were several di-*ortho* PCBs collected in the tetra- and tri-*ortho* PCB fraction. The fractionation of the relatively high amount of Clophen A50 showed that the PX-21 carbon column has a high separation efficiency for this type of complex PCB mixture. The amounts of PCBs in an environmental sample are normally lower.

The relative level of PCB 77 obtained in this experiment was approximately one magnitude higher than PCB 126 and three magnitudes higher than PCB 169. This pattern of non-*ortho* PCBs in Clophen A50 resembled the pattern found in other commercial PCB mixtures [22–24,29]. The pattern of mono-*ortho* PCBs (PCB 105, 118, 156, 167) in Clophen A50 was also in agreement with data from the literature [24,49].

The advantages of the developed HPLC PX-21 carbon column fractionation method are as follows: the inert carbon material with a large active surface, the short fractionation time, the re-use of the column, and the possibility to easily change the solvent conditions for the optimisation of other target compounds. A PYE column can separate the PCBs in a similar way as the PX-21 carbon column [30]. The sample must however be free from lipids to obtain a proper separation of PCBs with the PYE column and this is disadvantageous compared to the carbon method. One of the advantages of this PX-21 carbon column method presented here is the easy handling of the column. Other methods in the literature for example used four different open tubular carbon columns, two aluminium oxide columns and a complex scheme, including pooled fractions, for the separation of Clophen A50 into subgroups containing poly-*ortho* PCBs, mono-*ortho* PCBs, non-*ortho* PCBs, and PCDFs/PCNs [22]. Our results for the Clophen A50 fractionation on the HPLC PX-21 carbon column together with the recovery experiments show that it is possible to accomplish quantitative separations of mono-*ortho* PCBs and non-*ortho* PCBs using the HPLC PX-21 carbon column method.

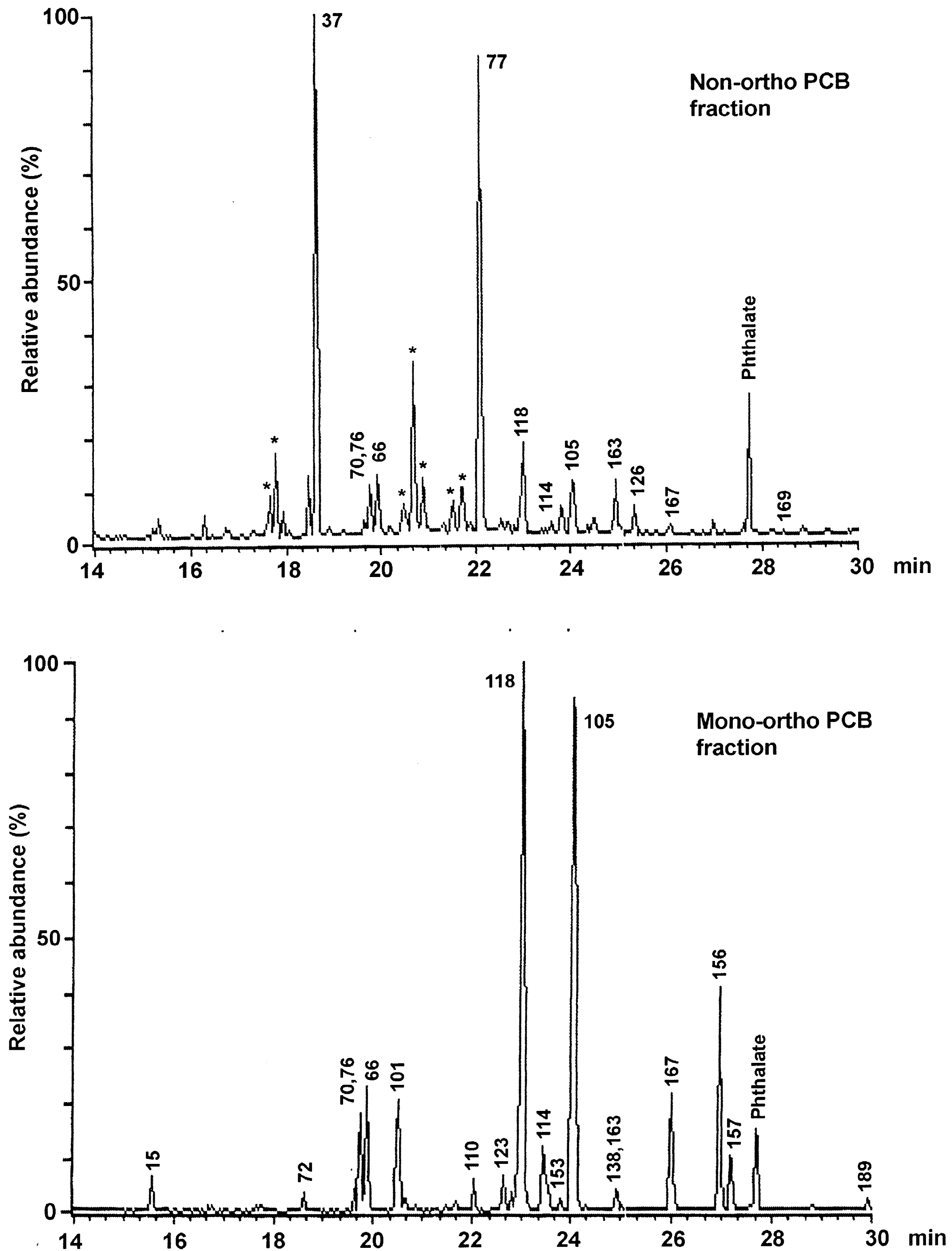

Fig. 4. HRGC–LRMS analysis of Clophen A50 fractionated on the carbon column. The elute was divided into four fractions and analysed separately by full scan (m/z 100–500). The two chromatograms shown are the non-*ortho* PCB fraction (Fraction 17, Fig. 2) and the mono-*ortho* PCB fraction (Fractions 8–16, Fig. 2). Marked (*) peaks are PCB congeners that originate from background contamination.

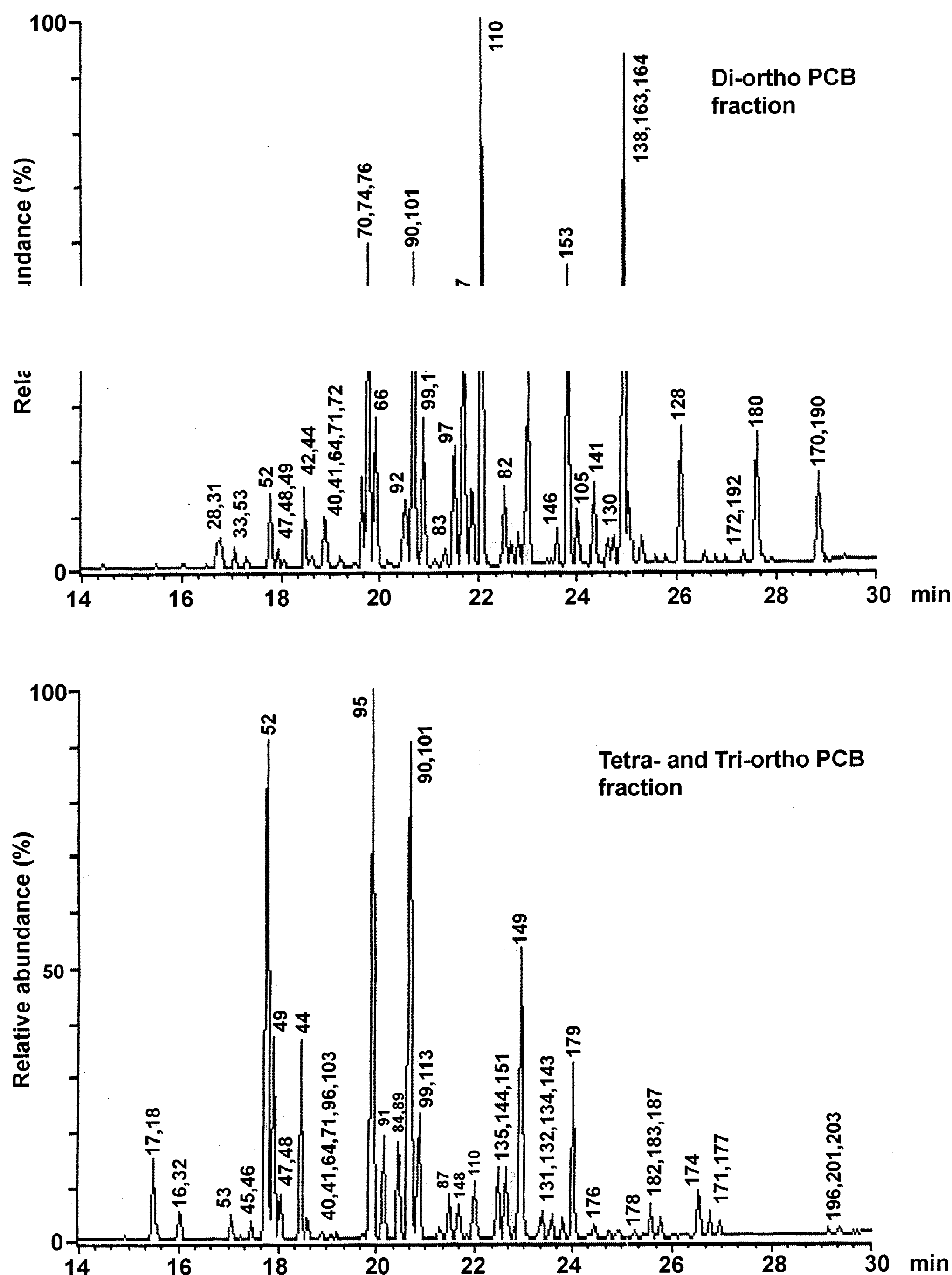

Fig. 5. HRGC–LRMS analysis of Clophen A50 fractionated on the carbon column. The eluate was divided into four fractions and analysed separately by full scan (m/z 100–500). The two chromatograms shown are the di-*ortho* PCB fraction (Fractions 2–7, Fig. 2) and the tetra-*ortho* PCB and tri-*ortho* PCB fraction (Fraction 1, Fig. 2).

3.4. Method validation with samples

Herring oil from a pooled herring sample netted offshore from Skellefteå, Sweden was used for the validation of the method. This particular herring oil has been used in an inter-laboratory study involving 19 laboratories [50]. Clean-up and optimised fractionation of herring oil samples (5 g) followed by the final analyses with HRGC–MS produced quantitative mono-*ortho* PCB (ng/g lw) and non-*ortho* PCB (pg/g lw) levels.

The average levels of mono-*ortho* PCBs and non-*ortho* PCBs with the HPLC carbon column method together with the results obtained in the inter-laboratory study are given in Table 4. The PCB levels obtained by the HPLC method are in good agreement with the average levels analysed in the same herring oil in the inter-laboratory study. The levels of mono-*ortho* and non-*ortho* PCBs were all within one standard deviation compared to the results obtained in the inter-laboratory study except for PCB 77.

The chromatograms were relatively free from interferences, only minor carryovers of two di-*ortho* PCBs (PCB 153 and 138) were found in the mono-*ortho* PCB fraction. Levels of these PCBs are however several magnitudes higher than the planar PCBs in the original sample. The recoveries determined for the internal ^{13}C-labelled PCB 77, 118, 126 and 169 were in the range of 57–78%. The results of the analyses of the herring oil samples were satisfactory and the fractionation of real samples produced similar results as the fractionation of pure standards and Clophen A50.

A major advantage of the PX-21 carbon column is that the fractionation is relatively unaffected by matrix effects. Other lower surface area HPLC packings used for PCB fractionations often have problems with lipids or other co-extracted compounds [31].

The same clean-up procedure as for the herring oil samples was applied to a cod liver oil sample (6 g). For this sample the levels of the five most abundant di-*ortho* PCBs were determined. The concentration of PCB 153 (271 ng/g lw) obtained with the HPLC carbon column method followed by HRGC–LRMS was very close to the certified concentration (276 ng/g lw) in the cod liver oil. The concentrations of other di-*ortho* PCBs found (PCB 101, 138, 170, and 180) were somewhat lower compared to the certified concentrations by about 20–40%.

Table 4
Levels of mono-*ortho* PCBs and non-*ortho* PCBs in herring oil

PCB	Inter-laboratory study (ng/g lw)	HPLC carbon column method (ng/g lw)
Non-*ortho*-PCBs		
77	0.71±0.18	0.61±0.06
126	0.46±0.10	0.42±0.05
169	0.087±0.021	0.090±0.010
Mono-*ortho*-PCBs		
105	20±7.3	19±3
118	52±13.0	55±4

A comparison between an inter-laboratory study involving 19 laboratories [50] and the present HPLC PX-21 carbon column method. Average levels in ng/g lw and standard deviations are given for triplicate samples.

4. Conclusions

A fast and quantitative method for the fractionation of mono-*ortho* and non-*ortho* PCBs (WHO-PCBs) using a HPLC PX-21 carbon column step before the final HRGC–MS analysis was successfully developed. The method was validated by several separation and recovery experiments of individual PCB standards. The validation also included fractionation of a technical PCB mixture and analyses of biological samples. The validation of the carbon column showed that this method is well suited for the quantification of the target analytes. The automatic HPLC carbon column method separated mono-*ortho* and non-*ortho* PCBs from poly-*ortho* PCBs into discrete fractions and interferences from coeluting PCBs on a DB-5 type GC capillary column were minimised. The method presented here can be used to increase the sample throughput in laboratories and at the same time minimise the use of organic solvents. The large active surface area of 100 mg PX-21 carbon used was sufficient for a wide range of PCB concentrations. The method can easily be incorporated in existing clean-up procedures in most environmental laboratories performing congener specific high-resolution gas chromatography determinations of PCBs. The presented method also showed

great potential for the determination of PCBs other than the WHO-TEF assigned PCBs. After the method validation, more than 100 successful analyses of various environmental samples have been carried out at our laboratory on a single HPLC PX-21 carbon column.

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