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Determination of 15 isomers of chlorobenzoic acid in soil samples using accelerated sample extraction followed by liquid chromatography

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ABSTRACT

A study was conducted to elaborate a fast, simple and efficient method for determination of 15 isomers chlorobenzoic acids (CBAs) in soil using HPLC-UV. Artificially contaminated soil samples were extracted using accelerated solvent extraction (ASE) with 1% acetic acid in a mixture of hexane and acetone (1:1, V/V) under a pressure of 10.34 MPa and temperature of 150 °C. The recovery of the ASE method was above 82%. The extracts were concentrated; dimethyl sulfoxide was used to prevent CBA volatilization and the final analysis was performed with a C18 XBridge HPLC column employing a mobile phase consisting of acetonitrile and 0.1% trifluoracetic acid in water. A HPLC procedure with gradient elution and UV detection was developed and validated. The method exhibited a linear range for 2-CBA; 2,6-CBA; 3-CBA; 4-CBA; 2,3-CBA; 2,3,6-CBA; 2,5-CBA; and 2,4-CBA from 5 to 120 μg/mL with a limit of quantification (LOQ) of 5 μg/mL, RSD from 2.42 to 9.42% and accuracy from 82 ± 2 to 103 ± 3%. The linear range of determination of 2,4,6-CBA, 3,4-CBA, 2,3,5,6-CBA, 3,5-CBA, 2,3,5-CBA, 2,3,4,5,6-CBA and 2,3,4,5-CBA was 10–120 μg/mL with LOQ 10 μg/mL, RSD from 0.74 to 5.84% and accuracy from 94 ± 1 to 114 ± 1%. The optimized analytical procedure was finally applied on two historically PCB contaminated soils and 9 CBAs were quantified in the samples.

1. Introduction

Chlorinated organic compounds are a class of serious environmental pollutants due to their ecotoxicity and environmental persistence. Chlorinated benzoic acids are widespread environmental pollutants resulting primarily from microbial biodegradation of polychlorinated biphenyls (PCB), reviewed e.g. in Field and Alvarez [1] and some herbicides [2]. CBAs are significantly more soluble than their parent compounds and therefore can enter into the aqueous phase from the contaminated soil of polluted sites. Some mono-, di-, and trichlorobenzoic acids (CBAs) have been shown to cause genomic damage to tobacco plants [3], and to be toxic to aquatic organisms such as ciliate [4], Daphnia [5], algae [6] and fish [5]. Zhao et al. and Muccini et al. suggested that the dissociation of CBAs is an important factor in their toxicity and the nonionized forms of CBAs are usually less toxic than the ionized analogues [4,5]. 2,3-CBA, 2,3,6-CBA, 2,4,6-CBA and monochlorinated isomers were also found to possess estrogenic disrupting activity [7]. Although it was found that CBAs are not very toxic toward bacteria, substantial negative effects of their presence on the transformation of PCBs have been reported [8]. In this way, they can inhibit bioremediation processes and therefore understanding the fate of CBAs is of environmental importance.

In order to determine the concentrations of analytes in environmental matrices using chromatography, it is usually necessary to include an extraction step. Liquid–liquid extraction methods have been described for aqueous samples containing CBAs, using methyl *t*-butyl ether [9] and ethyl acetate [10] as extraction solvents.

Several extraction methods have also been described for extraction of CBAs and other organochlorinated pollutants from solid environmental samples. Gentry et al. described simple liquid extraction of soil containing CBAs using detergent (not specified Zwittergent) with hexametaphosphate [11]. Other articles have deal with comparisons of the efficiency, time and the material cost of the various extraction methods, such as classical Soxhlet extraction, microwave extraction and accelerated solvent extraction (ASE) for various organochlorinated pollutants in soil matrices [12–14]. Traditional Soxhlet extraction attains acceptable efficiency, but is time-consuming and requires large amounts of solvents. Ultrasound extraction is an alternative method to Soxhlet. Advanced extraction methods, such as supercritical fluid extraction, microwave-assisted extraction and accelerated solvent extraction (ASE), are less time-consuming and usually require smaller amounts of solvents.HPLC connected

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with UV or MS detection and gas chromatography (GC) belong among classical methods for determination of organic acids, including CBAs. To determine CBAs by GC, it is necessary to derivatize their carboxylic group, usually using diazomethane [15] or bis(trimethylsilyl)trifluoracetamide [16]. In the HPLC mode, CBAs were separated on a C18 reverse phase [9,17] or ODS-AQ column [18]. The low pK_a of these organic acids usually requires acidification of the mobile phase used in the HPLC system [9,17,18].

Several chromatographic methods for determination of CBAs are described in the literature. These methods are typically used for quantification of several CBA isomers during degradation experiments or for determination of their residual concentrations in environmental matrices. However, none of the described methods includes an analysis of a wider range of representatives simultaneously including mono-, di-, tri-, tetra- a pentachlorine CBA isomers.

The main objective of our work was to develop a robust and efficient LC method for determination of 15 CBA isomers (mono-, di-, tri-, tera-, and penta-CBA) and to evaluate different HPLC columns and mobile phases. Another goal of this work was to develop and validate a simple, rapid and efficient extraction method for CBAs from soil samples using ASE. This method represents fast, low-solvent consumption and a reproducible method suitable for soil samples containing a complex matrix. ASE was then employed to determine the presence of CBAs in artificially contaminated soils.

2. Experimental

2.1. Standards and chemicals

2-CBA; 2,3-CBA; 3,4-CBA; 3,5-CBA; 2,3,5-CBA; 2,4,6-CBA; 2,3,5,6-CBA; 2,3,4,5,6-CBA were obtained from Sigma–Aldrich (Steinheim, Germany). 3-CBA; 4-CBA; 2,4-CBA; 2,5-CBA and 2,6-CBA were from Merck (Darmstadt, Germany). 2,3,6-CBA was purchased from Supelco (Steinheim, Germany), 2,3,4,5- from TCI Europe (Zwijndrecht, Belgium) and 2,3-dichlorophenol (internal standard, IS) from Reidel-de Haën (Steinheim, Germany).

Acetone and *n*-hexane for residue and pesticide analysis were provided by Chromservis (Prague, Czech Rep.). Methylene chloride for organic trace analysis was obtained from Merck. Anhydrous sodium sulfate (Na_2SO_4) was obtained from Lachner (Prague, Czech Republic). Glacial acetic acid and formic acid were obtained from Reidel-de Haën.

Acetonitrile (ACN, gradient grade) was purchased from Fisher Scientific (Leicestershire, UK), trifluoroacetic acid (TFA) from Fluka (Steinheim, Germany) and methanol (Chromapur GG) from Chromservis (Czech Republic). All the other chemicals were of analytical grade and were obtained from Sigma–Aldrich.

2.2. Stock solutions

Standard solutions containing 2 mg/mL of each of the 15 benzoic acid compounds were prepared in ACN and stored in a refrigerator. The stock standard solutions with the appropriate concentrations were prepared by mixing all 15 CBAs and diluting the stock solution with ACN.

2.3. HPLC conditions

HPLC analyses were performed using the Alliance Waters system (Prague, Czech Republic) equipped with a PDA detector and Empower software was used for data processing. Separation of the CBA mixture was tested on four reversed phase columns: XBridge C18 (250 mm × 4.6 mm I.D., particle size 3.5 μm), Waters; SynergyPolar RP (250 mm × 4.6 mm I.D., particle size 4 μm), Phenomenex; LiChroCart – LiChroSphere RP-18e (250 mm × 4 mm I.D., particle size 5 μm), Merck and LiChroCart – Superspher 100 RP-18e (250 mm × 4 mm I.D., particle size 4 μm), Merck. The column temperature was maintained at 35 °C, and the flow rate was tested from 0.8 to 1 mL/min for all the columns. Sample volumes of 10 μL were injected. CBAs were detected at the wavelengths corresponding to their absorption maxima: 212.2 nm for 2,3,4,5-CBA; 213.4 nm for 2,3,4,5,6-CBA; 239.9 nm for 4-CBA and 209.9 nm for the other CBAs.

Linear gradient programs using a mobile phase consisting of 10% (V/V) ACN (A) and 100% ACN (B) or MeOH (B) were employed to separate the CBAs. Several additives were used as pH modifiers in order to suppress CBA dissociation: acetic acid (0.1%), formic acid (0.1% and 0.2%), orthophosphoric acid (0.1%), and trifluoroacetic acid (0.05% and 0.1%). Each analysis was followed by a column equilibration step (8 min).

2.4. Soil sample preparation

The sandy–loamy soil collected from the garden of the Academy of Sciences of the Czech Republic was used for extraction tests. Its main properties were as follows: total organic carbon 0.8%, total organics 1.4%, pH 5.3, water-holding capacity 31% and granulometric composition: sand 50.9%, fine sand 31.2%, silt 10.8%, and clay 7.1%. 10 g of the homogenized soil was spiked 10 times with 100 μL of acetone solutions of the CBAs (100 μg/mL of each CBA), resulting in a final concentration 10 μg/mL of each CBA. The small volume steps were employed in order to avoid any contact of the solvent with the glassware and the soil was dried at laboratory temperature and was homogenized with a spoon after each step.

Two real historically PCB contaminated soils were collected in the Czech Republic for an application of the optimized analytical method. One of the soils was collected in central Bohemia in a refused heap in the town Lhenice (Soil A) and the second was from a former Soviet army military base Milovice from central Bohemia (Soil B). The soils were provided kindly by AECOM CZ s.r.o. and Dekonta a.s. companies. PCB concentration in the Soil A was 82 mg/kg (sum of PCB congeners 28, 52, 101,118, 153, 138, 180 according to US EPA method 8082) and the Soil B contained 175 mg/kg of PCBs in dry soil.

2.5. Extraction procedure

The extractions were carried out using Dionex 200 ASE extractor (Palaiseau, France). ASE samples were prepared by weighing 3.5 g of spiked soil, humidified with 350 μL H_2O and mixed with 3.5 g of Na_2SO_4 placed in the ASE cell (11 mL volume). In addition, the appropriate amount of washed sand was added at the bottom and on the top of the sample to fill the dead volume in the cell. Glassfiber filters were placed at the bottom and the top of each cell. The extractions were performed at temperatures of 100 °C and 150 °C and pressures of 10.34 MPa and 13.79 MPa with various solvent systems: methylene chloride, hexane/acetone (1:1, V/V), 1% acetic acid in hexane/acetone (1:1, V/V) and 1% formic acid in hexane/acetone (1:1, V/V). The details of the extraction conditions are summarized in Table 1. All the extraction conditions were performed in triplicate.

2.6. Pre-chromatographic sample adjustment

500 μL of DMSO was added to the collected organic extracts (approximately 20 mL in each vial) to avoid volatilization of the CBA analytes and the extracts were concentrated using a vacuum rotary evaporator at 60 kPa and 40 °C to approximately 1.5 mL. 50 μL of internal standard (IS, 2,3-dichlophenol 0.9 mg/mL in ACN) were added to each sample and the IS was used to calculate the vol-

Table 1
Description of the 3 ASE methods.

	Method A	Method B	Method C
Temperature (°C)	150	100	100
Pressure (MPa)	10.34	13.79	10.34
Cell preheat (min)	Off	Off	1
Cell heat up (min)	5	5	5
Static time (min)	7	7	7
Flush volume (%)	60	60	60
Purge time (s)	60	60	60
Static cycles	3	3	3

ume extracts. 1 mL of each sample was then centrifuged (9000 rpm, 10 min) and analyzed by HPLC.

2.7. Method validation

Selectivity and matrix effect. The selectivity of the HPLC method was determined by comparing the chromatograms of the CBA standards and a blank sample containing the soil matrix. This blank sample was prepared by extraction of the uncontaminated soil using ASE. The matrix effect was then evaluated by employing the method of analyte additions to an extract of uncontaminated soil at three different concentration levels, 5 (10), 40, and 80 μg/mL for CBAs and at concentration levels of 9, 90, and 180 μg/mL for the IS. The chromatograms were then compared in order to evaluate any possible interfering effect of the sample matrix with CBAs.

Calibration curve. The eight-point calibration curves over linear ranges were measured for all 15 CBAs from 5 μg/mL (2-CBA; 2,6-CBA; 3-CBA; 4-CBA; 2,3-CBA; 2,3,6-CBA; 2,5-CBA; 2,4-CBA; 2,4,6-CBA) and from 10 μg/mL (3,4-CBA; 2,3,5,6-CBA; 3,5-CBA; 2,3,5-CBA; 2,3,4,5,6-CBA; 2,3,4,5-CBA) to 120 μg/mL The calibration curve of the IS ranged from 3 to 300 μg/mL. Each point on the calibration curve represents the arithmetic mean of six values.

Limit of quantification. LOQ was determined as the lowest calibration standard level for each CBA and IS quantified with precision (relative standard deviation, RSD) and accuracy lower than 20%. Six replicates of CBAs (5 or 10 μg/mL) mixture and IS (9 μg/mL) were measured.

Accuracy and precision. To evaluate the precision and accuracy, quality control samples were prepared at concentrations of 5 (10), 20 and 80, 3, 90 and 180 μg/mL for 15 CBA mixtures and the IS, respectively. RSD was taken as a measure of the precision, and the percentage difference between the determined and spiked amounts was considered a measure of the accuracy.

3. Results and discussion

3.1. Optimization of the HPLC conditions

In this paper, four chromatographic columns filled with different sorbents from various providers (Synergy Polar RP, Xbridge C18 and LiChroSphere RP-18e and Superphere 100 RP-18e) were tested for separation of the 15 CBA isomers. Methanol appeared to not be a suitable component in the mobile phase due to its generally low ability to separate CBAs and a substantial negative effect on the peak shape of CBA that prevented complete separation of several CBAs (data not shown). Therefore, a further optimization was performed with ACN alone. The separation of the target compounds was optimized under acidic pH conditions in order to suppress CBA dissociation. The tested additives were employed at slightly different concentrations due to the pH stability of the tested columns, which was found to be crucial, especially for Lichrosphere and Supersphere columns. Due to the lack of stability of these columns, the data obtained from their application for the separation of CBAs, even at pH above 2, were not reproducible and the separation efficiency decreased rapidly after several chromatographic runs. The modifiers were added to the organic phase (B), to the water phase (A) or to both and the best resolution and peak shapes were generally achieved with 0.1% TFA in the aqueous phase, where other compositions mostly had a negative effect on the peak shapes. The order of suitability of the compounds was as follows: TFA ≥ formic acid > acetic acid > ammonium acetate > orthophosphoric acid. The peak shapes were similar when TFA and formic acid were used; however, formic acid significantly worsened the CBA separation (data not shown). Further testing was performed with XBridge C18 and Superspher 100 RP-18e columns, because of the satisfactory peak shapes of the separated CBAs. Several gradient programs (approximately 15 for the columns, data not shown), based on a multiple, stepwise increase in the acetonitrile ratio, were applied to the columns in order to obtain the separation of the individual CBAs. The best separation was obtained with the XBridge column when all the analytes were completely separated. A mixture of all 15 CBAs and IS were finally separated with 0.1% TFA in A using the best tested gradient conditions (min/%B): 0/17; 30/17; 60/34; 70/50. The duration of the analysis was 70 min and the applied flow rate was 0.8 mL/min. The results of the separation are displayed in Table 2.

There are a limited number of papers in the literature dealing with chromatographic determination of CBAs and those few published articles described only the HPLC separation of several mono- and dichlorinated derivatives. To our best knowledge, no publication is available on the separation of a larger group of

Table 2
Analyte retention characteristics and HPLC method efficiency.

Analyte	t_R (min)	t_R repeatability (%RSD)	Area repeatability (%RSD)	Resolution	Absorption maxima (nm)	LOQ (μg/mL)
2-CBA	12.96	0.15	4.49	–	210	5
2,6-CBA	14.81	0.13	6.64	3.06	210	5
3-CBA	24.00	0.17	4.68	11.42	210	5
4-CBA	25.77	0.11	6.23	2.29	240	5
2,3-CBA	27.70	0.24	6.69	2.64	210	5
2,3,6-CBA	28.89	0.18	7.08	1.45	210	5
2,5-CBA	30.27	0.06	6.60	1.63	210	5
2,4-CBA	37.72	0.19	6.60	8.70	210	5
2,4,6-CBA	39.70	0.25	6.50	2.24	210	10
IS	42.78	0.17	8.04	4.46	210	10
3,4-CBA	49.40	0.10	3.63	10.03	210	10
2,3,5,6-CBA	50.48	0.09	5.29	1.72	210	10
3,5-CBA	52.86	0.09	8.37	4.21	210	10
2,3,5-CBA	54.61	0.09	7.95	3.46	210	10
2,3,4,5,6-CBA	66.17	0.05	3.60	42.04	213	10
2,3,4,5-CBA	68.48	0.04	2.90	9.41	212	10

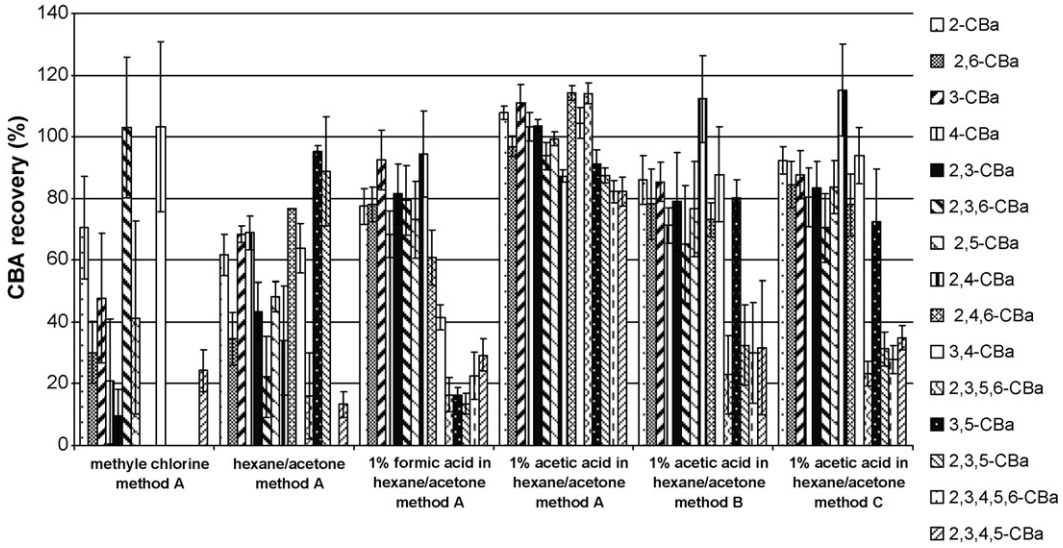

Fig. 1. Comparison of various solvents and various ASE methods for the determination of 15 isomers of chlorobenzoic acid.

CBA isomers. One of the first HPLC methods was published by Dietz et al. [9] where these authors elaborated an HPLC method in order to determine the concentrations of 2-CBA, 3-CBA and 4-CBA together with benzoic acid and chlorendic acid in landfill leachate using a Waters Nova-Pak C18 column and mobile phase of 0.2% acetic acid in water and acetonitrile. Another HPLC method for determination of the PCB degradation products including eleven CBAs (mono–tri chlorinated) using a Luna C18 column and methanol–water–H_3PO_4 mixture was also described by Macková et al. [17]. However, the authors analyzed CBAs only as single compounds and only an isocratic mode was employed. Adebusoye et al. [18] studied the influence of the presence 2-CBA, 3-CBA, 4-CBA and 2,3-CBA on the growth and degradation potential of PCB-degrading bacterial strains. The CBAs in this study were analyzed in liquid culture medium using HPLC with YMC-Pack ODS-AQ column and phosphate buffer–methanol–acetonitrile mobile phase. The results of our study showed that the tested classical particle sorbent columns were not able to separate the studied CBA mixture, in contrast to the XBridge column, which uses octadecyl silica Bridged Ethyl Hybrid (BEH) sorbents. This column also provided generally higher back pressure, which finally allowed us to decrease the flow rate to 0.8 mL/min. This caused a certain extension of the chromatographic run, particularly, leading to better separation without any negative influence on the peak shapes, as was observed for the other columns. A possible explanation could be the outstanding endcapping of the sorbent with BEH technology, enabling successful separation of CBAs; however, this was not found to be useful for other chlorinated pollutants, e.g.

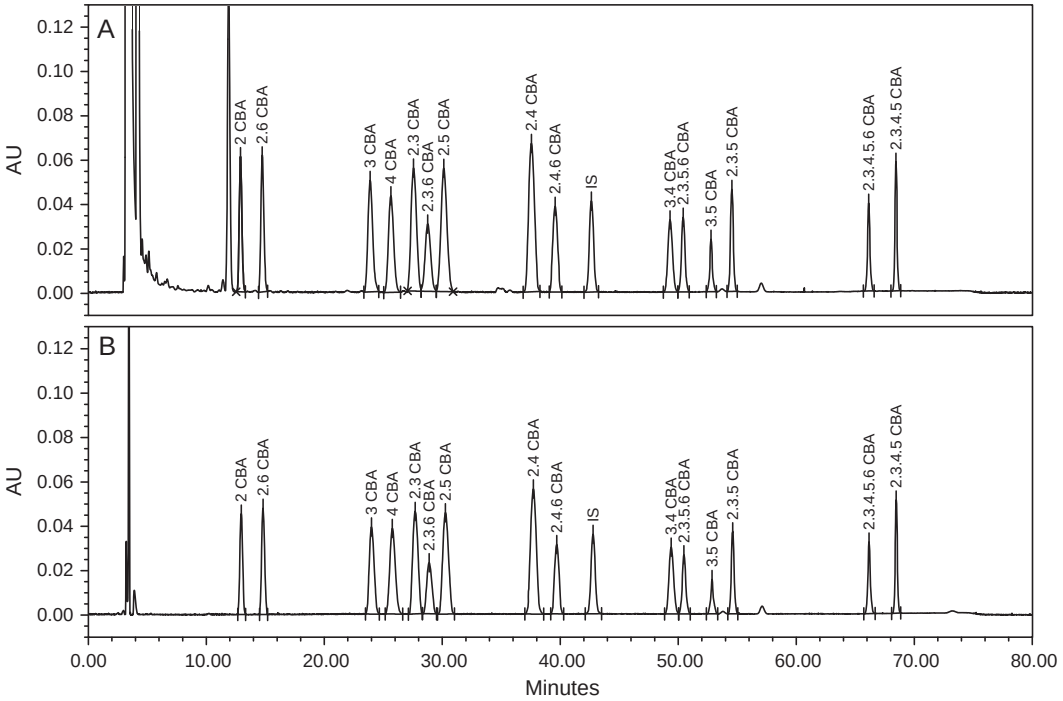

Fig. 2. Chromatogram of a spiked soil extract (A) and standard solution in acetonitrile (B) at the 35 μg/mL level. The chromatogram is displayed in the MAX Plot mode at its maximal absorption wavelengths (for CBAs see Table 2).

Table 3
Calibration curves, determination coefficients, accuracy and precision of 15 CBAs and IS in the HPLC method.

Analyte	Calibration curve	r^2	μg/mL	Accuracy (%)	%RSD
2-CBA	$y = 4.13 \times 10^4 x + 2.48 \times 10^4$	0.9996	5	95.4 ± 7.8	8.17
			40	99.1 ± 1.4	1.42
			80	98.5 ± 1.5	1.54
2,6-CBA	$y = 5.06 \times 10^4 x + 2.43 \times 10^4$	0.9997	5	90.9 ± 6.6	7.30
			40	99.8 ± 0.5	0.51
			80	100.1 ± 0.9	0.93
3-CBA	$y = 5.92 \times 10^4 x + 4.84 \times 10^4$	0.9996	5	90.5 ± 6.0	6.66
			40	98.8 ± 2.0	1.98
			80	101.3 ± 1.7	1.68
4-CBA	$y = 6.96 \times 10^4 x - 7.39 \times 10^4$	0.9991	5	95.2 ± 8.1	8.53
			40	101.0 ± 2.1	2.07
			80	102.4 ± 1.9	1.85
2,3-CBA	$y = 9.34 \times 10^4 x + 3.33 \times 10^4$	0.9994	5	96.8 ± 3.6	3.68
			40	100.0 ± 0.8	0.82
			80	98.5 ± 2.3	2.27
2,3,6-CBA	$y = 5.62 \times 10^4 x + 6.93 \times 10^3$	0.9990	5	96.9 ± 9.1	9.42
			40	99.8 ± 1.0	1.03
			80	100.4 ± 1.1	1.12
2,5-CBA	$y = 9.23 \times 10^4 x + 4.77 \times 10^4$	0.9994	5	81.5 ± 1.9	2.28
			40	100.1 ± 0.7	0.66
			80	100.8 ± 1.6	1.56
2,4-CBA	$y = 1.00 \times 10^5 x - 3.74 \times 10^5$	0.9992	5	102.5 ± 2.5	2.42
			40	101.4 ± 1.3	1.28
			80	98.6 ± 2.8	2.78
2,4,6-CBA	$y = 9.35 \times 10^4 x - 5.33 \times 10^5$	0.9990	10	108.6 ± 2.3	5.84
			40	101.1 ± 1.2	1.22
			80	96.7 ± 1.3	1.25
3,4-CBA	$y = 8.69 \times 10^4 x - 7.30 \times 10^5$	0.9989	10	107.5 ± 2.4	2.20
			40	101.3 ± 1.3	1.27
			80	95.0 ± 1.2	1.26
2,3,5,6-CBA	$y = 9.68 \times 10^4 x - 1.03 \times 10^6$	0.9989	10	103.0 ± 1.5	1.42
			40	101.9 ± 1.1	1.08
			80	91.9 ± 1.8	1.91
3,5-CBA	$y = 7.95 \times 10^4 x - 1.08 \times 10^6$	0.9992	10	114.2 ± 1.4	1.22
			40	101.7 ± 1.4	1.41
			80	91.9 ± 4.0	4.34
2,3,5-CBA	$y = 1.10 \times 10^5 x - 1.26 \times 10^6$	0.9989	10	114.3 ± 0.8	0.74
			40	101.8 ± 1.1	1.03
			80	93.0 ± 1.7	1.79
2,3,4,5,6-CBA	$y = 1.06 \times 10^5 x - 1.26 \times 10^6$	0.9993	10	114.4 ± 1.4	1.20
			40	101.7 ± 0.8	0.74
			80	93.3 ± 1.0	1.12
2,3,4,5-CBA	$y = 8.47 \times 10^4 x - 8.45 \times 10^5$	0.9990	10	112.2 ± 0.8	0.85
			40	101.6 ± 0.7	0.70
			80	92.4 ± 1.8	1.90
IS	$y = 8.95 \times 10^4 x - 3.32 \times 10^5$	0.9999	10	94.4 ± 1.0	1.10
			40	93.0 ± 1.2	1.25
			80	90.9 ± 0.3	0.34

polychlorinated biphenyls [19]. Another advantage of this column is its pH stability, which was documented by other authors utilizing XBridge (TM) HPLC columns for method development at extreme pH values [20] and which we found to be crucial for successful CBA separation.

3.2. Optimization of sample preparation

Sample preparation to ensure quantitative transfer of the analytes from the sample matrix represents another analytical step that is of equal importance to effective chromatographic separation. The extraction recovery experiments were performed using artificially spiked soil samples (at a level of 20 μg of each CBA per gram of soil).

For the development of the rapid and quantitative extraction method, various solvent systems (methylene chloride, hexane/acetone, 1% acetic acid in hexane/acetone and 1% formic acid in hexane/acetone) and various conditions were employed (see Table 1). It was found that the pressure had a negligible influence on the extraction recoveries in the tested range, in contrast to the temperature, where significantly better results were obtained at 150 °C. However, the selection of the solvents for ASE had a crucial influence on the recovery results. The results were compared on the basis of percent amount yield (amount of CBA in the extract/grams of sample). Fig. 1 shows an overview of the CBA recovery results mainly for the ASE method A (150 °C, 10.34 MPa) and also a comparison of the best solvent system (hexane–acetone, 1% acetic acid) applied at other pressures and temperatures. From

the results it is clear that much better recoveries were generally achieved after acidification of the organic solvents and the best conditions were obtained with hexane–acetone by adding 1% acetic acid. Using methylene chloride led to incomplete extraction of CBAs from the soil, even after addition of formic or acetic acid (data not shown). Using semi-polar solvent mixtures consisting of hexane and acetone led to an increase in the extraction efficiency, but pentachlorobenzoic acid was still absent in the extract. Reducing the extraction temperature during the ASE process had a significant negative impact on the tetra- and pentachlorine substituted benzoic acid recovery. Consistently with US EPA method for pressured fluid extraction [21] it was found that a pressure increase from 10.34 to 13.79 MPa in the extraction process had no or negligible influence on the recovery of the chlorinated analytes. The best results were then obtained at an extraction pressure of 10.34 MPa and temperature of 150 °C (method A). In this case, the recoveries were higher than 82% for all the tested CBAs. Carrying out each determination in triplicate clearly illustrates the variability, which was about 3–5%. The results also indicate that the selection of a pH modifier must be taken into consideration. The pH must be set to as low value in order to suppress CBA dissociation and to improve CBA solubility in the extraction solvent. However, the results show, that a simple selection of a stronger acid (i.e. formic acid), need not necessarily lead to higher recovery yields. This could possibly be because lower pH conditions charge the soil matrix, causing another interaction between the matrix and CBAs. An alternative explanation of the recovery changes after the applications of different modifiers, could lie in the type of matrix–analyte interactions and a possible competition of the modifiers with the analytes for active sites of the soil matrix. Similar behavior of analytes (nitro polycyclic aromatic hydrocarbons) was observed using ASE by other authors [22]. They observed an improvement in the extraction recovery after addition of acetic acid to the extraction solvent, suggesting possible competition between the acid and the analytes for active sites of the matrix.

3.3. Method validation and real sample analyses

The method selectivity and the matrix effect study were performed to verify the optimal conditions for quantification of 15 CBAs in the ASE soil extracts. Under the chromatographic conditions described in this paper, all 15 CBAs (including IS) were sufficiently separated (for resolution, see Table 2). On the basis of visual comparison of the two chromatograms alone, it is evident from Fig. 2 that no detectable general matrix effect was observed. Thus, no interfering components of the sample matrix were detected by the UV detector under the chromatographic conditions employed.

The calibrations curves were prepared over a linear range from 5 μg/mL for 2-CBA, 2,6-CBA, 3-CBA, 4-CBA 2,3-CBA, 2,3,6-CBA, 2,5-CBA, 2,4-CBA and 2,4,6-CBA and from 10 μg/mL for 3,4-CBA, 2,3,5,6-CBA, 3,5-CBA, 2,3,5-CBA, 2,3,4,5,6-CBA, and 2,3,4,5-CBA at eight (seven) concentration levels, 5, 10, 20, 30, 40, 60, 80 and 120 μg/mL. The IS calibration curve was prepared over a linear range from 9 to 300 μg/mL at six concentration levels, 9, 20, 40, 80, 150 and 300 μg/mL. The representative regression equations and determination coefficients are given in Table 3.

The accuracy and precision were determined by analyzing CBA samples at three concentrations levels in six replicates and the results are shown in Table 3. The lowest concentration also represents the LOQ.

The recovery of 15 CBAs in the matrix was tested at three concentration levels, LOQ (5 or 10 μg/mL), medium level (60 μg/mL) and high level (120 μg/mL) in six replications for each level. The matrix recovery for LOQ ranged from 85 ± 2 to 116 ± 0, for the medium level from 88.0 ± 1.1 to 116.0 ± 0.4 and for the high level from 86.9 ± 0.6 to 108 ± 1. The recovery of the IS was tested analogically to CBA at three concentrations levels (9, 90 and 180 μg/mL). The recovery in the sample matrix was for LOQ 94 ± 1, for the medium level 93 ± 1 and for the high level 91 ± 0.

Table 4
Results of CBA concentrations in real historically contaminated soils.

	Soil A (μg/g)	Soil B (μg/g)
2-CBA	n.q.[a]	n.q.
2,6-CBA	0.09	0.08
3-CBA	0.13	0.05
4-CBA	0.29	0.09
2,3-CBA	0.52	0.02
2,3,6-CBA	0.40	0.12
2,5-CBA	0.07	n.q.
2,4-CBA	0.62	0.08
2,4,6-CBA	0.34	0.64
3,4-CBA	n.q.	n.q.
2,3,5,6-CBA	n.q.	0.35
3,5-CBA	n.q.	n.q.
2,3,5-CBA	n.q.	0,19
2,3,4,5,6-CBA	n.q.	n.q.
2,3,4,5-CBA	n.q.	n.q.

The values were below the respective LOQ.
[a] Not quantified.

The two real historically contaminated soils were finally extracted and analyzed with the optimized methods. Due to low concentrations of CBA in the samples, we used a higher amount of soil for extraction (30 g) when also the volume of the extraction solvents was proportionally increased. The injection volume was also increased to 50 μL, resulting finally in 100 times concentrations of the samples. The results are displayed in Table 4. The results indicate that during the time of PCB contamination (decades of years) certain amounts of PCBs were transformed into CBAs in the soils. Such information could be taken into consideration when properties and e.g. self-treating potentials of a contaminated area are evaluated.

4. Conclusion

In this article, four various HPLC columns were tested for separation of 15 chlorobenzoic acid isomers. The best separation was obtained with the XBridge column when all the analytes were completely separated within 70 min, where the other tested columns generally exhibited poorer separation efficiency or lower stability for the optimized mobile phase. The optimized mobile phase consisted of water–acetonitrile and 0.1 TFA, where the use of other pH modifiers and methanol were found to have negative effects, either on the CBA separation or on the peak shapes. The newly developed HPLC method was partially validated and exhibited a linear range of detector response for quantification of CBAs, with acceptable correlation coefficients. The application of a UV detector allowed us to determine and verify LOQ at concentrations of 5 or 10 μg/mL. The accelerated solvent extraction method was tested with an artificially contaminated soil sample and its parameters were also partially optimized. Finally it was found that the use of hexane–acetone (V/V) as the extraction solvent with addition of 1% acetic acid at 10.34 MPa and 150 °C yielded an extraction recovery of greater than 82%. The extraction method was also verified and it was documented that no soil matrix compound interfered with CBAs during HPLC analysis. Certain problems were found with volatilization of CBAs during concentration of the samples and these difficulties were resolved by adding a small amount of DMSO to the extracts. To our knowledge, this is the first article describing HPLC separation of a larger group of CBAs. The optimized extraction and sample preparation process, together

with the chromatographic determination, allow analysis of CBAs in environmental soil samples, as demonstrated in the cases of two real PCB historically contaminated soils. The method can be used directly, especially by experts studying PCB biodegradation, where the presence of CBAs as "dead-end metabolites" was identified as a substantial problem because of their inhibiting effects on further PCB transformation.

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References

[1] J.A. Field, R.S. Alvarez, Environ. Pollut. 155 (2008) 1–12.
[2] S.R. Sorensen, M.S. Holtze, A. Simonsen, J. Aamand, Appl. Environ. Microbiol. 73 (2007) 399–406.
[3] T. Gichner, P. Lovecká, B. Vrchotová, Mutat. Res. 657 (2008) 140–145.
[4] M. Muccini, A.C. Layton, G.S. Sayler, T.W. Schultz, Bull. Environ. Contam. Toxicol. 62 (1999) 616–622.
[5] Y.H. Zhao, G.D. Ji, M.T.D. Cronin, J.C. Dearden, Sci. Total Environ. 216 (1998) 205–215.
[6] P.Y. Lee, C.Y. Chen, J. Hazard. Mater. 165 (2009) 156–161.
[7] K. Svobodová, M. Plačková, V. Novotná, T. Cajthaml, Sci. Total Environ. 407 (2009) 5921–5925.
[8] D.H. Pieper, M. Seeger, J. Mol. Microbiol. Biotechnol. 15 (2008) 121–138.
[9] E.A. Dietz, N.J. Cortellucci, K.F. Singley, J. Liq. Chromatogr. 16 (1993) 3331–3347.
[10] A.J. Lambo, T.R. Patel, Curr. Microbiol. 53 (2006) 48–52.
[11] T.J. Gentry, G. Wang, C. Rensing, I.L. Pepper, Microb. Ecol. 48 (2004) 90–102.
[12] D. Drăgan, S. Cucu-Man, R. Mocanu, A. Covaci, Rev. Roum. Chim. 52 (2007) 597–601.
[13] W. Wang, B. Meng, X. Lu, Y. Liu, S. Tao, Anal. Chim. Acta 602 (2007) 211–222.
[14] O. Zuloaga, N. Etxebarria, L.A. Fernández, J.M. Madariaga, Fresen. J. Anal. Chem. 367 (2000) 733–737.
[15] V. Niedan, H.F. Scholer, Chemosphere 35 (1997) 1233–1241.
[16] I. Kamei, R. Kogura, R. Kondo, Appl. Microbiol. Biotechnol. 72 (2006) 566–575.
[17] M. Macková, B. Vrchotová, K. Francová, M. Sylvestre, M. Tomaniová, P. Lovecká, K. Demnerová, T. Macek, Eur. J. Soil Biol. 43 (2007) 233–241.
[18] S.A. Adebusoye, F.W. Picardal, M.O. Ilori, O.O. Amund, C. Fuqua, Biodegradation 19 (2008) 145–159.
[19] J. Olšovská, Z. Křesinová, M. Flieger, T. Cajthaml, Talanta 80 (2010) 1849–1855.
[20] K. Jenkins, D. Diehl, D. Morrison, J. Mazzeo, LC GC Eur. – The Application Notebook, September (2005) 42–43.
[21] US EPA, Method 3545A, Pressurized fluid extraction (PFE), Environmental Protection Agency, 2007, http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3545a.pdf.
[22] K. Oukebdane, F. Portet-Koltalo, N. Machour, F. Dionnet, P.L. Desbene, Talanta 82 (2010) 227–236.