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Simultaneous determination of chlorophenols, chlorobenzenes and chlorobenzoates in microbial solutions using pentafluorobenzylbromide derivatization and analysis by gas chromatography with electron-capture detection

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Abstract

A sensitive method was developed for the simultaneous determination in aqueous media of pentachlorophenol (PCP), 2,4,6-trichlorophenol (TCP), 2,4-dichlorobenzoic acid (DCBA), hexachlorobenzene (HCB) and 1,2,4-trichlorobenzene (TCB), model compounds for biodegradation research. Compounds were extracted into dichloromethane, solvent was evaporated, PCP, TCP and DCBA were derivatized using pentafluorobenzylbromide and all compounds were determined by gas chromatography with electron-capture detection. Florisil clean-up was developed for use when necessary. Linear calibration curves (HCB, 0.5–5.0 μg/l; others, 0.1–10 mg/l) were obtained in culture media in the presence and absence of the Gram negative bacterium, S14. The method is suitable for laboratory studies of bioremediation.

Keywords: Derivatization, GC; Environmental analysis; Organochlorine compounds; Chlorophenols; Chlorobenzenes; Chlorobenzoates

1. Introduction

Chlorinated monoaromatic compounds have been used in large quantities as solvents, lubricants, insulators, insecticides, herbicides and plasticizers [1]. This usage coupled with inadequate storage and waste treatment has resulted in accidental or deliberate release of chloroaromatics and ultimate contamination of the environment, where these pollutants tend to persist [2,3]. Low aqueous solubilities, high octanol–water partition coefficients and both deactivation and steric hindrance due to the number and position of chlorines on the aromatic ring all contribute to resistance to biodegradation [1]. Mixtures of compounds are always encountered in contaminated sites and waste treatment processes. There have been few investigations of the biodegradation of mixtures of chloroaromatics, especially of more than one class of compound [4]. Studies on the biodegradation of mixtures containing chlorinated benzenes, phenols and benzoates at aqueous solubility levels require a quantitative method for their simultaneous determination over a working range of 1–100% of the aqueous solubilities of the compounds in the presence of at least 10^6 colony forming units of bacteria per millilitre (cfu/ml).

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Gas chromatography with electron-capture detection (GC–ECD) is exquisitely sensitive to polyhalogenated compounds and has been used for analysis of chlorobenzenes in biodegradation studies [5,6]. Oliver and Nicol [7] reported detection limits for hexachlorobenzene (HCB) of 0.05 ng/g in sediments and 0.01 ng/l in water by GC–ECD. Adequate sensitivity for chlorinated phenols and benzoates by GC–ECD, needed for biodegradation studies at solubility levels, has not been reported. Lindström and Nordin [8] were able to detect 2,4,6-trichlorophenol (TCP) and 2,4-dichlorophenol (DCP) in kraft pulp mill effluent by GC coupled to flame ionization detection (FID) but they could not detect DCP in similar samples by GC–ECD.

When phenols and benzoates are analysed on GC columns suitable for the analysis of chlorinated benzenes, non-specific interactions between the polar groups of those molecules and the stationary phase often cause severe peak tailing. Derivatization of the polar groups with reagents containing halogens can generate volatile derivatives, readily detected by ECD. Flophemesyl derivatives of alcohols, phenols and carboxylic acids [9,10] and pentafluorobenzylbromide (PFBBr) derivatives of carboxylic acids [11,12] have been determined in the picogram to femtogram range. Extraction and derivatization with PFBBr allowed determination of chlorinated phenols in urine at 0.5 μg/l [13].

This paper describes an analytical method for the simultaneous determination of pentachlorophenol (PCP), TCP, 2,4-dichlorobenzoic acid (DCBA), 1,2,4-trichlorobenzene (TCB) and HCB at concentrations down to 1 μg/l for the latter and 100 μg/l for others by GC–ECD. The method allows the biodegradation of mixtures of model chloroaromatic compounds to be conveniently studied.

2. Experimental

2.1. Internal standards

To each sample (10.0 ml), containing HCB, TCB, PCP, TCP and DCBA in minimal salts medium (MSM) at pH 7 in 12-ml culture tubes was added 5 μl of a mixture of internal standards in ethyl acetate: 1,2-dichloro-4-fluorobenzene (DCFB; 10 mg/ml), 2,6-dichloro-4-fluorophenol (DCFP; 10 mg/ml), 2-chloro-4-fluorobenzoic acid (CFBA; 10 mg/ml) and 1,4-dibromobenzene (DBB; 0.02 mg/ml).

2.2. Solvent extraction and evaporation

Each sample was adjusted to pH$\leq$2 with 2.5 *M* H_2SO_4, and extracted for 1 min with 1 ml of dichloromethane (DCM). The mixture was centrifuged for 5 min and the organic phase separated. Extraction was repeated with three further 1-ml aliquots of DCM. The extracts were combined, evaporated under a stream of clean dry nitrogen to 5 μl and transferred to a 100-μl vial.

2.3. Derivatization with PFBBr

Acetonitrile (10 μl), 8 μl of N,N-diisopropylethylamine and 8 μl of PFBBr (25%, v/v, in acetonitrile) were added to each vial and derivatization was allowed to proceed at room temperature for 40 min. This was a modification of the method of Hofmann et al. [11]. The mixture was evaporated under a stream of nitrogen to 5–10 μl then diluted to 100 μl with ethyl acetate.

2.4. Instrumental finish

The ethyl acetate solution (100 μl) was diluted 1:100 in hexane, then 1:1 with the injection standard, (octafluoronaphthalene, 2 μg/ml in hexane) prior to injection of an aliquot of 1 μl into the GC system.

2.5. Sample clean-up

Clean-up is not required unless samples are particularly contaminated or are to be analysed by on-column injection. Florisil (3.5 g, activated by heating overnight at 650°C) was placed in a chromatography column (20 cm$\times$1 cm) and a 1 cm layer of sodium sulfate was placed on top. The solution containing TCB, HCB and PFBBr derivatives of PCP, TCP and DCBA was added to the column and eluted with hexane (5 ml), 6% diethyl ether in hexane (20 ml) and 30% diethyl ether in hexane (15 ml). Eluate was pooled, evaporated to 5 ml and diluted to 10 ml with hexane.

2.6. Method calibration and validation

Mixtures of PCP, TCP, DCBA and TCB at 0.1–10.0 mg/l and HCB at 0.1–5.0 μg/l in MSM, in the presence and absence of 10^8 cfu/ml of the Gram-negative bacterium S14 [14], were analysed by the method. Recoveries from MSM of HCB at 2.5 and 5.0 μg/l and other compounds at 0.5 μg/l and 10 mg/l were determined.

2.7. PFBBr derivatization of metabolites

Potential interference with detection of the five chlorinated aromatic compounds by other materials reported as biodegradation metabolites was investigated. Succinic acid, salicylic acid, chlorohydroquinone, 3-chloro-4-hydroxybenzoic acid, chloroacetic acid, 2-chloropropionic acid, tetrachlorocatechol, dichloromalealdehydic acid, 4-hydroxybenzoic acid and catechol were dissolved separately in acetonitrile (1 mg/ml). Ten micrograms of each compound were derivatized and analysed as above.

2.8. Chemicals

PCP (99%), TCP (98%), HCB (99%), DCFB (99%), DCFP (99%), CFBA (99%), octafluoronaphthalene (96%), PFBBr (>99%), flophemesyl chloride (97%), N,N-diisopropylethylamine (99%), 3-chloro-4-hydroxybenzoic acid (98%), tetrachlorocatechol (98%); chloroacetic acid (>99%), Florisil (250–420 μm) and sodium sulphate were obtained from Aldrich (Milwaukee, WI, USA). TCB (97%), DCBA (97%), DBB (99%), chlorohydroquinone (80–90%), succinic acid (>99.5%), catechol (99%), salicylic acid (>99.5%) and 4-hydrobenzoic acid (99%) were obtained from Fluka (Buchs, Switzerland). Dichloromalealdehydic acid (99%) and 2-chloropropionic acid (96%) were purchased from Merck (Munich, Germany). Flophemesyl amine (>98%) was sourced from Lancaster (Eastgate, UK). Solvents were nanograde and supplied by Rhone-Poulenc (Leon, France).

2.9. Minimal salts medium

MSM contained the following chemicals (mg/l) in distilled water: $NaH_2PO_4 \cdot 2H_20$ (1560), $MgSO_4 \cdot 7H_2O$ (500), KNO_3 (210), $CaCl_2 \cdot 2H_2O$ (100), $FeSO_4 \cdot 7H_2O$ (4.5), EDTA (192), $CuCl_2 \cdot 2H_2O$ (0.015), $NiCl_2 \cdot 6H_2O$ (0.025), $Na_2MoO_4 \cdot 2H_2O$ (0.025), $ZnCl_2$ (0.07), $MnCl_2 \cdot 4H_2O$ (0.1), $CoCl_2 \cdot 6H_2O$ (0.12), $FeCl_3$ (4), *p*-aminobenzoic acid (0.05), pyridoxine–HCl (0.1), thiamine–HCl (0.05), riboflavine (0.05), nicotinic acid (0.05), *d*-calcium pantothenate (0.05), lipoic acid (0.05), nicotinamide (0.05), vitamin B_{12} (0.05), biotin (0.02) and folic acid (0.02). All ingredients were analytical grade.

2.10. Chromatography

Samples were analysed using a Perkin-Elmer Autosystem GC equipped with a nickel (^{63}Ni) (ECD System). The fused-silica capillary column was a SGE bonded phase BP5 (25 m×0.22 mm I.D.) with a film thickness of 0.25 μm. The carrier gas was helium (1 ml/min) and the make-up gas was nitrogen (20–30 ml/min). The injector and detector temperatures used were 250°C and 300°C, respectively. The temperature profile for analysis was isothermal at 60°C for 5 min followed by a temperature ramp of 10°C/min to 180°C and a second ramp of 5°C/min to 240°C which was maintained for 5 min. GC was run in split mode with a split ratio of 100:1. Data were analyzed with a Perkin-Elmer 1020 GC Plus integrator.

3. Results and discussion

3.1. Solvent extraction

Four 1-ml aliquots of DCM extracted the model chlorinated aromatic compounds from aqueous samples with extraction efficiencies of 84 to 100% (Fig. 1). DCBA, the most polar compound, was extracted least efficiently and was the only one to require a fourth DCM aliquot.

3.2. Derivatization with PFBBr

Following the method of Hofmann et al. [11], PFBBr derivatives of TCP, PCP, DCBA and the internal standards DCFP and CFBA were readily obtained, sensitively detected by GC–ECD and well resolved chromatographically (Fig. 2). The concen-

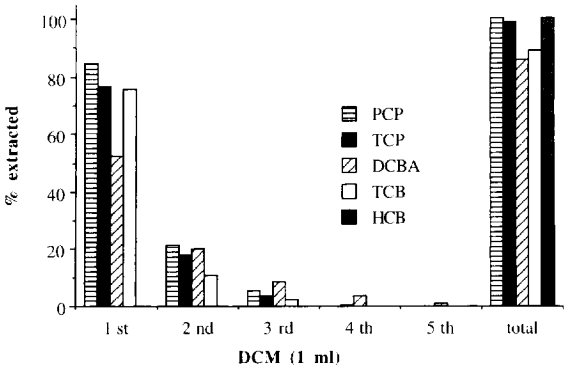

Fig. 1. PCP, TCP, DCBA and TCB at an initial concentration of 10 mg/l were extracted separately from MSM by five 1-ml aliquots of dichloromethane (DCM). Extraction efficiencies were determined by UV absorption at the absorption maxima: TCB, 280 nm; DCBA, 284 nm; TCP, 287 nm and PCP, 300 nm. HCB (0.5 μg/l) was extracted by four DCM aliquots which were pooled, evaporated under nitrogen, diluted in hexane and analysed by GC–ECD.

tration and volume of PFBBr, the volume of N,N-diisopropylethylamine and incubation time were optimized for derivatization. Optimal derivatization was found at 40 min for a mixture of 100 μg of PCP, TCP and DCBA. Derivatization of PCP increased by 50% between 20 and 30 min reaching a limit at 40 min whereas the majority of DCBA and TCP were derivatized in the first 15 min. Linear calibration

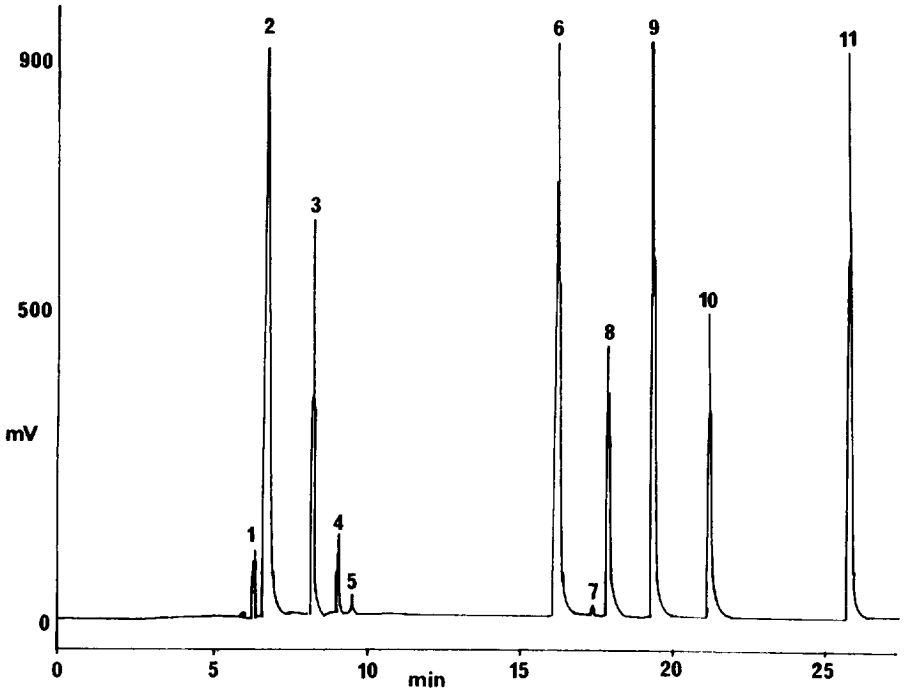

Fig. 2. Chromatogram of chloroaromatic compounds and standards: (1) DCFB (internal standard for TCB); (2) PFBBr (derivatization reagent); (3) octafluoronaphthalene (injection standard); (4) TCB; (5) DBB (internal standard for HCB); (6) DCFP (internal standard for PCP); (7) HCB; (8) CFBA (internal standard for DCBA); (9) TCP; (10) DCBA and (11) PCP. Phenolic and carboxylic analytes were present as PFBBr derivatives.

curves (correlation coefficients >0.99) were found for PCP, TCP and DCBA derivatized by 8 μl of 25% PFBBr for 40 min.

PFBBr derivatization as described here is simpler and faster than that of Veningerová et al. [13], who determined chlorophenols in urine by derivatizing compounds with PFBBr for 3 h at 60°C in the presence of acetone and K_2CO_3, followed by replacement of acetone with hexane as solvent.

3.3. Derivatization with flophemesyl amine

Flophemesyl amine was also investigated as a derivatization reagent for DCBA, PCP and TCP following Francis et al. [10]. TCP was successfully derivatized but we were unable to derivatize PCP or DCBA despite numerous modifications to the method, including the use of flophemesyl chloride as catalyst or derivatization reagent.

3.4. Florisil clean-up

The loss of chloroaromatics during the clean-up procedure was investigated by collecting and analysing 5 ml volumes of the eluate. All of the HCB and TCB were eluted from the column in the first 5 ml of hexane (Table 1). The majority of the TCP was eluted in the second 5-ml volume of 6% diethyl ether in hexane; a marginal increase in recovery was obtained with a further elution. The second and third 5-ml volumes of 6% diethyl ether in hexane eluted most of the PCP, and almost all of the DCBA was eluted in the first and second 5-ml volumes of 30% diethyl ether in hexane. Small losses of the derivatives of the chlorinated phenols (11–15%) were found to occur during the clean-up procedure, but will be compensated for during calibration.

3.5. Method calibration and validation

Calibration curves for PCP, TCP, DCBA, HCB and TCB in MSM were linear, both in the absence and presence of bacteria (Table 2). The gradient of the regression line was reduced in the presence of bacteria for PCP and HCB but increased slightly for the others. This indicates that, for biodegradation research, calibration curves must be constructed using samples containing the bacteria being studied. Between 96% and 103% of PCP, TCP, DCBA and TCB at 10 mg/l and 100% of HCB at 2.5 μg/l were recovered by the proposed method (Table 3).

3.6. Internal standards

Fluorinated and brominated internal standards were chosen with chemical and physical properties closely related to the parent compounds: 2-chloro-4-fluorobenzoic acid for 2,4-DCBA, 1,2-dichloro-4-fluorobenzene for 1,2,4-TCB, 2,6-dichloro-4-fluorophenol for 2,4,6-TCP (and PCP) and DBB for HCB.

Table 1
Recovery of chlorinated aromatic compounds[a] from Florisil for clean-up procedure

Eluent	Eluate[b]	% HCB[c] recovery	% TCB recovery	% PCP recovery	% TCP recovery	% DCBA recovery
Hexane	1st	100	100	0	0	0
6% Diethyl ether in hexane	1st	0	0	0	0	0
	2nd	0	0	39	86	0
	3rd	0	0	34	3	0
	4th	0	0	8	0	1
30% Diethyl ether in hexane	1st	0	0	3	0	19
	2nd	0	0	1	0	76
	3rd	0	0	0	0	2
	4th	0	0	0	0	0
Total		100	100	85	89	98

[a]Masses added were: HCB, 50 μg; TCB, 145 μg and PFBBr derivatized PCP, TCP and DCBA, 60 μg each.
[b]Eluate was collected in 5-ml volumes.
[c]Recoveries were determined by GC–ECD.

Table 2
Regression data for analysis of PCP, TCP, DCBA, HCB and TCB in MSM

Compound[a]	Slope	Intercept	No of data points	Standard error	Correlation coefficient
PCP (−)	0.084	−0.015	15	0.28	1.00
PCP (+)	0.043	0.011	12	1.04	0.94
TCP (−)	0.107	0.029	15	0.32	0.99
TCP (+)	0.141	0.044	12	0.45	0.99
DCBA (−)	0.120	0.024	15	0.16	1.00
DCBA (+)	0.171	0.019	12	0.61	0.98
HCB (−)	0.240	0.038	12	0.24	0.99
HCB (+)	0.219	0.077	12	0.33	0.98
TCB (−)	0.248	0.056	15	0.28	1.00
TCB (+)	0.292	0.001	12	1.21	0.96

[a] HCB (0.5, 1.0, 2.5 and 5.0 μg/l) and others (0.1, 0.5, 1.0, 5.0 and 10.0 mg/l) were analysed as mixtures in MSM. (−) in the absence of bacteria. (+) in the presence of 10^8 colony forming units per millilitre of bacterium S14, a Gram-negative environmental isolate.

Table 3
Recovery of chlorinated aromatic compounds by the proposed method from MSM

Compound	Concentration added (mg/l)	Concentration found (mg/l)	Recovery (%)
PCP	0.500	0.543	109
	10.0	9.99	100
TCP	0.500	0.506	101
	10.0	10.3	103
DCBA	0.500	0.519	104
	10.0	9.75	98
TCB	0.500	0.517	103
	10.0	9.60	96
HCB	0.00250	0.00251	100
	0.00500	0.00495	94

These compounds could not be produced as metabolites of chloroaromatic compounds.

3.7. Derivatization of potential metabolites

Potential metabolites were investigated for interference with compounds of interest (Table 4). PFBBr derivatives of all compounds were well separated.

3.8. Sensitivity and applicability of method

The calibration plot was linear between 10 mg/l and 100 μg/l for PCP, TCP, DCBA and TCB. Biodegradation over this range represents 99% removal of the original material, and the sensitivity of the method is therefore sufficient for such studies.

Table 4
Retention times of PFBBr derivatives of potential metabolites

Metabolite[a]	Retention time (min)
Chloroacetic acid	11.45
Chloropropionic acid	11.54
Salicylic acid	17.78
Catechol	22.87
Succinic acid	23.08
Dichloromalealdehydic acid	25.44
Chlorohydroquinone	28.21
4-Hydroxybenzoic acid	30.95
Tetrachlorocatechol	33.55
3-Chloro-4-hydroxybenzoic acid	34.70

[a] Metabolites (10 μg) in 15 μl of acetonitrile were derivatized by PFBBr (8 μl, 25% in acetonitrile) in the presence of N,N-diisopropylethylamine (8 μl) at room temperature for 40 min.

Mueller et al. [2] reported the limit of detection of PCP as 100 μg/l by GC of its trimethylsilyl derivative. Our method has similar sensitivity and could be made more sensitive with a modification to the final dilution step. Gerritse and Gottschal [15] investigated the biodegradation of 2,3,6-trichlorobenzoic acid and reported a linear response to the methylated derivative at 2.25 mg/l by GC–FID. Without modification, our method detected DCBA with greater sensitivity.

HCB response was linear between 0.5 and 5.0 μg/l. If required, improved sensitivity to HCB can be obtained by carrying out the solvent extraction under alkaline conditions (pH≥11), with hexane in place of DCM as the solvent. The pooled hexane fraction containing HCB can be evaporated to a small volume and injected directly into the GC system. The deprotonated polar analytes remain in the aqueous phase. If desired, analysis can continue as before, after adjustment of the sample pH to 2.

The recent report of the biodegradation of a mixture of chlorobenzenes and chlorophenols in slow sand filters [4] did not describe the analytical procedures for these individual components. A method devised for the detection of organic contaminants in drinking water reported the detection of TCP, PCP and HCB at 0.05 μg/l [16]; however, the large sample size (400 ml) would prohibit its use for most biodegradation research. Furthermore, drinking water contains comparatively low bacterial numbers and the effect of a high bacterial load is unknown.

Chlorinated pollutants are encountered as mixtures in contaminated sites and during waste treatment processes. There is, however, a dearth of information on the biodegradation of such mixtures. This method for simultaneous determination of chlorinated benzenes, phenols and benzoic acids in the presence of bacteria will be useful for laboratory studies of bioremediation and may be extended to monitoring of contaminated sites.

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