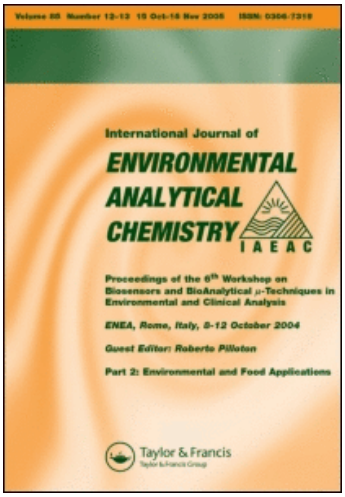

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

GC-FID Determination of Chlorobenzene Isomers in Methanogenic Batch-Cultures from River Sediments

H. -J. Stan[a]; N. -H. Kirsch[a]
[a] Institute of Food Chemistry, Technical University of Berlin, Berlin, Germany

To cite this Article Stan, H. -J. and Kirsch, N. -H.(1995) 'GC-FID Determination of Chlorobenzene Isomers in Methanogenic Batch-Cultures from River Sediments', International Journal of Environmental Analytical Chemistry, 60: 1, 33 — 40
To link to this Article: DOI: 10.1080/03067319508027225
URL: http://dx.doi.org/10.1080/03067319508027225

Intern. J. Environ. Anal. Chem., Vol. 60, pp. 33–40
Reprints available directly from the publisher
Photocopying permitted by license only

GC-FID DETERMINATION OF CHLOROBENZENE ISOMERS IN METHANOGENIC BATCH-CULTURES FROM RIVER SEDIMENTS

H.-J. STAN and N.-H. KIRSCH

Institute of Food Chemistry, Technical University of Berlin, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany

(Received, 3 March 1994; in final form, 24 November 1994)

The investigation of the biological reductive dechlorination of chlorobenzenes by methanogenic consortia in river sediments is dependent on reliable analytical procedures. Two different extraction methods for the determination of chlorobenzenes from sediment samples were developed. Lower chlorinated benzenes up to the trichloro isomers were extracted with satisfactory recoveries by thoroughly mixing the sediment with an equal volume of acetone. After centrifugation, the supernatant was subjected to solid phase extraction (SPE) using RP-C18 adsorbent. The tetra-, penta- and hexachlorobenzene isomers could only be satisfactorily recovered by liquid-liquid extraction with ethyl acetate. With both methods, 2,4-dichlorotoluene was added as surrogate standard. Determination was carried out by gas chromatography using a thick film megabore column coated with methyl silicone containing 35% phenyl groups and flame ionization detection. Applying a temperature program, all chlorobenzene isomers including benzene were found to be well separated and exhibiting good peak shape with the exception of 1,2,3,5- and 1,2,4,5-tetrachlorobenzene which formed a critical pair under all chromatographic conditions. Recovery values for most analytes at the 10 µg/ml concentration level applying the internal standard method for quantitative analysis were found to be better than 75% for both extraction methods.

KEY WORDS: Chlorobenzenes, extraction, river sediment, reductive dechlorination.

INTRODUCTION

Chlorobenzenes (CBs) are regarded as ubiquitous pollutants in the environment[1]. They have been found in waste soil[2] and waste water[3], in river water[4], drinking water, sediments[3] and fish[5,6]. The toxicity of these compounds was reported to correlate to the bioconcentration factors and to increase with increasing chlorine substitution[7,8]. All dichlorobenzenes (DCBs), 1,2,4-trichlorobenzene (1,2,4-TrCB) and hexachlorobenzene (HxCB) have been classified as priority pollutants by the United States Environment Protection Agency (US EPA)[1] as well as by the European Community (EC)[9] in 1991.

Due to the ubiquitous occurrence of CBs the properties of microbiological degradation became a field of interest during the last two decades. It was shown that complete mineralization is possible for some CBs by different bacteria[10]. In general, aerobic microorganisms do possess the more efficient enzymes for mineralization by ring fission and subsequent dechlorination but are able to degrade only lower chlorinated CBs up to tri-/ tetra-chlorobenzenes[11,12].

Highly chlorinated benzenes, however, can be attacked by anaerobic organisms. The reactions involved are summarized under the term "reductive dechlorination"[13].

Adaptation of undefined methanogenic consortia by batch technique is an important tool for the enrichment of efficient cultures to use in bioreactors. For the evaluation of the substrate specificity and dechlorination rates of the cultures, an analytical procedure of sufficient reliability involving simple extraction methods is required to cope with the high number of samples. To follow the changes in the concentration profile of chlororbenzenes during successive dechlorination, the use of a FID instead of the frequently used ECD is recommended, because the FID offers equal responses to chlororbenzenes with varying numbers of chlorine atoms. The varying response factors of the ECD for dichloro- to hexachloro-compounds within the range of 10^3–10^4 lead to highly different detection limits which represents -with regard to the limited linearity of the ECD signal- a vast disadvantage for reliable routine analyses.

Additionally monochlorobenzene shows a very poor response to the ECD, whereas benzene is not detectable at all. Finally it is not possible to use a single appropriate internal standard for the analysis of all chlorobenzenes with ECD detection.

In this paper we present two simple extraction procedures and an optimized gas chromatographic method for the analysis of all chlorobenzene isomers in sludge.

EXPERIMENTAL

Materials

Benzene, 1,2-, 1,3-, 1,4-dichlorobenzene, 1,2,3-, 1,2,4-, 1,3,5-trichlorobenzene, 1,2,3,4- and 1,2,4,5-tetrachlorobenzene as well as penta- and hexachlorobenzene were of highest purity (> 99.9%) from Promochem, Wesel, Germany. Monochlorobenzene (99,9%) was purchased from Dr. Ehrensdorfer, Augsburg, Germany, and 2,4-dichlorotoluene used as surrogate standard (ISTD) from Aldrich, Steinheim, Germany.

These chemicals were used for the calibration of the GC system and for the recovery experiments. Degradation studies with the methanogenic cultures were carried out with chlorobenzenes p.A. (> 98% purity) purchased from Merck, Darmstadt, Germany.

Solvents acetone, n-hexane and ethyl acetate were Pestanal® grade from Riedel de Hâen, Seelze, Germany. Sodium sulfate p.A., from Merck, was dried at 400°C for at least one hour before use. Silica gel, 40–60 μm mesh, from Merck, was dried at 130°C overnight and deactivated with 5% water before use. Solid phase extraction (SPE) was performed with modified silica gel RP-C18, 40 μm, 60 Å from Baker, Deventer, The Netherlands, which was purified by Soxhlett extraction with n-hexane for at least 6 hours before use. SPE was performed with an extraction bank SPE-12G and borosilicate extraction cartridges Bakerbond 7328–03 from Baker.

Ultrasonic treatment was performed with a Sonorex RK 514 M from Bandelin, Berlin, Germany, with a high frequency of 35 kHz and an output range from 225–450 W. Further instruments were a Biofuge 22 R from Heraeus Sepatech, Hanau, Germany and a Vortex from Heidolph, Germany.

Treatment of sludge samples

The river sediment was stored in one litre blood serum bottles, sealed by a cap with a rubber septum. The sampling procedure started by shaking each bottle for a few minutes in order to homogenize the sludge within the aqueous phase. The bottle was then fixed in

a horizontal position and 2 ml of the homogenate was drawn through the spetum. Samples were taken by a glass syringe with a steel needle of 2 mm ID. Spike solutions were also added through the septum. The syringe contents were transferred quantitatively into a 10 ml glass centrifugation tube with teflon cap. 1 ml of the aqueous internal standard (usually 10 µg 2,4-dichlorotoluene/ml) was then added and mixed thoroughly using a Vortex shaker for about one minute. Further treatment differed according to the extraction procedure:

Solid phase extraction (SPE): 3 ml of acetone were added to the tube containing the sludge homogenate and internal standard and shaken with a Vortex shaker for 5 minutes. The samples were centrifuged at 3000 rpm and 8°C for 10 minutes; subsequently the supernatant phase was percolated through a preconditioned SPE cartridge containing 200 mg RP C-18 material. Conditioning included washing with 3 ml of methanol followed by 3 ml of double distilled water. The flow through the column during conditioning and sampling was held at 5–8 ml/min. The column was afterwards washed with a few ml of water and subsequently percolated with 1 ml of n-hexane by means of a gentle stream of nitrogen. The eluate was collected in autosampler glass vials of 1.8 ml volume. The co-eluting water was manually removed from the bottom of the vial by means of a Pasteur pipette. The capped vials were stored at 4°C until measured by GC.

Liquid extraction (LE): 2 ml of ethyl acetate were added; shaking and centrifugation were identical to the SPE-procedure. After centrifugation, the greenish coloured supernatant (about 1.5 ml) was transferred to a small clean-up column. This was carried out simply by using a SPE-cartridge with a teflon fit at the bottom and filling the cartridge with 0.5 g deactivated silica gel overlayered by 1 g dried sodium sulfate. The column was eluted with 0.5 ml n-hexane and the eluate was collected in an autosampler vial which was capped ready for GC analysis. However, when benzene was included in the analysis, n-hexane was used as liquid extraction solvent with poorer recovery rates for higher chlorinated benzenes.

Recovery experiments

Recovery experiments were performed with river sediment cultured for more than a year under anaerobic conditions. The methanogenic batch cultures were fed with fermentable substrates and chlorobenzenes over the entire cultivation period and showed good dechlorination activity. Studies were performed by spiking two bottles of 1 litre volume with 5 ml of an acetone/methanol solution containing all CBs except 1,2,3,5-tetrachlorobenzene in a range of 9,5–13,5 mg for each analyte, resulting in a concentration of about 10 µg/ml. This concentration is relevant with respect to the biotransformation process in bioreactors. The bottles were mechanically shaken for 6 hours. The biological activity of one bottle was inhibited by poisoning the culture with 10 g sodium azide 5 days before spiking with chlorobenzenes. The other bottle contained an active dechlorinating culture, adapted to all 3 trichlorobenzene isomers. Samples were taken in duplicate from each bottle immediately before spiking to determine the initial concentration of CBs from cultivations.

After spiking, samples were drawn from both bottles and six parallel experiments with both extraction procedures were carried out. The same analyses were performed 6 and 13 days after spiking. Rates of recovery were determined for the first day experiment with both bottles, whereas the later recovery experiments could only be evaluated with the

poisoned culture because of the rapid biotransformations of chlorobenzenes observed in the active culture.

Gas chromatographic procedures

A Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and an automatic liquid sampler HP 7673 with an on-column injector was used.

Injection volume was usually 3 µl, but in some experiments up to 6 µl. A fused silica megabore column of 30 m length and 0.53 mm ID coated with a cross-linked and surface-bonded methyl silicone film with 35% phenyl and 1.5 µm thickness was used (Rtx-35 from Restek corporation; Bellfonte, PA, USA). The analytical column was connected to a deactivated and uncoated retention gap of 2 m lenght and the same ID using a press fit connector.

Carrier gas was helium with a flow rate of 17 ml/min. Gas flows for the FID were 28 ml/min for hydrogen, 340 ml/min for air and 14 ml/min for make-up nitrogen. The detector temperature was held at 300°C.

a) Temperature program for CB analyses including benzene (hexane as solvent): 40°C (2 min); 8°C/min up to 120°C (0.5 min); 10°C/min up to 230°C (10 min).
b) Temperature program for CB analyses without benzene (hexane or ethyl acetate as solvent): 70°C (2 min); 6°C/min up to 120°C (0.5 min); 10°C/min up to 230°C (10 min).

Digitized detector output signals were transferred to a Personal Computer running with MS DOS operating system and processed by Nelson Analytical Software 5.1., generating reports based on the internal standard method. The results obtained with the automatic integration program were generally controlled by inspection of the chromatograms and, when necessary, checked by manual integration using the same software.

RESULTS AND DISCUSSION

Gas chromatography

Methyl silicone with 35% phenyl (Rtx–35) was found to be the most suitable coating for the analysis of all chlorobenzene isomers including benzene. Benzene was included in the analytical method as it was observed to be the final dechlorination product of the investigated anaerobic batch cultures as will be published elsewhere[14].

When benzene was analyzed, hexane was used as the extraction solvent, because ethyl acetate overlaps with benzene in the gas chromatogram. Hexane is not however suited for higher chlorinated benzenes because of the lower recovery rates at the concentration levels required for the degradation experiments. As long as monochlorobenzene was not present or detectable at significant amounts in the anaerobic sludge, benzene was not formed in our experiments as a degradation product.

In Figure 1, a chromatogram of a calibration mixture containing all chlorobenzene isomers is shown. All isomers were found to be well separated with the exception of 1,2,3,5-TeCB and 1,2,4,5-TeCB which coeluted at any temperature program tested. These two isomers also failed to be separated on less polar methyl silicone phases but can be resolved on phases containing 50% phenyl (OV-17). This type of column was not

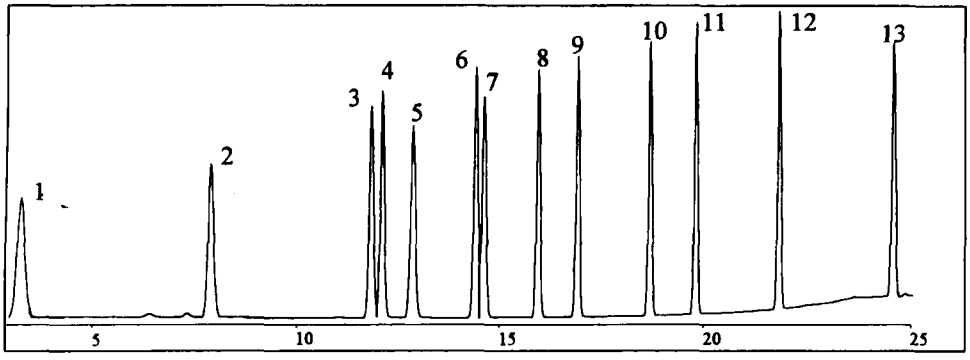

Figure 1 FID-gas chromatogram of a 0.1 mmolar calibration mixture of chlorobenzenes dissolved in n-hexane; temperature program a). [1=benzene (7.78 ng/µl) 2=MCB (11.17 ng/µl) 3=1,3-DCB (14.62 ng/µl) 4=1,4-DCB (14.78 ng/µl) 5=1,2-DCB (12.65 ng/µl) 6=2,4-dichlorotoluene; surrogate standard (16.30 ng/µl) 7=1,3,5-TrCB (18.45 ng/µl) 8=1,2,4-TrCB (18.24 ng/µl) 9=1,2,3-TrCB (18.16 ng/µl) 10=1,2,4,5-TeCB (21.60 ng/µl) 11=1,2,3,4-TeCB (21.90 ng/µl) 12=PeCB (25.30 ng/µl) 13=HxCB (29.42 ng/µl)].

however available with the desired film thickness at the time the method was under development. A phase containing 35% phenyl was therefore chosen as a good compromise. The temperature program was optimized with respect to analysis time and separation of all other isomers on Rtx-35. The final temperature of 230°C was held for 10 minutes after the latest eluting CB, hexachlorobenzene, appeared in order to ensure that less volatile matrix compounds did elute from the column before the beginning of the next run. Flame ionization detection was chosen to obtain similar molar response to all compounds under investigation as can also be seen by Figure 1 which shows a chromatogram of a calibration mixture of about 0.1 mmolar concentration of all compounds.

On-column injection was chosen for its better reproducibility in quantitative analysis. In addition, the internal standard was added to each sample to correct extraction variations and to control the chromatographic system in routine analysis. The combination of on-column injection with a megabore retention gap enabled the sample volume to be increased up to 6 µl without impairing resolution and peak shape. The higher sample volumes were used when minor components in the dechlorination experiments were to be analyzed with greater reliability.

Recovery experiments

By using 2,4-dichlorotoluene as surrogate standard (ISTD) which underwent the whole extraction procedures, the rates of recovery of the poisoned culture were determined as shown for the series after 13 days in Table 1. The data obtained from the other experiment after 6 day incubation as well as the samples of the active culture taken at the first day were within the same range. It was found to be important to add the ISTD to the samples as an aqueous solution before the extraction process was started and not dissolved in the organic solvent added for extraction. When 2,4-dichlorotoluene was added dissolved in the organic phase used for extraction, larger areas for the ISTD peak and consequently smaller recoveries for the chlorobenzenes based on internal standard report were obtained. Obviously the contact of 2,4-dichlorotoluene with the sludge particles was neccessary to reflect the adsorption of the CB isomers to the organic matter. This is in accordance with the observation that liquid-liquid extraction as

Table 1 Recoveries obtained from the sludge of the poisoned culture extracted by SPE and LE.

	SPE			*LE*		
Compound	*Rec. (%)*	*Sd (%)*	*VC*	*Rec. (%)*	*SD (%)*	*VC*
MCB	100.4	2.5	2.5	96.9	1.0	1.1
1,3-DCB	90.0	2.2	2.5	78.8	0.7	0.9
1,4-DCB	92.0	2.6	2.6	78.9	0.8	1.0
1,2-DCB	103.5	3.3	3.2	85.0	1.1	1.3
1,3,5-TrCB	81.9	1.9	2.4	90.3	0.9	1.1
1,2,4-TrCB	82.4	2.1	2.6	75.4	0.6	0.8
1,2,3-TrCB	94.7	2.5	2.7	82.3	0.9	1.0
1,2,4,5-TeCB	62.8	1.2	2.0	79.8	0.8	1.1
1,2,3,4-TeCB	70.9	1.6	2.2	81.5	0.9	1.0
PeCB	45.3	1.1	2.5	87.9	1.3	1.5
HxCB	16.1	0.7	4.3	100.2	5.7	6.4

(abbreviations: Rec. = recovery; SD = standard deviation; VC = variation coefficient).

described with the LE-method results in the same good recovery values for the poisoned sludge independent on the time of contact between CBs and organic matter.

The peak area measured of the ISTD was found to be a good criterion for the quality of the extraction. Small standard deviations for the analytes were usually observed with the ISTD, as can be seen by Table 1.

When comparing the results shown in Table 1 it becomes evident that the SPE-method yielded recovery rates greater than 80% and SD's as well as VC's better than 3.3% for chlorobenzenes up to the trichloro isomers. The recovery values of the higher chlorinated benzenes were however found to be unsatisfactory. In particular, the recovery of hexachlorobenzene was very low. Even with the addition of acetone, no improvement could be achieved with the SPE-method.

The data of Table 1 demonstrate recovery values higher than 75% and small standard deviations between 0.5 and 1.5% for all compounds extracted with the LE-method, with the exception of hexachlorobenzene which shows an increasing SD of 5.7%. With the higher chlorinated benzenes a homogenous distribution within the sludge is unlikely because they tend to be adsorbed strongly by the organic matter.

Since the eluate of the SPE-cartridge was found to be nearly free of matrix compounds as can be seen in Figure 2, this extraction method seems to be very selective,

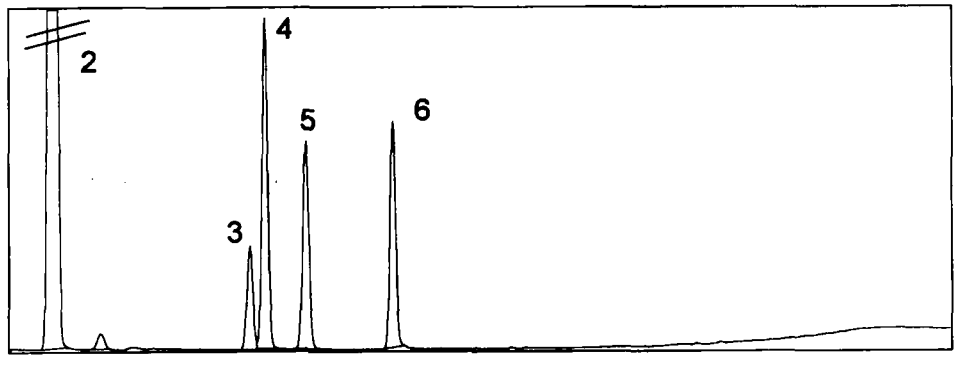

Figure 2 Gas chromatogram obtained from the spiked sample of the active culture after 13 days by using the SPE-method.

producing no load on the chromatographic system. No additional peak can actually be seen in the chromatogram recorded with the universal FID. This method should therefore be considered when chlorobenzenes with up to 3 chlorines are going to be measured and a high number of analyses have to be performed.

In parallel, the same sludge was extracted with ethyl acetate with subsequent clean-up on silica gel following the LE-method. The chromatogram obtained with the same gas chromatographic system is shown in Figure 3. Both the chromatograms show four peaks representing the chlorobenzenes with the same peak size, but the extract resulting from liquid-liquid partitioning contains additional compounds from the matrix and a small peak that shows a small remaining portion of hexachlorobenzene as confirmed by GC-MS.

The additional treatment of the samples using an ultrasonic bath for 10 minutes showed no positive effect. Neither the measured areas of the chlorobenzenes nor the rates of recoveries were significantly changed by this procedure. The chromatogram from a spiked sludge sample of the poisoned culture extracted with the LE-method is presented in Figure 4. At a relevant concentration level of 10 ng/µl all CBs could be detected and quantitatively determined because all analytes were found to be separated from the extracted matrix compounds. Note the varying peak size of the individual isomers due to nearly equal weights of the analytes in the sample in contrary to the equal molar concentrations shown in the chromatogram in Figure 1.

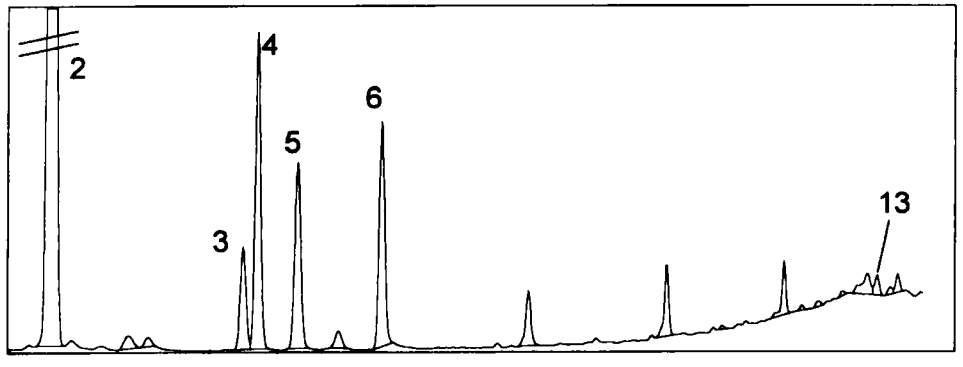

Figure 3 Gas chromatogram obtained from the active culture after 13 days by using the LE-method.

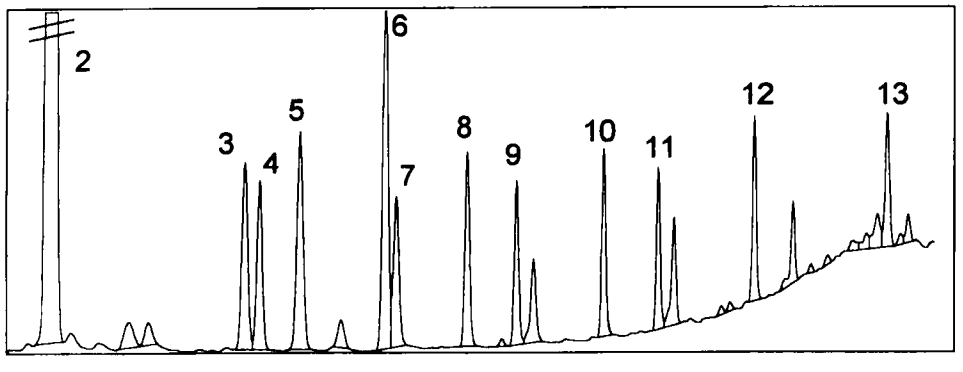

Figure 4 Gas chromatogram obtained from a recovery experiment using the poisoned culture and 13 days incubation (extraction following the LE-method). Concentration of CBs: 10 ng/µl.

In Figures 2 and 3, chromatograms are shown from experiments with the bioactive sludge fed with 10 ppm of all chlorobenzenes. Within 13 days all the higher chlorinated isomers were completely dechlorinated to dichlorobenzenes and monochlorobenzene. The latter accumulated under anaerobic conditions. This explains why monochlorobenzene presents such a large peak in the gas chromatograms shown in Figures 2, 3 and 4; the sediment cultures were adapted to chlorobenzenes by continous addition of mixtures of polychlorinated benzenes. The results of these studies will be described elsewhere[14].

CONCLUSIONS

Two extraction methods for the investigation of chlorobenzene biotransformation in anaerobic river sediments were established. All chlorobenzene isomers can be extracted with good yield by liquid-liquid extraction with ethyl acetate. The extract needs a further clean-up on silica gel.

The lower chlorinated isomers up to trichlorobenzenes can be satisfactorily recovered from the sludge by thorough mixing with equal volumes of acetone and subsequent SPE of the supernatant applying RP-C18 adsorbent. This extraction procedure was found to be very selective showing no co-extractives from the matrix in the chromatograms. The method allows a higher number of samples to be analyzed and is recommended when analyzing for the lower chlorinated benzenes up to the trichloro isomers. The determination of all chlorobenzenes can be easily performed by gas chromatography using megabore columns coated with methyl silicone containing at least 35% phenyl and relative thick films (1.5 μm). With the application of on-column injection combined with an optimized program, all CBs yielded well differentiated peaks with the exception of 1,2,3,5- and 1,2,4,5-tetrachlorobenzene which formed a critical pair. 2,4-dichlorotoluene was found to be a suitable surrogate standard improving the reproducibility of quantitative determinations.

Acknowledgements

The financial support by the German Research Council (DFG, Bonn) as part of the SFB 193 ("Biological Treatment of Industrial Waste Water") is gratefully acknowledged.

References

1. EPA; Health Assessment Document for Chlorinated Benzenes EPA 600/8-84-015F (1984).
2. K. H. Shafer, T. L. Hayes, J. W. Brasch and R. J. Jakobsen, *Anal. Chem.*, **56**, 237–240 (1984).
3. B. G. Oliver and K. D. Nicol, *Environ. Sci. Technol.*, **16**, 532–536 (1982).
4. M.-J. Wang and P.-Z. Lang, *China Environ. Sci.*, **10**, 241–249 (1990).
5. M.-J. Wang, P.-Z. Lang, F.-S. Long, Y.-Z. Ding and X. Yuan, *China Environ. Sci.*, **10**, 81–88 (1990).
6. M.-J. Wang, P.-Z. Lang, F.-S. Long, Y.-Z. Ding and X. Yuan, *Bull. Anal. Testing*, **7**, 38–40 (1988).
7. I. C. Figuera and M. S. Simmons, *Environ. Toxicol. Chem.*, **10**, 323–329 (1991).
8. B. G. Oliver and A. J. Niimi, *Environ. Sci. Technol.*, **17**, 287–291 (1983).
9. K. C. Jones and S. R. Wild, Water Research Report Number FR0169, Water Research Centre, Medmenham, U.K. (1991).
10. W. Reineke and H.-J. Knackmuss, *Ann. Rev. Microbiol.*, **42**, 263–287 (1988).
11. M. van der Meer, W. Roelofsen, G. Schraa and A. J. B. Zehnder, *FEMS Microbiol. Ecol.*, **45**, 333–341 (1988).
12. P. Sander, P. Fortnagel and W. Francke, *Appl. Environ. Microbiol.*, **57**, 1430–1440 (1991).
13. W. W. Mohn and J. M. Tiedje, *Mircobiol. Rev.*, **56**, 482–507 (1992).
14. J. Nowak, N.-H. Kirsch, W. Hegemann and H.-J. Stan, *Appl. Environ. Microbiol.* submitted.