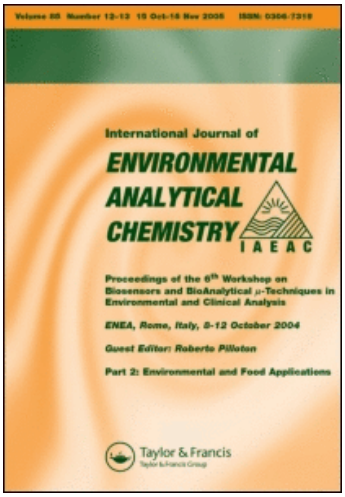

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Phase Systems and Post-Column Dithizone Reaction Detection for the Analysis of Organo-Mercurials by HPLC

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To cite this Article Gast, C. H. and Kraak, J. C.(1979) 'Phase Systems and Post-Column Dithizone Reaction Detection for the Analysis of Organo-Mercurials by HPLC', International Journal of Environmental Analytical Chemistry, 6: 4, 297 — 312
To link to this Article: DOI: 10.1080/03067317908081221
URL: http://dx.doi.org/10.1080/03067317908081221

Intern. J. Environ. Anal. Chem., 1979, Vol. 6, pp. 297–312
0306-7319/79/0604-0297 $04.50/0

Printed in Great Britain

Phase Systems and Post-Column Dithizone Reaction Detection for the Analysis of Organo-Mercurials by HPLC

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(*Received September 11, 1978*)

The suitability of HPLC (normal and reversed phase adsorption) with UV or post-column reaction detection for the analysis of organomercurials was investigated systematically. The separation of organomercurials is best carried out on a reversed phase system with a C-8 bonded phase material as the stationary phase and acetonitrile-aqueous sodium bromide mixtures as the mobile phase.

The precision and detection limit of the method and the efficiency of the extraction procedure were established. For the alkylmercury compounds the lowest limit of detection (80 ppb) was obtained with the dithizone reaction detection and for the phenylmercury compounds with UV detection (60 ppb). A chromatogram of a spiked fish (2 ppm Hg) and a river water sample (50 ppb Hg) is shown.

INTRODUCTION

It is well known that inorganic mercury brought into the environment by pollution or by natural geological activities, is converted into organomercury compounds by micro-organism.[1,2,3] Also synthesized organomercury compounds, such as ethylmercury bromide and phenylmercury acetate, are frequently used in agriculture for mould control.

The organomercurials and in particular methylmercury were found to be very toxic for man, especially because of the long biological half life in the body.[2] The accumulation of methylmercury in fish, which was used for consumption by people in the Minamata district in Japan, was the reason for the outbreak of the so-called Minamata disease in the early fifties.[1,2]

Therefore there is a need for a rapid and sensitive method to determine organomercurials in biological materials. Several methods have been applied for the determination of inorganic and organic mercury in biological materials.[4,5,6] In order to determine separately the different

forms of organomercurials, chromatographic methods such as PLC[3], TLC[3] and GC[3] have been applied. Especially GC is used for the analysis of methylmercury in biological materials.[3,7-12] However, investigations with GC-MS and labeled organomercury compounds showed that several organomercurials are thermally unstable, decompose, or are converted into other organomercury complexes in the GC-column;[13,14,15] this invalidates strongly the quantitative analysis of these substances. Liquid chromatography is a separation technique which is usually performed at moderate temperatures. Its potential as separation technique for the separation of test mixtures of organomercurials was demonstrated recently.[16,17]

In the present paper the results of an investigation of the suitability of HPLC (normal and reversed phase) for the determination of organomercury compounds in biological materials, in combination with UV and a post-column reaction detection with dithizone, will be described.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a reciprocating membrane pump (Orlita DMP 1515, Giessen, GFR), a flow-through manometer as damping device, a high pressure sampling valve (Chromatronix HPSV 20, Berkeley, U.S.A.) Equipped with a sample loop of 80 μl, and a variable wavelength detector (Perkin Elmer LC 55, Norwalk, U.S.A.). The dithizone reagent delivery system consisted of a syringe pump (Varian 4100, Walnut Creek, California).

In all experiments stainless steel 316 columns (3 mm I.D., 6.4 mm O.D.) of different lengths were used. The mixing manifold consisted of a modified 1/16″ T union (Swagelok®)[18] and the reactor consisted of a stainless steel 316 column (length 50 mm, I.D. 3 mm) filled with glass beads with a mean particle size of 150 μm.

For UV detection the wavelength was adjusted to 205 nm and for the post-column reaction detection to 480 nm.

Chemicals and materials

In all experiments double distilled water and organic solvents of analytical grade were used. The halogenated aryl mercury compounds were a gift from Dr. J. Wolters from the State University of Leiden (Leiden, The Netherlands). The propylmercury bromide was synthesized according to Slotta and Jacobi.[19] The other used organomercury compounds were

commercially available (Merck, Darmstadt, GFR, and Fluka, Switzerland).

The column materials applied were commercially available C-8 (RP-8 mean particle size 5 and 10 μm, Merck) used as delivered, and porous silica (SI 60, Merck) ground and classified by means of an air-classifier to a particle size range of 7–8 μm. For the TLC experiments commercially available TLC plates (Polygram Sil G/UV 254, Macherey-Nagel & Co., GFR) were used.

Procedures

Chromatography

The separation columns were packed by a pressurized balanced slurry method.[20,21] The capacity ratios (κ_i) of the selected organomercurials were determined from their retention times and that of an unretained compound (sodium nitrate and benzene in reversed phase respectively normal phase experiments). The eluent was prepared by mixing weighed amounts of solvents. The eluent was ultrasonicated in order to remove air.

Extraction

For the isolation of organomercurials from homogenised fish-samples, we used the washing and extraction procedure as described by Watts *et al.*[11] Toluene was used for the extraction instead of benzene. In order to back extract the organomercury salts in an aqueous phase, the cysteine method of Westöö[8] was applied. By addition of concentrated HBr (final concentration 1 M), after the cysteine extraction, the organomercury-cysteine complexes were converted into organomercury bromide and free cysteine. However, the free cysteine interfered seriously with the dithizone reaction detection and was therefore removed from the aqueous solution. This was accomplished on another C-8 column (250 mm length and 4.6 mm I.D.); a combination with 0.1 M HBr as mobile phase was found to be suitable. The complete aqueous solution (4.5 ml) was injected onto this column by means of a 6 ml sample loop.

The eluate was collected in a calibrated glass cylinder. After collection of 8 ml eluate (containing the cysteine), the flow was stopped. In order to produce a step gradient, the 6 ml loop was filled with acetonitrile. Then the pump was switched on and the acetonitrile was injected onto the column, by which the organomercurials were eluted from the column within the first two ml eluate. These two ml are brought to the eluent composition with 2 ml 0.1 M NaBr solution and aliquots of 80 μl of this solution were analyzed by HPLC.

For the isolation of organomercurials from water, the following scheme was used: 40 ml water sample was acidified with HBr to a final concentration of 0.1 M HBr; than the water sample was extracted two times with 40 ml toluene. The combined toluene fraction was treated as described for the fish sample.

RESULTS AND DISCUSSION

Organomercury salts have polar as well as hydrophobic moieties and therefore both normal and reversed phase adsorption systems might be suitable for their separation. In order to determine which phase system is to be preferred, both types were investigated.

Adsorption chromatography on silica

In order to find the most suitable mobile phase composition, use was made of TLC experiments with a large number of eluents. The organomercurials were visualized by spraying a dithizone solution in acetone after development of the plates. From the results of these TLC experiments the following conclusions could be drawn: (i) all the organomercurials are strongly retarded when using alkanes, toluene, dichloromethane, methanol or water as eluent; (ii) no retardation of the organomercurials was found when using butanol, ethanol, THF, or acetonitrile as eluent; (iii) the strong retardation of the organomercurials when using water as eluent could be reduced by the addition of sodium halides. The effect of sodium halides on the retention of the organomercury salts must be attributed to their effect on the degree of dissociation of organomercury salts. Addition of sodium halides to the eluent decreases the dissociation[19,22,23] and also the affinity for the silica stationary phase.

From the results of the TLC experiments it was decided to use hexane-butanol mixtures as eluents for the column experiments. However, all organomercurials, with the exception of diphenylmercury ($\kappa = 0$), were strongly retained on the column, even with pure butanol as eluent.

In order to exploit the favourable effect of the presence of sodium-halides in the eluents as found by the TLC experiments, a saturated NaCl solution in 9:1 (w/w) hexane:butanol was tried as the eluent. Due to the presence of NaCl, which probably depresses the dissociation of the organomercurials and/or deactivates the silica, moderate retention was observed. Moreover, the retention could be adjusted by the percentage of butanol, as can be seen from Table I. Unfortunately, this phase system showed some disadvantages:

i) poor reproducibility of the retention when refreshing the eluent;

ii) decomposition of diphenylmercury into phenylmercurychloride (identified by degree of retention (Table I) and by reaction with dithizone). The decomposition of diphenylmercury into other organomercurials was observed earlier.[15]

As the sodiumchloride content ($\approx$10 ppm) of the mobile phases is difficult to control (e.g. it is strongly dependent on the water content of the eluent), the effect of the addition of organic salts (tetramethylammoniumchloride (TMAC) and hexadecyltrimethylammoniumbromide (HTAB)) to the hexane-butanol mixtures on the retention of organomercurials was also investigated. The results of these measurements are given in Table II.

TABLE I

Capacity ratios of organomercurials measured with different butanol/*n*-hexane mixtures saturated with sodium chloride as eluent and silicagel SI 60 as stationary phase.

	Eluent (saturated with sodium chloride)		
Organomercury compound	5% butanol	10% butanol	15% butanol
Diphenylmercury	0	0.59	0.30
Phenylmercury chloride	0.90	0.59	0.30
Ethylmercury chloride	1.35	1.05	0.64
Methylmercury chloride	2.10	1.59	0.97

As can be seen from Tables I and II, a complete different elution pattern occurs when replacing NaCl by organic salts. The alkylmercury salts are eluted in front of the phenylmercury compounds when using the organic salts in contrast to what is observed with NaCl. Moreover, the capacity ratios are significantly larger with organic salts than with NaCl. The mechanism responsible for this retention behaviour has not yet been investigated. Further, no decomposition of diphenylmercury and a good reproducibility of the retention were found when using eluents with organic salts. The suitability of a phase system containing TMAC for the separation of a test mixture of organomercurials is shown in Figure 1.

Reversed phase adsorption

To determine the optimal chromatographic conditions for the separation of organomercurials with octylmodified silica (RP-8) as adsorbent and

TABLE II

Capacity ratios of organomercurials measured with 10% butanol in *n*-hexane saturated with TMAC and HTAB as eluent and silicagel SI 60 as stationary phase.

Organomercury compound	Eluent (10% butanol in *n*-hexane)	
	Saturated with TMAC (≈0.01%)	Saturated with HTAB (≈0.01%)
Diphenylmercury	0.15	0.48
Propylmercury bromide	1.79	2.44
Ethylmercury chloride	2.90	3.50
Methylmercury chloride	4.70	5.0
Phenylmercury chloride	11.2	14.5
2-Bromo-phenylmercury acetate	13.1	>20
2-Chloro-phenylmercury acetate	20.2	>20

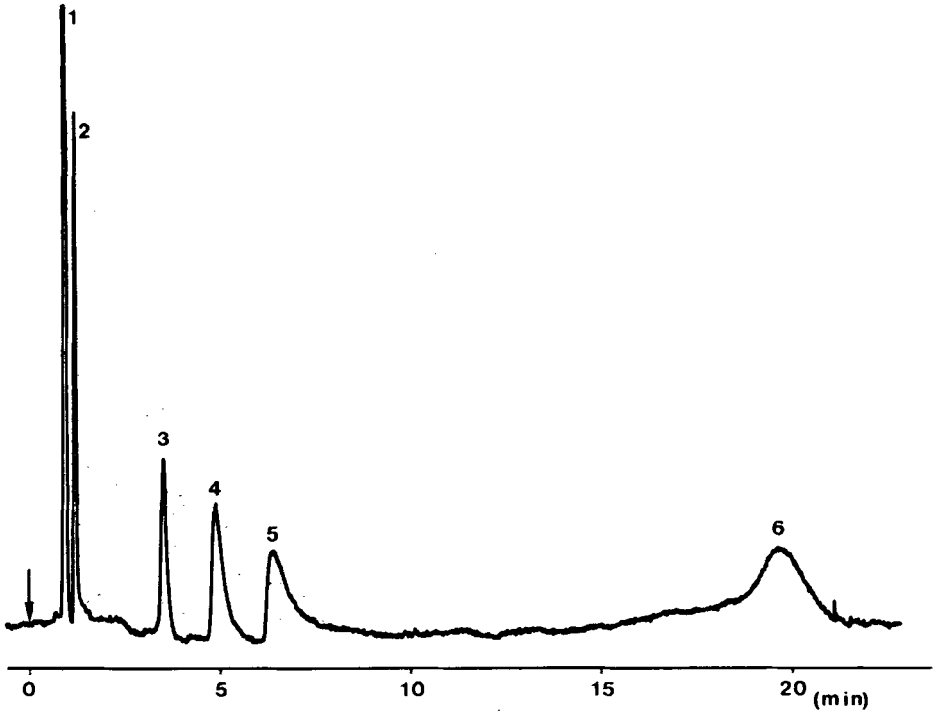

FIGURE 1 Separation of a test mixture of organomercurials. Stationary phase: Silica gel SI 60 (7–8 μm). Mobile phase: 10% butanol in *n*-hexane, saturated with TMAC (≈0.01%). Column: 250 × 3 mm. Sample: 1. benzene; 2. diphenylmercury; 3. propylmercury; 4. ethylmercury; 5. methylmercury; 6. phenylmercury.

water organic solvent mixtures as eluent, a number of experiments were carried out. The effects of the type and concentration of the organic modifier and sodiumhalide, of pH and of temperature on the capacity ratio of the organomercurials were investigated. Primary experiments with water-methanol mixtures as eluent showed that the capacity ratio of the organomercurials were not constant and seemed to depend on the number of preceding injections of methylmercurychloride, while, moreover, the peak shapes were very asymmetric. Also in this case the addition of sodium halides had a favourable effect, resulting in symmetrical peak-shapes and reproducible retention. Therefore, in all further experiments sodium halides were always present in the eluent.

Influence of the type and concentration of the organic modifier

Figure 2 shows the effect of the type of organic modifier on the capacity ratio of the organomercurials. For alcohols the capacity ratio decreases with increasing lipophility of the alcohol, as is commonly found in reversed phase systems. No significant selectivity changes can be noticed between the different types of alcohols. With acetonitrile, however, significant changes in retention behaviour were found. For instance, compared with methanol the alkylmercury compounds are more retained with acetonitrile, while the retardation of the phenylmercury compounds and in particular diphenylmercury is remarkably smaller.

The effect of the organic modifier concentration on the capacity ratio of the organomercurials was investigated with methanol, and can be seen in Figure 3. The logarithm of the capacity ratio of the organomercurials decreases linearly with the methanol percentage, as is usually found in reversed phase systems. However, the individual slopes are different, which indicates also a change in selectivity when varying the methanol percentage.

For all modifiers investigated the retention order of the alkylmercury compounds was methyl < ethyl < propyl (see Figure 6), as is commonly found for homologue series in liquid chromatography. Our results, however, are in contrast with those reported by Brinckman *et al.*,[17] who found an elution order of propyl < methyl < ethyl.

Influence of sodium halides

The influence of the type and concentration of sodium halides on the capacity ratio of organomercurials is shown in Figure 4. Two main trends can be noticed from this figure:

i) the capacity ratios decrease sharply when adding NaCl or NaBr up to 0.05 M to the eluent and become more or less constant at larger salt concentrations;

ii) the capacity ratios are larger with Br^- than with Cl^-, while moreover the capacity ratio of phenylmercury significantly drops at larger

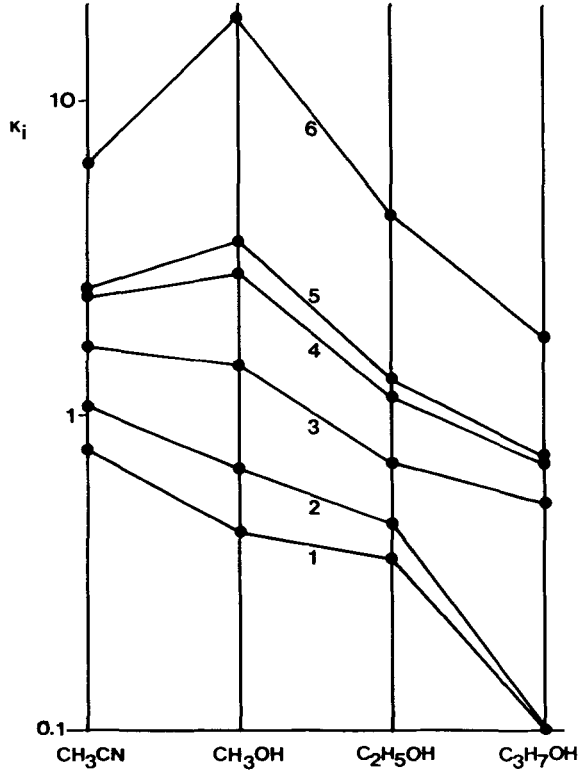

FIGURE 2 Effect of the type of moderator on the capacity ratio of organomercurials. Stationary phase: LiChrosorb RP-8 (10 μm). Mobile phase: organic solvent [50% (w/w)] + 0.05 M aqueous sodium chloride (pH = 3.5). Sample: 1. methylmercury; 2. ethylmercury; 3. phenylmercury; 4. 3-chlorophenylmercury; 5. 2-bromophenylmercury; 6. diphenylmercury.

Br^- concentration. The larger κ_i found with Br^- agrees with the solubility order of the halides, which is $Cl^- > Br^- > J^-$. The drop of κ_i of phenylmercury at larger Br^- concentration might be attributed to the formation of $\phi HgBr_2^-$ complexes[22] which increases the solubility again.

Brinckman *et al.*[17] also found a significant influence of the type and concentration of the anion on the retention of organomercurials. However,

our results are in contrast to theirs. They found no retardation of the organomercurials when halides are present. They state that presumably charged halide complexes are formed. According to Schwarzenbach *et al.*,[22] however, it does not seem likely that charged halide complexes are formed at halide concentrations below 0.2 M.

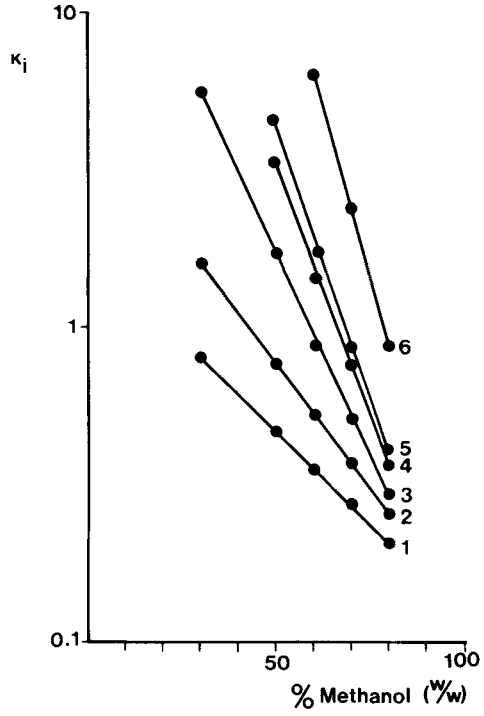

FIGURE 3 Effect of the methanol concentration on the capacity of ratio of organomercurials. Stationary phase: LiChrosorb RP-8 (5 μm). Mobile phase: 0.05 M aqueous sodium chloride + methanol [30–80% (w/w)] (pH = 3.5). 1 up to 6: see Figure 2.

Influence of pH

The effect of pH was investigated by measuring the capacity ratio of the organomercurials at different pH. No significant changes in κ_i were noticed in the pH range 2–5. At higher pH, however, κ_i increases while very asymmetric peaks were found.

Influence of temperature

Figure 5 shows the effect of temperature on κ_i. As can be seen, κ_i decreases with increasing temperature as is commonly found in liquid chromatography. No significant selectivity effects can be noticed. However, at higher temperatures the column efficiency improved significantly.

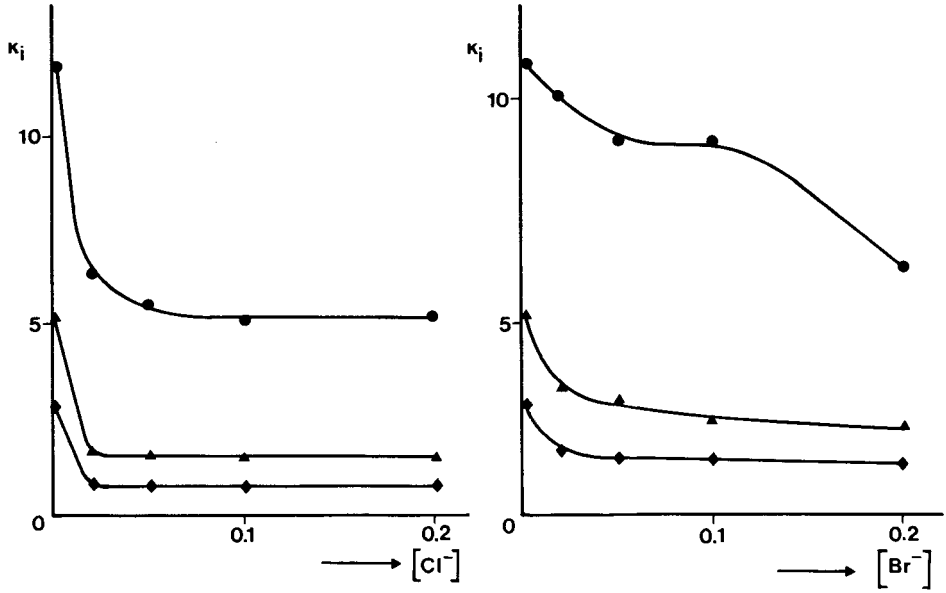

FIGURE 4 Effect of sodiumchloride and sodiumbromide concentration on the capacity ratio of some organomercurials. ◆ methylmercury; ▲ ethylmercury; ● phenylmercury. Stationary phase: Lichrosorb RP-8 (5 μm). Mobile phase: methanol [30% (w/w)] + sodium halide solution (0–0.2 M) (pH = 3.5).

Post-column reaction detection with dithizone

Alkylmercury compounds show a low molar absorption in the UV region.[24,25] In order to find a more sensitive and selective method of detection, a post-column reaction was applied. As the reagent dithizone (diphenylthiocarbazone) ($\lambda_{max} = 610$ nm), which forms coloured complexes ($\lambda_{max} = 480$ nm) with organomercury salts,[26] was chosen.

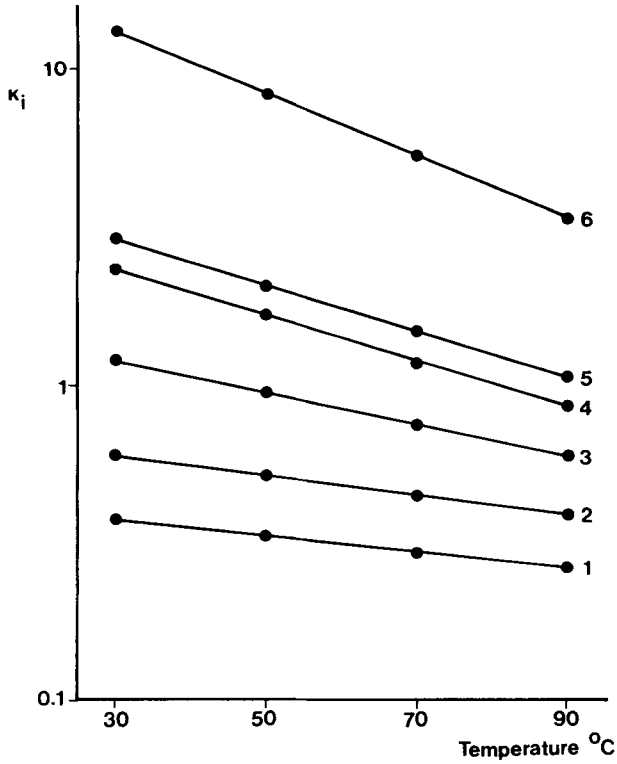

FIGURE 5 Effect of temperature on the capacity ratio of organomercurials. Stationary phase: LiChrosorb RP-8 (5 μm). Mobile phase: methanol [50% (w/w)] + 0.05 M aqueous sodium chloride (pH = 3.5). 1 up to 6: see Figure 2.

Combination of the dithizone reaction with the normal phase systems containing TMAC or HTAB in the eluent suffered from a low sensitivity. This is due to a shift in λ_{max} of dithizone to 480 nm, which is also the absorption maximum for the organomercury dithizonates. With reversed phase systems, however, this problem was not encountered and therefore the post-column reaction detection was further investigated with the developed reversed phase systems. By considering the different parameters investigated for reversed phase systems we selected a mobile phase, consisting of 40% (w/w) acetonitrile in 0.1 M NaBr, buffered to pH 3.5 with phosphate (0.001 M) and ambient temperatures for the analysis of organomercury salts in fish and water with post-column detection.

The mercury dithizone complex is formed within 10–15 sec, allowing a short residence time in the bed-reactor,[18] filled with 150 μm glass beads.

The main problem encountered was the solubility of the dithizone and mercurydithizonates in aqueous mixtures, which is very small.[26] However, in acetonitrile about 1 g/l dithizone dissolves.[26] Therefore as reagent solvent acetonitrile was chosen, which contains 2.10^{-4} M/l dithizone. This concentration was a compromise between the background caused by the absorption of dithizone itself, its solubility when mixed with the eluent, and the linear range of the post-column reaction detection. The reagent/eluent flow ratio was adjusted to 0.4, avoiding precipitation of dithizone or mercurydithizonates.

The standard deviation of the first chromatographic peak, without reactor, was found to be 50 μl. After installing the reaction system, allowing for a 20 sec. reaction time, the standard deviation was found to be 58 μl.

With respect to the effect of pH on the formation of mercury dithizonates, a pH = 3.5 was found to be optimal. A chromatogram of a test sample with the selected phase system combined with the post-column reaction detection is given in Figure 6.

Quantitative aspects of the method

Precision and linearity

The precision of the determination of organomercurials and the linear range using UV and post-column reaction detection were determined by injection of a constant volume (80 μl) of solutions of some organomercurials at different concentrations (0.2–40 ppm Hg) and peak height measurements. Figure 7 shows the proportionality of injected amounts of methylmercury versus peak height for postcolumn reaction detection. The dashed lines show the confidence limits for ± 3 times the standard deviation (99.7% reliability). The relative standard deviations were found to be $\pm 0.7\%$ at 10 ppm Hg ($n = 3$) and $\pm 8\%$ at 0.2 ppm Hg ($n = 3$). At Hg concentrations > 10 ppm the calibration curve of the dithizone reaction detection deviates from linearity as a result of sub-stoichiometric concentration of reagent at the used dithizone concentration. The limit of detection for a signal-to-noise ratio of 3, for methylmercury and phenylmercury, was found to be 0.18 ppm Hg respectively 0.06 ppm Hg with UV detection, and 0.08 ppm Hg, respectively 0.11 ppm Hg when using the dithizone reaction detection, for the given injection volume of 80 μl. This result shows that the dithizone reaction detection is superior for alkylmercury salts, and UV is superior for the detection of the phenylmercury compounds.

Recovery and reproducibility of the extraction

The recovery and reproducibility of the extraction procedure were determined by HPLC and extraction of known amounts of methyl- and ethylmercurychlorides added to river-water and homogenized fish samples, as described by Watts *et al.*[11] For water samples containing CH_3HgCl and C_2H_5HgCl (50 ppb Hg of each) a recovery of $75 \pm 3\%$ ($n=3$), and for

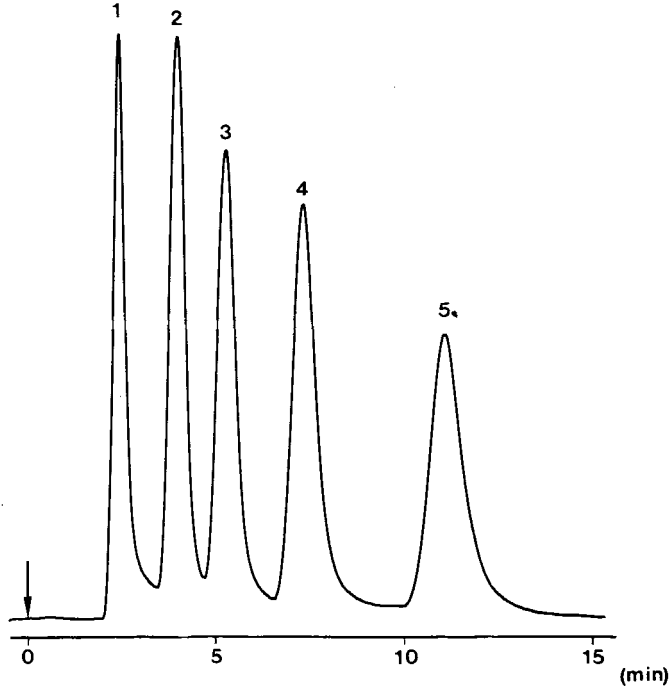

FIGURE 6 Separation of a test mixture of organomercurials using reversed phase adsorption and post column reaction detection. Stationary phase: RP-8 (10 μm). Mobile phase: acetonitrile [40% (w/w)] + 0.1 M aqueous sodium bromide (pH = 3.5). Column: 125 × 3 mm. Sample: 1. mercuricchloride; 2. methylmercury; 3. ethylmercury; 4. propylmercury; 5. 3-chlorophenylmercury.

fish samples containing 2 ppm Hg of each alkylmercury a recovery of $65 \pm 3\%$ ($n=3$) was found. Higher recoveries were found when the toluene extraction is performed three or more times. Figures 8 and 9 show the separation of methyl- and ethylmercury added to a river-water and a homogenized fish sample (50 ppb Hg and 2 ppm Hg of each respectively) and extracted as described in Procedures.

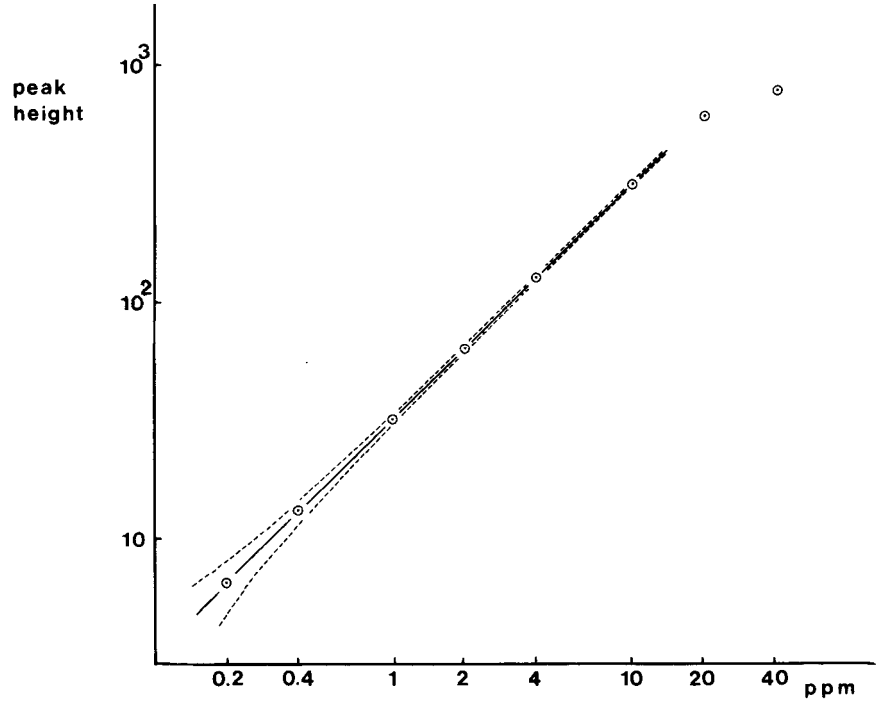

FIGURE 7 Precision and linearity of the determination of methylmercury with dithizone post-column reaction detection. The dashed lines show 3 times the standard deviation (99.7% reliability).

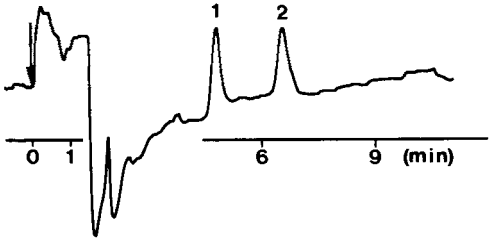

FIGURE 8 Chromatogram of an extract of river-water, spiked with methylmercury (1) and ethylmercury (2) (50 ppb Hg of each).

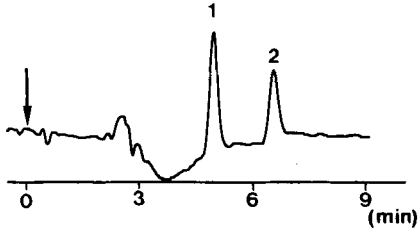

FIGURE 9 Chromatogram of an extract of homogenized fish, spiked with methylmercury (1) and ethylmercury (2) (2 ppm Hg of each).

Conclusion

The studies described show that HPLC, and in particular reversed phase liquid chromatography in combination with UV and/or a post-column reaction detection with dithizone, is very suitable for the separation and determination of organomercurials in natural samples. More profit of the method can be expected when element-specific detection systems, such as atomic absorption or inductively coupled plasma (ICP) can be used. Work in this direction is in progress.

Acknowledgement

The authors wish to thank Dr. J. Luten from TNO, IJmuiden (The Netherlands), for supplying the homogenized fish samples, and Dr. H. Poppe for his valuable discussions during the preparation of the manuscript.

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