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ANALYSIS OF ORGANIC MERCURY COMPOUNDS BY GAS CHROMATOGRAPHY

Part I Analytical and Extraction Method of Organic Mercury Compounds

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Indexing Words

analysis of organic mercury; gas chromatography; alkyl mercury; methyl mercury; phenyl mercury; electron capture detector; extraction of organic mercury
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Kimiaki SUMINO. *Analysis of Organic Mercury Compounds by Gas Chromatography. Part I Analytical and Extraction Method of Organic Mercury Compounds.* Kobe J. Med. Sci. 14, 115-130, June 1968—Formerly, it was impossible to determine trace amounts of organic mercury compounds and to clarify their chemical forms. The author, utilizing a gas chromatograph with an electron capture detector, successfully performed ultra-trace determinations of alkyl and phenyl mercury. In alkyl mercury analysis DEGS and 1.4-BDS were used as stationary phases, packed in glass on-columns. In phenyl mercury analysis 5% DEGS was used with on-column injection techniques. After scrutinizing various conditions, a detection limit for methyl mercury chloride was reached at 10^{-12} g and for phenyl mercury chloride at 10^{-11} g.

Isolation of organic mercury from different materials was performed by separation of organic mercury combined with SH radical in 1N-HCl solution. The solution was then extracted with benzene, and this benzene solution re-extracted with glutathione. After acidification of the resulting solution with HCl, final extraction with benzene was performed. This clean-up method was employed in the identification of organic mercury and was a very useful technique for its extraction.

INTRODUCTION

Following the outbreak of Minamata Disease which occurred in 1953 on the coast of Minamata Bay, Kumamoto Prefecture, it was reported in 1965 that several individuals developed symptoms similar to those of Minamata Disease, near the mouth of the Agano River, Niigata Pref.^{1,6)} After clinical, analytical and epidemiologic investigations it was confirmed that this was a second case of Minamata Disease, caused by waste discharge from a factory.^{1,8)}

At about the same time, problems of residual toxicity in foods were being discussed in the Diet, and social anxiety arose among the people because of the occasionally exaggerated news concerning this matter. Hence, research on organic mercury poisoning, and especially of its chronic toxicity, together with microanalysis of the detailed chemical form of organic mercury, was earnestly looked for.

Among the many organic mercury compounds, the causative substances of Minamata Disease, and compounds which are related to mercury toxicity such as

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pesticides, medicaments and antiseptics, are alkyl and phenyl compounds. With regard to LD₅₀, namely acute toxicity, there is very little difference between these compounds in their effect.^{10,14)} However, great differences exist in their chronic toxicity. For instance, organic mercury compounds capable of giving rise to Minamata Disease are confined to methyl, ethyl and n-propyl mercury.^{11,12)} Hence butyl, amyl and higher alkyl mercury, like lower alkyl mercurys, such as metoxyethyl, metoxypropyl and i-propyl, are unable to cause the disease, as also are phenyl mercury and other organic mercury compounds. It has been reported that in prolonged oral administration of phenyl mercury and inorganic mercury to experimental rats phenyl mercury acetate was 100 times as poisonous as mercuric acetate.⁸⁾ In research on residual mercury in foods, on trace quantities of mercury in waste discharge from factories using mercury catalysts, and on general organic mercury toxicity, greater stress should be laid on the study of the chemical form of mercury than on the measurement of total mercury.

Concerning the analysis of organic mercury compounds, several methods have been reported. These include methods employing distribution between solvents,^{5,6,9)} paper chromatography,¹⁾ thin layer chromatography (TLC)^{7,15)} and others. However, their accuracy of determination and detection limits have been open to question. In 1961, gas chromatography (GC) was first introduced by Brodersen et al. for separation of alkyl mercury.²⁾ However this method cannot be used for micro-determination owing to its poor practicability.

After trying various kinds of column packings and examining methods of extraction from biological materials, the author established a method for ultra trace determination of alkyl and phenyl mercury compounds, utilizing a high-sensitivity detector.

MATERIALS AND METHODS

2-1 Compounds Investigated

Constitutional formulas and abbreviations of the organic mercury compounds used are shown in Table 1.

Table 1. Organic mercury compounds

| Compound | Constitutional formula | Abbreviation |
|-----------------------------|--|--------------|
| Methylmercuriiodide | CH ₃ HgI* | MMI |
| " chloride | CH ₃ HgCl* | MMC |
| " hydroxide | CH ₃ HgOH* | MM(OH) |
| " acetate | CH ₃ HgOCOCH ₃ | MMA |
| " sulfate | (CH ₃ Hg) ₂ SO ₄ * | MMS |
| Ethylmercurichloride | C ₂ H ₅ HgCl* | EMC |
| " phosphate | (C ₂ H ₅ Hg) ₂ HPO ₄ | EMP |
| n-Propylmercurichloride | C ₃ H ₇ HgCl | PrMC |
| n-Butylmercurichloride | C ₄ H ₉ HgCl | BMC |
| n-Amylmercurichloride | C ₅ H ₁₁ HgCl | AMC |
| Methoxyethylmercurichloride | CH ₃ OC ₂ H ₄ HgCl | MtEMC |
| Phenylmercuriiodide | C ₆ H ₅ HgI | PMI |
| " chloride | C ₆ H ₅ HgCl | PhMC |
| " acetate | C ₆ H ₅ HgOCOCH ₃ | PMA |

* synthesized in our laboratory

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Each of these compounds was dissolved in organic solvents such as benzene, chloroform, ethyl acetate, at concentrations of from 1mg/ml to 0.1 μ g/ml. 1-5 μ l of this solution was injected into the GC using a Hamilton 10 μ l syringe.

2-2 Apparatus

Shimadzu Gas Chromatograph Model GC-1C, Model GC-2C and Model GC-3AE were used.

2-3 Column and Column Packing

U-shaped stainless steel columns, 3 mm i. d., 75 cm long, and straight stainless steel columns, 3 mm i. d., 37.5 cm long, were used, combined in a variety of lengths. Some glass columns, 4 mm i. d., 40cm, 50 cm, 120 cm, 175 cm, 330 cm in length were used in the on-column injection techniques. The 16 kinds of liquid phases employed for packed columns are shown in Table 2.

Table 2. Column packings

| Wt. | Liq. phase | Support | Mesh |
|------|------------|--------------|--------|
| 1.5% | SE-30 | Chromosorb-W | 60/80 |
| 5% | DC-11 | Shimalite-W | " |
| 25% | DC-200 | Shimalite | " |
| 7% | Apiezon-L | " | " |
| 15% | Thermol-3 | " | " |
| 1% | XE-60 | C-W | " |
| 3% | SE-52 | " | " |
| 3% | OV-17 | S-W | 80/100 |
| 25% | DC-550 | S | 30/60 |
| 5% | QF-1 | C-W | 60/80 |
| 1% | NGS | " | " |
| 5% | 1.4-BDS | S-W | " |
| 10% | " | C-W | " |
| 10% | CHDMS | " | " |
| 5% | DEGS | " | " |
| 15% | " | S | " |
| 20% | " | Celite 545 | " |
| 25% | " | S | " |
| 25% | " | " | 40/50 |
| 25% | " | " | 30/60 |
| 25% | PEG-20M | C-W | 60/80 |
| 10% | PPE | Celite 545 | " |

ESTABLISHMENT AND DESCRIPTION OF ANALYTICAL METHODS

3-1 Selection of Column and Column Packing

Using the column packing shown in table 2, chromatograms for each of the materials were recorded, varying the column temperature, the column material and length, and the flow rate and kinds of carrier gas. The results were examined, for

consideration of the analytical time, status of separation, sharpness of response peak height.

Favorable results were generally obtained when highly polar liquid phases were used. As stationary phases suitable for micro-determination, the author selected DEGS (diethyleneglycol succinate), PEG-20M (polyethyleneglycol), and 1.4-BDS (butanediol succinate) in alkyl mercury analysis, and DEGS of low concentration liquid phase in phenyl mercury analysis. In alkyl mercury analysis a glass on-column is desirable but a stainless steel column could be used. However, in the micro-determination of alkyl compounds by means of stainless steel columns, ghost peaks sometimes appear. In phenyl mercury analysis, a glass on-column must be employed, since when stainless steel is used, the phenyl mercury sometimes breaks down and bad reappearance occurs.

As to the column length, a short one can give satisfactory separating ability. Generally the detection limits for a short column are lower than those for a long one. In the case where a long column is used, phenyl mercury requires a long elution time, and so a short column is desirable.

3-2 Instrumental Conditions

Column temperature was varied up to 200°C in view of the relation between the detector temperature and the maximum temperature of column packing. Alkyl mercury, especially MMC, did not undergo any change in the range 120 to 200°C. PhMC decomposed at 230 to 250°C but good results were obtained in the range 170 to 195°C.

Detector temperature was held at 200 to 210°C, in consideration of the column temperature to be used. There was no change in sensitivity within this temperature range.

Nitrogen, argon or helium was used as carrier gas, and the highest sensitivity obtained with nitrogen. A somewhat higher flow rate and low column temperature also gave higher sensitivity.

3-3 Detector and Sensitivity

With regard to detectors, both a flame ionization detector (FID) and an electron capture detector (ECD) were used for comparison. Each of these produced some increase in sensitivity to alkyl mercury; however, that of ECD was generally several thousands times higher than that of FID. Hence, it is essential that ECD be employed in micro-analysis. The instrument was operated with a detector voltage of pulse height 40 volts, and an amplifier and recorder range of 4×10^{-9} A/FD to 8×10^{-10} A/FD and a chart speed of 1cm per minute.

3-4 Identification and Determination of Organic Mercury

Identification was made from the retention time (Rt). The sample was compared with standard substances using the addition method. In the case of alkyl mercury analysis various column packings were tried. The clean-up method, mentioned in 4-3, has a specificity for mercury and is highly effective in its identification. It is possible to direct materials coming out of the column vent into a

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mercury vapor meter so allowing confirmation of the results. The organic mercury of a similar sample to that used in the gas chromatography analysis was also ascertained by thin layer chromatography. In animal experimentation a clear difference was found between the experimental samples and the controls

In quantitative determination, peak height and peak area obtained by chart weighing were measured. (Fig. 1)

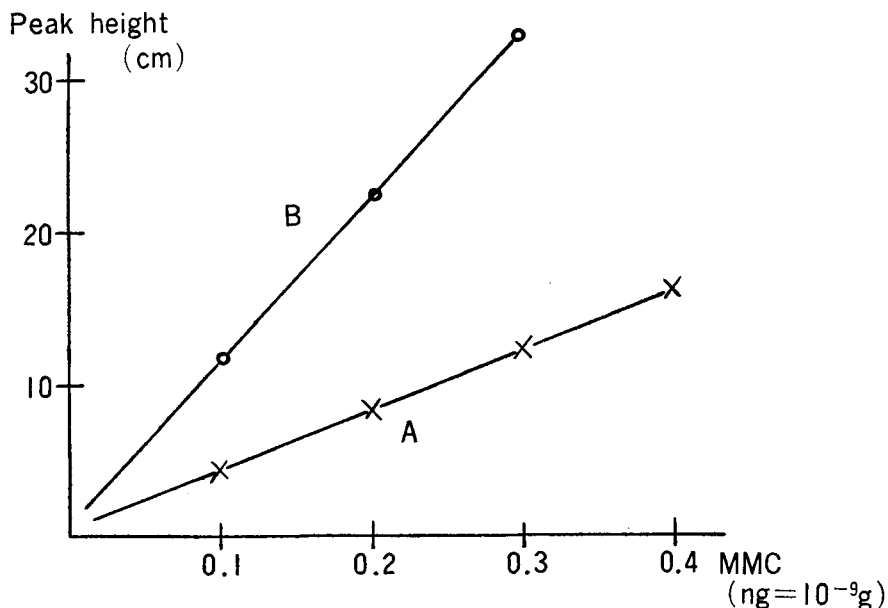


Fig. 1. Calibration curves of MMC.

A : 25% DEGS 4mm × 40cm, 160°C, N₂ ; 80ml/min.

B : 5% 1.4-BDS 4mm × 1.2m, 160°C, N₂ ; 60ml/min.
(glass on-column)

Both showed good linearity but the dynamic range remained within 10⁸. The calibration curve cannot be used for a long period of time. Quantitative determination can usually be made by comparing the peak height of the sample with that of a standard solution of about the same organic mercury concentration.

3-5 Results

DEGS, PEG-20M, and 1.4-BDS were selected for alkyl mercury analysis after examination of the chromatograms which had been recorded using the packed columns shown in Table 2, under the various conditions mentioned above. Two examples of such sets of conditions are shown in Table 3, their chromatograms in Fig. 2, and the relative retention times of alkyl mercury compounds in Table 4.

Table 3. Analytical conditions of alkyl mercury compounds.

| Apparatus | GC-1C | GC-3AE |
|----------------------------|--|--|
| Column | 4mm × 40cm glass on-column 160°C | 4mm × 1.2m glass on-column 160°C |
| Packing | DEGS | 1.4-BDS |
| Wt. % | 25 | 5 |
| Support | Shimalite | Shimalite-W |
| Mesh | 60/80 | 60/80 |
| Carrier gas | N ₂ | N ₂ |
| Flow rate | 80ml/min | 60ml/min |
| Detector | ECD 210°C | ECD 160°C |
| Applied Volts. | pulse height 40V | pulse height 40V |
| Amplifier range | 4 × 10 ⁻⁹ A/FS | 8 × 10 ⁻¹⁰ A/FS |
| Chart speed | 10mm/min | 10mm/min |
| Detection limit for MMC | 2 × 10 ⁻¹² g | 1 × 10 ⁻¹² g |

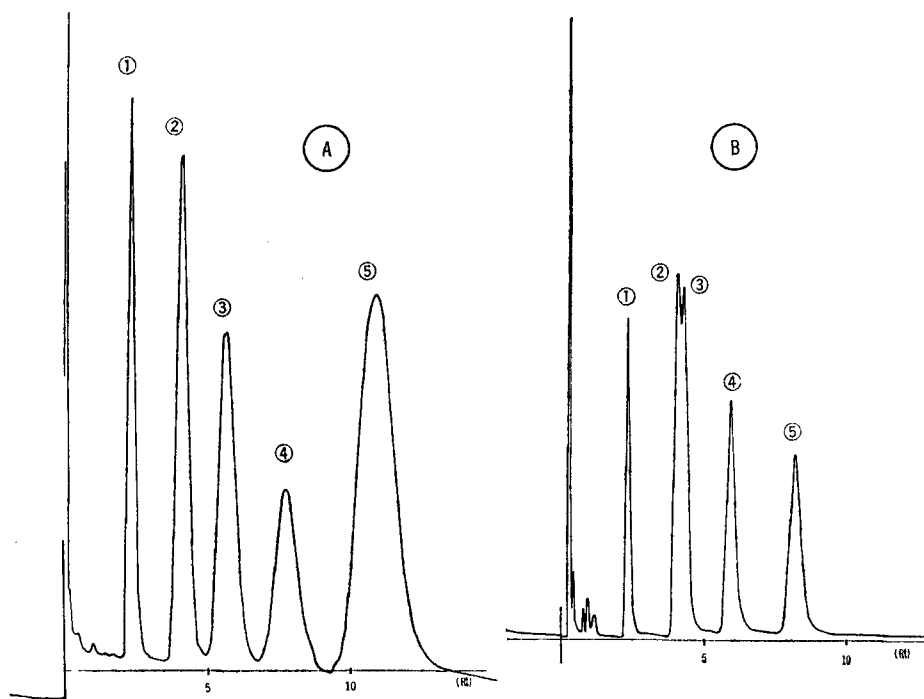


Fig. 2. Chromatograms of alkyl mercury compounds.

Ⓐ : 25% DEGS 4mm × 40cm 160°C
N₂ ; 80ml/min

① MMC ② EMC ③ BMC ④ AMC
⑤ MtEMC

Ⓑ : 5% 1.4-BDS 4mm × 1.2m 160°C
N₂ ; 60ml/min

① MMC ② EMC ③ Pr-MC ④ BMC
⑤ AMC

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Table 4. Relative retention time of alkyl mercury compounds.

| Compound | R. Rt (A) | R. Rt (B) |
|----------|-----------|-----------|
| MMI | 0.98 | 0.97 |
| MMC | 1.00 | 1.00 |
| MM(OH) | 1.00 | 1.03 |
| MMA | 1.00 | 1.06 |
| MMS | 1.00 | 1.05 |
| EMC | 1.79 | 1.75 |
| EMP | 1.80 | 1.74 |
| PrMC | 1.80 | 1.83 |
| BMC | 2.43 | 2.54 |
| AMC | 3.30 | 3.57 |
| MtEMC | 5.06 | 4.11 |

(A) ; column 25% DEGS 160°C 40cm

MMC=2.35 min.

(B) ; column 5% 1.4-BDS 160°C 1.2m

MMC=2.30 min.

The compounds eluted from all these three columns in order of increasing carbon number. MMC, MMI, MM(OH), MMA and MMS, all methyl mercury compounds, showed about the same Rt. In R-HgX type compounds, the same Rt was again obtained, regardless of X, so long as the carbon number was the same.

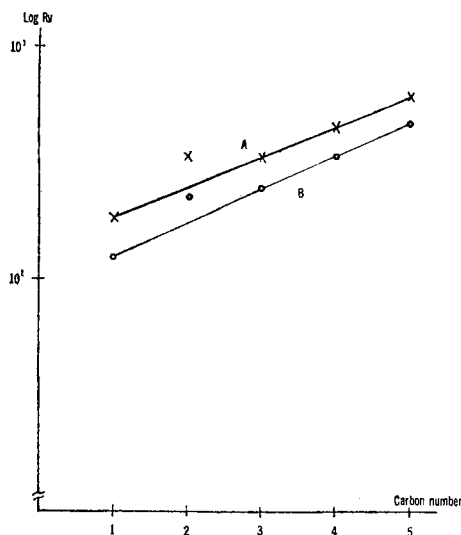


Fig. 3. Carbon number and logarithmic retention volume.

(GC conditions are described in Table 3)

These phenomena have been discussed by Nishi et al.⁸⁾

Separation of EMC and PrMC was difficult with DEGS and PEG-20M, even when a long column was employed. However, separation was possible when 1.4-BDS was used. Nevertheless, even in the case of a very long column (3.3m) perfect separation was impossible.

In order to know their relationship to one another, carbon number and retention volumes were measured in the cases of DEGS and 1.4-BDS. The logarithm of retention volume was plotted on the Y-axis and the carbon number of the alkyl radical on the X-axis. A good linearity was recognized for carbon numbers 1, 3, 4, 5. (Fig. 3)

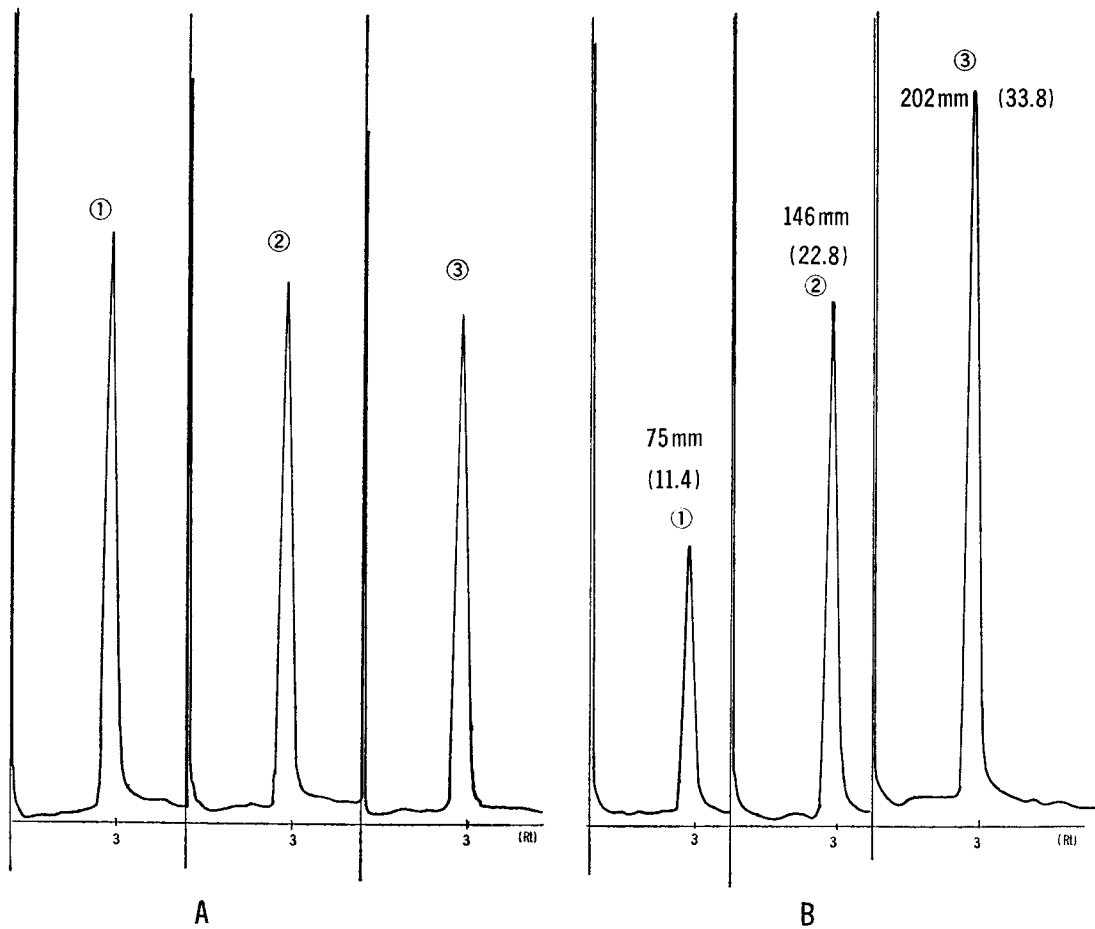


Fig. 4. Chromatogram of phenyl mercuric compounds.

① PhMC ② PMA ③ PMI

Calibration curve of PhMC peak height and (peak area).

① 0.5ng (5×10^{-10} g) ② 1.0ng ③ 1.5ng

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Greatest efficiency of separation was recognized in the case of 1.4-BDS. Its theoretical plate number for MMC was 900, as opposed to 500 for DEGS. A short column (40cm) also provided good separation. With a glass column, higher theoretical plate numbers and sensitivity were obtained, i. e. compared with a stainless steel column. Under optimum conditions the detection limit for MMC reached at 10^{-12} g. This was made possible by controlling the degree of column aging, and by using a low column-temperature, so reducing the noise level. The relative response peaks for methyl mercury did not show any prominent quantitative variation (MMC : MMI : MMA = 1 : 1.05 : 0.85).

In the analysis of phenyl mercury, good results were obtained when a low concentration of DEGS (5%) was used for the column packing. In order to shorten the analysis time a 40cm column was tried. This column had excellent separating ability. It was not possible to use stainless steel columns due to tailing of the peak and bad reappearance. For R-HgX the same retention times were observed, regardless of X. Both peak height and peak area showed an extremely good linearity, as in alkyl compounds. (Fig. 4)

The detection limit for PhMC was reached at 10^{-11} g under the conditions indicated in Table 5.

Table 5. Analytical conditions of phenyl mercury compounds.

| | |
|--------------------------|--|
| Column | 4mm × 40cm glass on-column 185°C |
| Packing | DEGS |
| Wt. % | 5 |
| Support | Shimalite-W |
| Mesh | 60/80 |
| Carrier gas | N ₂ |
| Flow rate | 80ml/min |
| Detector | ECD 210°C |
| Applied Volts. | pulse height 40V |
| Amplifier range | 4×10^{-9} A/FS |
| Chart speed | 10mm/min |
| Detection limit for PhMC | 1×10^{-11} g |

With generally low polar column packing, neither alkyl nor phenyl compounds gave a good result.

In the GC analysis of organic mercury compounds some points worthy of attention are as follows:

1) ECD should be employed as detector and the apparatus always be kept switched on for stability of ECD condition.

2) The on-column injection technique should be employed and the material of the column be glass.

- 3) The column packing should be aged for over 48 hours.
- 4) Nitrogen should be used as carrier gas, with a high flow rate and somewhat low column temperature.
- 5) Ghost peaks occasionally appear after the injection of solvent containing a large quantity of organic and inorganic mercury.

ESTABLISHMENT AND DESCRIPTION OF EXTRACTION METHODS

4-1 Solvent

MMC and PhMC in aqueous solution can be removed completely by extracting three times with organic solvents such as benzene, chloroform, ether, ethyl acetate, hexane etc. It has been reported that PhMC decomposes in strong acid solution.⁹⁾ However in the author's experiment PhMC in 0.5N, 1N and 2N-hydrochloric acid can be extracted completely and never breaks down.

In the analysis of alkyl mercury, ghost peaks sometimes appear if extraction solvents such as ether and ethyl acetate are used, so that the utilization of benzene is desirable. The number of interfering peaks was found to be smallest when benzene was used. Since this phenomenon never occurs in phenyl mercury analysis, mixed solvents of benzene and ether can be used. Such a solvent permits rapid concentration and therefore is a suitable one.

4-2 Extraction from Biomaterials

Since organic mercury compounds easily combine with the SH radical, they occur as mercaptides in biological materials.⁴⁾ Therefore, after washing the cut and homogenized materials with water, acetone and ethyl acetate etc., they were transferred to 1N-hydrochloric acid, and the organic mercury separated from the proteins. Following this, they were extracted repeatedly with organic solvents. As a result, organic mercury could be extracted completely, and was free of disturbant substances.

Aqueous solution of glutathione (GSH) containing MMC was extracted with benzene. The results are given in Table 6.

Table 6. Effect of pH on the extraction of MMC from GSH-solution (100 ppm) with benzene.

| No. | GSH-solution | | Added MMC(μ g) | Benzene (ml) \times times | MMC | |
|-----|--------------|------|------------------------|--------------------------------|---------------------|-----------------|
| | (ml) | (pH) | | | Found (μ g) | Recovery (%) |
| 1 | 20 | 7 | 2.00 | 5 \times 2 | 0 | 0 |
| 2 | 20 | 5 | 2.00 | 5 \times 2 | 0 | 0 |
| 3 | 20 | 3 | 2.00 | 5 \times 2 | 0 | 0 |
| 4 | 20 | 2 | 2.00 | 5 \times 2 | 0.35 | 17.5 |
| 5 | 20 | 1.5 | 2.00 | 5 \times 2 | 1.96 | 98.0 |
| 6 | 20 | 1 | 2.00 | 5 \times 2 | 2.00 | 100.0 |
| 7 | 20 | 0.5 | 2.00 | 5 \times 2 | 1.99 | 99.5 |

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This table shows the following points. i) When the pH of the solution is higher than 3, MMC which is combined with GSH cannot be separated from the latter. ii) For complete separation, a pH of less than 1.5 is required. iii) The ratio of aqueous solution of GSH to benzene was 20 : 5; however, all MMC was extracted by performing the extraction twice. iv) In each extraction, shaking for five minutes is sufficient.

Similar results were obtained with phenyl mercury. Diluted and undiluted benzene extract was injected into the GC. Methyl mercury was estimated adopting MMC as standard substance, since the Hg-S bond in methyl mercury is broken by the HCl solution used here, forming $\text{CH}_3\text{Hg}\cdot\text{Cl}$. This method was used for extractions from the following materials : Hair of patients with Minamata Disease, the fish and shellfish contaminated with MMC which gave rise to Minamata Disease, and other samples which seemed to contain large quantities of organic mercury. (See Part II)

The clean-up method, discussed in 4-3, should be performed in the extraction from materials which had been sprayed with pesticides and from waste discharged from factories (both of which seem to contain ultra trace amounts of methyl mercury), and also in the extraction from "non-contaminated" marine products and materials requiring strict identification.

Washing with solvents is highly effective in extraction from materials rich in fats and pigments, but this process may be omitted in the case where the following clean-up method is performed. The suspension of homegenized, acidified material is shaken in a separating funnel with benzene. Centrifugation at 6,000 to 10,000 rpm is required where an emulsion is produced.

As an alternative method, the material was transferred to 1N-HCl after homogenization and washing with solvents. Organic mercury can be eluted by occasional stirring of the solution in a half hour period. However, in this case elution of organic mercury into the HCl becomes complete only after repeating the procedure 3 times. The author has found this method convenient when it is employed in the case of hair and dried biomaterial, since the process of centrifugation not required.

4-3 Purification

The clean-up method is required in cases where a large amount of interfering substance is present in the extracted benzene solution mentioned above. Peaks similar to the standard MMC peak then appear on the chromatogram. In the case where ultra trace amounts of organic mercury are analyzed from a large quantity of material this clean-up method is also required. In a neutral solution GSH is capable of combining completely with isolated organic mercury compounds such as MMC, PhMC and so on. Thus, separated organic mercury within the benzene layer can be extracted completely with an aqueous solution of GSH. Table 7 shows the results of this experiment.

These can be summarized as follows. 1) 0.1-5 μg of added MMC can be transferred completely from the benzene layer to the GSH solution by 2 extractions. 2) The percentage recovery of MMC is good with 100, 50 and 10 ppm GSH solution.

Table 7. Extraction of MMC in benzene with GSH-solution.

| No. | Benzene (ml) | Added MMC(μg) | GSH-solution (ppm) | (ml) \times times | Found MMC (μg) | Recovery (%) |
|-----|-----------------|-------------------------------|-----------------------|---------------------|-----------------------------------|-----------------|
| 1 | 50 | 5.00 | 100 | 10 \times 2 | 4.90 | 98.0 |
| 2 | 50 | 5.00 | 50 | 10 \times 2 | 5.08 | 101.6 |
| 3 | 50 | 5.00 | 10 | 10 \times 2 | 5.00 | 100.0 |
| 4 | 50 | 1.00 | 100 | 10 \times 2 | 1.01 | 101.0 |
| 5 | 50 | 0.10 | 100 | 10 \times 2 | 0.098 | 98.0 |

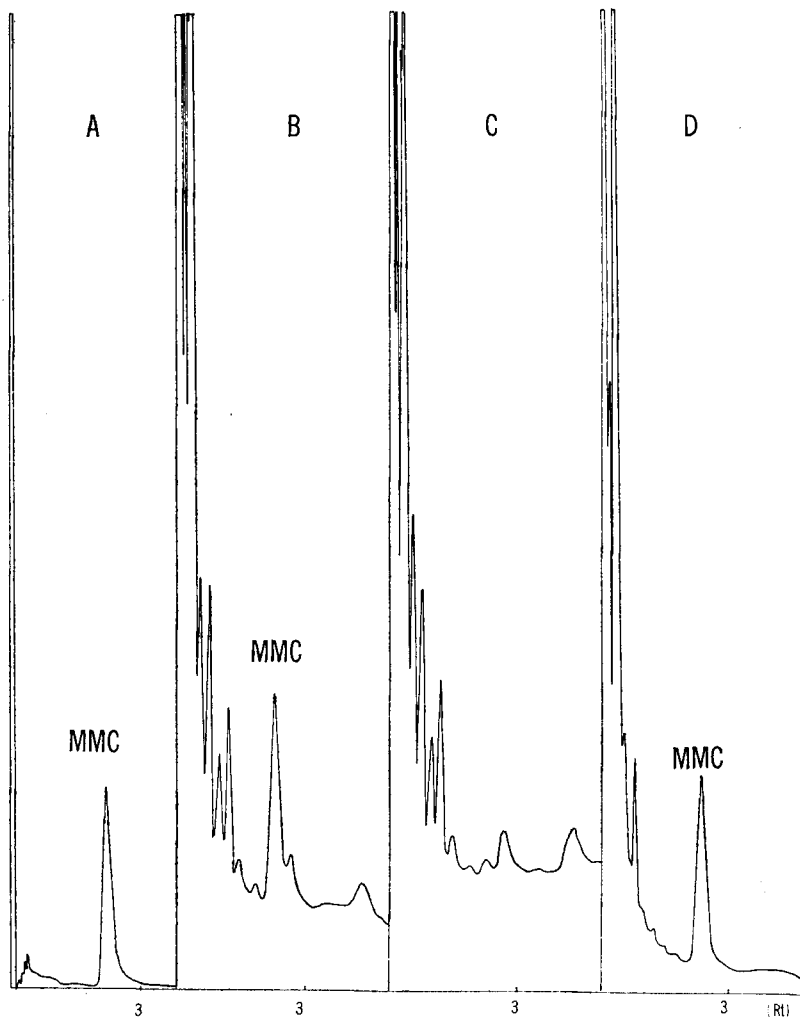


Fig. 5. Chromatogram before and after purification.

A ; standard CH_3HgCl :0.2ng. B ; river fish first benzene extracts (5g \rightarrow 20ml). C ; residual first benzene layer after GSH treatment of B. D ; second benzene extracts after acidifying GSH solution.

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3) A 5-minute shaking time is sufficient in the case where the ratio of the volume of benzene layer to GSH solution is 5 : 1.

After acidification of the GSH solution containing organic mercury (with HCl), the resulting solution was re-extracted with benzene. Comparison of the chromatogram obtained before purification with that obtained after purification, showed that interfering substances had been removed. (Fig. 5)

Moreover, the use of this technique also provided an excellent means for the identification of organic mercury.

4-4 Extraction from Troublesome Materials

If troublesome materials are used for extraction by the methods mentioned in 4-2, 4-3, various substances dissolve in the solvent which may interfere the GC analysis. In such cases TLC is better employed. After applying the extract to a preparative thin-layer silica gel chromatoplate as continuous streak, a small amount of the same sample was added to both edges as indicator. The material was developed according to Nakayama's method and the index sample then visualized with dithizone.⁷⁾ Confirmation of an organic mercury spot was made and, taking

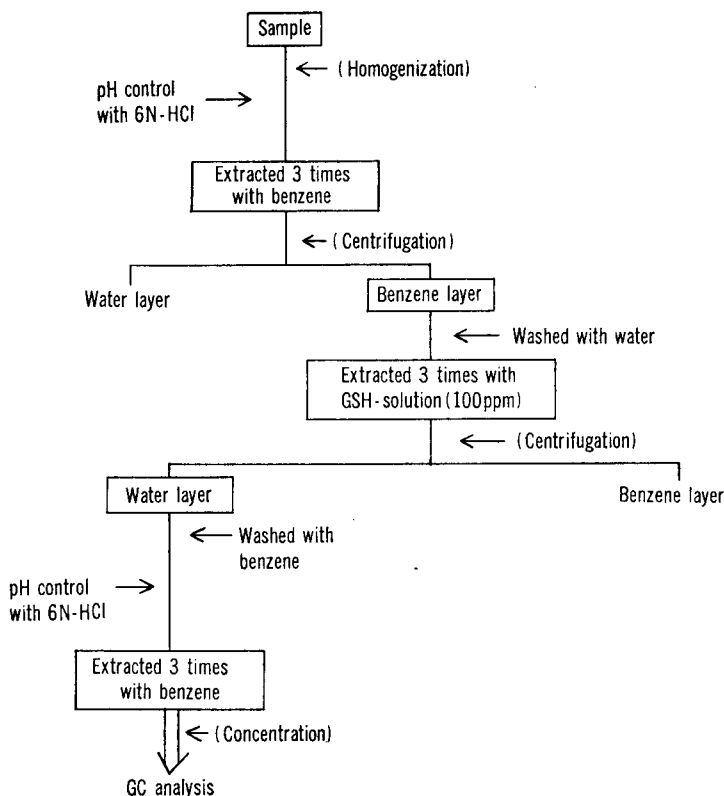


Fig. 6. Method for isolation and estimation of organic mercury compounds.

this spot as standard, a portion of silica gel containing organic mercury, namely the objective substance, was scraped off with a spatula. After elution of this material with a solvent such as benzene the sample was ready for GC analysis.

4-5 Results

The extraction method to be used should be chosen properly according to the materials. The flow chart Fig. 6 shows the sampling method for body fluids, water-soluble and other materials. A convenient extraction method for hair and dried biomaterials is given in Fig. 7.

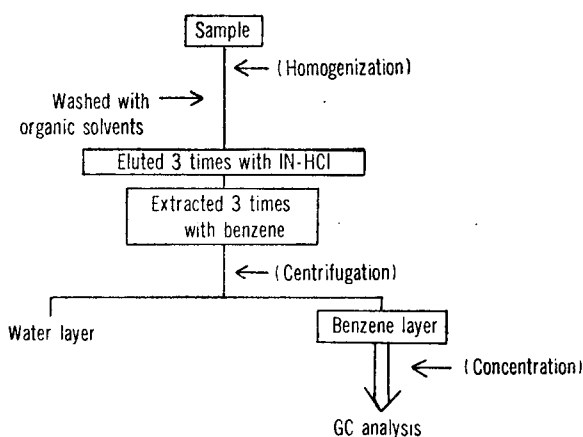


Fig. 7. Method for isolation and estimation of organic mercury compounds.

(dried biomaterial powder ; without purification)

SUMMARY

1) Micro-analysis of organic mercury compounds was performed by means of gas chromatography.

2) In alkyl mercury analysis DEGS, PEG-20M and 1.4-BDS were chosen as stationary phases. The use of 5% DEGS and a short but suitable glass on-column permitted the analysis of phenyl mercury.

3) The detection limit for MMC was reached at 10^{-12} g and for PhMC at 10^{-11} g, in the case of the use of an electron capture detector.

4) Extraction of organic mercury from different animal and plant materials was performed with organic solvents, after isolation of the compounds with HCl.

5) In the clean-up method benzene extract was reextracted with GSH solution, and the organic mercury then separated from acidified GSH solution for purification. This method was found to be useful not only in the elimination of interfering peaks but also in the identification of organic mercury itself.

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