LITERATURE CITED

Chambers, J. E., Yarbrough, J. D., *Bull. Environ. Contam. Toxicol.* **11,** 315 (1974).
Culley, D. D., Ferguson, D. E., *J. Fish Res. Board Can.* **26,** 2395–2401 (1969).
Dziuk, L. J., Plapp, F. W., *Bull. Environ. Contam. Toxicol.* **9,** 15–19 (1973).
Fabacher, D. L., Chambers, H., *Bull. Environ. Contam. Toxicol.* **6,** 372–376 (1971).
Ferguson, D. E., Bingham, C. R., *Trans. Am. Fish. Soc.* **95,** 325–326 (1966).
Ferguson, D. E., Ludke, J. L., Murphy, G. G., *Trans. Am. Fish. Soc.* **95,** 335–344 (1966).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265–275 (1951).
Ludke, J. L., Ferguson, D. E., Burke, W. D., *Trans. Am. Fish. Soc.* **97,** 260–263 (1968).
Moffett, G. B., Yarbrough, J. D., *J. Agric. Food Chem.* **20,** 558–560 (1972).
Vinson, S. B., Boyd, C. E., Ferguson, D. E., *Science* **139,** 217–218 (1963).
Wells, M. R., Ph.D. Thesis, Mississippi State University, 42 pp, 1971.
Wells, M. R., Yarbrough, J. D., *J. Agric. Food Chem.* **20,** 14–16 (1972).
Yarbrough, J. D., Wells, M. R., *Bull. Environ. Contam. Toxicol.* **6,** 171–176 (1971).

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A Method for the Estimation of Methylmercuric Compounds in Fish

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The methylmercuric ion ($MeHg^+$) liberated by alkaline hydrolysis from methylmercuric compounds in fish is partitioned into benzene as methylmercuric chloride (MeHgCl). This reacts with cysteine to form a MeHg sulfur complex; it is reextracted into benzene as MeHgCl and assayed using gas–liquid chromatography (GLC) and a ^{63}Ni electron capture detector (ECD). Mean recoveries for MeHgCl added to three species of fish at 0.2- to 0.6-ppm levels ranged from 73.3 to 87.2%, with percent coefficients of variation (% CV) of 8.1 to 14.1. The % CV for assay of naturally occurring MeHg compounds was 7.8. The lower limit of detectability is 0.02 ppm. Comparative assays using other methods are given. Statistical estimates include tests to examine the normal distribution of peak heights, instrumental stability, confidence limits of individual assays, and the number of assays required to provide a result with a known confidence limit.

The presence of mercury in food fish is widespread and levels exceeding 0.1 ppm are quite common in certain species of fish (Simpson et al., 1974; Uthe et al., 1972). Part of the mercury in the fish flesh has been isolated as the methylmercuric ion, $MeHg^+$, and identified as MeHgCl (Westoo, 1966; Johansson et al., 1970). Alkylmercuric compounds, including MeHg salts, cause irreversible neurological disturbances in the human (Hunter, 1969) and are considerably more toxic than other chemical forms of mercury such as the metallic, inorganic, or arylmercuric compounds.

In other methods used for the assay of MeHg compounds in fish the $MeHg^+$ is extracted as a halide into an organic solvent from the sample after the addition of a mineral acid and/or inorganic salts (Newsome, 1971; Rudling, 1971; Sumino, 1968; Uthe et al., 1972; Westoo, 1966, 1967). For any given fish sample, assays for the total mercury content usually indicate higher concentrations than that extractable as $MeHg^+$ by existing methods (Bache et al., 1971; Elkins, 1972; Uthe et al., 1972). The purpose for the development of this method, where the $MeHg^+$ is extracted as MeHgCl from fish samples after alkaline hydrolysis, includes the following: (1) to seek information on the chemical form of the mercury in the fish not extracted as a MeHg halide by other methods; and (2) to ascertain that the MeHg halide in the final step of the extraction procedures used in other methods is in fact in the fish flesh as MeHg compounds and was not synthesized in the course of the chemical reactions used to isolate and purify the MeHg halide for assay. Synthesis of the C–Hg bond of $MeHg^+$ can occur under fairly mild aqueous conditions if Hg^{2+} is present along with a protonable carbanion or an olefin (Makarova and Nesmeyanov, 1967). The rates for cleavage of the C–Hg bond of organomercuric compounds by mineral acids vary from one class of compounds to another (Makarova and Nesmeyanov, 1967). As an example, aromatic derivatives, such as the phenylmercuric ion, are decomposed at acidities exceeding 3 *M* HCl (Polley and Miller, 1952). For this reason, in the method described here, care was taken to maintain acidities of 1 *M* HCl or less in all the chemical reactions required for preparation of a benzene-soluble MeHgCl suitable for GLC assays. As a rule, alkalis do not cleave the C–Hg bond of either alkyl- or arylmercuric ions (Makarova and Nesmeyanov, 1967). Therefore, it can be assumed that alkaline hydrolysis does not alter the chemical form of the mercury as it existed in the fish.

Details of the instrumental conditions for the assay of nanogram (ng) to picogram (pg) concentrations of organomercuric halides by GLC with electron capture detection (ECD) need to be very explicit. For example, Nishi and Horimoto (1968) observed thermal degradation of the alkylmercuric compounds if stainless steel columns were used. Tatton and Wagstaffe (1969) described conditions to prevent "poisoning" of tritiated foil detectors by the mercury compounds, and Uthe et al. (1972) modified the detector design to make it adaptable to disassembly for cleaning. With the extraction method described here, sufficient instrumental stability was achieved so that data accumulated over several months could be combined for the statistical estimates. Detector disassembly for cleaning was not required.

The method consists of an eight-part extraction procedure involving four chemical reactions and a determinative step (see Scheme I).

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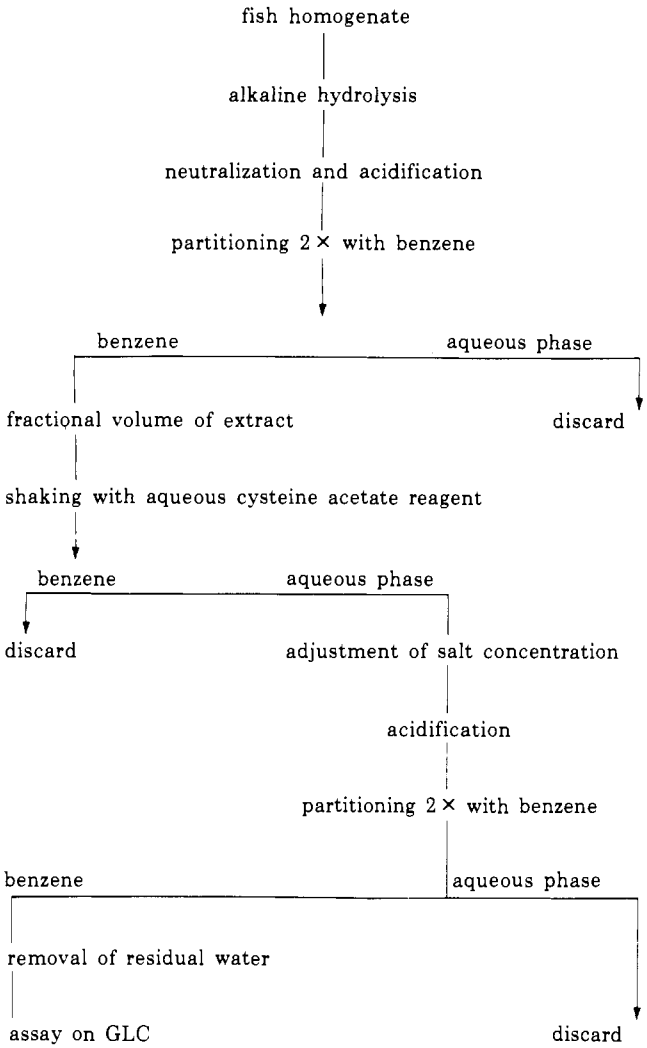
Scheme I. Outline for Extraction of MeHg Compounds from Fish



EXPERIMENTAL SECTION

Samples. Sheepshead, a product of the Great Lakes, was purchased on the retail market. The whole fish, shipped to Cincinnati, Ohio, from Chicago, Ill., had been held under commercial storage conditions for 4 to 5 days before being filleted, skinned, and homogenized. Four fish, the smallest available, were combined to prepare the one sample used for recovery data. The trout sample was one 1-lb lake trout with known exposure history to mercury compounds, provided by the Great Lakes Fisheries Laboratory, Ann Arbor, Mich. It was filleted within 1 hr after the fish was killed, and was skinned and homogenized within a 20-hr period. Canned tuna in oil was purchased on the retail market. The tuna sample for the comparative assays, BF 251, was a portion of a 251-lb blue fin tuna canned especially by the National Canners Association for a collaborative study.

Reagents and Apparatus. Benzene and 2-propanol contained a minimum concentration of impurities that respond to gas chromatography employing an electron capture detector. This grade of solvent is available from several different chemical companies. Water was purified with a Millipore Super-Q system equipped with prefiltration, organic absorption, ion exchange, and Millipore filtration cartridges. The MeHgCl was purchased from K&K Laboratories, Jamaica, N.Y. All other chemicals were reagent grade. The Na_2SO_4 was washed with benzene and allowed to air dry. Activated carbon (Darco G-60) was added to the 7 *M* HCl (3 g/l.) and removed by filtration after 12 to 24 hr. This purified 7 *M* acid was prepared fresh every 7 days and was diluted for the 1 *M* HCl. Aqueous saturated solutions of $HgCl_2$ and the aqueous cysteine acetate reagent were prepared for each set of assays. A 3-ml volume of the cysteine acetate reagent contained 5.2 mg of L-cysteine hydrochloride monohydrate, 6.0 mg of sodium acetate trihydrate, and 20.0 mg of sodium sulfate. MeHgCl in benzene (0.50 to 1.00 mg/ml) was used to spike the samples and to prepare the spiking standards. Suitable dilutions of it were made for the calibration solutions. These solutions were prepared every 4 to 6 weeks. Separatory funnels (250 ml) equipped with Teflon stopcocks were calibrated at the 120-ml level. Glass-stoppered (12 ml) centrifuge tubes with conical tips were calibrated at the 5.25-ml level. An alcoholic KOH rinse followed by a distilled water rinse was used on all glassware. A gas chromatograph equipped with a ^{63}Ni ECD (product of MicroTek Co.) and a 3.5 mm i.d. × 210 cm glass column packed with 5% phenyldiethanolaminesuccinate on 60–80 mesh Chromosorb W (HP) was used. The column, injector, and detector temperatures were 150, 205, and 270°C, respectively, and the nitrogen flow rate was 80 ml/min. The precolumn was Molecular Sieve 5A (60–80 mesh). Peak heights were used to prepare calibration curves.

Procedure. Association constants (Simpson, 1961) of $MeHg^+$ are included for each chemical reaction.

Step 1; Preparation of Sample. Nine-gram portions of fish muscle were used. The skinless fillets of fresh fish or canned tuna were combined with an equal weight of water and mixed in a blender until homogeneous. The 18-g portions were collected in tared 150-ml beakers. 2-Propanol (15 ml) was added, and the beakers were covered with parafilm. All samples required for recovery data were prepared from one composite. Samples to be extracted within 60 hr were held at 0–5°C; the remainder were stored at −20°C until assayed. For the recovery studies, 2 to 8 μl of MeHgCl in benzene (0.50 to 1.00 mg/ml) was added to one sample and to a 20-ml volume of benzene for the spiking standard. One sample with no added MeHgCl, the control, was extracted along with each spiked sample.

Step 2; Alkaline Hydrolysis, Probably Resulting in the Formation of MeHgSR (Fujiki, 1970). A Teflon-covered magnetized stir bar and 30 ml of 8 *M* KOH were added to each sample, and the beakers were covered with watch glasses. Each sample was stirred at minimum speed on a magnetic stirrer for a few seconds and then placed on an electric steam bath. After complete digestion (about 45 min) each sample was cooled for a minimum of 15 min in an individual ice–water bath.

Step 3; Addition of $HgCl_2$ to Tie Up S^{2-} (pK_s for HgS = 52.4), Followed by Adjustment of Acidity of Solution to 0.8 M HCl with the Formation of MeHgCl (Log K = 5.45). A saturated aqueous solution of $HgCl_2$ (6 ml) was added to each sample, in the individual ice–water bath, while it was being stirred. Approximately 30 ml of 7 *M* HCl (at 0.5°C) was added from an automatic buret to produce a solution of pH 1. Dual tint test paper, pH 1.0 to 4.3 in 0.3 pH unit, was used for this pH adjustment. The rate of addition of acid was controlled to prevent an excessive temperature increase. About 4 min was required. Ten milliliters of additional acid (7 *M* HCl) was added.

Step 4; Partitioning of MeHgCl from Aqueous Phase to Benzene. Immediately after acidification, the sample was transferred quantitatively to a 250-ml separatory funnel containing 50 ml of benzene. An additional 10-ml portion of benzene was added along with sufficient dilute acid (1 *M* HCl) to adjust the aqueous volume to 120 ml. After 5 min on a reciprocating shaker, the phase separation was rapid and complete. The aqueous phase, after removal of the benzene, was shaken with a second 60 ml of benzene. The two benzene extracts were combined and mixed.

Step 5; Formation of a Water-Soluble MeHgSR Complex Involving the Transfer of the $MeHg^+$ from a 30-ml Volume of Benzene to a 3-ml Volume of Aqueous Cysteine

Table I. Recovery of MeHgCl Added to Homogenate of Fish

Sample	Control fish MeHgCl,[a] ppm	MeHgCl added as spike, ppm	Number of recoveries,[b] n	Av % recovery	Std dev, s	% CV[c]
Sheepshead	0.02	0.16 and 0.23	19	73.3[d]	6.0	8.1
Trout	0.16	0.24 to 0.41	10	87.2	11.7	13.4
Tuna	0.29	0.34	9	85.5	12.0	14.1

[a] MeHgCl in final benzene extract from the MeHg compounds occurring naturally in fish. [b] Recoveries were calculated as follows: (concentration (ng/2 μl) in spiked sample − concentration (ng/2 μl) in control sample)/concentration (ng/2 μl) in spiking standard. [c] The % CV's for all three species are satisfactory, <15. [d] The average percent recovery for sheepshead is lower than that for trout and tuna by >10%.

Acetate (Log K = 15.7 when R = Cysteine). One-fourth of the benzene extract (30 ml) was transferred to a 40-ml separatory funnel containing 3 ml of aqueous cysteine acetate reagent. The funnel was shaken by hand for 10 min. With all samples, a 30- to 40-min time interval was sufficient for phase separation. Centrifugation was not used.

Step 6; Acidification to 0.9 M HCl and Adjustment of Salt Concentration with KCl to Convert the Sulfur Complex to MeHgCl. The aqueous phase was drained from the separatory funnel into a glass-stoppered, 12-ml centrifuge tube containing 0.3 g of KCl. Water was used to quantitate this transfer step and to give a final volume of 5.25 ml. Acidification with 0.75 ml of 7 *M* HCl resulted in conversion of the MeHg–cysteine complex to MeHgCl.

Step 7; Partitioning of the Halide from Aqueous Phase to Benzene. Two extractions, each with 2.5 ml of benzene and a 5-min shake (either by hand or with the shaker), were sufficient to partition the MeHgCl into benzene. Phase separation was rapid with all samples. The two benzene extracts were combined in a glass-stoppered centrifuge tube with a conical tip and diluted with benzene, if necessary, to give MeHgCl concentrations within the range of the calibration curve, 0.025–0.25 ng/2 μl. For the data reported here, 5-ml volumes of sheepshead and trout and 15-ml volumes of tuna were used.

Step 8; Removal of Residual Moisture from the Benzene Extract. Storage for at least 10 min at 0–5°C was sufficient. For all assays reported here, the extracts were held overnight at 0–5°C and assayed the following day.

Step 9; The Determinative Step, Using GLC and an ECD. Data for each assay and recovery were collected from a preconditioned column and immediately after a calibration curve. The sequence of injections for one assay was as follows: (1) two 5-μl portions of a benzene solution containing 1 mg of $HgCl_2$/ml, (2) one 2-μl portion of the benzene extract of the sample to be assayed, (3) one 2-μl portion of benzene (to ascertain, from the appearance of the chromatogram, that the instrumental conditions were sufficiently stable for quantitative assays), (4) four to five different dilutions of the calibration solutions (2-μl portions of each), and (5) one 2-μl portion of the benzene extract of the sample. For each recovery, the injections 1 through 4 were made followed by (5) one 2-μl portion of the benzene extract of the fish sample spiked with MeHgCl, (6) one 2-μl portion of the benzene solution of MeHgCl (the spiking standard that was prepared at the time that the sample was spiked); and (7) a 2-μl portion of the benzene extract of the fish sample serving as the control. Only injections 4 through 7 were used for computations.

Calculations. The following assumptions were made: (1) the peak heights produced by MeHgCl on the chromatograms were linearly related to the concentration; (2) the sample variance, s^2, was homogeneous for each set of samples over the range of concentrations; (3) the recovery of $MeHg^+$ from naturally occurring MeHg compounds was the same as that for MeHgCl added to samples prior to assay; and (4) the peak heights of MeHgCl produced from benzene solutions of pure MeHgCl, MeHgCl formed from naturally occurring MeHg compounds in fish muscle, and MeHgCl recovered from spiked samples were distributed normally and independently with mean, μ, and variance, σ^2. A test for the latter assumption (D'Agostino and Pearson, 1973) is described in the Supplementary Material (see paragraph at end of paper).

Calibration curves were prepared by least-squares analyses (Ostle, 1963) and used to estimate the concentration of MeHgCl (ng/2 μl) in each sample. The linear model used for the least-squares analyses was

$$y = b_0 + b_1 x \quad (1)$$

where y = observed peak height (cm), x = concentration (ng/2 μl), b_0 = estimated intercept, and b_1 = estimated slope. The concentration of MeHgCl, in parts per million, for any given sample was calculated in the following manner:

$$\text{MeHgCl in ppm} = A/B \times C/D \times E/F \quad (2)$$

where A = μg of MeHgCl (concentration from calibration curve in ng × 10^{-3}), B = grams of fish in sample (9 g), C = volume of final benzene extract (5 or 15 ml in samples reported), D = volume injected in step 9 (0.002 ml), E = total volume of benzene used in step 4 (120 ml), and F = volume of benzene used for steps 5 through 9 (30 ml for samples reported). Recovery data were calculated from the following ratio:

$$\hat{\theta} = (x_3 - x_2)/x_1 \quad (3)$$

where x_3 is the concentration of MeHgCl in the spiked replicate (ng/2 μl), x_1 in the spiking standard, and x_2 in the control replicate. Variations among sets of samples and among recoveries were estimated as percent coefficient of variation (% CV = $(s/\bar{x})100$). Since concentration is the known variable in the calibration curves and peak heights are known variables in the assay of samples, equations that are the inverse of eq 1 had to be used for the estimation of confidence limits for predicted concentrations of mercury from naturally occurring MeHg compounds. The development of these equations and an example using them are described in the Supplementary Material.

RESULTS AND DISCUSSION

Table I presents data on the concentrations of MeHgCl from MeHg compounds occurring naturally in fish samples and on the recovery of MeHgCl added to the fish. The mean percent recovery of added MeHgCl and % CV in replicate analyses were 73.3 and 8.1 for sheepshead, 87.2 and 13.4 for trout, and 85.5 and 14.1 for tuna, respectively. The recovery for sheepshead is 10 to 14% lower than the recoveries for trout and tuna. The range in % CV for the three species of fish is satisfactory, since a % CV of 15 or less was considered acceptable for the isolation of this chemically active organometallic ion from a complex biological medium such as fish flesh. The precision of the assay for naturally occurring MeHg compounds was in the same range as that for the recovery studies, 7.8% CV for the tuna sample used in the comparative assays (see Table II). Data for each recovery were prepared from data for one spiked sample

Table II. Comparison of Procedures for the Assay of MeHgCl Extracted from Canned Tuna Fish in Oil (BF 251)[a]

	Inter-laboratory study[b-d]	National Canners Association laboratory[b,d]	This study[e]
Number of replicates		8	9
Mean, ppm	0.79	0.83	0.81
Std dev	0.183	0.048	0.062
% CV	23.2	5.8	7.8

[a] Portion of a 251-lb blue fin tuna canned especially by the National Canners Association for a collaborative study. [b] Assay results provided by Elkins (1972). [c] Five laboratories. [d] Modifications of Westoo's method, using acid hydrolysis. [e] Alkaline hydrolysis.

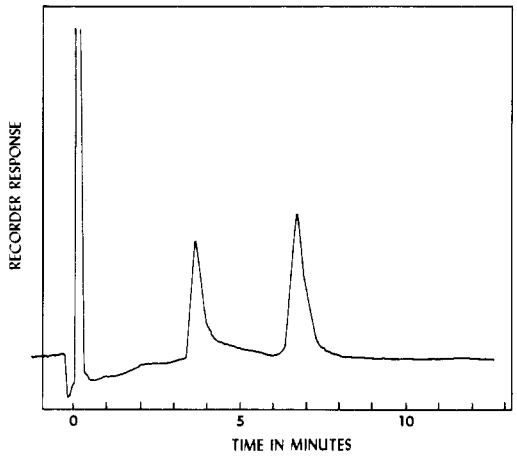


Figure 1. Typical chromatogram of benzene solution of 0.21 ng of MeHgCl and 0.25 ng of EtHgCl. Instrumental conditions are described in text.

and one control sample extracted on the same day. If data for two recoveries were collected on one day, four samples were extracted, two were spiked with MeHgCl, and two served as controls. A spiking standard was prepared each time that samples were spiked. By using a benzene solution of MeHgCl to spike the samples, it was possible to use an identical volume of the same solution to prepare the spiking standard. This procedure minimized volumetric errors associated with the spiking process itself. Data for the 19 sheepshead recoveries were collected at preset time intervals, that is, two recoveries per day and four recoveries per week. The data from two levels of spike, 0.16 and 0.23 ppm of MeHgCl, were combined for the recovery and precision estimates. Since the concentration of MeHgCl from the naturally occurring MeHg compounds in this sample of sheepshead gave peak heights $\leq$ 0.5 cm, no attempt was made to estimate the precision for this assay. Data for the ten trout recoveries were collected at irregular intervals over a 14-week period. The amount of MeHgCl added as a spike varied from 0.24 to 0.41 ppm. All recoveries were combined for the computation of our average recovery and the precision. Any given recovery was estimated from a control and a spiked sample that were extracted on the same day.

The comparison of data obtained by analysis of one tuna sample using this method and alternate methods (Elkins, 1972) is shown in Table II. This agreement among assays indicates that the naturally occurring $MeHg^+$ in this sample of tuna could be isolated from either an acid or an alkaline hydrolysate.

Solutions of MeHgCl in benzene ranging from 0.025 to 0.25 ng/2 μl were used for the calibration curves. A typical chromatogram of a benzene solution of pure MeHgCl and ethylmercuric chloride (EtHgCl) is shown in Figure 1. The EtHgCl was included in this solution because it has a retention time identical with a compound observed in many different fish samples assayed by the method described here. The instrumental conditions remained stable for several months during the assay of benzene extracts from alkaline hydrolysates. For example, the efficiency of the column for the MeHgCl, 16 (retention time/peak width)^2, was 560 theoretical plates on the first day of the sheepshead recovery study and 510 on the last day (the 34th day). In the interim, 60 injections of benzene extracts of sheepshead had been put on the column. The constancy of the least-squares analyses for the 19 calibration curves generated during these 34 days is illustrated with the following data: the estimated intercept, b_0, ranged from -0.16782 to 0.17760; the estimated slope, b_1, ranged from 17.51381 to 21.11876; and the coefficient of determination, R^2, was $\geq$0.990 for all curves. This latter estimate, R^2, represents the proportion of sum of squares due to the linear regression (Ostle, 1963). A value of 1.000 indicates a perfect fit where all points are observed to lie on a straight line. With an $R^2 \geq 0.990$, straight lines drawn visually seemed to yield results similar to those estimated from the least-squares analyses. However, prolonged experience with GLC assays using an ECD suggests that the least-squares method should be used to prepare the calibration curves for quantitative assays of MeHgCl.

At least two factors contributed to the instrumental stability and sensitivity reported in this study: (1) the conditioning of the column before each assay and (2) the age and quality of the reagents, including the water, used in preparation of the samples for assay. The injection of benzene solutions of $HgCl_2$ to condition the columns before assay may not be a suitable technique with detectors of other geometry and alternative stationary phases. However, this step in the conditioning procedure was required for the stability and sensitivity reported here. For some as yet unknown reason, some extracts of the fish samples influenced the sensitivity of the detector for MeHgCl. This phenomenon necessitated the inclusion of one injection of the fish extract in the column conditioning before the injections for the calibration curve and the injection used for the computations. The similarity in precision estimates for recovery data collected at preset time intervals (19 sheepshead recoveries within a 34-day period) and on a random basis (10 trout recoveries over a 14-week period) indicates that the time intervals for the replacement of reagents (as described in the Experimental Section) are adequate. Specific contaminants that affect recoveries and instrumental stability include hydrocarbons and/or moisture in an occasional cylinder of nitrogen, a spent Molecular Sieve precolumn, a spent charcoal absorption cartridge in the Millipore Q system, and unclean glassware.

Recoveries should be run along with all quantitative assays for naturally occurring MeHg compounds. Since no explanation is available at this time for the variation in recoveries with the species of fish that was observed in the studies reported here, it is recommended that recovery data be collected on each species of fish to be assayed. The arithmetic mean for 10 recoveries, with a % CV < 15, is the preferred correction factor for any given assay rather than the actual value for the recovery extracted on the same day that the sample is assayed. The assumptions and restrictions used to ascertain that 10 recoveries are adequate for this correction factor are described in the Supplementary Material.

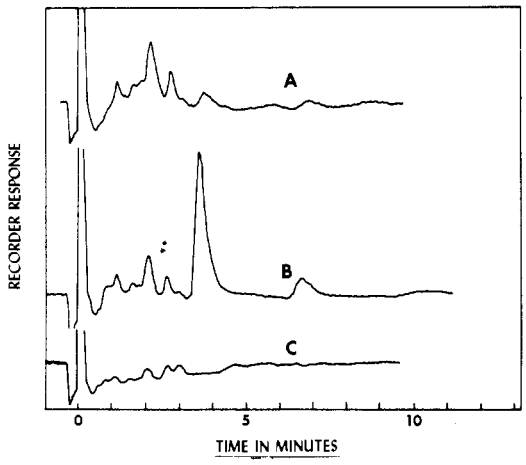


Figure 2. Typical chromatograms of MeHgCl extracted from fish: (A) benzene extract of sheepshead fillet; (B) benzene extract of trout fillet; (C) benzene extract of trout after shaking it with an aqueous saturated solution of Ag_2SO_4.

The chemical form of $MeHg^+$ in the alkaline hydrolysate is not known. However, either CuCl or $HgCl_2$ had to be added in step 3 for the quantitative formation of MeHgCl after acidification of the hydrolysate. Fujiki (1970) reported that sulfides of methyl mercury could not be changed easily to MeHgCl in 1 *N* HCl unless CuCl, $HgCl_2$ or AgCl was present in the aqueous solutions. Nishi and Horimoto (1968) observed that Na_2S or $Na_2S_2O_3$ interfered with the extraction of MeHgCl from 0.1 *N* HCl to benzene unless an excess amount of $HgCl_2$ was present. There is a real possibility, based on these observations, that the $MeHg^+$ is present in the alkaline hydrolysates of fish as MeHgSR and/or $(MeHg)_2S$. The extraction procedure, steps 3 through 7, is an adaptation of procedures described in the literature (Nishi and Horimoto, 1968; Kamps and McMahon, 1972; Newsome, 1971; Westoo, 1967, 1968).

A lower concentration of cysteine was used in step 5 of the procedure described here (5.2 mg of cysteine to complex 0.04 to 2.0 μg of MeHgCl) as compared to the concentration recommended in the other methods (160 to 512 mg of cysteine to complex >1 μg of MeHgCl). With the lower concentration of cysteine, KCl had to be added in step 6 to transfer the MeHgCl quantitatively to benzene from the aqueous solution < 1 *M* in HCl. Substitution of penicillamine acetate for cysteine acetate in step 5 had no observable effect on recoveries or precision. Gas chromatographic techniques described by Nishi and Horimoto (1968), Kitamura et al. (1966), Rudling (1971), and Tatton and Wagstaffe (1969) were used to achieve instrumental stability in these assays.

The identity of MeHgCl in the final benzene extracts from all fish samples was verified according to the procedure described by Jensen (1969). Figure 2 shows a chromatogram of a benzene extract of trout before and after it was shaken with an aqueous solution of Ag_2SO_4. The peak, with a retention time longer than that for the MeHgCl that is removed with this subtractive technique, has a retention time identical with that for a benzene solution of pure EtHgCl. This is the only confirmation available at this time for the identity of EtHg compounds in fish. The observed concentration of EtHg compounds, however, might account for some of the discrepancy between the assays for total mercury and MeHg compounds, as discussed earlier.

2-Propanol was added to samples in step 1 to preserve them until they were assayed and to prevent loss of any volatile methylmercuric compounds that might be formed during alkaline hydrolysis. No detectable synthesis occurred when 2-propanol was used in step 1, even when $HgCl_2$ was added to the samples before hydrolysis at levels as high as 75 ppm.

Other evidence that synthesis did not occur during hydrolysis and extraction includes the following: (1) substitution of CuCl for $HgCl_2$ in step 3 had no effect on the final assay; and (2) the agreement of assays for the one tuna sample with assays by alternative procedures, as shown in the comparative study.

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Supplementary Material Available. The details of the statistical procedure used to test the assumption that the peak heights were distributed normally (D'Agostino and Pearson, 1973), a summary table of the data from the peak heights generated by the spiking standards and fish extracts from 49 recoveries that were used to test this assumption, procedures for estimating confidence limits of individual assays from the regression coefficients of the least-squares analyses, and procedures for estimating the approximate number of replicates required to provide an assay with a 99% confidence interval will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JAFC-75-1079.

LITERATURE CITED

Bache, C. A., Gutenmann, W. H., Lisk, D. J., *Science* **172** (1971).
D'Agostino, R., Pearson, E. S., *Biometrika* **60,** 613 (1973).
Elkins, E. R., National Canners Association, Washington, D.C., private communication, 1972.
Fujiki, M., *Bunseki Kagaku* **19,** 1507 (1970).
Hunter, D., "The Diseases of Occupations", 4th ed, Little, Brown and Co., Boston, Mass., 1969, p 289.
Jensen, S., *Nord. Hyg. Tidskr.* **50,** 85 (1969).
Johansson, B., Ryhage, R., Westoo, G., *Acta Chem. Scand.* **24,** 2349 (1970).
Kamps, L/ R., McMahon, B., *J. Assoc. Off. Anal. Chem.* **55,** 590 (1972).
Kitamura, S., Tsukamoto, T., Hayakawa, K., Sumino, K., Shibata, T., *Igaku To Seibutsugaku* **72,** 1 (1966).
Makarova, L. G., Nesmeyanov, A. N., in "Methods of Elementoorganic Chemistry", Vol. 4, Nesmeyanov, A. N., Kocheshkov, K. A., Ed., North-Holland Publishing Co., Amsterdam, 1967.
Newsome, W. H., *J. Agric. Food Chem.* **19,** 567 (1971).
Nishi, S., Horimoto, Y., *Bunseki Kagaku* **17,** 1247 (1968).
Ostle, B., "Statistics in Research", 2nd ed, Iowa State University Press, Ames, Iowa, 1963.
Polley, D., Miller, V. L., *Anal. Chem.* **24,** 1622 (1952).
Rudling, R., Report C7A of the Institutet for Vatten-Och Liftvardsforskning, Swedish Water and Air Pollution Research Laboratory, Stockholm, Sweden, Aug, 1971.
Simpson, R. B., *J. Am. Chem. Soc.*, **83,** 4711 (1961).
Simpson, R. E., Horwitz, W., Roy, C. A., *Pestic. Monit. J.* **7,** 127 (1974).
Sumino, K., *Kobe J. Med. Sci.* **14,** 115 (1968).
Tatton, J. O'G., Wagstaffe, P. J., *J. Chromatogr.* **44,** 284 (1969).
Uthe, J. F., Solomon, J., Grift, B., *J. Assoc. Off. Anal. Chem.* **55,** 583 (1972).
Westoo, G., *Acta Chem. Scand.* **20,** 2131 (1966).
Westoo, G., *Acta Chem. Scand.* **21,** 1790 (1967).
Westoo, G., *Acta Chem. Scand.* **22,** 2277 (1968).

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