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GAS LIQUID CHROMATOGRAPHIC (GLC) ANALYSIS OF METHYLMERCURY IN FISH AND ITS COMPARISON WITH TOTAL MERCURY

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A gas liquid chromatographic (GLC) method is presented for reliable analysis of methylmercury in fish samples. Modified extraction and clean-up methods for methylmercury from fish samples are described. Decomposition studies of methylmercury has been done. A new calculation method has been developed to get better precision and accuracy. Selected fish samples were analysed for methylmercury by GLC and total mercury by voltammetry and cold vapour atomic absorption spectrometry and results compared.

Key words: Gas liquid chromatography, Methylmercury, Fish, Total mercury.

INTRODUCTION

Toxic metals constitute a significant hazard for man [1]. There is not enough detailed knowledge and reliable data on their occurrence, fate and behaviour in the various stages of their participation in biogeochemical cycles [2, 3]. Most of the toxic metals make their way into sea through inland waters and rain. In the sea, these are accumulated significantly by marine organisms (planktons, mussels, crustaceans and fish) [4] and thus cycled back to man through sea food. Only mercury may occur at risk levels [5] in fish meat, while other metals such as Cd, Pb and Cu are accumulated in the intestines of fish [6] and crustaceans [7]. Now it is well known that the bulk of mercury in fish is present as methylmercury [8, 9].

For the analysis of total mercury in fish Atomic Absorption Spectroscopic [10, 11] voltammetric [12] and other methods [13] are used. However for the estimation of methylmercury in fish gas-chromatographic method is still preferred [14]. Although some other methods for the estimation of organic mercury are reported [15] which are not specific for methylmercury.

Methylmercury being more toxic thus its analysis in sea-food is very important. Recent studies have shown [16-18] that methylmercury may be decomposed during extraction and clean-up procedures resulting in measurement errors.

A gas-liquid chromatographic method developed for reliable analysis of methylmercury in fish samples was used for comparative studies using other analytical techniques for total mercury and methylmercury.

EXPERIMENTAL

Reagents and apparatus. All chemicals used were of Merck p.a. grade if otherwise not mentioned. Benzene, toluene, sod. acetate, anhydrous Na_2SO_4, conc, HC1, conc. HBr, NaC1, KBr, copper sulfate, NaOH, H_2SO_4 and triply distilled water were used. CH_3HgC1, CH_3HgBr, C_2H_5HgC1 and dichlorophenol (DCP) were used to prepare standards. Cystein 1% solution was prepared by dissolving 1.0 gm cystein hydrochloride monohydrate + 0.8 gms sod. acetate ($3H_2O$) + 12.5 gm anhydrous Na_2SO_4 in water and diluted to 100 ml.

Gas Chromatograph Model 5710A equipped with Electron Capture Detector (ECD) Model 1813A, automatic sampler Model 7671A, Integerator Model 18652A, Computer Model 21MX series, sample control module, Model 18653B and Recorder all of Hewlett Packard, U.S.A. were used. Silent 700, Electronic Data Terminal of Texas Instruments Incorporated U.S.A. was used.

In addition to necessary laboratory glassware, homogenizator, centrifuge machine, shaking machine, rotary pumps, vacuum pump, oxysorb, molecular sieve, sample vials and gases (N_2) and (Ar + CH_4) were also used.

Procedure. Tuna fish were caught off Sicily, Italy and other fish bought fresh from the market in West Germany. Length and weight of the fish were recorded. Dissection was either done immediately or the whole specimen was stored deep frozen at -80° and then half thawed before dis-

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section. Samples of muscle at the time of dissection were taken with quartz instruments with precautions against contamination [5, 6]. Between 2 to 10 gms of fresh fish or 0.5 to 2.5 gms dried fish were taken and homogenized. For lower concentrations of methylmercury larger amounts of fish may be taken. Homogenate was transferred in a bottle, HC1 added making upto a concentration in the homogenate ≤ 0,5N, 10% KBr and 0.1M $CuSO_4$, were also added. An equal volume of Toluene was added. Solution was shaken for 10-15 minutes and centrifuged. Organic phase was separated and 6 ml of 1% cystein was added, shaken and centrifuged. Aqueous phase was separated and 1 ml of 1.0M KBr, 0.2N HC1 and O.1M $CuSO_4$ were added alongwith an equal volume of Toluene. The mixture was shaken and centrifuged. Organic phase separated and dried over anhydrous sod. sulfate. Calibration solutions were prepared taking known amounts of methylmercury. Extraction and clean-up procedure are given in Table 1.

Table 1. Extraction scheme of methylmercury from fish samples.

Fish sample (2-10 g wet weight)

↓

Homogenisation

↓

HCl (0.2 to 0.5N) + KBr (10%) + $CuSO_4$ (0.1M) + Toluene (equal volume)

↓ shake and centrifuge

Organic phase + 1% cystein solution (6 ml)

↓ shake and centrifuge

Aq phase + KBr (1 ml of 1.0M) + $CuSO_4$ (0.1M) + HCl (0.2N) + Toluene (equal volume)

↓ shake and centrifuge

Take organic phase, dry over anhydrous Na_2SO_4 and analyse by GLC.

After extraction and clean-up methylmercury was analysed by gas-liquid chromatography. The instrument operating conditions are presented in Table 2 alongwith corresponding retention times for methylmercury and the internal reference dichlorophenol. The column and filling material were prepared according to the procedure described by M. Donike [19]. The column was conditioned overnight at 100 ml/min. flow rate of carrier gas.

Two volumes of 2 µl and 4 µl were adjusted in the auto-sampler. Then required number of injections from each bottle, number of washings and the time interval between injections, calculation of peak areas and identification of peaks was done.

Table 2. Optimum conditions for the analysis of methylmercury by GLC.

Column:	Coiled pyrex glass, 1.70 m length, 3.0 mm i.d.		
Packing:	2.5% carbowax 20M on chromosorb G 80-100 mesh.		
Instrument settings:			
	Inlet	Column	Detector
Temperature °	250°	160°	300°
Carrier gas flow rate: 90 ml/min, Ar (95%) + CH_4 (5%)			
Retention times:			
MellgBr	6.50 min.		
Dichlorophenol	11.75 min.		

RESULTS AND DISCUSSIONS

Decomposition of methylmercury. In literature [14, 20] sodium chloride and HC1 are extensively used for extraction of methylmercury by organic solvents (toluene, benzene) and subsequent determination of methylmercury by gas liquid chromatography. Decomposition studies of methylmercury in the presence of these reagents in pyrex glass flasks covered with A1-foil were performed (Fig. 1). High concentrations of HCl or NaCl alone did not decompose methylmercury quickly, but when (15% NaCl + 2.2M HC1) was added, up to 40% of the methylmercury was de-

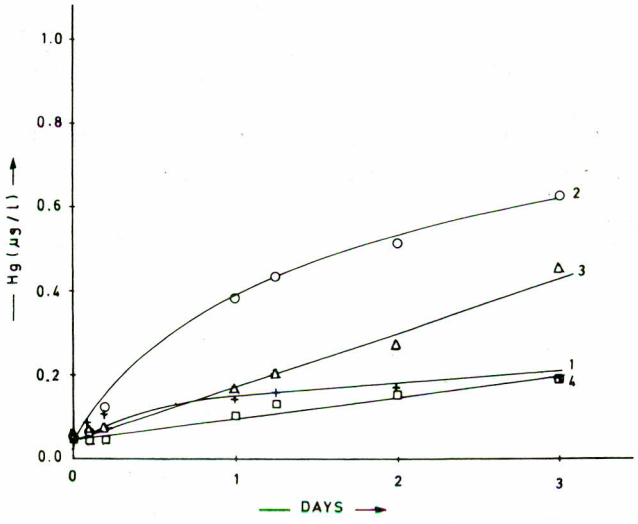

Fig. 1. Decomposition of methylmercury chloride (1.03 µgl⁻¹) in pyrex glass flasks covered with Al-foil in the presence of: 1, 15% NaCl + 0.2M HCl; 2, 15% NaCl + 2.2M HCl 3, 15% NaCl + 5.0M HCl; 4, 5% NaCl + 2.2M HCl.

composed within a day. This very combination has been extensively used for the extraction separation of methylmercury [14]. It is clear from Fig. 1 that the presence of these reagents losses of methylmercury may occur if more time is taken for the extraction, In the presence of 15% NaCl + 5MHCl the rate of decomposition of methylmercury is less than that with 15% NaCl + 2.2N HCl; may be due to low solubility of NaCl in presence of 5M HCl. Studies has shown that use of Bromide salt instead of chlorides and addition of $CuSO_4$ makes little difference on decomposition of methylmercury [21]. Light, particularly UV-light decomposes methylmercury very quickly [22], therefore care must be taken to protect the samples and extraction from light.

Extraction and clean-up. Benzene, used frequently [14, 20] for extraction purposes for its better extraction efficiency than toluene (Table 3) [23], but benzene being carcinogen its use is not advisable. Secondly with benzene, partition of methylmercury from aqueous to organic phase was not complete in presence of chloride ions alone [23] but it is nearly so when $CuSO_4$ was added. Furthermore it is reported that substitution of chloride ions by bromide ions considerably increases extraction efficiency by organic solvents [24]. In the presence of chloride ions at a concentration of 2.5M and 1.0M the partition coefficient of methylmercury chloride is $K_D = 10^{1.09}$ and $K_D = 10^{1.07}$. For methylmercury bromide, K_D is found to be $10^{1.69}$ at both these levels of ionic strength. In the present work toluene was used as a solvent and its extraction efficiency was enhanced by the using bromide salts and copper sulfate. The method adopted for the extraction of methylmercury from fish is described in Table 1. Comparative study of another recommended method [25] and developed method proved better extraction efficiency of the later (Table 4).

Table 3. Extraction of methylmercury by toluene and benzene from aqueous solutions in the presence of 2.2N HCl and 15% NaCl.

S. No.	CH_3HgCl conc. (µg/ml)	Pack heights after extraction with		% Extraction by Toluene compared to benzene
		Toluene	Benzene	
1.	0.067	5.25	6.75	78%
2.	0.13	10.5	12.9	81%
3.	0.33	30.0	37.25	81%
4.	0.50	46.2	61.0	77%
5.	0.67	63.5	79.5	80%

Average % extraction by toluene as compared to benzene = 70.4 ± 1.8

Selection of a column. Different column packing materials are recommended [14, 26]. Carbowax 20M was found better for routine work (Fig. 2) and could be operated at 160° for both methylmercury and dichlorophenol an internal standard of calculation. The column provided excellent sample peak resolution at short retention times, minimizing column bleed extending column life without excessive loss of detector sensitivity.

Table 4. Comparison of extraction capability between the developed method and recommended method (25).

S. No.	CH_3HgCl added (µg)	% recovery by developed	% recovery by another method
1.	1.0	96	70
2.	2.0	97	75
3.	5.0	99	80
4.	10.0	98	82

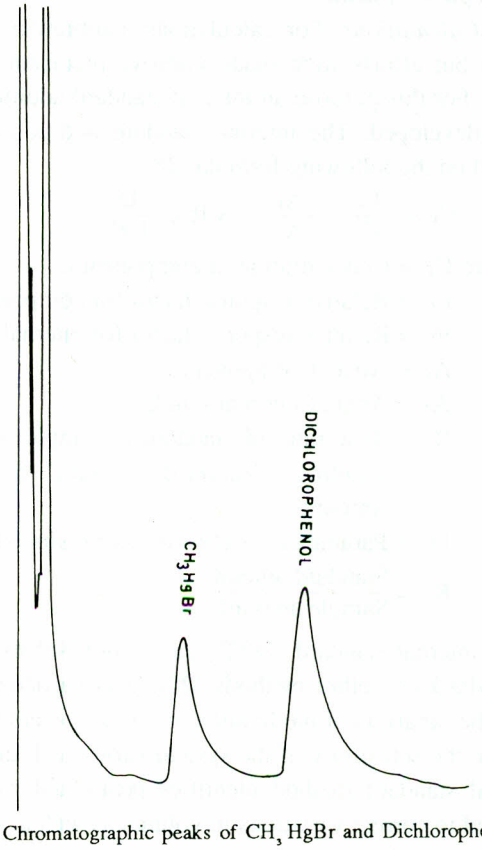

Fig.2. Chromatographic peaks of CH_3HgBr and Dichlorophenol on 2.5% carbowax 20M column.

Carrier gas. A mixture of argon and methane containing 5 to 10% methane was used as carrier gas. Experiments were done to find whether carrier gas could be replaced by nitrogen. It was found unsuitable as Nitrogen gave metastable peaks in the instrument used, because the sensitivity of the detector decreases with increasing flow rates of carrier gas. This was apparent from both peak area and peak height at relatively higher concentrations of methylmercury

halide i.e; at 50 pg levels or higher. At lower concentrations i.e; at less than 50 pg levels the sensitivity of the detector was not much changed by flow rates of carrier gas. Moreover higher flow rates have some advantage, The peak was better shaped and well defined at higher flow rate. In addition, peak height versus concentration curves were relatively linear than the curves at low flow rates of carrier gas. The optimum flow rate was found to be 90 ml min.

Electron capture detector (ECD). In the detector used for this work a 15 mc of Ni^{63} radioactive source is plated on the interior surface of one half of the cell. β-particles emitted by the radioactive source collide wsith carrier gas molecules and produce low energy electrons, which may be captured by sample molecules [27]. Electron capture detector is very sensitive and has very wide linearity range, because of its pulse system.

Calculations. For calculations, calibration method is good but efforts were made improve precision of the results. For this purpose an internal standard addition method was developed. The internal standard addition method is based on the following formula [28].

$$Ci = \frac{Fi}{F_s} \frac{Ai}{A_s} \times R \times \frac{D}{100}$$

Where Ci = Concentration of component i.
Fi = Relative response factor for component i.
Fs = Relative response factor for internal standard.
Ai = Area of component i.
As = Area of internal standard.
R = True ratio of standard to sample obtained by dividing "Standard Amount" by "Sample Amount"
D = Parameter "% Dil-Ftr", normality 100.

$$R = \frac{\text{Standard amount}}{\text{Sample amount}}$$

The internal standard (ISTD) calculation solves the major drawbacks of other methods [28]. It is particularly useful for the' analysis of methylmercury by chromatography, because the sensitivity of the system varies with time. An internal standard method identifies peaks and can be calibrated to correct variations in volume as well as in detector response and sensitivity of ECDduring measurements. Further details about ISTD method are given else where [28].

Dichlorophenol (DCP) was used as internal reference. DCP is quite stable and inert towards methylmercury chloride and methylmercury bromide on long standing. Response of the ECD towards DCP was also linear (Fig. 3). Ethylmercury halide or phenylmercury halide were not preferred as internal standard, due to their presence in the environmental samples. The retention time of phenylmercury halide is also relatively more. A known amount of Internal

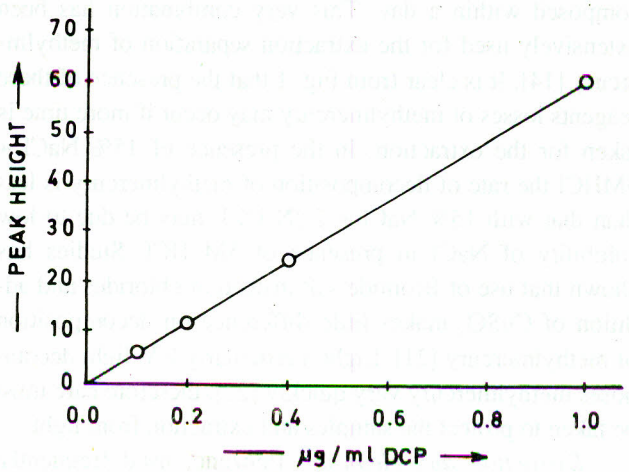

Fig. 3. Linearity curve for dichlorophenol.

Standard Reference (DCP) was added to the standards and samples before measurement. It was found that the calculations by ISTDmethod were more accurate than calculations by peak height or peak area alone. Standard deviations calculated by ISTDwere at least twice better than the standard deviations calculated by ISTD were at leas twice better than the standard deviations calculated from peak heights (Table 5).

Table 5. Comparison between different calculation methods.

S. No.	Methylmercury concentration standard (µ/g)	Pack height	Atten	Methylmercury measured by ISTD method
1.	0.1	15.3	8	0.097
2.	0.1	15.1	8	0.089
3.	0.1	17.0	8	0.093
4.	0.1	17.8	8	0.096
5.	0.1	18.3	8	0.090
6.	0.1	18.0	8	0.091
7.	1.0	30.8	32	1.014
8.	1.0	32.0	32	1.016
9.	1.0	34.2	32	1.07
10.	1.0	34.8	32	1.108
11.	1.0	36.4	32	1.056
12.	1.0	38.3	32	1.044

Relative standard deviation from peak height (0.1 µg) = 7.54
Relative standard deviation from ISTD method (0.1 µg) = 3.21
Relative standard deviation from peak height (1.0 µg) = 7.33
Relative standard deviation from ISTD method (1.0 µg) = 4.33
Atten. = Attenuation
ISTD = Internal standard

Linearity and precision. For methylmercury concentrations from 10 pg to 17 ng electron capture detector is quite linear (Fig. 4). Detector is certainly linear for higher concentrations of methylmercury halide, but concentrations

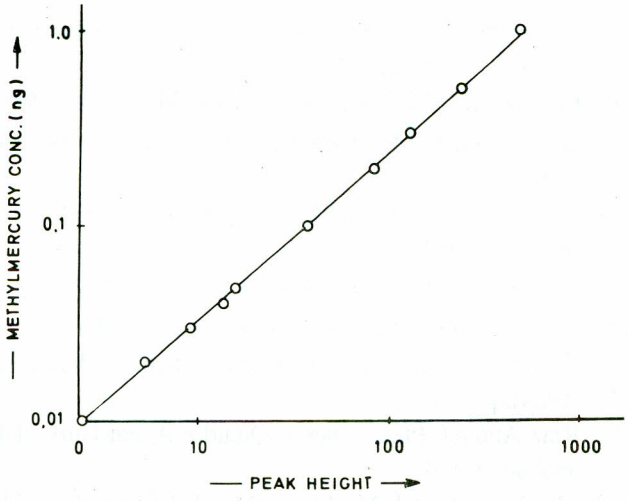

Fig. 4. Linearity curve for methylmercury.

more than 17 ng were not tried, as the fish samples had lower concentrations of methylmercury, in the final extracts and secondly the injection of higher concentrations of methylmercury created contamination problems. Detection limit of the detector is 2 pg of methylmercury halide for pure standard methylmercury chloride solutions and 50 µg/Kg methylmercury in fresh fish samples.

With the improved extraction and clean-up, recovery of methylmercury is nearly 100%. The overall recovery of methylmercury added to homogenized fish samples was 100 ± 5% by the method evolved. Relative standard deviation for the analysis of methylmercury in this method is 3% for fish sample containing about 0.1 µg/g of mercury as methylmercury (Table 6) on fresh weight basis, and 0.55 µg/g on dry weight basis.

Table 6. Precision of methylmercury determination in Halibut fish samples by GLC.

S. No.	Fish sample No.	Fish type	Methylmercury by ISTD method
1.	H 1	Halibut	0.54 µg
2.	H 2	"	0.56 µg
3.	H 3	"	0.54 µg
4.	H 4	"	0.58 µg

Methylmercury in fish samples in relation to total mercury. After developing the method satisfactorily for the analysis of methylmercury, different fish samples were selected. Samples were finely homogenized, and in duplicate for methylmercury. Fish selected were tuna well known for high mercury contents and extensively used for food. Tuna fish were caught from mediterranean off Italian coast. Mediterranean a relatively polluted sea, being surrounded by industrial countries. Mackerel and Halibut with lower concentrations of mercury were bought from West Germany. Concentrations of mercury and methylmercury in fish samples are given in Table 7. In tuna high concentrations of mercury was measured using voltametric method [12]. In other samples mercury was estimated using cold vapour atomic absorption spectrometry [21]. Both the methods are quite good and accurate for this analysis. It is apparent from Table 7 that in tuna fish nearly 90% mercury is present as methylmercury. But in mackerel fish methylmercury content is nearly 70% of the total mercury whereas in Halibut the methylmercury content is nearly 90%. Average methylmercury content of analysed fish samples is nearly 87% of the total mercury. It can be assumed from the results that major portion of mercury in fish is present as methylmercury. Since methylmercury is much more toxic than inorganic mercury, therefore to monitor sea food samples particulrly fish muscle for levels of mercury and methylmercury to avoid possible toxicity hazards is necessary.

Table 7. Mercury contents in fish samples.

S. No.	Fish type	as Methyl-mercury µg/g	Mercury mercury µg/g	Total % Methyl-mercury
	Tuna fish			
1.	T_1	2.01 ± 0.11	226 ± 0.06	88.9
2.	T_2	2.40 ± 0.08	2.96 ± 0.13	81.1
3.	T_3	2.71 ± 0.07	2.82 ± 0.04	96.1
4.	T_4	0.87 ± 0.03	1.05 ± 0.06	83.2
5.	T_5	2.03 ± 0.10	2.26 ± 0.04	89.8
6.	T_6	2.91 ± 0.14	2.36 ± 0.14	123.3
7.	T_7	2.29 ± 0.07	2.77 ± 0.10	82.7
8.	T_8	2.35 ± 0.11	2.56 ± 0.04	91.8
9.	T_9	0.70 ± 0.03	0.72 ± 0.03	97.2
10.	T_{10}	2.77 ± 0.10	3.31 ± 0.11	83.7
11.	T_{11}	2.94 ± 0.06	3.01 ± 0.14	97.7
12.	T_{12}	2.61 ± 0.04	2.83 ± 0.10	92.2
	Mackerel			
13.	M_1	0.31 ± 0.03	0.54 ± 0.03	57.4
14.	M_2	0.27 ± 0.02	0.42 ± 0.03	64.3
15.	M_3	0.14 ± 0.01	0.16 ± 0.01	87.5
16.	M_4	0.13 ± 0.01	0.20 ± 0.01	65.0
17.	Halibut fresh	0.10 ± 0.01	0.12 ± 0.01	83.3
18.	Halibut dry	0.55 ± 0.02	0.54 ± 0.01	101.8

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