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Improvement of Sample Pretreatment for Gas Chromatographic Determination of Methylmercury in Marine Biota

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Existing methods for analysis of methylmercury in biota consist of an initial extraction of the determinand at low pH with an organic solvent, followed by clean-up and gas chromatographic determination. The complex biological matrix causes considerable interferences in the extraction process and makes the calibration of the method a difficult task. Results from the calibration method (standard addition) will only be correct if the calibrant added and the determinand are in the same chemical form and homogeneously distributed in the sample to be analysed. It is shown here that this presupposition is fulfilled if the sample is pretreated with an aqueous solution of sodium hydroxide (alkaline hydrolysis). In order to avoid uncontrolled losses of methylmercury at high pH, cysteine is added at the beginning of the pretreatment procedure. As a reagent forming strong complexes with mercury compounds, it protects methylmercury from disintegration during alkaline hydrolysis of the sample.

Keywords: Methylmercury, biota, gas chromatography, calibration, sample pretreatment, alkaline hydrolysis

INTRODUCTION

Inorganic and organic mercury compounds are known to interconvert in aquatic systems by both biochemical and microbial pathways.[1,2] The accumulation of mercury by marine organisms apparently favours the methyl form (monomethylmercury). The preferential incorporation of this mercury species and its longer biological half-life result in both an increased percentage of methylmercury at higher trophic levels and a higher total mercury concentration. From the ecotoxicological point of view, methylmercury is considered more dangerous than inorganic forms of mercury. This emphasizes the need for appropriate analytical procedures that allow the distinct determination of methylmercury in various biological matrices.

Analytical procedures involving extraction and separation of methylmercury from organic matrices followed by gas chromatographic determination are well established. Based on principles developed by Westöö,[3] the following methodological approach has been used for our own investigations:[4,5]

(a) liberation of methylmercury from the organic matrix by treatment of the sample with hydrochloric acid;
(b) extraction of the methylmercury as a chloride complex with an organic solvent (toluene);
(c) separation of the methylmercury from interfering co-extracted impurities (matrix components) by back-extraction with an aqueous solution containing a thiol compound (cysteine);
(d) dissociation of the methylmercury–thiol complex in the aqueous phase by treatment with hdyrochloric acid;
(e) re-extraction of the methylmercury chloride complex with an organic solvent (toluene);
(f) gas chromatographic determination (electron capture determination—ECD).

Sample components of different chemical structures and physicochemical properties exert a considerable influence on the kinetics of the solvent extraction process outlined above. This well-known 'matrix effect' makes reliable quantitative analysis difficult.

Multiplicative influences of the sample matrix on the extraction yield can be compensated by using the method of standard addition. The results from this calibration method will only be

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correct, however, if the added standards are homogeneously distributed in the sample, and if they are in the same chemical form as the determinand. In order to meet these demands, the sample must undergo a preteatment procedure. In the following it is shown that an appropriate such procedure is alkaline hydrolysis of soft tissues of marine biota in the presence of cysteine as an agent protecting the carbon–metal bond from cleavage.

EXPERIMENTAL

Apparatus

A gas chromatograph Hewlett–Packard, Model 5710 A with a packed column (1.8 m glass, i.d. 2 mm, DEGS-PS 5%, Supelcoport 100–120 mesh) was used. The column was preconditioned by repeated injections of 20 µl $HgCl_2$ in toluene (5 mmol l^{-1}) according to Hight and Corcoran.[6] Gas chromatographic conditions were: carrier gas, argon–methane (95 + 5) at 30 ml min^{-1}; column temp. 155 °C; injector 200 °C; ECD 300 °C.

Establishment of a standard addition calibration function for fish muscle tissue

Method 1

Six equal and accurately weighed portions (0.15 g) of freeze-dried fish muscle tissue were transferred into 10-ml conical centrifuge tubes. Then calibration standard solutions, each 10 µl, containing 75.3, 150.6, 225.9, 301.2 and 376.5 ng CH_3HgCl respectively in high-purity water, were added to five of the centrifuge tubes. The sixth tube received no standard solution (zero sample). After addition of 3 ml diluted hydrochloric acid (3 mol l^{-1}) and 3 ml toluene, the centrifuge tubes were tightly capped and vigorously shaken on a shaking machine for 10 min. After centrifugation for 5 min at 2000 rpm the supernatant organic phases were transferred with a piston pipette into another set of 10-ml centrifuge tubes. The extraction/centrifugation procedure was repeated with 3 ml toluene.

The combined organic phases were re-extracted by shaking for 10 min with 1 ml cysteine solution (2 mol l^{-1}). After separation of the phases, the toluene layer was discarded, 1 ml diluted hydrochloric acid was added, and the solvent extraction procedure was repeated with 2 ml toluene. The aqueous phase was carefully drawn off with a piston pipette and discarded. Sodium sulphate (Na_2SO_4) (1 g) was added to the remaining organic phase. The dried toluene phase (test solution) was submitted to analysis by gas chromatography (3 µl injected).

Method 2

Six equal and accurately weighed portions (0.15 g) of freeze-dried fish muscle tissue were transferred into 10-ml conical centrifuge tubes. Identical calibration standard solutions to those described under method 1 were added, then 2 ml of an aqueous solution of NaOH (6 mol l^{-1}) was added to each tube, following which they were tightly capped and kept at room temperature for 24 h.

The light-yellow solutions obtained were acidified with 5 ml HCl (6 mol l^{-1}) and submitted to solvent extraction and clean-up as described under method 1.

Method 3

The procedure was identical to that described for method 2, with the difference that the NaOH solution contained cysteine hydrochloride at a concentration of 2 mol l^{-1}.

RESULTS AND DISCUSSION

The performance characteristics of the three methodological variants are summarized in Table 1. Methylmercury concentrations in the fish muscle tissue determined by the three variants are given in the same Table.

A significant effect of the alkaline sample hydrolysis (methods 2 and 3) was an increase of the measured methylmercury concentration in relation to the value obtained by the procedure without such a pretreatment (method 1). Further, an increase of the slope and intercept of the respective calibration functions (Table 1) indicated that an improvement of the extraction yield was gained.

Analysis of certified reference materials (dogfish muscle and liver) indicated that accurate values were obtained only by method 3. The standard addition method without alkaline sample pretreatment (method 1), and with alkaline pretreatment in the absence of cysteine (method 2) gave results significantly lower than the certi-

Table 1 Regression analysis of three variants of the method of standard addition for methylmercury determination in fish muscle tissue

Linear model: $y = a + bx$
Dependent variable (y): mm peak height
Independent variable (x): pg CH_3HgCl injected

Method	a	Sa	b	Sb	Sy	R	$\bar{x}$	Sr
1	14.6	±0.943	0.1015	±0.0088	±1.3314	0.9963	0.639	±0.059
2	22.8	±1.18	0.1255	±0.0095	±1.3102	0.9962	0.807	±0.047
3	29.2	±1.02	0.1307	±0.0091	±1.2955	0.9975	0.993	±0.044

Definition of parameters:
a = intercept
Sa = standard deviation of intercept
b = slope
Sb = standard deviation of slope
Sy = standard error of estimation
R = correlation coefficient of the linear standard addition calibration line
$\bar{x}$ = analytical result (mg methylmercury per kg freeze-dried fish muscle tissue), mean of five replicate analyses with the reproducibility Sr

fied values. Table 2 summarizes the relevant information.

It is concluded from the investigations carried out that the analytical procedure developed by Westöö[3] is not a self-contained method. It must be critically evaluated in relation to the question of whether calibration is being performed appropriately. For calibration with the method of standard addition it is imperative that the calibrant has the same physicochemical properties as the determinand. This condition was obviously not fulfilled if the sample was directly exposed to hydrochloric acid and subsequently extracted by toluene. The determinand was more strongly associated with matrix components and thus less accessible for extraction by the organic solvent than the methylmercury added. The discrepancy in the extraction yield between calibrant and determinand led to systematic deviation from the true value.

Alkaline hydrolysis has repeatedly been used in the context of organomercury analysis in different environmental samples.[7–12] Since homogeneous sample solutions were obtained in many instances, this seemed to be a promising sample pretreatment procedure suitable for subsequent use of the method of standard addition. However, uncontrollable losses of methylmercury at high pH values must be taken into consideration.[9,10]

As demonstrated by Tables 1 and 2, the alkaline pretreatment (method 2) was an improvement in relation to method 1. Results obtained were higher and approached the true (certified) values of two reference materials. However, the

Table 2 Determination of methlmercury in certified reference material (CRM) by methods 1, 2 and 3

CRM[a]	Method	$\bar{x}$ (mg Hg kg^{-1})	T (mg Hg kg^{-1})	Certified value (mg Hg kg^{-1})
DORM-1	1	0.438	±0.076	0.731 ± 0.060
DORM-1	2	0.521	±0.060	0.731 ± 0.060
DORM-1	3	0.719	±0.068	0.731 ± 0.060
DOLT-2	1	0.427	±0.068	0.693 ± 0.053
DOLT-2	2	0.501	±0.052	0.693 ± 0.053
DOLT-2	3	0.664	±0.063	0.693 ± 0.053

[a] DORM-1 (dogfish muscle) and DOLT-2 (dogfish liver), certified reference materials for trace metals (National Research Council, Canada).
Definitions of parameters:
$\bar{x}$ = arithmetic mean of five replicate analyses
T = confidence interval for $\bar{x}$ (P = 95%)

alkaline medium favoured the cleavage of the carbon–metal bond of intermediately formed methylmercury hydroxide. Improvement gained by alkaline pretreatment was thus minimized by partial disintegration of the organic mercury. Following these considerations, an intermediate association of the methylmercury cation with a nucleophic ligand which was capable of increasing the C–Hg covalency and thus stabilizing the carbon–metal bond during the alkaline pretreatment process was essential, if uncontrollable losses were to be prevented. From the stability constants of methylmercury with different nucleophiles it is evident that thiol compounds such as cysteine are the strongest complexing agents.[13–15] Accordingly, products from the reaction of methylmercury with sulphydryl-containing ligands are much less liable to chemical demethylation than methylmercury hydroxide. Table 2 confirms the usefulness of cysteine as a stabilizing agent protecting methylmercury from disintegration during alkaline sample hydrolysis.

The pretreatment procedure outlined may also be useful for other materials. The optimum amount of sample and of reagents necessary will be dictated by the chemical composition of the sample and by the level of methylmercury present in the sample, and must be tested in separate experiments.

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