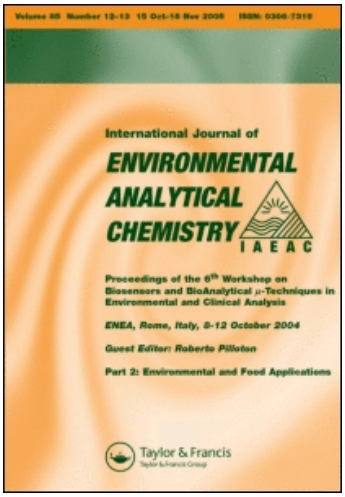

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Supercritical Fluid Carbon Dioxide Extraction and Liquid Chromatographic Separation with Electrochemical Detection of Methylmercury from Biological Samples

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SUPERCRITICAL FLUID CARBON DIOXIDE EXTRACTION AND LIQUID CHROMATOGRAPHIC SEPARATION WITH ELECTROCHEMICAL DETECTION OF METHYLMERCURY FROM BIOLOGICAL SAMPLES

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Using the coupled methods presented in this paper, methylmercury can be accurately and rapidly extracted from biological samples by modified supercritical fluid carbon dioxide and quantitated using liquid chromatography with reductive electrochemical detection. Supercritical fluid carbon dioxide modified with methanol effectively extracts underivatized methylmercury from certified reference materials Dorm-1 (dogfish muscle) and Dolt-2 (dogfish liver). Calcium chloride and water, with a ratio of 5:2 (by weight), provide the acid environment required for extracting methylmercury from sample matrices. Methylmercury chloride is separated from other organomercury chloride compounds using HPLC. The acidic eluent, containing 0.06 mol L^{-1} NaCl, insures the presence of methylmercury chloride and facilitates the reduction of mercury on a glassy carbon electrode. If dual glassy carbon electrodes are used, a positive peak is observed at -0.65 to -0.70 V and a negative peak is observed at -0.90V with the organomercury compounds that were tested. The practical detection limit for methylmercury is 5×10^{-8} mol L^{-1} (1×10^{-12} mol injected) when a 20 μL injection loop is used.

Keywords: Methylmercury; supercritical fluid extraction (SFE); liquid chromatography; electrochemical detection; biological samples

INTRODUCTION

Bioaccumulation of methylmercury (MeHg) is a persistent problem, the mechanism for which is not fully understood[1]. In the United States the number of areas are increasing where the levels of MeHg in fish limit, or prohibit, con-

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sumption by humans. For both regulatory reasons, and research purposes, it is important to develop accurate, rapid techniques for determining MeHg in environmental samples.

This method was developed to provide a rapid and clean separation of MeHg from biological samples resulting in an extract that is compatible with reductive electrochemistry after chromatographic separation. A long-term goal is to develop working conditions that are favorable for the general extraction of organometallic compounds from environmental samples that can be identified and quantified using reductive chemical techniques.

Methods used at the present time for low-level analysis of MeHg in environmental samples require isolation of MeHg by solvent extraction followed by extract clean-up, potassium hydroxide-methanol digestion, or distillation of methylmercury chloride (MeHgCl). Horvat *et al.*[2–3] have reported that the distillation of the mercury species is required before reaction with an ethylating reagent. It is possible that inorganic Hg carried over in the distillation process can react with the ethylation reagent to produce MeHg if the inorganic Hg is at much higher concentrations than MeHg in the sample[2].

Recent papers have reported the use of supercritical fluid extraction (SFE) for the isolation organometals from various matrices. Several investigators have added water plus complexing agents to simple materials spiked with solutions of heavy metals before extracting the complexed metals with supercritical fluid (SF) carbon dioxide (CO_2). Complexes of Cu^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} were extracted from filter paper, sand, or silica by Liu *et al.*[4]. Wai *et al.*[5] used CO_2 modified with a complexing agent, or neat CO_2, to extract inorganic Hg, or MeHgCl and dimethylmercury, from filter paper moistened with water. Other investigators have published SFE methods for the isolation of organotin[6–9], organolead[10], and organomercury[11] from sediments.

Dachs *et al.*[6] and Chau *et al.*[9] used carbon dioxide modified with MeOH doped with hydrochloric acid gas or carbon dioxide coupled with *in situ* derivatization with diethyldithiocarbamate to extract organotin compounds from certified reference material. Cai *et al.*[7] was able to extract organotin compounds from both certified standards and real sediment samples using *in situ* derivatization with hexylmagnesium bromide and SF carbon dioxide. Johansson *et al.*[10] extracted alkyl lead species using MeOH modified CO_2 from an urban dust sample used in an intercalibration exercise. Emteborg *et al.*[11] used neat CO_2 to isolate MeHg from certified and intercalibration samples of sediment.

After separation from an environmental matrix, MeHg can be quantified using gas chromatography with electron capture detection, atomic absorption or atomic emission spectrometry, or atomic fluorescence detection[12–14]. Quantitation of organomercury compounds using liquid chromatography (LC) has been done by

using a complexing agent in the chromatographic eluent. Several investigators have complexed organomercury species with a sulfur-containing ion-pairing reagent and used atomic emission spectroscopy in an inductively coupled plasma, ultraviolet detection, or atomic fluorescence spectrometry for detection[15–18]. Other investigators have detected Hg complexed with a sulfur-containing ion-pairing reagent by reductive electrochemistry (EC)[19–21]. These electrochemical methods require the use of a gold-mercury electrode that must be periodically polished and reamalgamated with Hg.

The glassy carbon electrode used in this method is more robust; its upkeep consists of occasional cleaning with a laboratory tissue. Also, the use of a dual glassy carbon electrode, with each electrode set at a different potential, provides confirmatory information that peaks with retention times of calibration standards are organomercury peaks in chromatograms of environmental samples.

Current methods for MeHg isolation and detection are laborious, multi-stepped, require pure solvents, produce waste that has a high cost of disposal, and can expose personnel to hazardous chemicals. A simple procedure requiring a minimum number of steps to minimize analyte loss coupled with a rugged method of detection that can accept samples with minimal clean-up would be welcome. SFE is an extraction technique that does not require the use of organic liquid solvents. Reductive electrochemical detection coupled with liquid chromatography does not require derivatization of MeHg and is sensitive to reducible compounds only. SFE and LCEC are compatible techniques that are easily coupled. This paper presents a procedure for extracting underivatized MeHg from biological tissues. The solution in which the MeHg is collected is ready for analysis by LCEC, without derivatization or further extract clean-up. The small number of preparation and analytical steps limits the loss of MeHg during the analytical process.

EXPERIMENTAL

In this paper the term methylmercury (MeHg) is used if the associated anion is not known (samples) and the term methylmercury chloride (MeHgCl) is used in reference to solutions prepared in the laboratory, or to the compound formed in the liquid chromatograph.

Reagents and Chemicals

Supercritical Extraction Grade CO_2 was purchased from Air Products Corporation†. HPLC grade acetonitrile was purchased from Burdick and Jackson†. Organomercury compounds were obtained from Ultra Scientific† (North Kings-

†The use of firm and trade names in this paper is for identification purposes only and does not constitute endorsement by the US Geological Survey.

town, RI). Stock solutions of the standards were made up in acetonitrile and stored at 4°C. Solutions of MeHgCl at concentrations of 100 μg L^{-1} or greater stored at 4°C are stable for more than 3 months.[22] Working standards were made up in acetonitrile. ACS grade anhydrous calcium chloride was obtained from Sigma Chemical Company†.

Dorm-1 and Dolt-2 are reference materials certified by the National Research Council of Canada. Dorm-1 is dogfish muscle and Dolt-2 is dogfish liver. Both are homogenized and acetone extracted to produce partially-defatted protein powders. The Dorm-1 and Dolt-2 material contain 5% and 24% fat, respectively. The total Hg concentrations are 0.780 $\pm$ 0.074 and 1.99 $\pm$ 0.10 mg kg^{-1} for Dorm-1 and Dolt-2, respectively. The certified MeHg concentrations are 0.731 $\pm$ 0.06 and 0.693 $\pm$ 0.053 mg kg^{-1} (reported as Hg) for Dorm-1 and Dolt-2, respectively.

Equipment

Supercritical fluid extraction

The equipment used in this work includes an ISCO† 260D syringe pump that produces CO_2 under pressures of up to 400 atmospheres (atm). A working pressure of 350 atm is used as a practical upper limit that provides a dense fluid without causing fittings to leak. At this pressure, the calculated density of fluid CO_2 is approximately 0.8 to 0.9 g mL^{-1} at 35°C. A second ISCO† 260D syringe pump is used to introduce methanol (MeOH) into the stream of CO_2. MeOH at a pressure of 300 atm is added to the flow-stream of CO_2 at a mixing tee. The ISCO† pump program is set to supply sufficient MeOH to provide a mixture of either 5% or 10% MeOH in CO_2. All tubing, including the restrictor, is 0.010 in ID $\times$ 0.062 inch stainless steel. There are two valves in-line between the fluid CO_2 and the collection vessel. The first, is a Swaglok† high-pressure shut-off valve between the reservoirs and the sample cartridge; the second is a 2-way through valve between the sample cartridge and the restrictor. All tubing that exits the column heater is wrapped with heating tape. A column heater preheats the CO_2 reservoir and the sample cartridge to a preset temperature (not less than 35°C) to insure that the CO_2 entering the extraction vessel is at a temperature where the CO_2 is a fluid, not a liquid. A thermocouple is used to determine the temperature of the sample cartridge.

The sample cartridges are threaded stainless steel tubes with screw-on polyetheretherketone (PEEK) caps having 0.5μ titanium frits (Upchurch Scientific†). The cartridge has a volume of 0.78 cc. A piece of precut filter paper (Schleicher and Schuell†, No. 740-E, thick filter pads having a retention pore-size of ap-

proximately 20–30 μm) is fitted in the bottom of one of the caps after it is screwed onto the cartridge. Fifty μL of water are pipetted onto the filter paper. The filter paper plus the frits act as barriers to the transfer of particles from the sample to the fluid stream. Approximately 0.15 to 0.25 g of dry, ground tissue is weighed in the cartridge. Granular calcium chloride ($CaCl_2$) is added to the cartridge to fill the volume not occupied by the sample. The sample and $CaCl_2$ can be thoroughly combined by transferring the contents of the cell to a piece of weighing paper and mixing them well before returning the mixture to the cartridge. The contents of the cartridge are covered with a second disk of the precut filter disk and 100 μL of water is pipetted onto the paper before the second cap is screwed onto the cartridge. Samples are prepared 2 hours before introduction into the SF CO_2 extraction set-up. Prior to putting the sample cartridge holder in the extraction set-up, both valves are closed. The sample cartridge is placed in the extraction set-up with the end of the cell containing the largest volume of water on filter paper connected to the inlet for the CO_2. The sample cartridge is heated to the desired temperature in the column heater before the valve separating the storage cell for the fluid CO_2 and the sample cartridge is opened, and the sample cell is pressurized. A static step (no flow of CO_2 or modified CO_2) of approximately 10 minutes precedes flow of the modified CO_2 which is initiated when the shut-off valve connected to the restrictor is opened. During the dynamic extraction the flow rates vary from 0.5 to 1 mL min^{-1}. Extraction volumes are 15 to 20 mL of CO_2. Commercially precut and cleaned tubing (Upchurch Scientific†) will not clog during extraction. CO_2 extraction volumes are 15 to 20 mL.

In the recovery experiments described below, the restrictor ends in 3 to 5 mL of acetonitrile contained in a 125-mL separatory funnel. The solvent in the narrow base of the teardrop-shape funnel has a depth of several centimeters. A cold-finger is seated in the top opening of the separatory funnel to minimize loss of volatile compounds released as the fluid CO_2 decompresses and becomes a gas. Using a separatory funnel, with a cold finger loosely fitted into its opening, provides a collection vessel in which the CO_2 exiting the restrictor circulates and repeatedly contacts the collecting solvent permitting the removal of residual extracted compounds from the gas before the gas escapes. A small Teflon centrifuge tube containing ice will fit snugly into opening, project into the separatory funnel, and function as an efficient condenser.

Liquid chromatography with electrochemical detection

The HPLC equipment that is used is a Bioanalytical Systems† (BAS) Model 480 Liquid Chromatograph with a LC-4C dual channel amperometric controller and a preheater module. The solvent delivery system is a BAS† PM-80 recip-

rocating dual piston pump. The mobile phase manifold provides rigorous deoxygenation. Eluent is filtered through a 0.2μm solvent filter prior to use. Oxygen is removed from the solvents by heating the eluent to 50°C while flushing with helium. The eluent is a 55:45 mixture of acetonitrile and a buffer of 0.08 mol L^{-1} monochloroacetic acid, 0.13 L^{-1} NaCl and 0.01 mol L^{-1} NaOH. Flow rates of 1.0 and 1.5 mL min^{-1} have been used successfully. The electrochemical cell consists of dual-channel glassy-carbon electrodes, a 0.002 inch Teflon gasket, a Ag-AgCl (3 mol L^{-1} NaCl) reference electrode, with a Teflon o-ring, and a stainless-steel auxiliary electrode. The electrochemical cell preheater is set at 40°C. The amperometric cell is set at either -0.65 or -0.70 V. Sensitivity is maintained by manual polishing of the electrode. This can be done easily by daily wiping the surface of the electrode with a laboratory tissue. The detector is routinely operated with a current amplification of 20 nanoamperes (nA). Background currents are less than 100 nA using these working conditions. The sample is degassed with helium, and helium continues to flow through the sample while the sample loop is filled manually. The injector is a Rheodyne† 7010 titanium valve with a 20 μL titanium loop into which the sample is drawn, rather than injected, to avoid introduction of oxygen into the system. The fingertight fittings are obtained from Upchurch Scientific†. Data is collected by, and managed with, the ChromGraph Data Reduction System from BAS†.

A 50 × 4.6 mm Altima† precolumn (5μm, C_{18}) is placed between the injection valve and the 250 mm × 4.6 mm Altima† column. Column packing is based on a metal-free silica that is double end-capped. The resin has a 100 Å pore size and is 16% carbon-loaded. The C_{18} resin is packed in a polyetheretherketone (PEEK) column with PEEK alloyed to Teflon frits. Both the column heater and the preheater module for the detector are set at 40°C. The cell temperature of the eluent is less than 40°C if the preheater module is not used.

The eluent is a premixed mobile phase that consists of 55% acetonitrile and 45% of a solution containing 0.08 mol L^{-1} monochloroacetic acid, 0.13 mol L^{-1} NaCl and 0.01 mol L^{-1} sodium hydroxide (NaOH). The pH of the buffer solution is 2.1. The ionic strength of this eluent is approximately 0.1. The system is run continuously at a flow rate of 1.0 mL min^{-1} with the mobile phase recycled back into the reservoir. Because the detector for the system is operated continuously, the reducible compounds are cleansed from the eluent when the system is not in use. The eluent is replaced when the retention times become excessively long due to preferential evaporation of acetonitrile. A flow rate of 1.5 mL min^{-1} can be used with samples containing few compounds that respond to reductive electrochemistry and, therefore, have uncluttered chromatograms.

TABLE I Working conditions and recoveries for methylmercury from Dorm-1 and Dolt-2 samples. Sample size was approximately 0.2 g. Samples were prepared approximately 2 hours before extraction. A static step of approximately 10 minutes preceeded the dynamic extraction. Carbon dioxide pressure was 350 atm and CO_2 volume was 20 mL for all extractions. Salt to water ratio was 2:5 (wt:wt). Volume of collecting solution was approximately 5 mL.

Sample	*Temperature*	*Water*	*Salt*	*Methanol*	*Percent Recovery*
Dorm-1	35°C				nd
Dolt-2	35°C				nd
Dorm-1	35°C	150 μL	$CaCl_2$		~10
Dolt-2	35°C	150 μL	$CaCl_2$		~10
Dorm-1	35°C	150 μL	$CaCl_2$	150 μL	50
Dolt-2	35°C	150 μL	$CaCl_2$	150 μL	85
Dorm-1	45°C	150 μL	$CaCl_2$	100 μL	75
Dolt-2	45°C	150 μL	$CaCl_2$	100 μL	95
Dorm-1	35°C	150 μL	$CaCl_2$	10%	100
Dolt-2	35°C	150 μL	$CaCl_2$	10%	100
Dorm-1	50°C	150 μL	$CaCl_2$	5%	75
Dolt-2	50°C	150 μL	$CaCl_2$	5%	100
Dorm-1	50°C	200 μL	$CaCl_2$	5%	95
Dorm-1	50°C	150 μL	$MgCl_2 \cdot 6H_2O$	10%	nd
Dolt-2	50°C	150 μL	$MgCl_2 \cdot 6h_2O$	10%	nd

Procedure

Supercritical fluid extraction

To test the efficiency of extraction set-up, Dolt-2 samples were spiked with MeHgCl in acetonitrile that was added to the sample and allowed to dry before extraction. Spikes of 0.12, 0.24 and 0.36 μg of MeHgCl were added to approximately 0.2 g of Dolt-2 reference material. Extraction conditions were 350 atm CO_2, 50°C, 5% MeOH added to the flow of CO_2, and an extraction volume of 20 mL (CO_2 + MeOH). This procedure was used to validate the performance of the extraction apparatus.

To test the time required for samples to interact with the added modifiers, extractions of Dorm-1 and Dolt-2 were done using samples prepared by adding approximately 60 mg of $CaCl_2$ and 150 μL H_2O to the samples before extraction at 35°C with 10% MeOH added to CO_2 at a pressure of 350 atm. With the same extraction conditions, allowing two hours to elapse between the time of sample preparation and introduction into the extraction unit resulted in MeHg extraction efficiencies of approximately 100% as compared with approximately 75% extraction efficiencies for samples for which 1 hour had elapsed between sample preparation and introduction into the extraction unit. It is postulated that time is required for diffusion of H_2O, reaction of $CaCl_2$, and release of MeHg from the matrix surfaces to occur before extraction is initiated.

Extraction conditions that were tested are summarized in Table I. The sample extraction conditions that were tested using CO_2 neat, or CO_2 with modifiers

added to the samples before extraction, included: (1) 100% CO_2 at 35°C, (2) addition of approximately 60 mg $CaCl_2$ and 150 μL H_2O before extraction at 35°C, (3) addition of approximately 60 mg $CaCl_2$, 150 μL H_2O, and 150 μL MeOH before extraction at 35°C, and (4) addition of approximately 60 mg $CaCl_2$, 150 μL H_2O, and 100 μL MeOH before extraction at 45°C. Sample extraction conditions that were tested using $CaCl_2$ or $MgCl_2$ and H_2O added to the samples before extraction, and MeOH added to the stream of CO_2 during extraction included: (1) addition of approximately 60 mg $CaCl_2$ and 150 μL H_2O before extraction at 35°C with 10% MeOH added to the CO_2, (2) addition of approximately 60 mg $CaCl_2$ and 150 μL H_2O before extraction at 50°C with 5% MeOH added to the CO_2, (3) addition of approximately 60 mg $CaCl_2$ and 200 μL H_2O before extraction at 50°C with 5% MeOH added to the CO_2 (Dorm-1 only), and (4) addition of approximately 60 mg $MgCl_2$ $6H_2O$ and 150 μL H_2O before extraction at 50°C with 10% MeOH added to the CO_2.

Liquid chromatography with electrochemical detection

Hydrodynamic voltammograms for MeHgCl were determined for potentials ranging from -0.40 V to -0.90 V. Data was obtained using four combinations of settings. The column heater was always set at 40°C. The cell heater was either off or on (set at 40°C). The displayed temperature for the detector was 3°C cooler (37°C) when the cell heater was turned off. The dual detectors were operated over a series of reducing potentials with either both detectors (E_1 and E_2) at the same potential, or with E_2 held at -0.90 V and E_1 varied over the series of potentials.

A comparison was made of eluents consisting of 40%, 50%, 55% and 60% of acetonitrile combined with the buffer. Retention times and separation of organomercury compounds were tested.

RESULTS AND DISCUSSION

Extraction of spiked samples showed that the extraction set-up is efficient at extracting and recovering MeHg. Figure 1 shows the recoveries of MeHg from Dolt-2. The amounts of MeHg that were extracted from Dolt-2 were equal to the sum of the MeHg in certified reference material Dolt-2 plus the spike when the amounts total MeHg were less than 0.4 μg as MeHgCl. This would correspond to a sample concentration of approximately 2 $\mu g\ g^{-1}$. Twenty mL of CO_2 plus methanol were used in the extraction. An increased extraction effi-

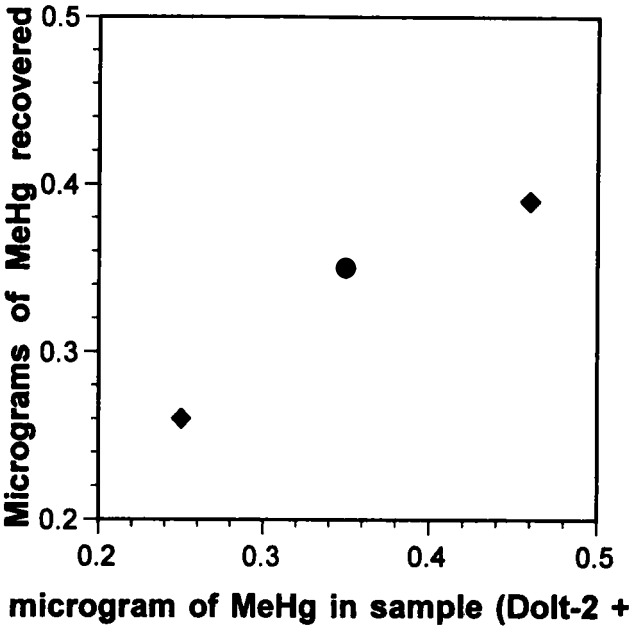

FIGURE 1 Extraction of ~0.2 g Dolt-2 spked with MeHgCl. Extraction conditions: 350 atm, 50°C, 5% MeOH. Extraction volume = 20 mL (CO_2 + MeOH). Spikes = 0.12, 0.24, and 0.36 microgram MeHgCl.

ciency for the sample spiked with 0.36 μg MeHgCl might have been obtained if a larger volume of CO_2 plus methanol were used for extraction.

The results of the extractions using various working conditions are given in Table I. Carbon dioxide at 350 atm and 35°C was not able to extract MeHg from the Dorm-1 and Dolt-2 samples. When $CaCl_2$ and 150 μL of MeOH were added to cartridges prior to extraction at 35°C, approximately 50% and 85% of the MeHg present in Dorm-1 and Dolt-2 were extracted. By increasing the extraction temperature to 45°C, and decreasing the volume of MeOH to 100 μL to improve the flow through the restrictor, extraction efficiencies increased to approximately 75% and 95% for Dorm-1 and Dolt-2, respectively. MeOH added to the stream of CO_2 by auxiliary pump was a more efficient extractant of MeHg from the biological tissues than MeOH added to the sample prior to extraction.

Extraction of Dorm-1 and Dolt-2 with CO_2 containing 10% by volume of MeOH at a pressure of 350 atm and a temperature of 35° results in 100% recovery of the reported concentrations of MeHg. Figure 2 shows the rate of recovery of MeHg from the two reference materials. Duplicate samples are marked A and B. Note that the matrix affects the rate of recovery, however, extraction of both samples was complete after 15 to 20 mL of CO_2 plus MeOH.

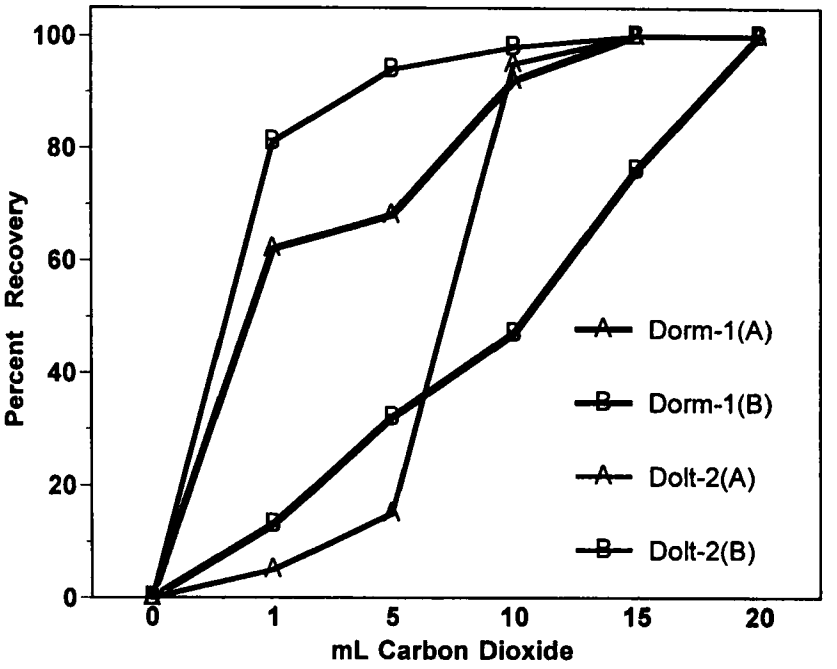

FIGURE 2 Recoveries of MeHg from Dorm-1 and Dolt-2 related to volume of SF Carbon Dioxide. A and B are replicate samples. Extraction conditions: 350 atm, 35°C, and 10% MeOH. Samples modified with 60 mg $CaCl_2$ and 150 μL H_2O.

Extraction of triplicate samples of the two reference materials resulted in average experimental concentrations that were 99.3 ± 1.2% of the reported value for Dorm-1 and 101.6 ± 2.9% of the reported value for Dolt-2. Extractions were done using samples prepared by adding approximately 60 mg of $CaCl_2$ and 150 μL H_2O to the samples before extraction at 35°C with 10% MeOH added to CO_2 at a pressure of 350 atm.

The use of 10% MeOH at approximately 35°C does not provide supercritical fluid MeOH for the extraction. Other investigators have reported that MeOH at a concentration of 5% is present in CO_2 as a fluid at pressures greater than 72 atmospheres and temperatures equal to or greater than 50°C[23–24]. Therefore, extraction at 50°C with 5% MeOH was tested to determine if supercritical fluid MeOH is a better extractant of MeHg than fluid MeOH. At a temperature of 50°C and a pressure of 350 atm, 5% MeOH in CO_2 represents a mole fraction of approximately 5.8. With the working conditions used, methanol should be a fluid rather than a liquid. Dorm-1 and Dolt-2 samples modified with 150 μL of water were extracted with 5% MeOH at 350 atmospheres of pressure and 50°C.

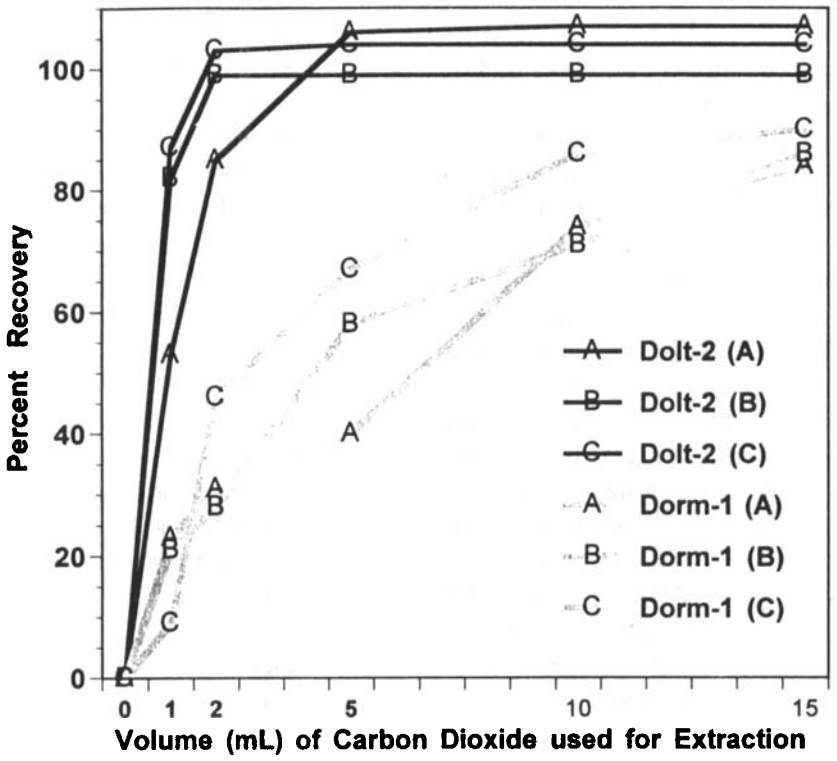

FIGURE 3 Recovery of MeHg from Dorm-1 and Dolt-2. Extraction conditions: 350 atm. 50°C, 5% MeOH. Dorm-1 modified with 200 μL H_2O and 80 mg $CaCl_2$; Dolt-2 modified with 150μL H_2O and 60 mg $CaCl_2$.

Extraction of the Dolt-2 samples was complete after a volume of approximately 5 mL of CO_2 were used (Figure 3). Triplicate samples are marked A, B, and C in Figure 3. Dorm-1 samples were only 75% extracted after 20 mL of CO_2 were used (data not presented). When the cartridges were opened the Dolt-2 samples were fully wetted top to bottom and the Dorm-1 samples were wetted to a depth of approximately half the sample volume. A second set of Dorm-1 samples was run using 200 μL of water as a modifier with approximately 80 mg of $CaCl_2$. The additional $CaCl_2$ is required to insure that the acidity of sample environment is maintained at approximately a pH of 1 (see below). The extraction of these samples with CO_2 and 5% MeOH at 350 atmospheres was 95% complete after the use of 20 mL of CO_2 (Figure 3). The samples were fully wetted when the cartridges were opened at the end of the extraction.

The extraction efficiencies using either 10% MeOH or 5% MeOH are similar. An advantage to the use of 5% MeOH is that the collecting solvents for the

extractions using 5% MeOH were less colored than the solutions collected using 10% MeOH. The chromatograms of the collecting solvents for the extractions using 5% MeOH have fewer peaks than the collecting solvents for extractions using 10% MeOH.

In this method, the extraction of MeHg from the Dorm-1 and Dolt-2 samples is facilitated by the use of $CaCl_2$. When $CaCl_2$ is combined in a ratio of approximately 20 mg to 50 μl, the pH of the wetted $CaCl_2$ is apparently less than 1 as indicated by the red color produced with either cresol red or bromocresol blue. The change from yellow to red is between pH's of 1.2 and 0.2 for cresol red and pH's of 1.8 and 1.2 for bromocresol blue. In the presence of a limited amount of water, the reaction between water and $CaCl_2$ apparently produces H^+ and Cl^-. It is postulated that the H^+ aids in the release of $MeHg^+$ from the matrix, and the Cl^- combines with the $MeHg^+$ to produce MeHgCl, a neutral molecule, which is readily extractable.

The requirement for an acidic environment for efficient extractions of MeHg is demonstrated by the results of extracting samples using $MgCl_2 \cdot 6H_2O$ in place of $MgCl_2$. The $MgCl_2 \cdot 6H_2O$ salt provides an environment with a pH greater than 1. $MgCl_2 \cdot 6H_2O$ produces yellow and red colors when cresol red and bromocresol blue, respectively, are added to the salt. Using $MgCl_2 \cdot 6H_2O$ as the modifier salt with water, no detectable MeHg was extracted (Table I). The use of water as matrix modifier and MeOH as a modifier of CO_2 will extract MeHg only if the sample environment is acidic.

$CaCl_2$ might serve other purposes. It could provide a salting-out affect[25] to aid in the transfer of MeHg from the H_2O-MeOH to nonpolar SF CO_2. The use of $CaCl_2$ lowers the vapor pressure of water to minimize the mixing of water with fluid CO_2. $CaCl_2$ also keeps the sample porous and prevents clumping of the sample in the extraction cell. The formation of $CaCO_3$ is not a problem because of the acidic environment in the cell since water in the presence of SF CO_2 has a pH of approximately 3[26].

Moderate pressures and low heat conditions used in this method require modifiers of water and MeOH to efficiently extract MeHg from biological samples. Because these conditions are mild, it is reasonable to expect that other organomercury compounds, such as ethyl- and dimethyl mercury, if present, are extracted along with MeHg.

Glassy-carbon electrodes have the advantages of having a high oxygen overpotential[27] and being a surface from which Hg is easily removed. The sensitivity of the detector is related to the condition of the glassy-carbon electrode which must be cleaned occasionally. This task is made easy by the fact that reduced (elemental) Hg^0 produced in a reductive electrochemical reaction can be wiped off the surface of glassy carbon using a laboratory tissue[28].

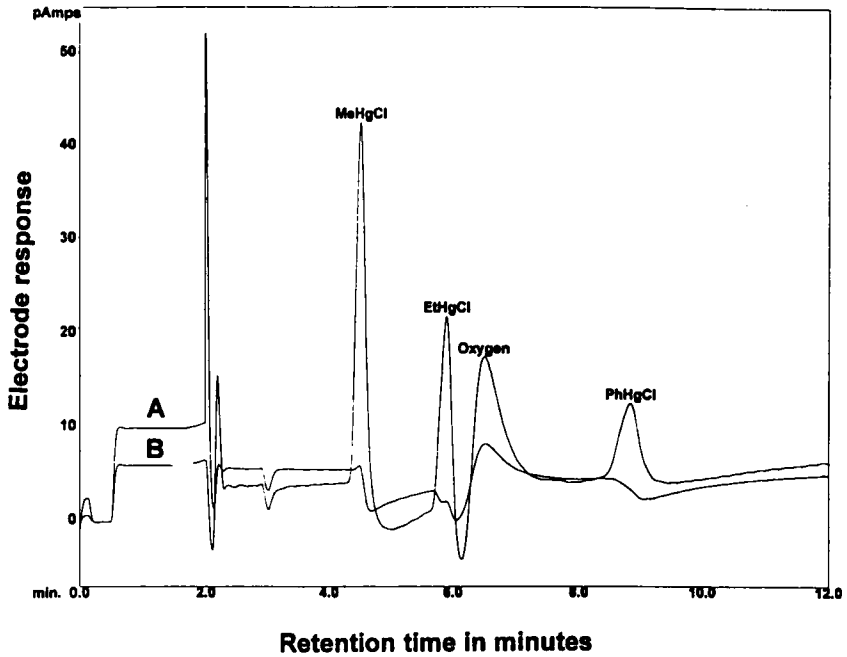

FIGURE 4 Chromatograms of a standard containing MeHgCl, EtHgCl, and phenyl Hg acetate. Chromatography conditions: 250 mm C_{18} column, eluent of 55:45 acetonitrile:buffer with flow of 1 mL min^{-1}, and temperature of 40°C. Chromatogram A is the response at −0.65 V and chromatogram B is the response at −0.90 V on a dual glassy carbon electrode.

The choice of 55% acetonitrile as the organic component of the eluent is based on a comparison of retention times obtained when 40%, 50, 55% and 60% of acetonitrile in the eluent were used to separate the compounds of interest. The retention times obtained with 40% acetonitrile gave poor separations for the organomercury compounds tested. The other percentages of acetonitrile easily separate phenylmercury from other organomercury compounds and from oxygen. When 60% acetonitrile is used, the retention time for MeHg is close to the void volume of the column. Using 50% acetonitrile, ethylmercury elutes less than 0.5 minute before oxygen. To achieve (a) the separation of MeHg from the void volume, (b) prevent ethylmercury from eluting with oxygen, and (c) maintain the separation of retention times of phenylmercury and oxygen, 55% acetonitrile is used. A 55:45, acetonitrile:buffer, eluent elutes MeHg approximately 0.5 minute after the void volume for the column, elutes ethylmercury more than 0.5 minute before oxygen, and elutes phenylmercury 3 minutes after oxygen. As expected, inorganic mercury (Hg^{2+}) comes off the column with the void volume. Figure 4 shows a chromatogram of a mixture of organomercury com-

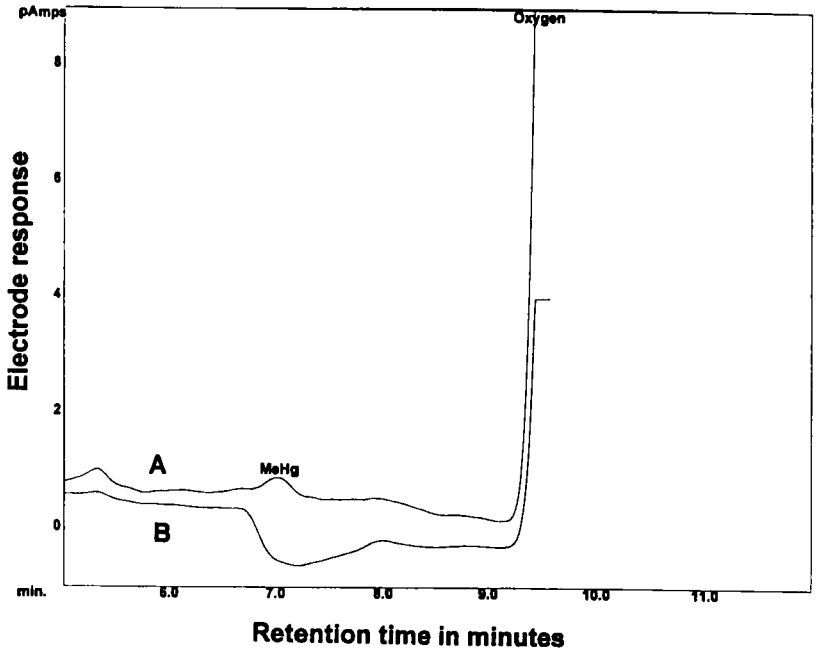

FIGURE 5 Chromatograms of a solution obtained when a vegetation sample was extracted using SFE. The instrumental conditions are the same as described in Figure 4 except the flow rate was adjusted to get good separation of the retention times for MeHgCl, EtHgCl and oxygen. In chromatogram A at the retention time for MeHgCl, the response at the electrode set at -0.65 V is a peak. In chromatogram B at the retention time for MeHgCl, the response at the electrode set at -0.90 V is a dip.

pounds. The compounds were dissolved in acetonitrile at a concentration of 100 μg L^{-1} MeHgCl and ethylmercury chloride and 50 μg L^{-1} phenylmercury acetate. The injected amounts were 2 nanograms (0.008 nanomol) of MeHgCl and ethylmercury chloride, and 1 nanograms (0.003 nanomol) of phenylmercury acetate. The chromatographic conditions are described in the experimental section. These compounds are well separated and oxygen does not interfere with the response for MeHg, ethylmercury or phenylmercury.

MeHg, ethylmercury and phenylmercury undergo reaction at similar reducing potentials. Dimethylmercury is not reactive until a reducing potential of approximately -1 V is applied. This is expected because dialkyl mercury compounds are generally not reducible and, when they are, a small signal is obtained[29]. For this reason dimethylmercury and diethylmercury were not included among the compounds included in this study.

Figures 4 and 5 show the difference in response of the dual glassy carbon electrodes. Figure 4 demonstrates the occurrence of negative peaks in the chro-

matogram for −0.90 V for all of the organomercury compounds that were tested. Figure 5 demonstrates a typical negative peak for real sample which in this case is the extract from a plant sample. The difference in response of organomercury compounds at reductive potentials of −0.65 V and −0.90 V is useful as additional evidence that a peak obtained at the retention time of an organomercury compound is correctly identified as an organomercury compound in an environmental sample. When a dual glassy-carbon electrode is used, one of the electrodes can be set at a potential of −0.90 V. At this potential, the flow of current is reduced below baseline when an organomercury compound reacts at the electrode. This dip in the chromatogram produced by the electrode set at −0.90 V provides evidence that the flow of current, apparent from a peak in the signal from the electrode set at −0.65 or −0.70 V, is indeed due to reduction of an organomercury compound. The information derived from the dip is used in the integration of the positive peak. The retention times that define the negative peak are used to define the baseline for the positive peak. This is particularly useful with environmental samples that contain peaks of unknown origin. Because the integration is dependent on the relation between the positive and negative peaks, peak height was not used to quantify peaks. The relative standard deviations calculated as the quotient of the standard deviation and the concentration is 0.07 and 0.06 with n = 3 for 25 and 50 $\mu g\ L^{-1}$ MeHgCl standards, respectively.

Hydrodynamic voltammograms provided the following information. The optimal reduction potential for MeHg is between −0.65 and −0.70 V using an eluent of 45:55 acetonitrile:buffer and a setting of 40°C for both the column heater and the detector preheater module. For a standard of less than 100 $\mu g\ L^{-1}$ MeHg in acetonitrile, a reducing potential of less than −0.55 V provides less than an optimal response at the electrode. At reducing potentials of approximately −0.65 to −0.70 V, MeHg causes the current flow at the electrode to increase briefly, producing a small peak and then, for a 100 $\mu g\ L^{-1}$ sample, decreases about 1 μamp before continuing the chromatogram. At reducing potentials of −0.75 V or more, there is no peak. The baseline dips approximately 1 μamp for a 100 $\mu g\ L^{-1}$ standard at the retention time for MeHg. At the retention time for MeHg, the magnitude of the dip at potentials more negative than −0.90 V is equivalent to the magnitude of the peak produced at −0.65 V. This dip occurs also in chromatograms obtained at potentials more negative than −0.90 V for ethylmercury and phenylmercury. Whether a positive or a negative signal is obtained could depend on which is greater, the current from reduction, or the decrease in residual current when adsorption occurs on the electrode. Residual current is greater at −0.9 V than at −0.65 V.

Organomercury compounds of the type RHgX with one carbon-mercury bond, and those of the type R_2Hg, with two carbon-mercury bonds, have been studied[29]. Hush and Oldham[30] examined the polarographic reduction of a number of alkylmercuric halides in ethanol-water mixtures. These show two-step polarograms the first step reversible, the second generally irreversible. Essentially the same results have been obtained in aqueous solutions[31]. It is postulated that the reactions at the electrode in this system could be either:

$$\text{(A)} \quad RHgCl + e^- \rightarrow RHg^{\cdot} + Cl^- \tag{1}$$

$$2RHg^{\cdot} \rightarrow (R)_2Hg + Hg^0 \tag{2}$$

or

$$\text{(B)} \quad RHg^{\cdot} + e^- \rightarrow R^- + Hg^0 \tag{3}$$

$$R^- + H^+ \rightarrow RH \tag{4}$$

Whether reaction set A, or reaction set B, were occurring at the electrodes, the formation of R_2Hg or RH at the electrode set at -0.90V would inhibit the flow of electrons and result in a dip rather than a peak in the chromatogram.

A typical calibration curve using the operating conditions described above is presented in Figure 6. The calibration for MeHg concentrations expected for extractions of biological samples is linear within an order of magnitude range. The detection limit of the method is 5×10^{-8} mol L^{-1} if a 20 μL loop is used. The response of the detector is approximately 1×10^{-12} amp per 1×10^{-12} mol of MeHgCl. When the extraction and detection techniques are combined, the expected detection limits for environmental samples are dependent on the size of the sample that is extracted, the volume of the collecting solution, and the background noise of the electrochemical detector. If 0.2 g of sample is extracted and the collecting solution has a volume of 5 mL, the method can detect approximately 0.015 μg g^{-1} MeHg in the sample.

This paper presents a procedure for extracting underivatized MeHg from standard reference materials of biological tissue. The solution in which the MeHg is collected is ready for analysis by LCEC, without derivatization or further extract clean-up. The organomercury compounds are not derivatized; they are

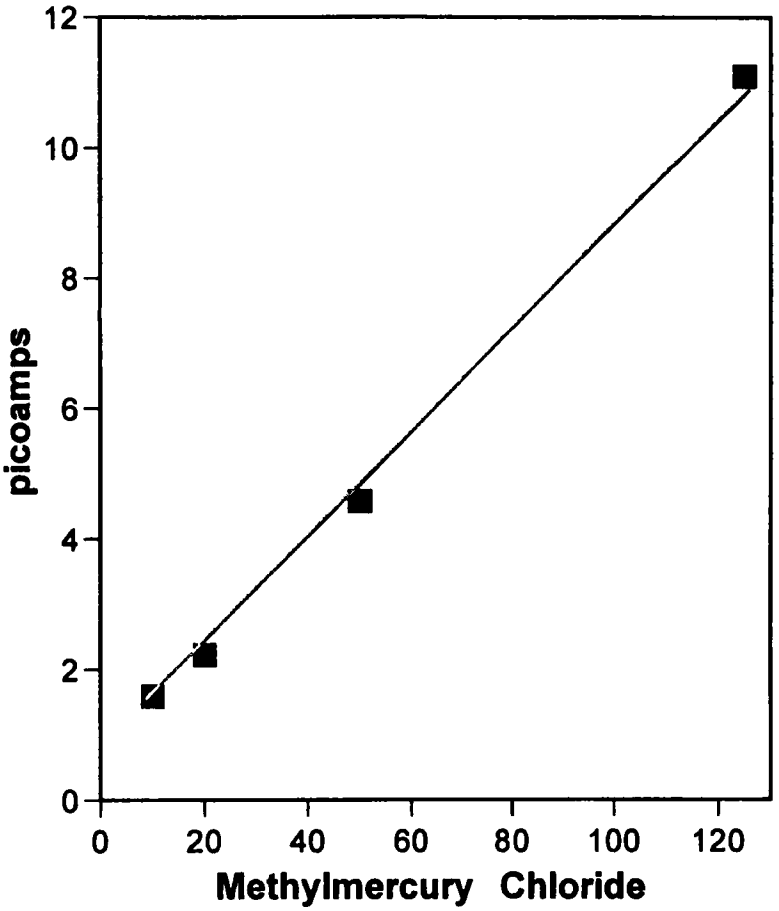

FIGURE 6 Calibration Curve for MeHgCl. Concentrations = μg L^{-1}.

maintained as their neutral chloride complexes. Verification of organomercury peaks is possible by the difference in behavior of organomercury compounds on a dual glassy carbon electrode set at reducing potentials of −0.70 and −0.90V. It would be possible to automate this method if an autosampler were used that would degas samples with helium while drawing sample volume into the sample loop.

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