Reprinted from BIOCONJUGATE CHEMISTRY, 1995, 6.

Development of Rapid Mercury Assays. Synthesis of Sulfur- and Mercury-Containing Conjugates

Ferenc Szurdoki, Horacio Kido, and Bruce D. Hammock*

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616.
Received October 3, 1994®

We have devised rapid analyses for mercury exploiting the high affinity of dithiocarbamate chelators for the mercuric ion. Our first assay is based on a sandwich chelate formed by a ligand supported on the well of an ELISA plate, Hg^{2+} ion of the investigated sample, and another ligand bound to a reporter enzyme. The second assay utilizes competitive binding of the analyte Hg^{2+} ions versus an organomercury conjugate to a chelating conjugate. Low ppb sensitivity and high selectivity for Hg^{2+} ions have been achieved in our pilot studies.

INTRODUCTION

Most instrumental methods for trace analysis and chemical speciation of hazardous metals (*1*–*3*) are not adaptable as low-cost, field-portable assays for monitoring a large number of environmental or biological samples. The development of immunoassays to detect metal ions has been a promising trend (*4*–*7*). However, this approach is based on highly specific monoclonal antibodies which usually are expensive to generate.

We have utilized the high affinity chelation of the mercurials by sulfur-containing ligands and the simple ELISA technique providing high signal amplification to devise sensitive and selective analyses of Hg^{2+} ion. Dithiocarbamates form stable complexes with a variety of metal ions except the alkali and alkaline earth metals (*8*). However, dithiocarbamates can be used to analyze Hg^{2+} selectively because Hg^{2+} replaces numerous metal ions from their dithiocarbamate chelates in fast exchange reactions (*9*). Mercury–dithiocarbamate complexes have a very high thermodynamic stability (*10*). Dithiocarbamates seem to have similar or higher avidity only for several noble metals (*1*, *8*, *10*). In our studies, dithiocarbamates formed from secondary amine moieties of biopolymers were used because similar conjugates obtained from primary amines were reported to be unstable (*11*). Dithiocarbamates are difficult to prepare in pure form (*12*). Thus, these chelator groups were generated in the last synthetic step because of the simplified isolation of the macromolecular products.

RESULTS AND DISCUSSION

Our first approach involved a sandwich chelate of the Hg^{2+} ion with one chelator (**1**) immobilized on an ELISA plate and another bound to a reporter enzyme (**2**, format 1, Figure 1). In analogy with sandwich ELISAs (*13*), the acyclic dithiocarbamate (**1**) having presumably higher avidity for the analyte (*10*) was employed as immobilized ligand, and the pyrrolidine derivative (**2**) with lower affinity for Hg^{2+} ion was conjugated to the reporter enzyme. Secondary amino groups were formed on the biopolymers and then the resulting products were treated

* To whom correspondence should be addressed. Phone: (916) 752-7519. Fax: (916) 752-1537. E-mail: bdhammock@ucdavis.edu.
® Abstract published in *Advance ACS Abstracts*, February 1, 1995.

1043-1802/95/2906-0145$09.00/0

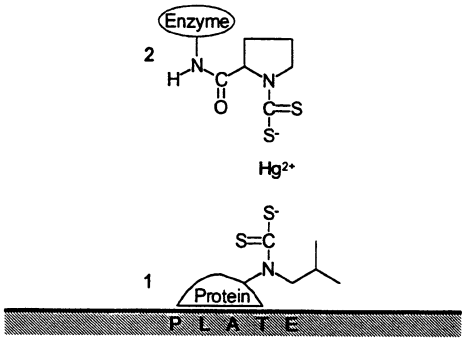

Figure 1. Chemical structures of the reagents involved in assay format 1.

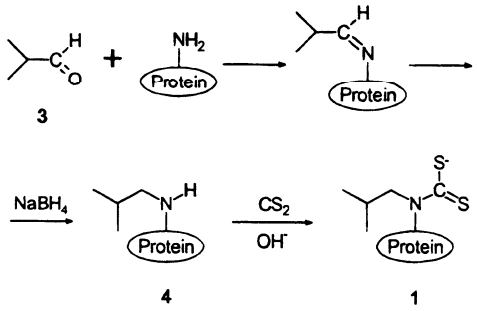

Figure 2. Synthesis of the immobilized chelator.

by carbon disulfide in alkaline solution to synthesize dithiocarbamate-bound protein and enzyme conjugates (Figures 2 and 3). Reductive alkylation (*14*) of ϵ-amino groups of lysine residues on conalbumin (CONA) with 2-methylpropionaldehyde (**3**) furnished the secondary amine intermediate (**4**–CONA) of the plate coating chelator (**1**–CONA, Figure 2). A protected secondary amino acid, *N*-(9-fluorenylmethyloxycarbonyl)-L-proline (Fmoc-Pro, **5**), served as a precursor for the preparation of the enzyme-linked chelator (**2**, Figure 3). This lipophilic acid (**5**) was efficiently conjugated to alkaline phosphatase (AP) by means of *N*-hydroxysulfosuccinimide (**6**) and a water soluble carbodiimide (**7**) (*15*). (The reagents were used in excess to ensure the completion of this coupling reaction.) The standard protocol for the cleavage of the *N*-FMOC group employs secondary amines in aprotic solvents (*16*, *17*). However, deblocking of FMOC-peptides by secondary amines in solvents like formamide, 5% TFA/DMF (*17*), and 5% AcOH/$CHCl_3$ (*18*) has recently been reported. In our experiments with Fmoc-Pro (**5**), the blocking group could be removed by 4-(aminomethyl)piperidine (**10**) in alkaline aqueous solution at a slow rate (pH 11, 4 °C, 24–36 h). Under these conditions, the activity of some enzymes (AP, HRP) was preserved. In the case of biopolymers, excess reagent **10** and water soluble side products (*16*) were eliminated by dialysis in a slightly acidic buffer (Figure 3). The cleavage of the protecting group of **9**–AP resulting in **11**–AP was confirmed by UV spectroscopy.

The sandwich chelate with Hg^{2+} ion, **1**–CONA, and **2**–AP (Figure 1) was formed in slightly acidic acetate buffer containing Tween 20 with simultaneous incubation of the reagents in the wells of the ELISA plates. The quality and the concentration of the assay buffer and of the detergent as well as other assay parameters exercised profound influence on the assay performance. Contaminating heavy metal ions were removed from the buffers used in the assay by a specific ion-exchanger resin. The standard curve (Figure 4) obtained with this system has an IC_{50} value of 0.54 ppb (2.7 nM) and a limit of detection

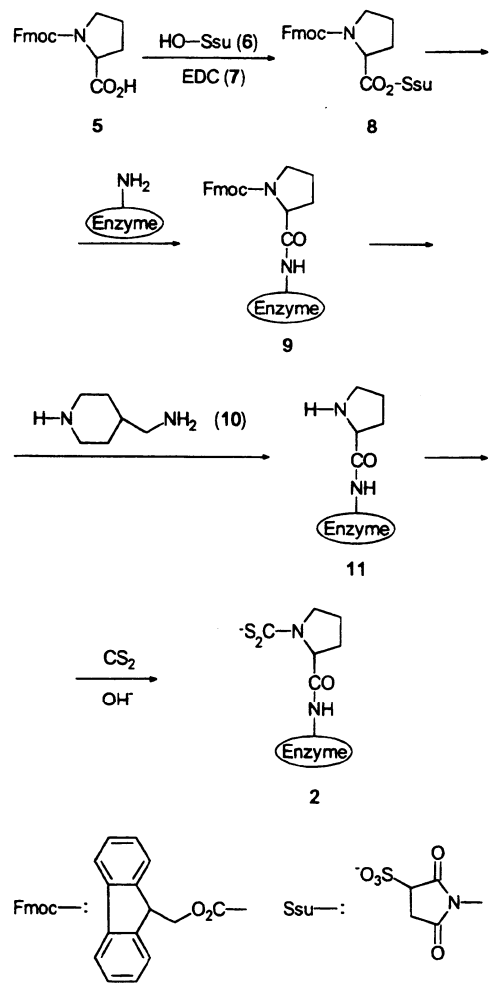

Figure 3. Synthesis of the chelator linked to the reporter enzyme.

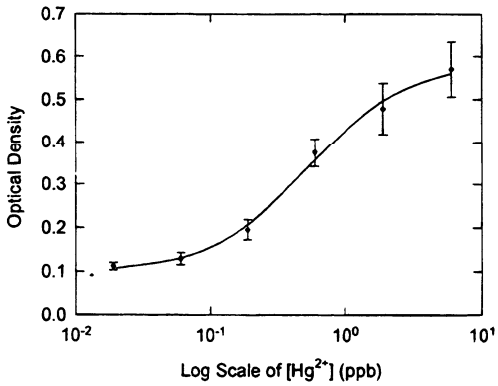

Figure 4. Standard curve of assay format 1.

(LD) of about 75 ppt (0.375 nM) of mercuric ion concentration. Most of the metal ions studied (e.g., Al^{3+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, Cr^{6+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}) did not significantly interfere up to about 3000 nM concentrations; however, cross-reactivities (CR = $100^{Hg(II)}IC_{50}/^{ion}IC_{50}$) with, e.g., Ag^{+} (7%), Au^{3+} (0.2%), Cu^{2+} (2%), Pd^{2+} (29%), and Zn^{2+} (0.3%) ions were noticed. No significant change in the IC_{50} value of the Hg^{2+} standard curve was observed when the assay buffer was supplemented with 1000 nM Zn^{2+}; thus, the interference from foreign zinc ions might be reduced this way. The signal of the assay fell below background level when methylmercury was given in increased concentrations. (This unexpected downward

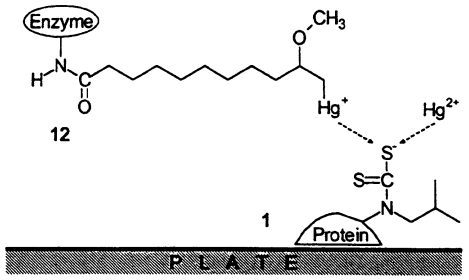

Figure 5. Chemical structures of the reagents involved in assay format 2A. Dotted lines indicate competition for the ligand bound to the plate.

trend, however, was not significant with an analogous system using different assay parameters.) Methylmercury may be competing with traces of heavy metals, remaining in the buffers and reagents, causing background signal. Total mercury content of a sample containing CH_3Hg^+ and Hg^{2+} can be determined with this assay after decomposition of the organic mercury species into mercuric ion.

Monoalkyl- and monoarylmercury salts are known to form highly stable 1:1 chelates with dithiocarbamate-ligands (*19–21*); the exact measure of the thermodynamic stability, however, is not reported to our knowledge. The second assay (format 2) is based on the competition between mercuric ions and an organomercury conjugate in binding to a chelating conjugate. The first application of this assay principle (format 2A) involved a mercury-linked reporter enzyme (**12**–AP, Figure 5) and the same immobilized chelator as used in the first assay (**1**–CONA, Figures 1 and 5). 10-Undecenoic acid (**13**) and mercuric acetate in the presence of methanol gave organomercury compound **14** (Figure 6) (*22*). This acid (**14**) was then conjugated to AP to yield mercury-containing tracer **12**–AP. The standard curve constructed with the **1**–CONA/**12**–AP assay system (format 2A, Figure 5), depicted in Figure 7, had an IC_{50} value of 40 nM (8 ppb) and a LD of 5 nM (1 ppb) of mercuric ion. This assay displayed remarkable selectivity for Hg^{2+}. No or only marginal interferences were observed with most metal ions (e.g., Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cr^{6+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}) up to about 3000 nM concentrations. Significant cross-reactivity with only the following metals were detected: Ag^+ (CR: 82%), Au^{3+} (CR: <1%), and Cu^{2+} (CR: 10%). The concentration–response curves with CH_3Hg^+ and Zn^{2+} ions displayed on unexpected, slightly increasing trend. The increasing tendency with methylmercury was diminished when the slightly acidic acetate assay buffer contained trioctylmethylammonium chloride (Aliquat 336), an unusual detergent employed to reduce background signal, instead of Tween 20. The cleavage of the mercury–carbon bond in conjugate **12**–AP with sodium tetrahydroborate (*22*) without inactivating the enzyme resulted in no signal.

Another variant (format 2B) of the second assay employed a mercury-linked protein conjugate **16**–CONA and a chelate-linked tracer **2**–AP (Figure 8). Reagent **16**–CONA, used for plate coating, was produced from a mercury-containing acid, 2-[*N*-[3-(hydroxymercurio)-2-methoxypropyl]carbamoyl]phenoxyacetic acid (Aldrich's mersalyl acid) in a manner analogous to the synthesis of tracer **12**–AP. In our preliminary studies with this assay, we prepared a standard curve (IC_{50}: 25 nM) similar to that obtained with system 2A. A cross-reactivity pattern similar to that of format 2A was found, and no significant interference with a number of metal ions (e.g., Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) up to 300 nM concentrations was

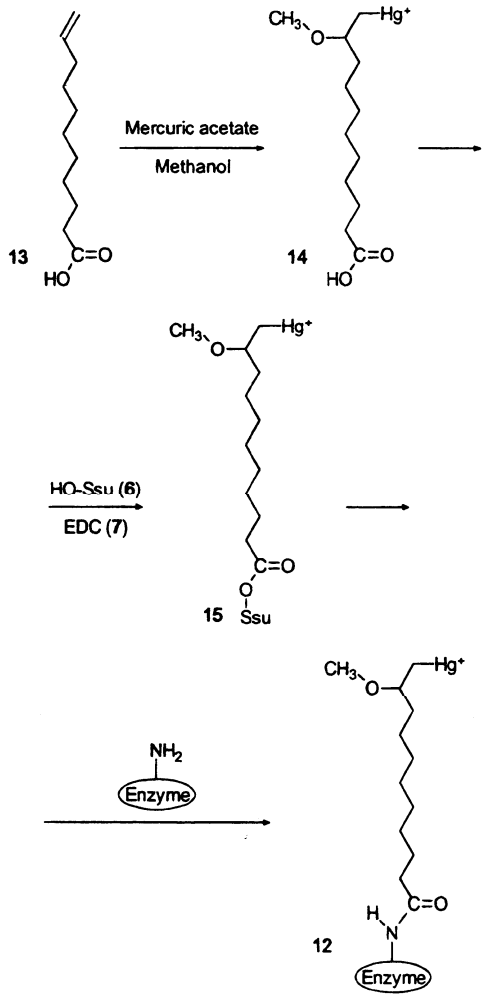

Figure 6. Synthesis of the mercury derivative linked to the reporter enzyme.

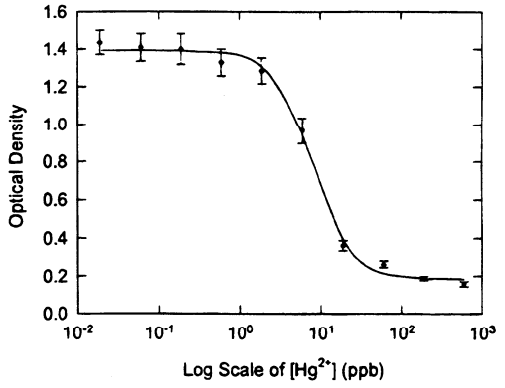

Figure 7. Standard curve of assay format 2A.

observed. Au^{3+} (CR: 11%) and CH_3Hg^+ (CR: 14%) displayed cross-reactivities.

CONA and intestinal AP were used to obtain our reagents. Intestinal AP's unpaired cysteine residues are hidden (*23*). All cysteines of CONA are blocked forming cystines (*24*). Thus, these biopolymers lack thiol groups, ligands with a very strong affinity for mercury, on their surfaces. Therefore, in experiments with CONA, AP, and intermediates instead of the corresponding reagents only low signals were observed.

Mercury is released to the environment largely in its inorganic forms. Methylmercury is produced by bacterial

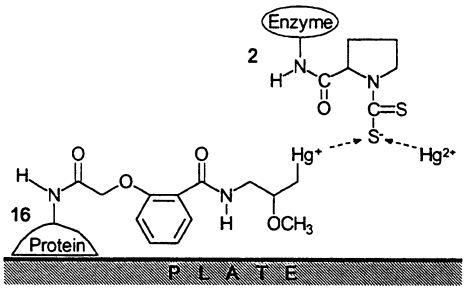

Figure 8. Chemical structures of the reagents involved in assay format 2B. Dotted lines indicate competition for the ligand bound to the reporter enzyme.

methylation of inorganic mercury in lake sediments (*25*). In natural waters, mercury is concentrated in fish, predominantly in the methylmercury form, and biomagnified through the food chain (*3*, *25*, *26*). Amongst the various mercury species which occur in real samples and which are reactive toward our chelators, Hg^{2+} and CH_3-Hg^+ are by far the most important. They are the most abundant mercury species in most environmental and biological matrices and have the greatest ecotoxicological significance (*25*–*29*). We thus focused on these two species during the preliminary characterization of our assays. The concentrations of various mercury species and of total mercury in lakes and seas far from pollution sites are usually in the low-ppt range or less (*28*, *29*). However, the amount of total mercury in rivers contaminated with industrial, mining, and urban waste occasionally reaches mid-ppt to low-ppb levels (*30*). An important application of rapid mercury assays could be to monitor whether the mercury concentrations in environmental water samples complies with the regulatory limit, 1 ppb in many countries (*30*, *31*). The sensitivities of some of our assays appear to be adequate for this use and are similar to those of the cold-vapor atomic absorption spectrometry and a current immunoassay method (*32*). (The amount of total mercury can be measured after decomposition of all mercury species to Hg^{2+}.) For the detection of lower concentrations of mercury, enrichment of the sample, e.g., by means of a specific ion-exchanger resin (*29*) *prior to* the analysis, is necessary. In natural waters, the concentrations of a number of ions are much higher than that of the mercury. For instance, while the large concentrations of alkali metals do not seem to cause problems, small amounts of other ions (e.g., Cu^{2+}) common in environmental samples may crossreact with some of the assays. These interferences might be eliminated by addition of specific masking agents to the assay buffer as well as by cleanup of the samples with selective ion-exchanger resins or with chelate-extraction. These remedies are currently being investigated. Studies with new ligands, tracers, assay formats, and with environmental samples are also in progress. We hope that eventually the principles of our new mercury assays will find further applications in the analysis of hazardous ions.

ACKNOWLEDGMENT

This work was supported in part by the California Department of Food and Agriculture, NIEHS Superfund Grant 2 P42 ES04699, U. C. Systemwide Toxic Substances Program, U.S. EPA Center for Ecological Health Research at U. C. Davis (R819658), and U.S. EPA Cooperative Agreement CR-814709-01-0. H.K. received a fellowship from NIEHS Center for Environmental Health Sciences ES05707. B.D.H. is a Burroughs-Wellcome Scholar in Toxicology.

LITERATURE CITED

(1) Lo, J.-M., and Lee, J.-D. (1994) Dithiocarbamate extraction and Au(III) back extraction for determination of mercury in water and biological samples by anodic stripping voltammetry. *Anal. Chem. 66*, 1242–1248.

(2) Jackson, K. W., and Mahmood, T. M. (1994) Atomic absorption, atomic emission, and flame emission spectrometry. *Anal. Chem. 66*, 252R–279R.

(3) Saouter, E., and Blattmann, B. (1994) Analyses of organic and inorganic mercury by atomic fluorescence spectrometry using a semiautomatic analytical system. *Anal. Chem. 66*, 2031–2037.

(4) Reardan, D. T., Meares, C. F., Goodwin, D. A., McTigue, M., David, G. S., Stone, M. R., Leung, J. P., Bartholomew, R. M., and Frincke, J. M. (1985) Antibodies against metal chelates. *Nature 316*, 265–268.

(5) Wylie, D. E., Lu, D., Carlson, L. D., Carlson, R., Babacan, K. F., Schuster, S. M., and Wagner, F. W. (1992) Monoclonal antibodies specific for mercuric ions. *Proc. Natl. Acad. Sci. U.S.A. 89*, 4104–4108.

(6) Barbas, C. F., III, Rosenblum, J. S., and Lerner, R. A. (1993) Direct selection of antibodies that coordinate metals from semisynthetic combinatorial libraries. *Proc. Natl. Acad. Sci. U.S.A. 90*, 6385–6389.

(7) Chakrabarti, P., Hatcher, F. M., Blake, R. C., 2nd, Ladd, P. A., and Blake D. A. (1994) Enzyme immunoassay to determine heavy metals using antibodies to specific metal-EDTA complexes: optimization and validation of an immunoassay for soluble indium. *Anal. Biochem. 217*, 70–75.

(8) Sachsenberg, S., Klenke, T., Krumbein, W. E., and Zeeck, E. (1992) A back-extraction procedure for the dithiocarbamate solvent extraction method. Rapid determination of metals in seawater matrices. *Fresenius J. Anal. Chem. 342*, 163–166.

(9) Lo, J.-M., Yu, J. C., Hutchison, F. I., and Wal, C. M. (1982) Solvent extraction of dithiocarbamate complexes and back-extraction with mercury(II) for determination of trace metals in seawater by atomic absorption spectrometry. *Anal. Chem. 54*, 2536–2539.

(10) Bond, A. M., and Scholz, F. (1991) Calculation of thermodynamic data from voltammetry of solid lead and mercury dithiocarbamate complexes mechanically attached to a graphite electrode. *J. Phys. Chem. 95*, 7460–7465.

(11) Valentine, W. M., Amarnath, V., Graham, D. G., and Anthony, D. C. (1992) Covalent cross-linking of proteins by carbon disulfide. *Chem. Res. Toxicol. 5*, 254–262.

(12) Giboreau, P., and Morin, C. (1994) Procedure for the preparation of pure dithiocarbamates. *J. Org. Chem. 59*, 1205–1207.

(13) Porstmann, T., and Kiessig, S. T. (1992) Enzyme immunoassay techniques. An overview. *J. Immunol. Methods 150*, 5–21.

(14) Means, G. E., and Feeney, R. E. (1968) Reductive alkylation of amino groups in proteins. *Biochemistry 7*, 2192–2201.

(15) Bekheit, H. K. M., Lucas, A. D., Szurdoki, F., Gee, S. J., and Hammock, B. D. (1993) An enzyme immunoassay for the environmental monitoring of the herbicide bromacil. *J. Agric. Food Chem. 41*, 2220–2227.

(16) Carpino, L. A. (1987) The 9-fluorenylmethyloxycarbonyl family of base-sensitive amino-protecting groups. *Acc. Chem. Res. 20*, 401–407.

(17) Larsen, B. D., and Holm, A. (1994) Incomplete Fmoc deprotection in solid-phase synthesis of peptides. *Int. J. Peptide Protein Res. 43*, 1–9.

(18) Kates, S. A., Daniels, S. B., and Albericio, F. (1993) Automated allyl cleavage for continuous-flow synthesis of cyclic and branched peptides. *Anal. Biochem. 212*, 303–310.

(19) Parkin, J. E. (1989) High-performance liquid chromatography of mercury and phenylmercury as the N-disubstituted dithiocarbamate complexes. *J. Chromatogr. 472*, 401–405.

(20) Parkin, J. E. (1991) High-performance liquid chromatographic assay of thiomersal (thimerosal) as the ethylmercury dithiocarbamate complex. *J. Chromatogr. 542*, 137–143.

(21) Sarzanini, C., Sacchero, G., Aceto, M., Abollino, O., and Mentasti, E. (1992) Simultaneous determination of methyl-, ethyl-, phenyl-, and inorganic mercury by cold vapour atomic absorption spectrometry with on-line chromatographic separation. *J. Chromatogr. 626*, 151–157.

(22) Brown, H. C., and Rei, M.-H. (1969) The solvomercuration–demercuration of representative olefins in the presence of alcohols. Convenient procedures for the synthesis of ethers. *J. Am. Chem. Soc. 91*, 5646–5647.
(23) Fosset, M., Chappelet-Tordo, D., and Lazdunski, M. (1974) Intestinal alkaline phosphatase. Physical properties and quaternary structure. *Biochemistry 13*, 1783–1788.
(24) Williams, J., Elleman, T. C., Kingston, I. B., Wilkins, A. G., and Kuhn, K. A. (1982) The primary structure of hen ovotransferrin. *Eur. J. Biochem. 122*, 279–303.
(25) Zillioux, E. J., Porcella, D. B., and Benoit, J. M. (1993) Mercury cycling and effects in freshwater wetland ecosystems. *Environ. Toxicol. Chem. 12*, 2245–2264.
(26) Bulska, E., Emteborg, H., Baxter, D. C., Frech, W., Ellingsen, D., and Thomassen, Y. (1992) Speciation of mercury in human whole blood by capillary gas chromatography with a microwave-induced plasma emission detector system following complexometric extraction and butylation. *Analyst 117*, 657–663.
(27) Liang, L., Bloom, N. S., and Horvat, M. (1994) Simultaneous determination of mercury speciation in biological materials by GC/CVAFS after ethylation and room-temperature precollection. *Clin. Chem. 40*, 602–607.
(28) Bloom, N. S., and Effler, S. W. (1990) Seasonal variability in the mercury speciation of Onondaga Lake (New York). *Water Air Soil Pollut. 53*, 251–265.
(29) Emteborg, H., Baxter D. C., and Frech, W. (1993) Speciation of mercury in natural waters by capillary gas chromatography with a microwave-induced plasma emission detector following preconcentration using a dithiocarbamate resin microcolumn installed in a closed flow injection system. *Analyst 118*, 1007–1013.
(30) Janjic, J., and Kiurski, J. (1994) Non-flame atomic fluorescence as a method for mercury traces determination. *Water Res. 28*, 233–235.
(31) Mariscal, M. D., Galban, J., Urarte, M. L., and Aznarez, J. (1992) Study and evaluation of a fluorimetric method for the determination of inorganic mercury in water. *Fresenius J. Anal. Chem. 342*, 157–162.
(32) Wylie, D. E., Carlson, L. D., Carlson, R., Wagner, F. W., and Schuster, S. M. (1991) Detection of mercuric ions in water by ELISA with a mercury-specific antibody. *Anal. Biochem. 194*, 381–387.

BC940107+