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Mercury speciation by liquid chromatography coupled with on-line chemical vapour generation and atomic fluorescence spectrometric detection (LC–CVGAFS)

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Abstract

Reverse phase chromatography (RPC) coupled on-line with UV–vis diode array detector (DAD) and cold vapour generation atomic fluorescence spectrometry (CVGAFS) is proposed for the speciation and determination of inorganic and organic mercury (methylmercury, ethylmercury and phenylmercury) in the form of cysteine, penicillamine and glutathione complexes. The mercury–thiol complexes are separated on a C_{18} Reverse Phase column and oxidized on-line with bromine, generated in situ by $KBr/KBrO_3$ in HCl medium, in order to fast convert organic mercury species to inorganic mercury in less than 2.5 s, at room temperature, in a 30 cm knitted coil. Hg(II) is selectively detected by AFS in a Ar/H_2 miniaturized flame after sodium borohydride reduction to Hg^0. Under optimized conditions, on-line bromine treatment gives recoveries of thiol-complexed methylmercury, ethylmercury and phenylmercury with respect to inorganic mercury ranging between 79 and 85%, 80 and 85%, 63 and 76%, respectively, depending on the complexing thiol employed.

Optimized elution conditions were provided in the three complexing agents. The detection limits (LODc) for inorganic mercury, methylmercury, ethylmercury and phenylmercury, in the optimized conditions complexed with thiols were about 16, 18, 18 and 20 pg (as mercury), respectively, a relative standard deviation (R.S.D) ranging between 1.5 and 2.0%, and a linear dynamic range between 0.1 and 100 ng injected. LC–DAD–CVGAFS method has been validated by analysing two certificate reference material, DORM-2 and NIES CRM 13, obtaining 98 ± 6 and $97 \pm 5\%$ of methylmercury recovered, respectively.

Keywords: Mercury speciation; Atomic fluorescence spectrometry; Chromatography; Thiols; Hyphenated technique

1. Introduction

The importance of mercury speciation in environmental and biological sample has been well assessed in many papers and reviews, as well as a big number of methods are described in literature based on liquid or gas chromatography or capillary electrophoresis coupled to sensitive, specific mercury detectors, including atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), inductively coupled plasma mass spectrometry (ICPMS), inductively coupled plasma atomic emission spectrometry (ICPAES) and microwave-induced plasma atomic emission spectrometry (MIPAES) [1–3].

Among them, the cold vapour (CV) generation technique coupled with atomic fluorescence spectrometric (AFS) detection represents the most sensitive, low-cost analytical tool for mercury determination [4]. CVGAFS has been previously employed as LC detector for mercury speciation by several authors [5–10] in conjunction with various on-

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line oxidation techniques for the conversion of organomercury species to Hg(II). Oxidation techniques generally comprehend the employment of $KBr/KBrO_3$ [9,11], potassium dichromate without [12] or with cadmium [13,14], UV irradiation [7,8,15,16] and acidic potassium persulfate in the presence of copper sulfate [5,17,18] associated to microwave digestion [10].

In particular, the $KBr/KBrO_3$ oxidization represents an effective procedures and it has been employed for on-line oxidation of mercury compounds in FIA experiments coupled to CVAAS [19–21] and CVGAFS detection [22,23], and in LC–CVGAFS experiments by using long reaction coils (3.4–19 m) and/or external heating sources [9].

In previous papers, we proposed a new hyphenated technique for the determination, separation and characterization of native, denatured and denatured-reduced thiolic proteins, based on pre-column derivatization of protein–SH groups with an organic mercurial probe (*p*-hydroxymercurybenzoate, PHMB), separation by hydrophobic interaction chromatography (HIC) followed by on-line digestion of the protein–mercury complexes and mercury detection by chemical vapour generation atomic fluorescence spectrometry (CVGAFS) [24–26]. On-line digestion was realised at room temperature in a 30 cm long reaction coil with bromine, generated in situ by $KBr/KBrO_3$ in HCl medium. Recently, the CVGAFS detection system has been optimized for the hyphenation with reverse phase chromatography (RPC) in the presence of methanol in the eluent phase and for the on-line digestion of complexed and uncomplexed PHMB [27].

In this paper, the LC–CVGAFS technique coupled to on-line post column oxidation with bromine generated in situ at room temperature has been optimized for the speciation and determination of inorganic mercury (Hg(II)), methylmercury (MeHg), ethylmercury (EtHg) and phenylmercury (PhHg) complexed with cysteine (CYS), penicillamine (PSH) and reduced glutathione (GSH) in the presence of CYS, PSH and GSH in the eluent phase, respectively. Complexing agents (CYS or β-mercaptoethanol) or ion-pairing agents are usually employed for the analysis of mercury compounds by RPC in order to improve chromatographic separation and/or to avoid adsorption of mercurial species to the stationary phase [1,2]. In this study, three thiolic compounds which form stable complexes with mercurial species in the order PSH > CYS > GSH [28], have been compared. A 30 cm knitted reaction coil guaranteed the conversion of MeHg, EtHg and PhHg to Hg(II) with a yield of 80–85 ± 2%, 81–85 ± 2% and 70–85 ± 2%, respectively, depending on the complexing thiol employed. The subsequent reduction of Hg(II) to Hg^0 was performed in a knitted reduction coil with $NaBH_4$ solution containing hydrazine in order to control the potential quenching effect of Br_2 on mercury fluorescence [25–27]. The use of short knitted coils guaranteed an effective mixing of reactants and a control of diffusion processes with resulting symmetric, well-shaped chromatographic peaks [29]. The optimized elution conditions, the results of calibrations experiments and the figures of merit are reported in the three complexing agents. The accuracy of the method was determined by the analysis of two certified reference materials (DORM-2 and CRM 13).

Despite the huge number of methods for mercury speciation proposed [5,7,9,10,30], no papers are reported which propose the use of post column on-line oxidation of organic mercury with bromine generated in situ at room temperature in a 30 cm knitted reaction coil with good yield of reduction, and detection limit comparable with those obtained by other expensive and/or more complex hyphenated techniques [31,32].

2. Experimental

2.1. Reagents and samples

Analytical reagent-grade chemicals were used without further purification. Stock solution of $1000 \pm 5\ \mu g\ mL^{-1}$ of inorganic Hg^{II} in the form of $Hg(NO_3)_2$ was purchased from Merck Laboratory Supplies (Poole, Dorset, UK). Methyl-, ethyl- and phenyl-mercury chlorides (ICN Biochemicals, Cleveland, OH, USA) were used as calibrants. A stock solution of such standards ($256\ \mu g\ mL^{-1}$ MeHg in methanol; $338\ \mu g\ mL^{-1}$ EtHg in ethanol; $354\ \mu g\ mL^{-1}$ PhHg in methanol) was used to obtain daily standards by suitable dilution in $5\ mmol\ L^{-1}$ thiols in water. All the stock solutions were protected against light and stored at 4 °C in the dark.

Stock solutions of L-CYS and GSH (Sigma Chemical Co., St. Louis, MO, USA), and DL–PSH (Fluka Chemie AG CH-9470 Buchs), were prepared in HCl $0.1\ mol\ L^{-1}$. The minimum assay of solid reagents was >99% for GSH and PSH and >99.5% for CYS. In order to prevent oxidation, standard solutions of thiols were prepared daily and kept cold (4 °C) and protected from light until used.

Methanol and toluene for RPC were purchased from Carlo Erba (Rodano, MI, Italy).

Solutions of $NaBH_4$ more concentrated than 1% (m/v) were prepared by dissolving the solid reagent (Merck, pellets, reagent for AAS, minimum assay >96%) into 0.3% (m/v) NaOH solution. The solutions were microfiltered through a 0.45 μm membrane and stored in a refrigerator. Dilute solutions of $NaBH_4$ were prepared by appropriate dilution of the stock solutions, the total NaOH concentration being kept at 0.3% (m/v), unless specified. In our experience, alkaline $NaBH_4$ stock and diluted standard solutions can be prepared and preserved at least for the working day (maximum time tested 8 h) without any particular care.

The 24–26% (m/m) hydrazine standard solution (53847) was purchased from Fluka Chemie and the optimized concentration added to $NaBH_4$ solution containing 0.3% (m/v) of NaOH (see Table 1).

An amount of $3.5\ mol\ L^{-1}$ HCl solutions were prepared by 37% (m/m) HCl (Carlo Erba).

Table 1
Optimized operating conditions for on-line digestion and determination of inorganic and organic mercury by LC–CVGAFS system

[Br_2]	0.045 mol L^{-1}
[HCl]	3.5 mol L^{-1}
[$NaBH_4$]	0.05 mol L^{-1}
[N_2H_4]	0.1 mol L^{-1}
Br^-/BrO_3^- flow rate	1.0 mL min^{-1}
Reducing mixture ($NaBH_4/N_2H_4$) flow rate	0.8 mL min^{-1}
HCl flow rate	1.0 mL min^{-1}
LC flow rate	1.0 mL min^{-1}
Bromine formation coil	100 cm, 1.5 mm i.d., reaction time 53 s
Oxidation coil (knitted)	30 cm, 0.79 mm i.d., reaction time 2.97 s
Reduction coil (knitted)	30 cm, 0.79 mm i.d., reaction time 2.34 s
Stripping coil (knitted)	30 cm, 1.6 mm id
Transfer line from the GLS to atomization cell	40 cm, 1.6 mm i.d.
Carrier Argon flow rate	475 mL min^{-1}
Hydrogen flow rate	75 mL min^{-1}

Working solution of Br^-/BrO_3^- was prepared by solid reagents (Carlo Erba) (0.075 mol L^{-1} Br^-, 0.015 mol L^{-1} BrO_3^-) keeping an approximate 5:1 molar ratio on the basis of stoichiometry of redox reaction. Addition of a moderate excess of Br^- guaranteed a complete conversion of bromate to Br_2.

Two certified reference materials (CRM), the dogfish muscle DORM-2 with a content of MeHg 4.47 ± 0.25 mg kg^{-1} (Institute for National Measurement Standard, National Research Council, Canada) and the human hair (CRM 13) with a content of MeHg 3.8 ± 0.4 mg kg^{-1} (National Institute for Environmental Studies, Ibaraki, Japan), were analysed for methylmercury, for speciation method validation.

Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

2.2. Chromatographic instrumentation

A narrowbore HPLC gradient pump (P4000, ThermoQuest) equipped with a mechanical degassing system (SC1000, ThermoQuest) and with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) and a 35 μL injection loop was used. A diode array detector (DAD, UV6000, ThermoQuest) equipped with a flow cell with a 5 cm path length was employed at the end of the column, just before and in series with the CVGAFS detection system. This detection system (DAD–CVGAFS) allowed the simultaneous acquisition of UV–vis absorbance and mercury-specific chromatograms.

2.3. Chromatographic conditions

Separations were carried out by a HyPurity C_{18} (Thermo Hypersil-Keystone, Thermo Finnigan Italia S.p.A, Milano, Italia) 250 mm × 4.6 mm RP column (silica particle size 5 μm). The pump flow was 1.0 mL/min. The elution conditions were different depending on the complexing agent employed:

(a) CYS–mercury complexes: 2.5 min isocratic elution in solvent A (5 mmol L^{-1} CYS) followed by a 2.5 min linear gradient from 100% A to 50% B (95% MeOH, 5% of 5 mmol L^{-1} CYS solution).
(b) PSH–mercury complexes: 10 min linear gradient from 100% solvent A (5 mmol L^{-1} PSH) to 50% solvent B (95% MeOH, 5% of 5 mmol L^{-1} PSH solution) followed by a 1 min linear gradient to 100% B.
(c) GSH–mercury complexes: 7 min linear gradient from 100% solvent A (10 mmol L^{-1} GSH) to 30% solvent B (95% MeOH, 5% of 10 mmol L^{-1} GSH solution) followed by a 1 min linear gradient to 50% B.

All the solutions were filtered by a 0.45 μm cellulose acetate filter (Millipore).

2.4. Sample preparation

Samples were prepared following essentially the Westöo procedure [33–36] and summarized in Fig. 1. Final aqueous extracts were diluted 1:1 in the initial mobile phase (5 mmol L^{-1} CYS in water) and injected.

2.5. Chemical vapour generation with AFS detection

A schematic diagram of continuous flow (CF) mercury chemical vapour generator modified for on-line digestion of organic mercury in a miniaturized Ar/H_2 flame is reported in Fig. 2 and a detailed description of the apparatus has been previously reported [25–27]. All reagent concentrations, reaction coil dimension, and flow rates were optimized and summarized in Table 1.

The mercury vapour coming from the gas–liquid separator was delivered into the atomizer, which was a miniature Ar/H_2 diffusion flame supported on a simple quartz tube (i.d. 4 mm). The employment of the flame was found to be necessary in order to remove the interferences in the atomization/detection step generated by volatile species arising from the complex matrix during the post-column chemical reactions.

A laboratory assembled non-dispersive atomic fluorescence (NDAF) detector [37] equipped with an EDL2 System Perkin Elmer and an EDL mercury lamp was employed. A description of the NDAF detector has been previously reported [24].

The output data from the lock-in amplifier were collected at 1 Hz with a personal computer (600 MHz Pentium®, Intel Corporation, Santa Clara, CA) equipped with a data acquisition card (Advantech PCL 718H, Taiwan, ROC) and its acquisition software (Advantech Genie 2.12). The UV data were averaged down to 5 points/s prior to saving; a 5 point boxcar averaging was applied for AF signal data. Raw data were saved and processed by Origin 6.1 Professional (OriginLab Corporation, Northampton, MA 01060, USA).

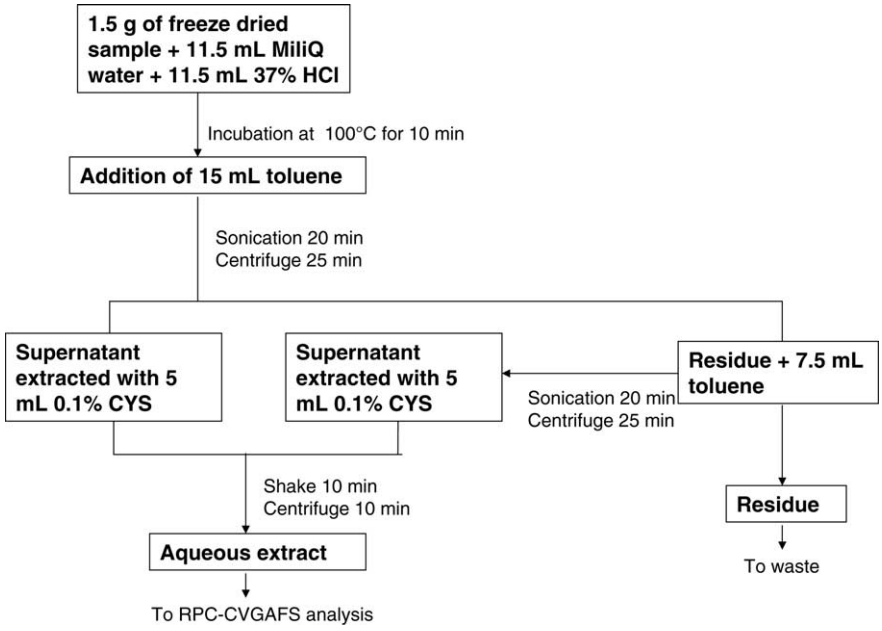

Fig. 1. Sample preparation scheme for methylmercury determination.

3. Results and discussion

3.1. Mercury speciation and figures of merit

Figs. 3–5 show the typical mercury-specific chromatograms obtained for a solution of inorganic mercury, MeHg, EtHg and PhHg complexed and eluted in CYS, PSH and GSH, respectively (3.5 ng of each species of mercury injected). In Fig. 3, the grey line shows, as example, the UV absorbance chromatograms at 254 nm of the CYS–mercury complexes, acquired simultaneously with mercury-specific traces. Selectivity of CVGAFS detection apparatus with respect to UV trace is evident. The UV chromatogram in Fig. 3 has been reported with the aim of showing the poor sensitivity (only EtHg and PhHg peaks are evident) and the absence of specificity of the UV detection (an unassigned major peak at 7.28 and other minor peaks are present). The baseline drift do to the solvent purity employed in the mobile phase is also

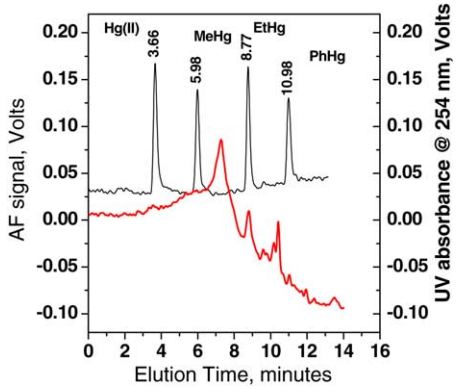

Fig. 3. LC–CVGAFS analysis of Hg(II)–$(CYS)_2$, MeHg–CYS, EtHg–CYS and PhHg–CYS complexes (3.5 ng injected). Chromatographic conditions: 2.5 min isocratic elution in solvent A (5 mmol L^{-1} CYS) followed by a 2.5 min linear gradient from 100% A to 50% B (95% MeOH, 5% of 5 mmol L^{-1} CYS solution) (flow rate = 1 mL/min).

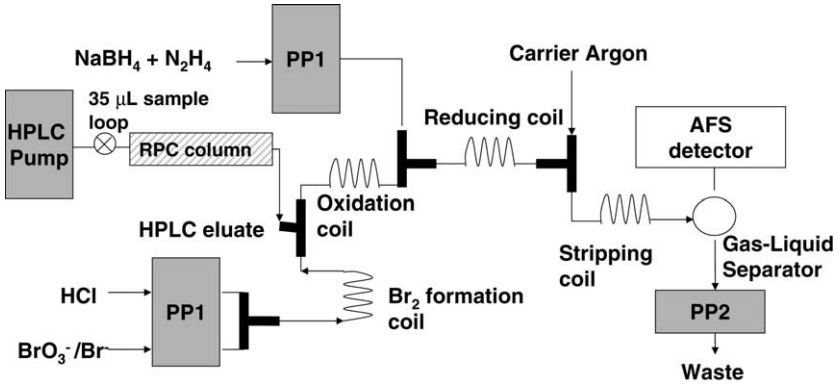

Fig. 2. Schematic diagram of the LC–CVGAFS system with post column on-line digestion at room temperature of organic mercury (PP, peristaltic pump).

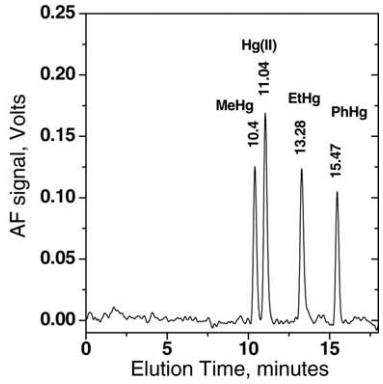

Fig. 4. LC–CVGAFS analysis of Hg(II)–$(PSH)_2$, MeHg–PSH, EtHg–PSH and PhHg–PSH complexes (3.5 ng injected). Chromatographic conditions: 10 min linear gradient from 100% solvent A (5 mmol L^{-1} PSH) to 50% solvent B (95% MeOH, 5% of 5 mmol L^{-1} PSH solution) followed by a 1 min linear gradient to 100% B (flow rate = 1 mL/min).

a drawback of UV detection, but it does not affect the AF signal. It is interesting to observe that no speciation or determination of mercury is pursuable at this concentration levels by UV detection. The effectiveness of the knitted coil system is also evident, being the bandwidth of EtHg peak, for example, in the CVGAFS trace comparable with that one of the EtHg UV absorbance peak.

Thiol–mercury species have a different hydrophobicity, as expected, depending of the thiol employed, requiring different elution conditions and giving different retention times (Figs. 3–5). Only in the case of PSH as complexing agent, the $Hg(PSH)_2$ complex was eluted after the MeHg(PSH) complex (Fig. 5). The separation of Hg(II), MeHg, EtHg and PhHg occurs in less than 12 min in the presence of CYS in the mobile phase (Fig. 3), between 10 and 16 min in the presence of GSH (Fig. 4), and between 7 and 15 min in the presence of PSH (Fig. 5). The coefficient of variation for the peak retention time is 0.5%.

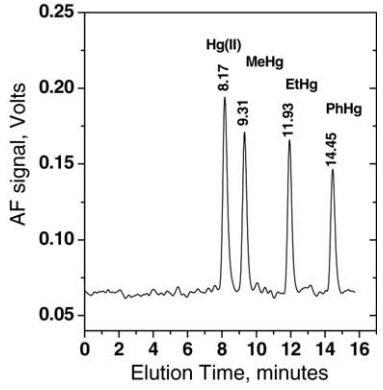

Fig. 5. LC–CVGAFS analysis of Hg(II)–$(GSH)_2$, MeHg–GSH, EtHg–GSH and PhHg–GSH complexes (3.5 ng injected). Chromatographic conditions: 7 min linear gradient from 100% solvent A (10 mmol L^{-1} GSH) to 30% solvent B (95% MeOH, 5% of 10 mmol L^{-1} GSH solution) followed by a 1 min linear gradient to 50% B (flow rate = 1 mL/min).

The effect of the concentration of thiols in the mobile phase on the LC chromatograms was studied in the range of 5–50 mmol L^{-1} (not shown for brevity). No effects were observed on the retention time in the investigated range, while the recovery of EtHg and PhHg decreased by 15–20% for thiol concentrations higher than 25 mmol L^{-1} because of the bromine partial consumption of the thiols. This effect was also observed as a loss of the typical colour of bromine in the oxidation coil. By adopting a 5 mmol L^{-1} concentration of thiols in the mobile phase, 0.045 mol L^{-1} bromine was the minimum concentration required in order to obtain the best oxidation yield. In our configuration, the decomposition time of organic mercury species was less than 2.5 s. In the case of GSH 10 mmol L^{-1} concentration, compatible with the employment of 0.045 mol L^{-1} bromine, was chosen. Indeed, at lower concentrations of GSH an additional peak at 12.95 min was present in the chromatogram due to the 1:1 complex Hg-GSH. It is not trivial to explain this result, taking in account that CYS is in large excess with respect to mercury. However, a binding competition between the complexing agent and the column stationary phase can be hypothesised.

Table 2 shows the fitting results of the calibration curves performed in the optimized conditions of CYS–, GSH– and PSH–mercury species obtained by plotting the areas of LC–CVGAFS chromatogram peaks as a function of mercury amount (ng) injected. Recoveries of MeHg, EtHg and PhHg have been calculated by the ratio of slope of calibration curves and that one of inorganic mercury, and they ranges between 79 and 85%, 80 and 85%, 63 and 76% for MeHg, EtHg and PhHg, respectively.

Repeatability was determined by performing five injections of a test mixture containing 3.5 ng of Hg(II), MeHg, EtHg and PhHg. The relative standard deviation of the peak areas was less than 2% for all the species. Calibration curves base on peak areas were linear over three decades between 0.1 and 100 ng injected. The detection limits (LODm) were

Table 2
Results of calibration experiments performed by the LC–CVGAFS system of Hg(II), MeHg, EtHg and PhHg complexed with CYS, PSH, GSH

Sample	Slope (V min pg^{-1})	R	N	Recovery (%)
CYS–mercury complexes				
Hg(II)	11 ± 0.3[a]	0.9984	5	–
MeHg	9.5 ± 0.2	0.9981	5	85 ± 3
EtHg	9.5 ± 0.2	0.9981	5	85 ± 3
PhHg	8.5 ± 0.1	0.9993	5	76 ± 2
PSH–mercury complexes				
Hg(II)	9.5 ± 0.2[a]	0.9999	5	–
MeHg	7.5 ± 0.1	0.9999	5	79 ± 2
EtHg	7.6 ± 0.1	0.9998	5	80 ± 1
PhHg	6.6 ± 0.1	0.9999	5	70 ± 1
GSH–mercury complexes				
Hg(II)	9.5 ± 0.2[a]	0.9999	5	–
MeHg	7.6 ± 0.1	0.9999	5	80 ± 2
EtHg	7.5 ± 0.1	0.9999	5	83 ± 2
PhHg	5.9 ± 0.1	0.9999	5	62 ± 1

[a] Standard deviation from linear fitting on three independent curves.

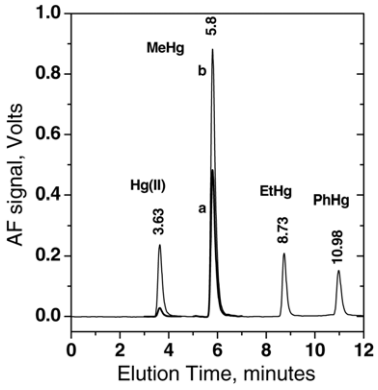

Fig. 6. LC–CVGAFS chromatogram of DORM-2. (a) sample 1:1 diluted in 5 mmol L^{-1} CYS (about 12 ng MeHg injected); (b) sample 1:1 diluted in 5 mmol L^{-1} CYS spiked with Hg(II), MeHg, EtHg, PhHg (10 ng each). Chromatographic conditions: see Fig. 3.

calculated from calibration curves for CYS–mercury complexes which have the best sensitivity factors (see Table 2), and based on the amount necessary to yield a net signal equal to 3σ. Standard deviation value was estimated as 1/5 of the integrated area of the baseline noise (1 min integration time). The absolute LODm were 16 pg for Hg(II), 18 pg for MeHg, 18 pg for EtHg and 20 pg for PhHg, comparable to or better than previously reported with similar LC–AFS techniques [5,7,9,10,30].

3.2. Accuracy of the method

In order to validate the accuracy of results obtained by the LC–CVGAFS hyphenated method and to test its applicability for practical analysis, the DORM-2 dogfish muscle and the human hair (CRM 13) were analysed for the content of MeHg in the form of CYS complex. CYS has been preferred as complexing agent in the analysis of CRM because of better recoveries and shorter retention times of the analyte (see Fig. 3 and Table 2). The samples were extracted as described in the Section 2 by incubation in a bath at 100 °C. A typical chromatogram of the DORM-2 dogfish sample is shown, as example, in Fig. 6, curve (a). Both in DORM-2 and CRM 13 sample, MeHg and traces of Hg(II) are present, despite the fact that procedure should not extract inorganic mercury, likely due to a partial decomposition of MeHg [38]. The concentrations of mercury compounds were determined by a standard addition method. In Fig. 6, the curve (b) shows the same sample spiked with 10 ng of each species of mercury. The amount of MeHg injected was approximately 12 and 10 ng for DORM-2 and CRM 13, respectively, and the recoveries of MeHg were 98 ± 6 and 97 ± 5%, respectively.

4. Conclusion

LC–CVGAFS apparatus coupled to on-line digestion with bromine at room temperature resulted a simple, low cost, fast, reliable tool for determination and speciation of mercury.

The detection limits obtained with the proposed LC–CVGAFS configuration (16 pg for Hg(II), 18 pg for MeHg, 18 pg for EtHg and 20 pg for PhHg) are lower or comparable with those obtained so far by CVGAFS detection coupled to liquid chromatography [5,7,9,10,30] and are also comparable with those obtained by CE–UV, CE–QICP–MS, CE–DFICP–MS [31,32].

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