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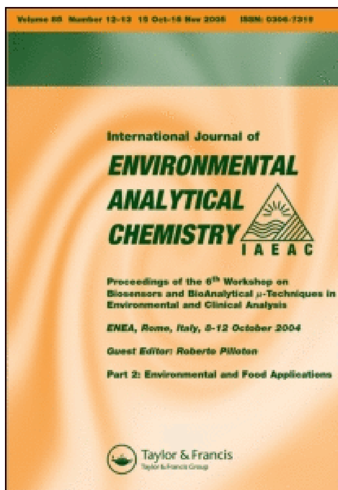

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MERCURY SPECIATION IN BIOLOGICAL SAMPLES

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An extraction method is proposed in order to evaluate mercury species in biological samples (e.g. fish, mushrooms). Sulphur-containing ligands have been considered and thiourea was chosen for the optimised extraction procedure according to the best recovery obtained (65.9%, 75.8%, 47.1% and 98.2% for CH_3Hg^+, $C_2H_5Hg^+$, $C_6H_5Hg^+$ and Hg^{2+} respectively) and the chromatographic performance. Thiourea solutions added to biological samples enabled extraction of organomercury species; the influence of reaction time and recovery yields were evaluated. The proposed procedure, coupled with an on-line preconcentration step, allows to reduce contamination problems and to obtain detection limits at ng/g levels. The method has been evaluate with reference materials (BCR).

KEY WORDS: Mercury speciation, organomercury, HPLC-CV-AAS, extraction procedure.

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INTRODUCTION

Mercury speciation is a difficult challenge in analytical chemistry, as its compounds usually occur at pg/g level in most samples; on the other hand, it is well-known that organomercury compounds show a biological and toxicological activity very different from inorganic mercury. The development of methods for the determination of species like methylmercury and ethylmercury at pg/g level is an important aim for environmental studies[1-3]. An interesting approach for speciation studies is hyphenation[4] and for mercury compounds, in particular, CV-AAS (Cold Vapour-Atomic Absorption Spectrometry)[5-8] is a good detection system for its selectivity and sensitivity. In our laboratory CV-AAS has been interfaced with a liquid chromatographic system and two separation methods were developed, based respectively on a reversed-phase mechanism[5] and an ion-exchange mechanism[6], for mercury species determination in aqueous samples.

Speciation in solid samples (e.g. biological samples) is even more difficult, because at present no analytical technique is known capable of allowing speciation in the solid state; an extraction procedure is required. Organic species have to be brought in solution without altering the molecular structure; organomercury compounds, however, are not very resistant. Treatment of the samples has to be performed very carefully, in order not to break carbon-mercury bonds. Acid digestion cannot be applied, since conversion to inorganic mercury would occur; thermal treatment or UV digestion would cause similar results. In the case of dialkylmercury compounds, conversion to monoalkylmercury is likely to occur.

Many extraction methods derive from the classical procedure by Westöö[9], involving conversion to halide derivative and the extraction of the organo-Hg halide with benzene, toluene or chloroform[10]. A ligand, such as dithizone[11] or diethyldithiocarbamic acid[12], was added to the organic extractant. To facilitate the breakage of S-Hg(R) bond, Cu^{2+} ions were used to mask sulphydryl groups[13,14], whereas urea[15] was added for its ability in denaturing proteins and exposing mercury-binding sulphydryl sites for cleavage (organomercury compounds are frequently associated with proteins, being bound to sulphydryl residues such as those of cysteine). Conversion to organomercury iodide with iodoacetic acid was performed by Decadt[16] and Lansens[17], followed by head-space injection into a gas chromatographic system. If the organic solvent is not suitable for the analytical technique employed, or if a clean-up procedure is necessary (organic matter excluded from the final extract), back-extraction with aqueous cysteine acetate[18,19] or sodium thiosulphate[14,20-22] has to be performed; then a new re-extraction step can be carried out in the same way as the first step. A different approach was developed by Holak *et al.*[23], in which organomercury compounds are extracted from the sample with a solid phase extraction procedure, using a diatomaceous earth column for retention and a thiosulphate solution for elution.

In the present work a new procedure has been developed, involving the use of thiourea as a reagent able to extract both inorganic and organic mercury compounds in the same extraction run, due to its sulphide group which has great affinity for Hg compounds. This procedure, coupled with HPLC separation and CV-AAS detection, allows speciation of several inorganic and organic mercury compounds.

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EXPERIMENTAL

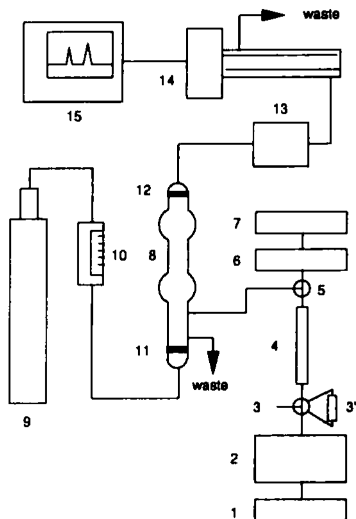
Instrumentation. The block diagram of the instrumentation utilised is reported in Figure 1. Two modifications were made in the system used in a previous work[5]: a Varian Model 9010 Solvent Delivery System was used instead of a Model LC 5000 instrument, and data were collected with an Axxiom chromatography Model 727 Data Station instead of chart recording. For centrifugation, a Beckman Spinco Ultracentrifuge Model L was used (with a batch rotor 1600).

Reagents and solutions. High purity water (HPW), obtained with a Milli-Q system (Millipore, Badford, MA USA), was used for the preparation of all solutions. All reagents employed were analytical grade and obtained from Merck, except for ethylmercury chloride (Alfa, Karlsruhe-Germany) and phenylmercury chloride (Aldrich, Steinheim-Germany).

The extracting solution was prepared dissolving 5.0 g of thiourea in 1.00 L of HPW; it was then filtered on 0.45 μm acetate cellulose filter.

A 1000 mg/L standard solution for AAS (Merck) was used for inorganic mercury. Stock standard solutions of methyl-, ethyl- and phenylmercury were prepared by dissolving respectively 1.25, 1.32 and 1.56 g of their chlorides in 1 L of CH_3CN, to yield a 1000 mg/L concentration (expressed as Hg). The working solutions were prepared daily by dilution with HPW of stock standard solutions. They were stored in the dark under refrigeration.

Ammonium pyrrolidine dithiocarbamate (APDC) $5.0 \cdot 10^{-2}$ M stock solution was prepared dissolving 0.41 g in 50.0 mL of a CH_3CN/H_2O mixture (50:50, v/v).



1 Eluent reservoir
2 Chromatographic module
3 Injection valve
3' Preconcentration microcolumn
4 Chromatographic column
5 T-mixer
6 Pump module
7 $NaBH_4$ reservoir
8 Flow cell
9 Carrier gas (N_2)
10 Flow meter
11,12 Glass frits
13 $Mg(ClO_4)_2$ trap
14 CV-AAS detector
15 Recorder

Figure 1 Block diagram of the instrumentation utilised.

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Chromatographic separation and detection. The separation was performed according to conditions as reported in a previous work[5] i.e. an RP-C18 (5 μm) separation column and a CH_3CN-H_2O (58:42, v/v) mobile phase containing $5.0 \cdot 10^{-4}$ M APDC. The eluent was buffered with ultrapure acetic acid and NaOH to pH 5.5. Flow rate was 1.5 ml/min. Eluate was continuously mixed with a solution of sodium tetrahydroborate (1.0 g/L adjusted to pH 11.5 with NaOH) to reduce the analytes to Hg(0) and to allow their detection by CV-AAS after stripping with N_2 in a glass flow-cell. Alkaline conditions in the cell should promote conversion of organic species to elemental mercury.

Dissolution of samples and total mercury determination. Samples were dissolved via acid digestion. 0.1 g of sample and 4.0 mL of HNO_3 (65%, w/v) were introduced in a Teflon bomb, which was sealed and put in a microwave oven (White Westinghouse, Model Wl 40) 1350 W, 2450 MHz. Two heating stages were then applied: the first for 3 min at 30% power, and the second 1 min at 40% power. After cooling, HPW was added to a final volume of 100 mL. CV-AAS has been used to evaluate total mercury concentration of the digested samples. The analytical procedure is the following: a well-defined volume of sample is introduced in the reaction cell (usually 25–100 mL) then 1 mL of 1% (w/v) $NaBH_4$ aqueous solution is added. The cell is closed and a nitrogen flow sweeps the reduced mercury, Hg(0), which is continuously collected on a gold trap. After 10 minutes the gold trap is heated, via Joule effect, Hg(0) vapour is released and carried into the detector (set at 253.7 nm) and its signal is registered by the computer.

Selenium determination. Selenium was determined by GF-AAS, using a Perkin-Elmer model 5000 spectrophotometer with an electrodeless discharge source lamp. The analytical wavelength was 196.0 nm.

Sulphur determination. Sulphur content was determined by ion chromatography, after oxidative digestion of the sample (see total mercury determination) in order to convert all S-containing compounds to SO_4^{2-}. Determinations were performed by a Model QIC ion chromatograph (Dionex, Sunnivale, CA, USA) and conductometric detection. A Dionex AS-4 anion-exchange analytical column was used for the separation; the eluent was 2 mM Na_2CO_3 + 1 mM $NaHCO_3$ aqueous solution at 1.0 ml/min flow rate.

Sample extraction. A Tuna fish certified sample (BCR T22) was directly processed, while mushrooms were lyophilised and homogenised before the extraction. The procedure, after optimisation, was performed in the following way: a proper amount of sample (4 ÷ 5 g) was weighed and poured in 450 mL of 0.5% (w/v) thiourea solution, then left overnight under stirring. Solid phase was removed by centrifugation (20' at 13000 rpm) and the solution was filtered on 0.45 μm cellulose acetate filters. A 1.0 mL $5.0 \cdot 10^{-2}$ M APDC solution was added to a 100.0 mL sample, which was then loaded on the preconcentration microcolumn (RP-C18) at 2 mL/min flow-rate. Finally, chromatographic separation took place (see below). Figure 2 shows the flow diagram for all manipulations occurring to the sample.

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RESULTS AND DISCUSSION

Two kinds of samples were chosen, representing distinct biological matrices in which the biochemical pathways are very different; animal (Tuna fish) and vegetable without chlorophyllian function (*Boletus edulis,* a mushroom). Fish and mushrooms have a marked tendency to preconcentrate mercury compounds[24,25]. Tuna fish was a BCR certified sample, labelled T22 (only total mercury concentration, equivalent to 3.52 ± 0.15 μg/g, was certified for this sample); Boletus edulis was obtained by purchasing a commercial product (dried mushrooms). To characterise samples both total mercury and different species concentration were evaluated.

Samples extraction. Experiments were performed to evaluate the extraction efficiency of thiourea on biological samples. Thiourea was chosen because, among the ligands with sulphide groups, it has high solubility in water and its presence does not affect the chromatographic performance.

Different concentrations were tested for a defined quantity of sample and at fixed volume of reagent. A 0.5% (w/v) thiourea solution resulted to be the best choice, according to its solubility, and gave the best results for extraction yield and reproducibility.

The influence of extraction time on recovery yield of analytes was evaluated by carrying out extraction experiments on the two samples for different lengths of time. It has been found that the yields of extraction obtained for inorganic and methylmercury do not change significatively by increasing the extraction time from 1 hour to overnight

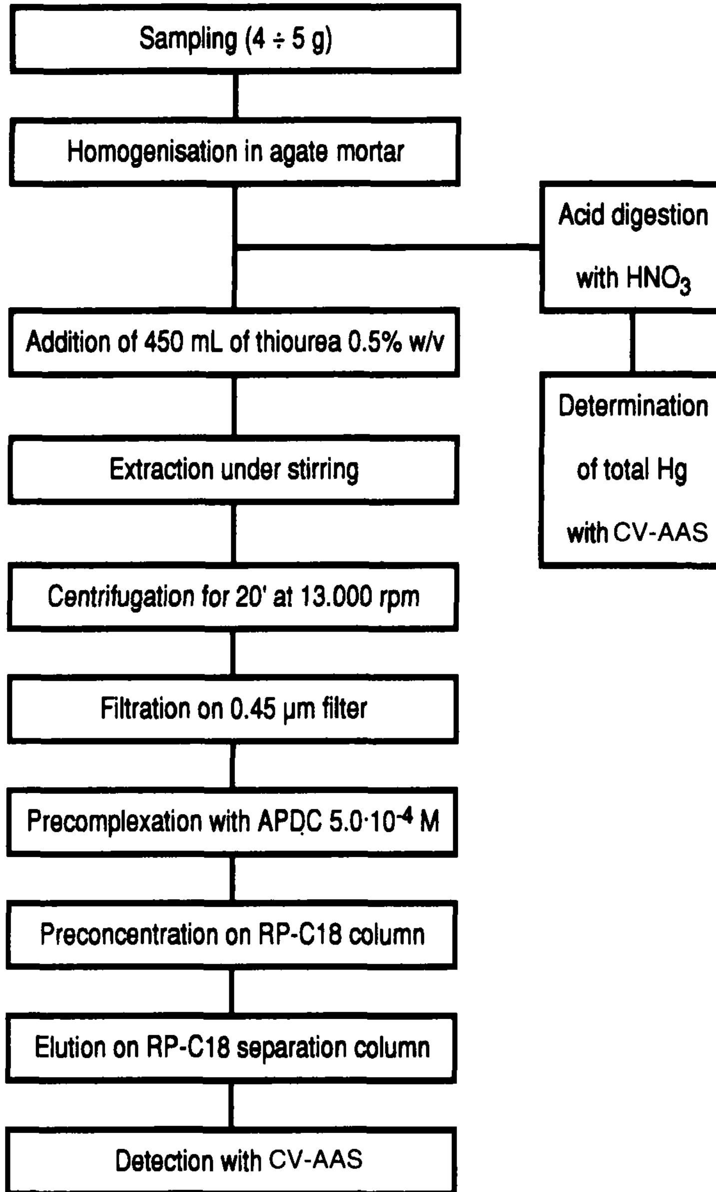

Figure 2 Flow diagram of sample manipulations.

period, whereas for ethylmercury no extraction occurred within 1 hour but the maximum yield was after 1 night, as it is shown in Figure 3. Overnight extractions were thereafter performed, also considering that, within such a period, stability of organomercurials in thiourea solution is guaranteed, as is demonstrated in Figure 4.

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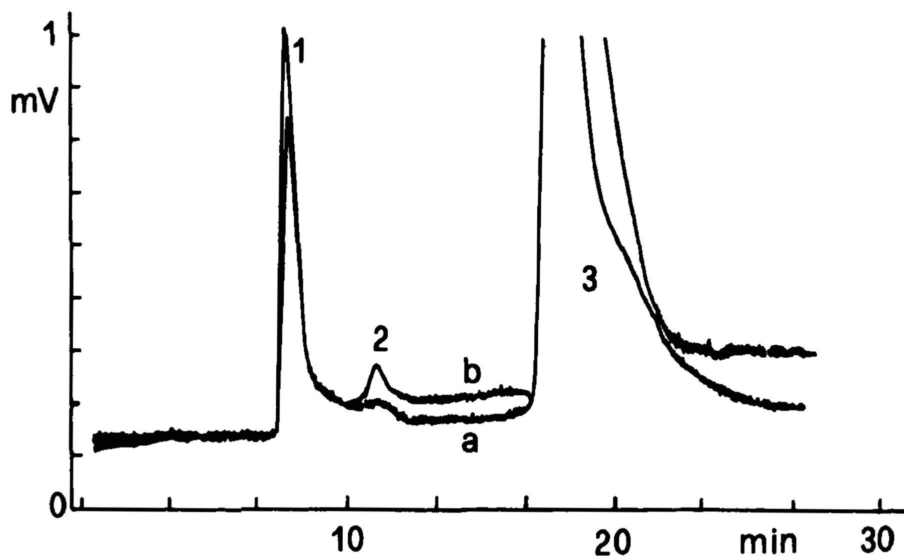

Figure 3 Comparison between one hour (a) and overnight (b) extraction on Tuna fish sample. Peaks: 1=CH_3Hg^+, 2=$C_2H_5Hg^+$, 3=Hg^{2+}; full scale 1.0 mV=0.001 a.u.. Chromatographic conditions: mobile phase, acetonitrile-water (58:42, v/v) containing 20.0 mM acetic acid, 0.5 mM APDC and sodium hydroxide up to pH 5.5; flow-rate 1.5 ml/min.

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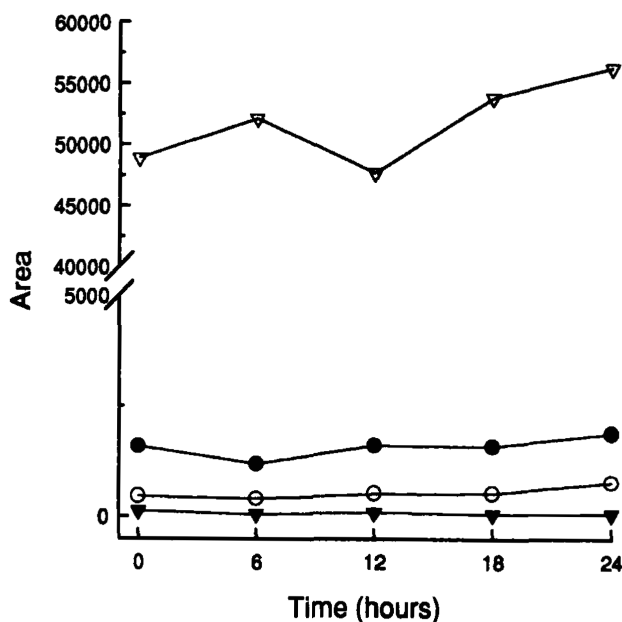

Figure 4 Contaçt time influence on organomercury compounds in a solution containing 0.5% thiourea. Chromatographic conditions: mobile phase, acetonitrile-water (58:42, v/v) containing 20.0 mM acetic acid, 0.5 mM APDC and sodium hydroxide up to pH 5.5; flow-rate 1.5 ml/min. 100 ml sample volume; ○ = CH_3Hg^+ (100 ng as Hg); ● =$C_2H_5Hg^+$ (100 ng as Hg); ▼ = $C_6H_5Hg^+$ (300 ng as Hg); ∇ = Hg^{2+} (300 ng as Hg).

Total mercury determination. As above mentioned, total mercury concentration was evaluated in order to compare the distribution of different species. Since the total concentration of mercury in the analysed samples was high, determinations were carried out adding 100–200 µL of sample to 25.0 mL of HPW (previously purified by nitrogen bubbling). Moreover we evaluated that a volume of reducing agent higher than usual (5 mL) was needed to yield more accurate determinations; this was probably due to the amount of oxidised matter present in the sample matrix after digestion with HNO_3. Quantitative determinations have been performed using standard additions of Hg^{2+} in the samples for at least three replicates. Results are reported in Table1.

Preconcentration of mercury compounds from thiourea solution. Since the total content of mercury was sufficient for its direct determination but some species, after their separation, could result below the detection limits, a preconcentration procedure was developed. Before analysing real samples, experiments were performed on standard solutions in order to evaluate preconcentration recovery as a function of sample volumes loaded (keeping constant the absolute amount of analyte) and reaction time influence on organomercurials stability. All trials were carried out in a 0.5% (w/v) thiourea solution and a RP-C18 microcolumn (4 x 4 mm i.d.) was used. The results are shown in Figure 5. In order to optimise the recovery of analytes, APDC was added to the sample solutions before loading into the column, as precomplexation reagent. As expected, better results were achieved due to the higher lipophilicity of the complex so formed, resulting in a higher interaction with the reverse-phase preconcentration column (see Table 2, also showing the better performances obtained with thiourea rather than with cysteine as extracting reagent).

Table 3 shows preconcentration yields for the mercury compounds considered. Figure 6 shows calibration graphs for on-line preconcentration; linearity is obtained up to 5 µg/L for all species considered. A great difference of sensitivities exists between inorganic

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Table 1 Total mercury concentrations in some biological samples.

Sample	*Total Hg (µg/g)*	*Certified values*
Tuna fish (BCR T22)	3.77 ± 0.15	3.52 ± 0.15
Boletus edulis	5.13 ± 0.50	–[a]

[a] uncertified sample

Table 2 Peak height signals for preconcentration of Hg^{2+} and CH_3Hg^+ standard solutions in different matrices (5.0 µg/L, 100.0 mL) on RP-C18 microcolumn. Signals are normalised assuming that peaks obtained from water were equivalent to 100 (arbitrary units).

Compound	*Water*	*Thiourea solution*	*Thiourea + APDC*[b]	*Cysteine*	*Cysteine + APDC*[b]
Hg^{2+}	100	313	801	12	487
CH_3Hg^+	100	56	139	–[a]	22

[a] no retention
[b] APDC concentration: $5.0 \cdot 10^{-4}$ M

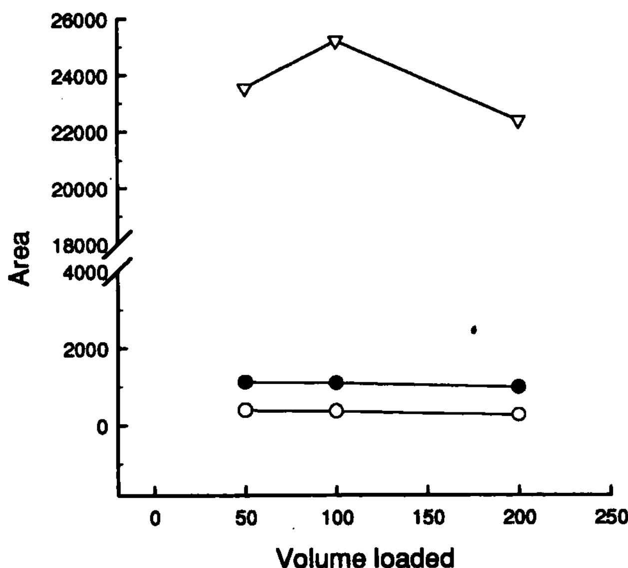

Figure 5 Variation of peak area signals with different volumes loaded. Chromatographic conditions: mobile phase, acetonitrile-water (58:42, v/v) containing 20.0 mM acetic acid, 0.5 mM APDC and sodium hydroxide up to pH 5.5; flow-rate 1.5 ml/min. Absolute analyte amount was 100 ng as Hg. O = CH_3Hg^+; ● = $C_2H_5Hg^+$; ∇ = Hg^{2+}.

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Table 3 Preconcentration yields of mercury compounds. They were evaluated comparing peak area signals obtained loading 100.0 mL of a 1.0 μg/L solution on a preconcentration column, with signals obtained from direct injection of 100.0 μL of a 1.0 mg/L solution (absolute amount of analyte is 100 ng – as Hg – in both cases).

Compound	*Yield (%)*
CH_3Hg^+	65.9
$C_2H_5Hg^+$	75.8
$C_6H_5Hg^+$	47.1
Hg^{2+}	98.2

mercury and organomercurials (see Figures 6a and 6b); this could be explained by assuming that the latter are not quantitatively reduced to Hg(0) by $NaBH_4$. Evidence is reported in literature[26,27] about possible formation of organic mercury hydrides (e.g. CH_3HgH), undetectable by CV-AAS, occurring mainly after $NaBH_4$ reaction at weakly acid pH values.

Quantitative determination on real samples. Determinations of the mercury species detected were carried out by the standard additions method:

– To determine the concentration of mercury compounds extracted in thiourea solution, standard additions were made before preconcentration;

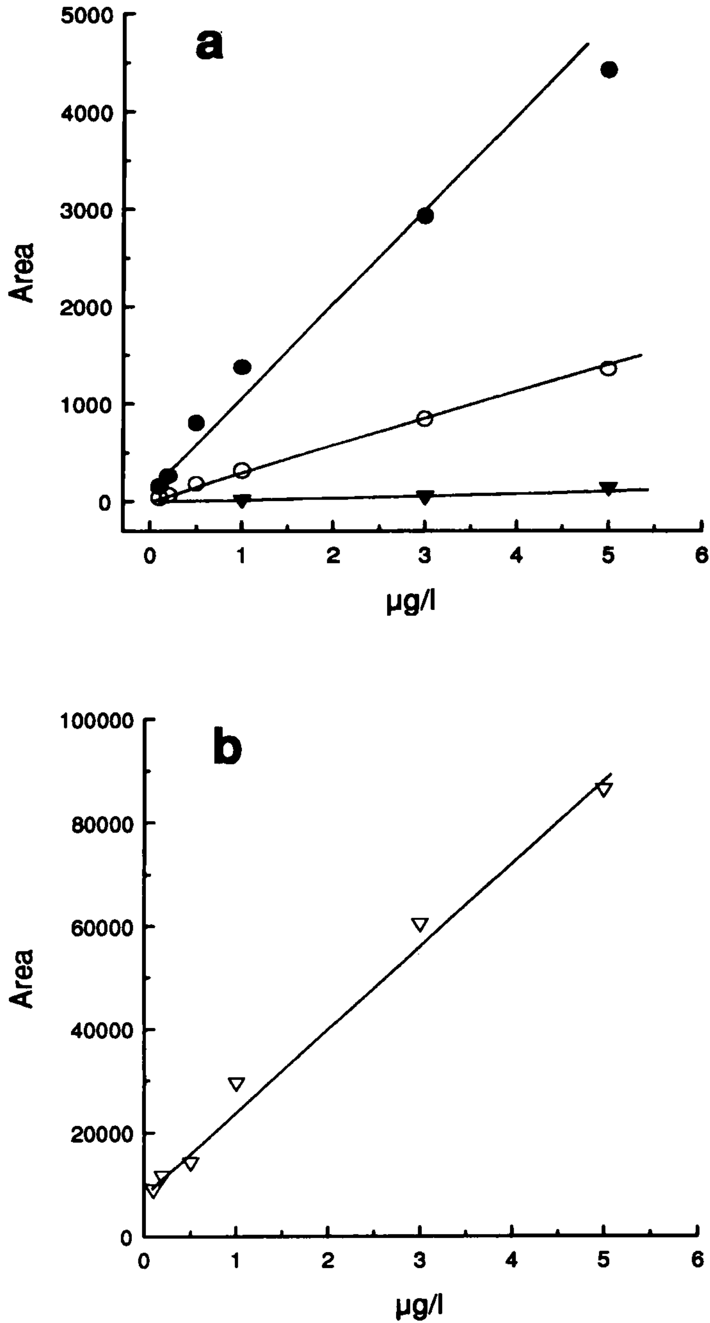

Figure 6 Calibration graphs for on-line preconcentration procedure. Chromatographic conditions: mobile phase, acetonitrile-water (58:42, v/v) containing 20.0 mM acetic acid, 0.5 mM APDC and sodium hydroxide up to pH 5.5; flow-rate 1.5 ml/min. 100 ml sample volume. (a) ○ = CH_3Hg^+; ● = $C_2H_5Hg^+$; ▼ = $C_6H_5Hg^+$; (b) ▽ = Hg^{2+}.

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– To evaluate the extraction recovery, standard additions were made before adding thiourea solution to solid samples.

The concentration of mercury species in the solid samples has been consequently calculated taking into account extraction and preconcentration yields. Figure 7 shows the chromatograms of (a) sample of Tuna fish as such and (b) the same sample spiked before preconcentration; (c) sample of *Boletus edulis*. The concentrations of the species detected in the two samples are reported with extraction yields in the tables 4 and 5. The differences in the recovery could be tissue-dependent, as one matrix can bind each mercury compound more strongly than another, and possible side-reactions depend on the matrix composition too.

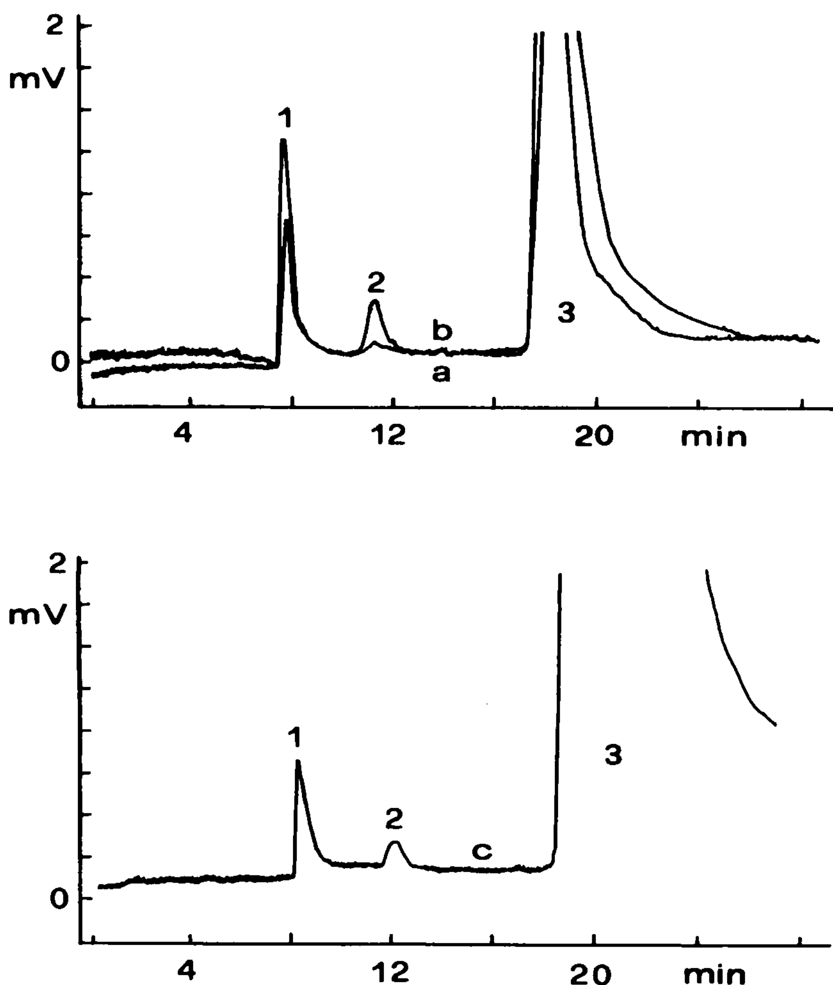

Figure 7 Chromatograms of real samples: Tuna fish (a) as such and (b) spiked with CH_3Hg^+ (1.5 μg/g), $C_2H_5Hg^+$ (1.0 μg/g), Hg^{2+} (1.0 μg/g); (c) *Boletus edulis* as such. Peaks: 1 = CH_3Hg^+; 2 = $C_2H_5Hg^+$; 3 = Hg^{2+}; full scale 2.0 mV = 0.002 a.u.. Chromatographic conditions: mobile phase, acetonitrile-water (58:42, v/v) containing 20.0 mM acetic acid, 0.5 mM APDC and sodium hydroxide up to pH 5.5; flow-rate 1.5 ml/min.

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Table 4 Concentration values of mercury species and their extraction yield in Tuna fish (BCR T22).

Species	*μg/g ± r.s.d. (%) (n=3)*	*Extraction yield*[a]
CH_3Hg^+	2.26 ± 7.6	30.3%
$C_2H_5Hg^+$	0.035 ± 12.5	31.2%
Hg^{2+}	0.046 ± 5.1	51.1%
Sum	2.34	–
Total Hg^{2+}	3.77 ± 0.15	–
Certified value	3.52 ± 0.15	–

[a] calculated through standard addition to the sample before extraction.

Table 5 Concentration values of mercury species and their extraction yield in *Boletus edulis*.

Species	*μg/g ± r.s.d. (%) (n=3)*	*Extraction yield*[a]
CH_3Hg^+	0.310 ± 3.1	36.4%
$C_2H_5Hg^+$	0.053 ± 17.5	38.2%
Hg^{2+}	1.40 ± 6.2	87.6%
Sum	1.76	–
Total Hg^{2+}	5.13 ± 0.50	–

[a] calculated through standard addition to the sample before extraction.

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Several certified concentration values of methylmercury and total mercury in biological samples are reported in literature[21,28]; under no circumstance the presence of ethylmercury is reported. To insure that the presence of this compound is not due to residual contamination, it should be considered that blank solutions run with the same procedure resulted to be absolutely free of alkylmercury impurities. Moreover, it must be remembered that every RP-C18 cartridge for sample preconcentration was used just once, to minimise possible contaminations from one sample to the following.

As shown before (Tables 4 and 5), recovery from the whole procedure is lower than expected, since concentration values for total mercury are higher than the sum of single species. This could be due to different phenomena:

– The presence of mercury compounds which could be either not extracted or not detected by our method (e.g. dimethyl- or diethylmercury), probably because the reduction reaction with $NaBH_4$ has a low efficiency.

– Strong interactions Hg-Se and Hg-S, that is, mercury existing as selenide and/or sulphide; these compounds could not be cleavable with the extraction procedure employed in the present work. Similar results have recently been reported[29]. A pharmacological study[30] tended to prove that HgS and HgSe are the stable inorganic mercury compounds in tissues; this assumption was suggested by experiments in which rats fed with mercury sulphide and selenide showed less toxicity symptoms than in the case of other Hg^{2+} salts.

The Se and S content of samples has been evaluated as above described (see Table 6). The results show an opposite behaviour in respect to the recovery yields obtained for mercury species. This fact agrees with the hypothesis made.

Analytical parameters of the method. Precision over the whole procedure was around 17% (for ethylmercury) and 7% or better (for methylmercury and inorganic mercury), as calculated by three different extraction-preconcentration-separation cycles. Detection limits on solid samples may vary in relation to the matrix considered (see above); anyway, values reported in Table 7 can be held as a reference. They were calculated according to the results obtained from the samples analysed, following the procedure described in the Sample extraction paragraph.

In conclusion the developed procedure allows the simultaneous determination of different mercury species in various biological matrices even at very low concentrations (ng/g level). Therefore it is possible to complete a selective determination of different organomercury species on solid samples.

Table 6 Concentration values for S and Se in the biological samples considered.

Sample	*So_4^{2-} (mg/g)*	*S (mg/g)*	*Se (μg/g)*
Tuna fish	4.60 ± 0.6	1.53	9.50 ± 0.2
Boletus edulis	9.81 + 0.8	3.27	37.1 = 1.2

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Table 7 Detection limits, calculated from three times the standard deviation of background signal (extraction and preconcentration yields are also accounted for).

Compound	*D. L. (ng/g)*
CH_3Hg^+	50
$C_2H_5Hg^+$	20
Hg^{2+}	1

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