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On line solid phase extraction-HPLC-ICP-MS system for Mercury and methylmercury preconcentration using functionalised carbon nanotubes for their determination in dietary supplements

Agustín Londonio[ab], Paul Emir Hasuoka[c], Pablo Pacheco[cd], Raúl Andrés Gil[*cd] and Patricia Smichowski[*ad]

[a] *Comisión Nacional de Energía Atómica, Gerencia Química, Av. Gral Paz 1499, B1650KNA-San Martín, Buenos Aires, Argentina.*

[b] *Instituto de Investigación e Ingeniería Ambiental (3iA), Universidad de San Martín, Martín de Irigoyen 3100, 1650-San Martín, Buenos Aires, Argentina.*

[c] *Instituto de Química de San Luis, Centro Científico Tecnológico San Luis, Ejército de los Andes 950, Bloque 3, PB-San Luis, 5700, Argentina.*

[d] Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Godoy Cruz 2290, C1425FQB, Buenos Aires, Argentina

Corresponding authors: P. Smichowski. E–mail: smichows@cnea.gov.ar; Fax: +54 11 6772 7886; Tel: +54 11 6772 7873 and R. A. Gil. E-mail: ragil@unsl.edu.ar; Fax: +54 11 6772 7886; Tel: +54 11 6772 7873

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A hyphenated system was developed for Hg(II) and methylmercury (MeHg) preconcentration and speciation analysis by the on-line coupling of solid phase extraction (SPE), high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS). Both analytes, Hg(II) and MeHg, were preconcentraed on a microcolumn filled with multiwalled carbon nanotubes (MWCNTs) functionalised with poly-*L*-methionine (polymet-MWCNTs). During the method development the sorbent material was carefully studied and the solid phase extraction conditions were optimised. An enrichment factors of 190 for Hg(II) and MeHg was obtained when 20 mL of sample were passed through the microcolumn. For the chromatographic separation, a mobile phase composed by a ternary mixture of 0.5% formic acid + 0.2% 2-mercaptoethanol + 20% methanol was used. Separation of both mercurial species was accomplished in ~ 10 min on a 250-mm C18 column. The detection limits of the SPE-HPLC-ICP-MS method were 15 ng L^{-1} for Hg(II) and 17 ng L^{-1} for MeHg. The relative standard deviations of peak area for 5 ng L^{-1} of each Hg species were below 5%. Recoveries of Hg(II) and MeHg were never less than 93%. For checking accuracy, BCR 643-tuna fish and TORT-3 Lobster hepatopancreas certificate reference materials were analysed. Mercury species were determined in spiked fish oil-based dietary supplements and Antarctic water.

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1. Introduction

Mercury is an element that raises concern from both the environmental and human health point of view. The high toxicity of Hg and its species is well known through the Minamata (Japan-1956) and Iraq diseases (1972) caused by the consumption of contaminated fish and grains seeds treated with organomercury fungicides, respectively.[1,2] Mercury is recognised as a global pollutant because it is distributed in many environmental compartments where it is found at very low concentrations.[3] As an example, typical total Hg concentrations (different dissolved Hg species) ranging from 0.03 to 90 ng L^{-1} in uncontaminated natural waters were reported in a comprehensive review by Leopold et al.[1] The chemical form of Hg controls its bioavailability, transport, persistence and impact on biota and humans.

Mercury emissions may be from anthropogenic or natural origin and are mainly in the form of elemental Hg (Hg^0), representing > 99% of the total Hg in the atmosphere.[1] When Hg is discharged into the environment as elemental Hg it can form methylmercury.[4] Due to their lipophilic nature, organic Hg compounds are more toxic than inorganic Hg species. Methylmercury (MeHg) can then bioaccumulate in the ecosystem to a level that can cause serious health hazards. It is known that Hg species can easily permeate biological membranes and for their affinity with sulfhydryl groups that are present in proteins and enzymes. Bioaccumulation occurs in different and specific organs such as brain, kidney, liver and central nervous system.[5]

The determination of Hg in various matrices, including environmental and food samples, requires the use of highly sensitive detection techniques. This situation is even more complicated when the selective determination of Hg species is required.

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Among the reported methods for the selective determination of Hg species in different matrices the most employed approaches have been the direct coupling of HPLC to ICP-MS. This combines the high resolving power of the former with the advantages of element specificity, wide dynamic range and excellent detection power of ICP-MS. In spite of these recognised advantages, the sensitivity that can be reached is not enough to determine Hg species at the levels normally found in natural waters and/or unpolluted environments. To improve the capability of the method it is advantageous to add a preconcentration step prior to the chromatographic separation. Gao et al[6] reported the on-line preconcentration and in situ photochemical vapor generation in coiled reactor for Hg(II) and MeHg determination by atomic fluorescence spectrometry. A detection limit of 0.004 μg L^{-1} for Hg(II) was achieved based on 40 s sample loading.

The on-line determination of inorganic Hg and MeHg in waters and fish tissues using a microcolumn filled with polyaniline (PANI) and flow injection-chemical vapour generation-inductively coupled plasma mass spectrometry (FI-CVG-ICP-MS) was successfully applied to the direct determination of iHg and MeHg in various water samples.[7] Preliminary studies showed that inorganic and MeHg species could be separated on the column in two different speciation approaches depending on pH.

Wang et al. developed an on-line high performance liquid chromatography-cold vapour generation-atomic fluorescence spectrometry method for the selective determination of Hg(II), MeHg and ethylmercury (EMC) using cysteine and ammonium acetate as mobile phase in the chromatographic separation, avoiding the use of an oxidation system.[8] The detection limit for the three species, based on three folds of baseline noise and 100 mL injection, were 0.05, 0.07, and 0.1 mg L^{1} , respectively.

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Other authors employed a thiol-material for preconcentration followed by ion chromatography and CV-AFS.[9] The addition of 10–60 mM thiourea (TU) quantitatively releases MeHg from water samples by forming a more labile complex (MeHgTU+) that quantitatively exchanges MeHg with thiol-functionalized resins at pH ~3.5 during column loading. A limit of detection of 0.007 ng L^{-1} (for MeHg) was reported.

In another approach, Brombach et al.[10] determined methylmercury in water samples at the pg L^{-1} level using an online preconcentration liquid chromatography cold vapour-atomic fluorescence spectrometry system. A column ccontaining sulfur based ligands anchored to a silica backbone was used in the preconcentration step. A detection limit as low as 40 pg L^{-1} MeHg (as Hg) was reached when 200 mL of sample were passed through the system.

Taking advantages of the well know properties of nanomaterials such as large surface area and excellent mechanical strength, Deng et al.[11] described the use of MWCNTs in an on-line solid phase dispersion-HPLC-ICP-MS system for the determination of Hg species in fish samples. Limits of detection of 9.9 ng g^{-1} and 8.4 ng g^{-1} were obtained for Hg(II) and MeHg, respectively. According to our experience, MWCNTs are very effective substrates for metals and metalloids preconcentration.[12-14]

The objective of this study was to investigate the potential of functionalised MWCNTs, specifically a sulfur-based sorption nano material, as an efficient and selective sorbent in an on-line SPE system coupled to HPLC-ICP-MS for the speciation analysis of Hg in a matrix much less studied such as fish oil-based dietary supplements. Antarctic waters were also analysed. The proposed solid phase extraction method is easy to operate and presents several advantages in comparison with other methods that include: extraction- clean-up-separation-species quantification into one step without Hg derivatisation.

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2. Experimental

Safety considerations

All species of Hg and 2-mercaptoethanol are toxic, thus in both cases contact with skin and eyes should be avoided. During the development of this study a well-ventilated fume hood was used for reagents and samples handling.

2.1. Instrumentation

The schematic instrumental set-up (preconcentration, separation and detection) of the hyphenated system used in this study for Hg speciation analysis is depicted in Fig. 1.

The preconcentration unit consisted of a six-port valve (Rheodyne 5020, Rheodyne, LP, Rohnert Park, CA, USA) (namely V1) where the sample loop was substituted with the preconcentration microcolumn filled with a mixture of MWCNTs functionalised with a mixture of poly-*L*-methionine (polymet-MWCNTs) and Epolene®. A peristaltic 6-channels pump, Minipuls 3 from Gilson (Villiers–Le–Bel, France) was used to deliver the sample (and the eluent) onto the preconcentration microcolumn at a constant flow rate. Tygon type pump tubing with internal diameters of 0.95 mm (Ismatec, Cole Parmer, Vernon Hills, IL, USA) were employed to propel the sample and the eluent.

The outlet of the preconcentration unit was directly connected to a syringe through a small hole made in the barrel. When V1 is switched, the eluate (1.0 mL) containing Hg species was delivered to the syringe and stored in the barrel during no more than ~ 1 min to avoid overfilling. Then, the plunger was manually pressed to inject the eluate retained in the syringe into the chromatographic unit through V2.

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The chromatographic unit for Hg speciation consisted of a reversed-phase C18 column Phenomenex SphereClone (Torrance, CA, USA) ODS (5µm, 4.6 mm i.d.; 250 mm long), a high pressure pump (HPLC pump) with a 0.01–5 mL min^{-1} flow rate range (PerkinElmer Series 200 LC Pump) and a six-port manual injector (namely V2) with a 100 µL sample PEEK loop (Rheodyne model 7725i). A HPLC mobile phase composed by 0.5% formic acid + 0.2% of 2-mercaptoethanol (ME) + 20% methanol at a flow rate of 0.8 mL min^{-1} was used.

PEEK tubing (0.030 in i.d.) was used to connect the chromatographic reversed-phase C18 column directly to the Type C glass concentric nebuliser (Meinhard Glass Products, USA) of the ICP-MS (PerkinElmer inductively coupled Ar plasma mass spectrometer model NexIon 300X), equipped with a Peltier cooler for ICP-MS cyclonic Spray chamber (PC^3 Peltier Cooler, PerkinElmer) cooled at 3 ºC to remove the coarsest aerosol droplets. Data was analysed by Chromera Speciation Software V4.1 from PerkinElmer.

The ICP-MS instrument was daily optimised using a multi-element "tune" standard solution containing 1.0 µg L^{-1} of ^{9}Be, ^{140}Ce, ^{28}Mg, ^{115}In and ^{238}U in 1% HNO_3. The optimised ICP-MS and HPLC operating conditions are shown in Table 1. During the study, the room temperature of the SPE–HPLC–ICP–MS system was kept at 20 °C. Calibration standards of Hg(II) and MeHg of 0.01-10 µg L^{-1} were prepared and peak area was used for quantification.

A MLS-1200 (Millestone-FKW, Sorisole, Bergamo, Italy) microwave apparatus equipped with ten Teflon-PFA vessels was used to digest samples.

2.2 Reagents

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All reagents were of analytical grade and the presence of Hg was not detected within the working range. A 1000 mg L^{-1} standard solution of Hg(II) was obtained from Chem-Lab (Chem-Lab NV, Zedelgem, Belgium). A 1000 mg L^{-1} standard solution of methylmercury chloride (CH_3HgCl) in water was obtained from Fluka (Germany). Analytical calibration standards of Hg species were prepared daily over the range 0.01–10.0 µg L^{-1} by step-wise dilutions of the stock solution. Additional chemicals for the speciation studies were HPLC grade: methanol and 2-mercaptoethanol (Sigma–Aldrich, USA). Formic acid was purchased from Sigma–Aldrich (USA).

Multiwalled carbon nanotubes (MWCNTs) were purchased from Sun Nanotech Co. Ltd., Jiangxi, China. All chemicals (Merck) used for MWCNTs functionalisation (HNO_3, H_2SO_4, $SOCl_2$, DMF, THF, poly-*L*-methionine) were of analytical reagent grade and were used without further purification.

Argon 99.99998% (minimum purity) from Indura (Buenos Aires, Argentina) was used for ICP-MS. Deionised distilled water (DDW) was produced by an arium® Water Purification System (Sartorius, Goettingen, Germany).

All glassware and plastic bottles used were cleaned by rinsing with DDW, soaking with a 10% (v/v) nitric acid solution for 24 h and then rinsing several times with DDW. All samples and standards were stored in polyethylene bottles (50 mL) or Falcon® tubes (Becton Dickison, Lincoln Park, NJ, USA).

2.3 Microcolumn substrate and immobilisation procedure

Functionalised MWCNTs were used as sorbent for their immense surface area, low cost and easy to get them. They are useful materials in preconcentration studies even when very small amounts are used. The functionalisation process is simple and enhances selectivity. The following steps were followed to obtain the sorbent material:

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Oxidation of MWCNTs was carried out in a mixture of H_2SO_4:HNO_3 (3:1). Commercial MWCNTs were soaked for 30 min in an ultrasound bath and then placed in a water bath at 55 ºC for 4 h. The resulting oxidised MWCNTs were dialysed with DDW until complete elimination of the residual acid that was checked by measuring the pH. Poly-*L*–methionine functionalised MWCNTs (polymet–MWCNTs) were obtained by the chemical attachment of poly-*L*–methionine using the following procedure: the oxidised MWCNTs were dispersed in thionylchloride ($SOCl_2$) with 2 mL of dimethylformamide (DMF) and the mixture was left to react while stirring at 70 ºC for 24 h. The resultant solid material (COCl–MWCNTs) was centrifuged and then washed with tetrahydrofuran (THF). Poly L–methionine and COCl–MWCNTs were mixed with THF at 45 ºC for 4 days. The resultant material, polymet–MWCNTs was resuspended with ethanol, filtered and dried. [12]

The sorbent resulted stable over a wide range of pH and the column packed with polymet–MWCNTs had a lifetime of (at least) 575 cycles of mercury retention/elution without demand for generation or modification of the initial conditions.

2.4 Preconcentration procedure

A peristaltic pump was used to load (*load* position) samples/standards (adjusted at pH 9) and the eluent onto the preconcentration microcolumn at a constant flow rate of 1.7 mL min^{-1} (Fig. 1-a). Once the sample was loaded onto the preconcentration microcolumn filled with polymet-MWCNTs, the six-port valve was manually changed from the *load* to the *inject* position and the eluent was delivered through the preconcentration microcolumn for Hg species elution. This eluate was delivered into a 1 mL syringe entering through a small hole made in the barrel (Fig. 1-b). The plunger was manually pressed and the preconcentrated sample was injected onto the chromatographic unit. After the injection, the valve V1 was switched back to allow the

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next sample to be loaded. About 6 min were necessary to complete a cycle (loading, elution and analysis) for 5 mL of sample volume.

Calibration standards were prepared in a range of 0.05–10 μg L^{-1} (blank and standards of 0.05, 0.1, 0.5, 1.0, 5.0 and 10 μg L^{-1}) for both Hg species, with a preconcentration volume of 5 mL. Peak area was used for quantitative evaluation of the data.

2.5 Sample preparation

Rigorous cleaning procedures were used for all devices, including sampling equipment, and laboratory ware in contact with the samples, especially for the speciation analysis of Hg at trace levels in waters. All devices and containers used were acid-washed and rinsed several times with deionised water. Water sample was analysed as soon as possible after collection to avoid contamination, especially by inorganic Hg.

Water samples: Two water samples collected in Antarctica were analysed following the developed procedure. Both samples were collected at the Carlini Station (ex Jubany), 25 de Mayo Island (62º14'18''S, 58º40'0''W). Collected samples were placed in poly(ethylene terephthalate), PET previously thoroughly cleaned with 10% HNO_3. Samples were transported to the laboratory and stored in a dry and dark place until analysis. Samples were filtered through a 0.45 μm polyvinyldifluoride syringe filter (Minisart–Sartorius, Göttingen, Germany). In a previous paper[13] we discussed the use of Teflon® and PET bottles for sampling and storage of water samples. Both materials didn´t introduce detectable levels of contamination, either by leaching from sampling vessels or metal losses, when they were tested for mercury.

Extraction and separation of Hg species from the matrix is one of the key steps in species determination because two conflicting issues need to be taken into account: (*i*) avoid contamination, and (*ii*) prevent losses when the species are separated from the

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matrix. To control these potential drawbacks, these sample treatment procedures were followed:

Dietary supplements: hydrochloric acid was used for the extraction of Hg and MeHg from dietary supplements. A sample aliquot of ~0.5 g was placed in a Falcon® tube and 5 mL of HCl 5 M were added. The resulting suspension was sonicated for 15 min and then centrifuged at 3500 rpm. After centrifugation, the supernatant was decanted and the residue was re-extracted as described above. Afterwards, both supernatants were combined and neutralised with 10 M NaOH to a final volume of 10 mL. All extractions were performed in triplicate and extraction blanks were prepared following the same procedure.[15,16]

Tuna fish: To check accuracy, Hg species were extracted from the certified reference material BCR® - 463 tuna fish (from the former Community Bureau of Reference of European Commission) with certified values for MeHg and total Hg. The CRM was used as provided. A portion of ~ 250 mg of the CRM was placed in a centrifuge tube. A volume of 5 mL of 5 M HCl was added and the mixture was sonicated for 15 min. After this period, the suspension was centrifuged at 3500 rpm for 5 min and the supernatant was transferred to a glass vial for further analysis. All extractions were performed in triplicate and extraction blanks were prepared following the same procedure.

Lobster hepatopancreas: The CRM TORT-3 (NRC-Canada) was treated following the procedure described above. This CRM has certified values for Hg and MeHg.

2.6 Total Hg determination

For total Hg determination in solid CRMs and oil capsule samples (~ 0.5 g) were subject to microwave assisted digestion (MW) with 8.0 mL of HNO_3 (c) + 2.0 mL of 30% H_2O_2 following these steps: 2 min at 250 W; 2 min at 0 W; 6 min at 250 W; 5 min

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at 400 W and 6 min at 600 W. For checking accuracy, aliquots of both certified reference materials were subject to the same treatment and included in the overall analytical process.

3. Results and discussion

The first step was the optimisation of the different parameters involved in the preconcentration process.

3.1 Microcolumn characteristics

In this study, we explored the performance of a sulphur-based substrate based on the well known affinity of sulphur compounds with Hg species.

With respect to the physical characteristics of the column and according to our previous studies[13,14], a careful evaluation of the optimum dimensions of the MWCNT microcolumn is necessary. To this end, a good compromise was to use a small (home–made) microcolumn of 2.3 mm (i.d) x 25 mm (net length of substrate: 15 mm and a nominal microcolumn capacity ~ 62 µL). To avoid aggregation and an unacceptable higher back-pressure due the tight packing of the filling material we filled the column with a mixture of the substrate (polymet-MWCNTs) and inert microparticles of Epolene® (Eastman Chemical Products, Inc., Kingsport, TN, USA), a low-density polyethylene wax that offers good high-temperature stability, low-temperature flexibility, and very good compatibility with the use of mineral acids.[13]

In this study a mixture of ~3.0 mg of polymet-MWCNTs and 30 mg of Epolene® was used to fill the microcolumn using the dry packing method.

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To check that Hg species were not retained by the low molecular weight polyethylene wax, a test microcolumn was filled only with ~ 30 mg of this material and inserted in the preconcentration system. This test was performed off-line. No retention was observed which evidenced that Hg species were only retained by the sorbent. In addition, we checked that the eluent could remove the retained Hg species from the substrate in a minimum volume to reach higher preconcentration factors.

3.2 Optimisation of solid phase extraction conditions

The optimisation of the SPE conditions for Hg(II) and MeHg retention on the polymet-MWCNTs microcolumn was performed off-line. To this end, Hg(II) and MeHg were passed separately through the microcolumn. The first parameter evaluated was pH because it plays a crucial role in sorption of different ions on MWCNTs. The sorption of metal ions on the functionalised MWCNTs increases as pH increases because the MWCNTs surface becomes more deprotonated, causing electrostatic interactions between metal ions and oxygen and sulphur functional groups. In this context, the isoelectric point (IEP) of MWCNTs shifts to the lower pH values. When the pH of the solution is higher than the IEP of the ox-MWCNTs, the negative charge on the surface provides electrostatic attractions that are favourable for adsorbing cations. The decrease of pH leads to the neutralisation of surface charge, so the adsorption of cations onto ox-CNTs decreases quickly.

In order to evaluate the effect of pH, a series of 10 μg L^{-1} sample solutions of Hg(II)/MeHg were adjusted to different pH values (between 3.0 and 10) and the preconcentration/elution was carried out. Aliquots of Hg(II)/MeHg solutions of 5.0 mL were loaded on the microcolumn at a flow rate of 1.7 mL min^{-1}. Species of Hg were eluted from the column with 2 mL of a 10% HCl solution. Mercury eluates were

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measured off-line by ICP-MS. The study indicated that Hg species were retained in a wide range of pHs and retention was maximum at pH 9. pHs higher than 10 were not tested in order to avoid metal hydrolysis. Accordingly to these findings, pH 9 was selected for further experiments. Fig. S1 (Supplementary Information) shows the effect of sample pH on Hg(II) and MeHg signals obtained off-line.

Hydrochloric acid was tested as eluent to promote Hg(II) and MeHg desorption from the sorbent material. The elution step involved exchange between analyte ions and H^+. In order to achieve a complete elution of both mercurial species with only 1 mL, a combination of HCl and ME was tested. The optimisation of eluent composition was performed by employing a full factorial design, where hydrochloric acid was examined within a wide range of concentration, from 1.0 to 20% v/v (five levels) and ME was examined at three levels of concentration: 0.05, 0.1 and 0.2% v/v. Maximum signal readouts for Hg(II) and MeHg were obtained at a concentration of 10% v/v HCl + 0.1% v/v ME (Fig. 2S-supplementary information).

The best flow rate for Hg species preconcentration and elution was selected by examining their effect over the range 0.5 and 5 mL min^{-1} while keeping the other conditions constant. No relationship between preconcentration and elution flow rates on Hg signal was observed over the studied range. As in previous studies, a flow rate of 1.7 mL min^{-1} was adopted.[13]

The effect of potential interferents on Hg(II) and MeHg signals was also examined. The individual effect of a variety of metals, metalloids and ions was assessed (Al, As, Cd, Cr, Fe, Mg, Sb, Se, Zn, Cl^-, NO_3^- and SO_4^{2-}). All analysed samples contained 10 µg L^{-1} of Hg/MeHg while the concentration of the potential interferent was 10 mg L^{-1} in all cases. Variations over ±5% in the analytical signal of mercurial species in the presence of other elements were taken as interference. Samples were analysed by

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the on-line preconcentration procedure developed. The study evidenced that no significant interference effect from coexisting ions in the on-line enrichment method was observed for most of the elements. Only, the presence of Fe and Zn produced a reduction of Hg(II) signal of 8 and 11%, respectively.

3.3 Link between the low pressure and the high pressure system

Initially, only one six-port valve was used. In these conditions, once the standard/sample was loaded onto the preconcentration column, the six-port valve was switched to the inject position and the eluate from the microcolumn was delivered directly to the chromatographic system. During these preliminary tests we observed overpressure in the system and a poor resolution of the chromatographic peaks. To relieve this pressure, a syringe (Fig. 1) was incorporated as a link between a low pressure system (preconcentration unit) and a high pressure system (HPLC unit). This syringe collected all the eluate in the barrel. Then, the operator pressed the plunger to introduce the liquid in V2. As the link between the two systems, the syringe keeps them separated allowing cleaning, conditioning and reusing the microcolumn while a sample is being analysed by HPLC-ICP-MS. The syringe has a small volume, just the same volume used to elute the retained mercurial species (1 mL), reducing dead volumes

3.4 Optimisation of HPLC conditions for Hg(II) and MeHg separation

Based on the conditions employed in a previous study for the determination of inorganic and organic species of Hg by HPLC-cold vapour-atomic fluorescence spectroscopy (HPLC-CV-AFS) [17], the variables influencing the chromatographic separation of Hg(II) and MeHg by HPLC were tested. In this approach ICP-MS was used for detection. On the base of the experimental results, the chromatographic separation of Hg species on a

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C18 column using a mobile phase containing 0.5% formic acid and 0.2% v/v of ME was adopted. ME is a useful reagent because thiolate group strongly bonds to Hg to form an ionic pair making possible the separation of the species in a reverse phase column. We also corroborated the findings of Li et al.[18] which demonstrated that when ICP-MS is used as detector, ME is an efficient reagent to reduce the possibility of Hg deposition in the walls of the system namely, spray chamber and transfer tubing. Also Bramanti and coworkers[19] observed that the employment of thiols as Hg complexing agent is necessary to avoid Hg adsorption to the chromatographic stationary phase on the Teflon and PEEK tubing. In our study we used ME and potential memory effects were checked and they resulted insignificant.

We tested the use of ME in a range of concentrations varying from 0.05 to 0.3 % and a concentration of 0.2% ME resulted in the best separation. Higher concentrations were not tested due to the toxicity of this reagent. The concentration of formic acid added to the mobile phase was maintained in the values reported previously because higher values did not show an increase resolution in the separation of the species.[17]

The addition of methanol to the mobile phase was necessary to obtain the chromatogram in a reasonable time. Methanol concentration was assessed between 1 and 20%, and 20% was the concentration adopted for further experiments. It is known that the addition of organic solvents to the ICPs can cause instability of the discharge. Even when a 20% of methanol was added to the mobile phase no noticeable flickering was observed and the plasma was stable enough to continue the experiments. The Peltier cooled cyclonic spray chamber of our instrument and the low flow rate used contribute to reducing solvent loading when volatile organic solvents are present in the plasma.

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In conclusion, a mobile phase composed by 0.5% formic acid + 0.2% ME + 20% methanol was adopted for further work.

The effect of the mobile phase flow rate was examined in the range of 0.5–1.0 mL min^{-1}. A compromise between peaks width and separation time was adopted and a good and fast separation was reached in less than 12 min at a mobile phase flow rate of 0.8 mL min^{-1}.

Under the above mentioned conditions the obtained chromatogram using a C18 column showed two satisfactorily resolved peaks of Hg(II) and MeHg as shown in Fig. 2. The first identified peak at 8.5 min retention time corresponds to MeHg and the other at 10 min retention time to Hg(II). This result confirms that the method has a reasonable sample throughput (5 samples h^{-1}).

3.5 Analytical performance

Under the optimal experimental conditions, the analytical performance of the SPE-HPLC-ICP-MS system for the determination of Hg(II) and MeHg species was evaluated. Calibrations were performed with a series of standards of Hg(II) and MeHg (0.05 – 10 μg L−1) injected in the coupled system. Limits of detection (LODs, 3σ criterion) were: 15 and 17 ng L^{-1} for Hg(II) and MeHg, respectively when 5 mL of sample/standard where passed through the system and 5 ng L^{-1} for both species when 20 mL were flushed. The enrichment factor (EF), defined as the ratio between the areas of a calibration standard with SPE to that without preconcentration, was 190 for both species when 20 mL of sample/standard were passed through the system. This is a high EF in comparison with other values reported in the literature[7,20,21]. EF can be further improved by increasing the sample loading. In this study we used a volume of 5 mL to avoid

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prolonging the analysis time and obtain the chromatogram in a reasonable time. When 5 mL of sample were passed through the system the EF was 48.

The relative standard deviations (RSDs) for 8 replicate determinations of both Hg species at 0.5 μg L^{-1} (as Hg) was in all cases < 6 %. Linearity was attained from levels close to the detection limit up to at least 100 μg L^{-1}.

The reference materials BCR® - 463 (tuna fish) certified for total Hg and MeHg and TORT-3 (certified for Hg and MeHg) were analysed to verify the accuracy of the method. No significant differences (at the 95% confidence level) were detected between the certified and found concentrations while the sums of the concentrations of the individual species were in good agreement with the certified total concentrations of Hg. Table 2 summarises the results of the accuracy test.

3.6 Analysis of real samples

The applicability of the developed procedure was examined by the analysis of two different categories of samples. Results are depicted in Table 3.

Waters: Levels of Hg(II) and MeHg species in Antarctic waters were below the respective detection limits even when 20 mL of water were passed through the system. Water sample 1 and Water Sample 2 were spiked with 5 μg L^{-1} of Hg and 5 μg L^{-1} of MeHg. As depicted in Table 3, the spike recoveries for the species under study were in the range 92–108% and the precision between sample replicates was better than 7% in all cases (n=3).

Fish oil: the main exposure pathway of Hg to humans is through the consumption of marine fishery products. In this context, six fish oil dietary supplements samples purchased in different countries were analysed. The extraction of Hg species from fish oil samples was described in Section 2.5. Neither Hg(II) nor MeHg was detected in the

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three dietary supplements samples. Samples were spiked as shown in Table 3 with different amounts of Hg(II) and MeHg. The spike recoveries for the species examined in fish oil samples were in the range 93-114%. The precision between sample replicates was better than 9% in all cases (n=3).

4. Conclusions

A procedure for the on-line preconcentration and speciation of Hg(II) and MeHg using a functionalised MWCNTs microcolumn and HPLC-ICP-MS hyphenated system was described. The use of solid phase extraction in on-line mode undoubtedly offered a speed saving in comparison to other techniques such as liquid-liquid extraction[22], cloud point extraction[23], stir bar sorptive extraction[24], coprecipitation[25]. The sorbents resulted stable over a wide range of pH and the column packed with functionalised MWCNTs had a lifetime of (at least) 575 cycles of mercury retention/elution without demand for generation or modification of the initial conditions. Microcolumns filled with nanosorbents lead to higher extraction efficiency and rapid dynamics of extraction due to the higher surface area to volume ratio and the short diffusion route in comparison with other filling materials, e.g.,thiol-funcionalised resin[9]. Another characteristic is the good selectivity towards the ions tested.

The mobile phase adopted (0.5% of formic acid + 0.2% 2-mercaptoethanol + 20% methanol) allowed a rapid separation of Hg(II) and MeHg that was accomplished in ~ 10 min on a 250-mm C18 column. Enrichment factors up to 190-fold were reached when passing 20 mL of sample through the system. A sample throughput of 5 samples h^{-1} was obtained. The method is simple, sensitive and cost-efficient.

Results presented provide evidence of the ideal properties of functionalised MWCNTs for the preconcentration of Hg(II) and MeHg to be separated and analysed

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on-line by HPLC-ICP-MS. Limits of detection obtained were comparable to others previously reported.[6,8] As additional advantages we can mention: (i) samples are analysed without derivatisation of Hg(II) and MeHg, (ii) minimum amount of substrate is required, (iii) instantaneous release of retained species, (iv) small volume (1 mL) of acid is necessary for species elution.

No Hg species were detected neither in Antarctic waters nor in dietary supplements. Therefore, due to the extensive consumption of dietary supplements worldwide it is necessary to further survey commercial market fish oil dietary supplements with a focus on the determination of dangerous compounds/species for human health.

Conflict of interest

There are not conflicts to declare

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References

1 K. Leopold, M. Foulkes and Paul Worsfold, *Anal. Chim. Acta*, 2010, **663**, 127–138.

2 D. Kocman, M. Horvat, N. Pirrone and S. Cinnirella, *Environ. Res.,* 2013, **125**, 160-170.

3 D. Kocman, S. J. Wilson, H. M. Amos, K. H. Telmer, F. Steenhuisen, E. M. Sunderland, R. P. Masson, O. Outridge and M. Horvat, *Int. J. Environ. Res. Public Health*, 2017, **14**, 138-154.

4 T. Tomiyasu, H. Kodamatani. R. Imura, A. Matsuyama, J. Miyamoto, H. Akagi, D. Kocman, J. Kotnik, V. Fajon and M. Horvat, *Chemosphere*, 2017, **184**, 244-252.

5 T. Syversen and P. Kaur, *J. Trace Elem. Med. Bio*., 2012, **26**, 215–226.

6 Y. Gao, W. Yang, Ch. Zeng, X. Hou and L. Wu, *J. Anal. At. Spectrom*., 2011, **26**, 126-132.

7 M.V. Balarama Krishna, K. Chandrasekaran and D. Karunasagar, *Talanta*, 2010, **81**, 462-472.

8 Z.H. Wang, Y.G. Yin, B. He, J.B. Shi, J.F. Liu and G.B. Jiang, *J. Anal. At. Spectrom.* 2010, **25**, 810–814.

9 B.R. Vermillion and R.J.M. Hudson, *Anal. Bioanal. Chem*., 2007, **388**, 341–352.

10 Ch.-C. Brombach, B. Chen, W. T. Corns, J. Feldmann and E. M. Krupp, *Spectrochim. Acta Part B*, 2015, **105**, 103–108.

11 D. Deng, S. Zhang, H. Chen, L. Yang, H. Yin, X. Hou and Ch. Zheng, *J. Anal. At. Spectrom*., 2015, **30**, 882-886.

12 B. Parodi, M. Savio, L. D. Martínez, R. A. Gil and P. Smichowski, *Microchem. J*., 2011, 98, 225-230.

View Article Online
DOI: 10.1039/C8JA00188J

13 B. Parodi, A. Londonio, G. Polla, M. Savio and P. Smichowski, *J. Anal. At. Spectrom*., 2014, **29**, 880-885.

14 B. Parodi, A. Londonio, G. Polla, P. Smichowski, *Microchem. J*., 2015, ***122***, 70-75.

15 B. Vallant, R. Kadnar and Walter Goessler, *J. Anal. At. Spectrom*., 2007, **22**, 322–325.

16 A. I. Cabañero Ortiz, Y. Madrid Albarrán and Carmen Cámara Rica, *J. Anal. At. Spectrom*., 2002, **17,** 1595–1601.

17 G. Acosta, A. Spisso, L. P. Fernández, L. D. Martinez, P. H. Pacheco, R. A. Gil, *J. Pharm. Biomed. Ana*l., 2015, **106**, 79-84.

18 Y. Li, Ch. Chen, B. Li, J. Sun, J. Wang, Y. Gao, Y. Zhao and Z. Chai, *J. Anal. At. Spectrom*., 2006, **21**, 94–96.

19 E. Bramanti, C. Lomonte, M. Onor, R. Zamboni, A. D'Ulivo and G. Raspi, *Talanta*, 2005, **66**, 762–768.

20 T. Hashempur, M.K. Rofouei and A.R. Khorrami, *Microchem. J.*, 2008, 89, 131–136.

21 Z. Fan, *Talanta,* 2006, 70, 1164–1169

22 X.Y. Jia, Y. Han, X.L. Liu, T.C. Duan and H.T. Chen, *Spectrochim. Acta B,* 2011, 66, 88–92.

23 J.G. Chen, H.W. Chen, X.Z. Jin, and H.T. Chen, *Talanta,* 2009, 77, 1381–1387.

24 R. Ito, M. Kawaguchi, N. Sakui, N. Okanouchi, K. Saito, Y. Seto and H. Nakazawa, *Talanta,* 2009, 77, 1295–1298.

25 Q. Zhang, H. Minami, S. Inoue and I. Atsuya, *Anal. Chim. Acta,* 2004, 508, 99-104.

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Table 1 Operating conditions of the SPE-HPLC-ICP-MS system for Hg and MeHg preconcentration and determination

Parameter	Conditions
ICP-MS	PerkinElmer NexIon 300X
RF power	1250 W
Argon flows	Nebuliser, 0.95; Plasma, 16; Auxiliary, 1.2 (all in L min^{-1})
Isotope monitored	^{202}Hg and ^{209}Bi
Dwell time	50 ms
Data acquisition mode	Time resolved analysis
HPLC system	
Analytical column	Phenomenex SphereClone ODS C18 (5 µm, 250 mm x 4.6 mm i.d.)
Mobile phase	0.5% (v/v) formic acid; 0.2% (v/v) 2-mercaptoethanol; 20% (v/v) methanol
Flow rate of mobile phase	0.8 mL min^{-1}
Preconcentration system	
Eluent	10% HCl; 0.1% (v/v) 2-mercaptoethanol
Eluent volume	1 mL
Sample/eluent flow rate	1.7 mL min^{-1}

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Table 2 Accuracy data (μg g^{-1}) obtained with BCR-463 (tuna fish) and TORT-3 (Lobster hepatopancreas) certified reference materials.

		HPLC-ICP-MS		MW digestion + ICP-MS
	Certified	Found	∑ species	Found
BCR-463				
Hg(II)	Non certified	0.018±0.001	--	--
MeHg *	3.04±0.16	3.13±0.19	--	--
Total Hg	2.85±0.16	--	2.93±0.18	3.01±0.19
TORT-3				
Hg(II)	Non certified	0.161±0.019	--	--
MeHg	0.137±0.012	0.141±0.013	--	--
Total Hg	0.292±0.022	--	0.305±0.035	0.287±0.034

* expressed as μg g^{-1} of MeHg

Concentrations are means of three measurements ± standard deviation

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Table 3 Recoveries and concentration of Mercury and Methylmercury in real samples. Results are expressed in μg L^{-1} (waters) and ng g^{-1} (fish oil)

Sample	Hg (II) found	Recovery/%	MeHg found	Recovery/%	Origen
Water 1	4.85±0.24	97	4.59±0.24	92	Antarctica
Water 2	5.35±0.28	107	5.39±0.36	108	Antarctica
Fish oil 1	11.14±0.57	114	10.98±0.72	110	Argentina
Fish oil 2	15.22±0.74	102	16.79±0.88	111	USA
Fish oil 3	4.79±0.31	96	4.66±0.29	93	China

Concentrations are means of three measurements ± standard deviation

Fish oil 1: spiked with 10 ng g^{-1} of Hg(II) and 10 ng g^{-1} of MeHg

Fish oil 2: spiked with 15 ng g^{-1} of Hg(II) and 15 ng g^{-1} of MeHg

Fish oil 3: spiked with 5 ng g^{-1} of Hg(II) and 5 ng g^{-1} of MeHg

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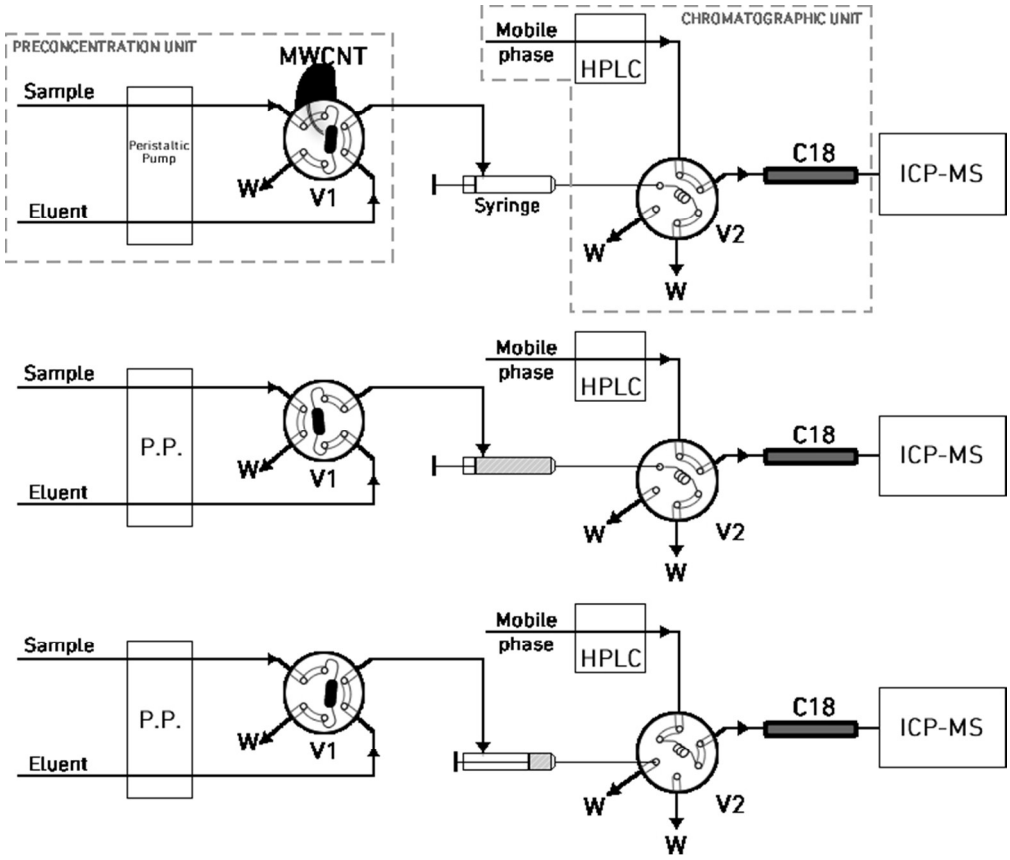

Figure 1: Operational scheme of the SPE-HPLC-ICP-MS system used for mercury speciation analysis. P.P: peristaltic pump; V1 and V2 six-port valves; W: waste; C18 chromatographic column.

277x235mm (72 x 72 DPI)

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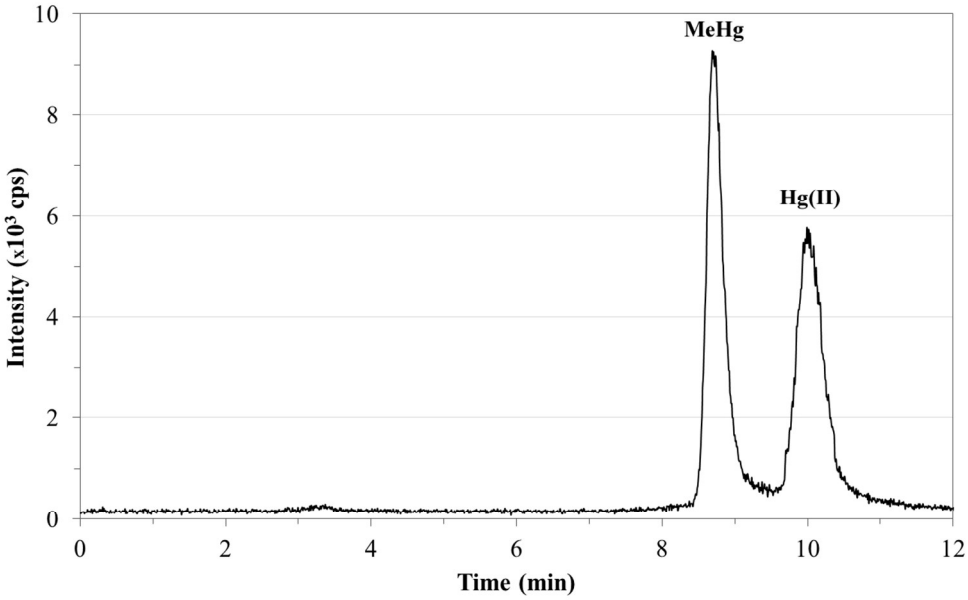

Chromatogram of Hg(II) and MeHg in Antarctic water sample spiked with 10 µg L-1 of each species. Experimental conditions used are described in Table 1

227x141mm (149 x 149 DPI)