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Mercury speciation in edible seaweed by liquid chromatography - inductively coupled plasma mass spectrometry after ionic imprinted polymer-solid phase extraction

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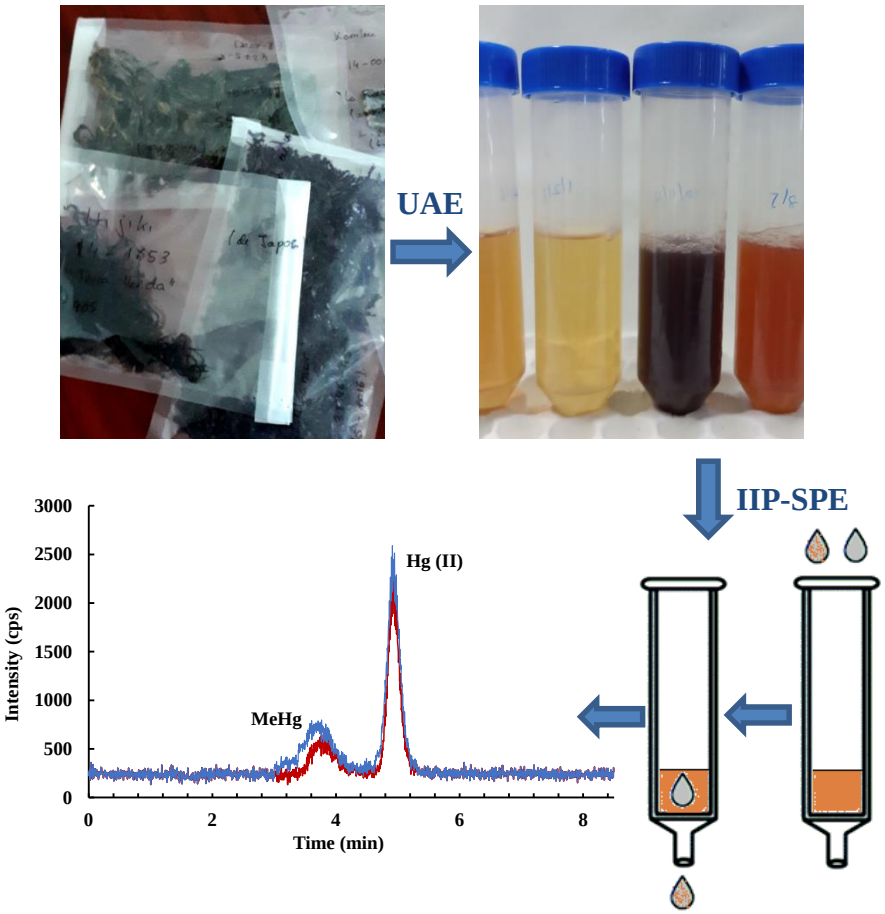
Highlights:

-Selective pre-concentration of mercury species by using an ionic imprinted polymer

- Trace levels of inorganic mercury and methylmercury are assessed in edible seaweed

- Robust and low-cost pre-concentration procedure for total mercury assessment and for mercury speciation

Graphical Abstract (for review)



Mercury speciation in edible seaweed by liquid chromatography - inductively coupled plasma mass spectrometry after ionic imprinted polymer-solid phase extraction

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Abstract

In contrast to most of essential and heavy metals, mercury levels in seaweed are very low, and pre-concentration methods are required for an adequate total mercury determination and mercury speciation in this foodstuff. An ionic imprinted polymer-based solid phase extraction (on column) pre-concentration procedure has been optimized for mercury species enrichment before liquid chromatography hyphenated with inductively coupled plasma mass spectrometry determination. The polymer has been synthesised by the precipitation polymerization method and using a ternary pre-polymerization mixture containing the template (methylmercury), a non-vinylated monomer (phenobarbital), and a vinylated monomer (methacrylic acid). Factors affecting the adsorption/desorption of Hg species (extract pH, loading and elution flow rates, volume of eluent, etc.), and parameters such as breakthrough volume and reusability, were fully studied. Mercury species were first isolated from seaweed by ultrasound assisted extraction using a 0.1% (v/v) HCl, 0.12% (w/v) L-cysteine, 0.1% (v/v) mercaptoethanol solution. Under optimized conditions, the limits of detection were 0.007 and 0.02 μg kg^{-1} dw for methylmercury and Hg(II), respectively. The pre-concentration factor (volume of 10 mL of

seaweed extract) was 50. Repeatability and reproducibility of the method were satisfactory with relative standard deviations lower than 16%. The proposed methodology was finally applied for the selective pre-concentration and determination of methylmercury and Hg (II) in a BCR-463 certified reference material and in several edible seaweeds.

Keywords

Ionic imprinted polymer, **s**olid-phase extraction, mercury speciation, edible seaweed

1. Introduction

Seaweed (benthic marine algae or macroalgae) are a source of food for humans since ancient times, and they have also been used in medicine and as animal fodder [1]. Seaweed can be classified into three main groups according to their dominant pigmentation: red (*Rhodophyta*), brown (*Phaeophyta*), and green (*Chlorophyta*) seaweed. According to the Food and Agriculture Organization (FAO) statistics, world seaweed mariculture production reached 24.9 million tons (valued about six billion USD) in 2014 [2]. As a food source, edible seaweed are rich in polysaccharides, proteins, dietary fibre, polyunsaturated fatty acids, vitamins (A, C, B2 and B12), iodine, and minerals (magnesium, sodium, and iron) [3,4]. Additionally, seaweed are used as low-calorie food (body weight control), and their consumption has been reported to prevent cancer, and gastrointestinal and cardiovascular diseases [4,5]. Another important application of seaweed is the production/extraction of derivatives such as agar, carrageenan, alginate, and bio-active compounds [5]. However, some studies are showing the safety hazard of seaweed due to their content of non-essential trace metals (Hg, Cd, Pb, As, etc.), radioactive isotopes, dioxins, and pesticides [6-8].

Mercury (Hg) is a highly toxic element that can be bio-accumulated and biomagnified through the food chain, especially in the marine environment [9]. The World Health Organization (WHO) has classified Hg as "one of the top ten chemicals or groups of chemicals of major public health concern" [10], and the Agency for Toxic Substances and Disease Registry (ATSDR) of the United States (US) ranked Hg on the third place of their substance's priority list [11]. The sources of Hg in the aquatic environment are several natural processes such as volcanism, weathering of rocks and degassing of the earth's crust; and several anthropogenic activities (e.g. coal combustion, mining industry and by-products, use of agriculture fertilizers, and waste incineration) [12,13]. The toxicity of Hg is related to the chemical form, and the path of entry into the organism. As an example, methylmercury (MeHg) has the greatest impact in the digestive tract, and it has shown the ability to affect the nervous system [14].

The levels of heavy metals in seaweed depend on several factors i.e. pH, salinity, presence of complex organic-inorganic molecules, temperature, light irradiation, oxygen, and nutrient concentration. According to the published scientific data, Hg concentration in seaweed varies with the type of seaweed and sampling location, and in contrast to other non-essential metals, Hg levels in seaweed are very low [15]. Therefore, many investigations have reported the Hg content as total Hg concentration (tHg), and the levels were frequently below the limits of detection of most of conventional instrumental techniques [6,16-18].

An adequate sample pre-treatment is very important when assessing ultra-trace levels of Hg (and Hg species), and accurate quantification of Hg and/or mercury species in seaweed usually requires a pre-concentration technique before instrumental analysis. Microwave assisted acid digestion has been typically used for seaweed solubilisation when total Hg is assessed [19-22]. Other authors have performed alkaline treatments by using aqueous KOH or KOH/methanol for MeHg isolation [23-25] when applying the US Environmental Protection Agency (USEPA) 1630 method [26] with cold vapour – atomic fluorescence spectrometry (CV-AFS) quantification (total mercury assessment, MeHg assessment, and inorganic Hg by difference)

[24,25]. On other occasions, organic solvents such as toluene [27] and HCl/toluene mixtures [28] have been proposed, although several additional stages (back extraction step with sodium thiosulfate and a final oxidation of MeHg with acidified BrCl for total Hg determination by CV-AFS) are required [27]. Few developments of hyphenated techniques can be found in the literature, and the speciation method by Brombach *et al.* [23] consists of CV-AFS coupling with liquid chromatography (LC) and a sample two-stages pre-treatment (microwave assisted extraction with aqueous KOH, followed of a treatment with concentrated HCl) for guaranteeing a quantitative extraction of mercury species.

In order to pre-concentrate and/or cleaning the extracts/digests from seaweed, some procedures, mainly based on solid phase extraction (SPE), have been proposed. Because of the great affinity of mercury ions for thiol groups, sulfydryl-based absorbents such as laboratory made sulfydryl cotton fiber (SCF) for total Hg determination by cold vapour atomic absoroption spectrometry (CV-AAS) [19], and thiol-thiourea on silica by LC-CV-AFS [23] have been used. Molecularly imprinted polymers (MIPs) and ionic imprinted polymers (IIPs) are promising absorbents for selective pre-concentration, matrix removal, and medium exchange [29], and some few applications have been developed for Hg determination/speciation [30]. Selective MIP-based SPE has been applied for pre-concentrating mercury species from seawater before LC-inductively coupled plasma – mass spectrometry (ICP-MS) [31], although most of MIP/IIP applications have been focused on total Hg determination or the assessment of a specific species (MeHg) in fish [20,21,28], wine [32], human hair [21,22], and human serum [33]. Similarly, IIPs have been also used for developing selective electrodes for potentiometric/voltammetric determination of total mercury in freshwater [34,35]. However, to the best of authors' knowledge, there are not developments of MIPs/IIPs for ultra-trace pre-concentration of Hg species from extracts from complex materials such as seaweed. In this work, a selective IIP for mercury species has been synthesized by the precipitation polymerization method, and a on column pre-concentration method combined

with LC-ICP-MS has been optimized for assessing low concentrations of Hg(II) and MeHg in edible seaweeds.

2. Materials and methods

2.1. Reagents

Methylmercury stock solution (1000 mg L^{-1}) was prepared from methylmercury chloride from Sigma Aldrich (St. Louis, MO, USA). This reagent was also used when preparing the IIP (MeHg as a template). The Hg (II) stock solution (1000 mg L^{-1}) was from Scharlab (Barcelona, Spain). Working standard solutions of MeHg and Hg (II) were prepared daily by appropriate dilution of the stocks. Methacrylic acid (MA), ethylene glycol dimethacrylate (EGDMA), and phenobarbital sodium salt (Sigma Aldrich), 2,2′-azobis(2-methyl propionitrile) (AIBN) from Fluka (Steinheim, Germany), and acetonitrile (Merck, Darmstadt, Germany) were used for IIP synthesis. The ammonium chloride/ammonium hydroxide (NH_4Cl/NH_4OH) buffer solution was prepared from NH_4Cl and NH_4OH from Merck. Multi-element standard solutions for the cross-reactivity study were prepared by combining single As, Ca, Co and Mg stock standard solutions (1000 mg L^{-1}) from Merck, single Cr, K, P, Pb and Zn stock standard solutions (1000 mg L^{-1}) from Scharlab, and single Cd, Cu, Fe and Na stock standard solutions (1000 mg L^{-1}) from Perkin Elmer (Shelton, CT, USA). Internal standard solutions (Ge, Sc, and Rh) were prepared from single-element standards (1000 mg L^{-1}) purchased from Perkin Elmer. Other reagents were hydrochloric acid (37%), nitric acid (Hyper pure, 69%), and 33% of hydrogen peroxide from Panreac (Barcelona, Spain), ammonia solution from Merck, thiourea from Sigma Aldrich, and ultrapure water (18.2 MΩ/cm resistivity) from a Milli-QA10 water purification system (Millipore Co., Bedford, MA, USA). Due to the non-availability of Hg certified reference material for Hg species in seaweed, a tuna fish certified reference material

(BCR-463) from the European Commission Joint Research Centre, Institute for Reference Materials and Measurements (Geel, Belgium) was used to evaluate the accuracy of the method. To avoid contamination with Hg throughout the study, all glassware and plastic-ware were thoroughly rinsed with ultrapure water, soaked 2 days in 10% (v/v) nitric acid, and finally rinsed several times with ultrapure water.

2.2. Instrumentation

A Perkin Elmer Nex-Ion 300X ICP–MS (Waltham, MA, USA) coupled with a Flexar LC (LC pump, column oven, and LC autosampler) from Perkin Elmer, was used for Hg speciation. A Kinetex C-18 100 Å analytical column (100 mm × 2.10 mm, 5 μm particle diameter) connected with a C-18 guard column from Phenomenex (Torrance, USA) was used for reverse-phase chromatographic separation. Polymerization was performed in a Boxcult temperature-controlled chamber (Stuart Scientific, Surrey, UK), with the support of a low-profile roller (Stovall, Greensboro, NC, USA). The IIP sorbent was packed into 5 mL syringes (Dispomed Witt OHG, Gelnhausen, Germany) between two Teflon frits (Supelco, Bellefonte, PA, USA). SPE was performed by using an 8 channel Minipuls 3 (Gilson, Middleton, WI, USA) peristaltic pump with 2-stop PVC tubing (1.52 mm i.d.) from SCP Sciences (Baie-D'Urfe, Quebec, Canada). IIPs characterization was performed by Fourier transform infrared spectrometry (FT-IR) using a Spectrum-Two FT-IR (Perkin Elmer). Other general instrumentation such as an USC60TH ultrasonic cleaner bath (45 kHz, 120 W) from VWR (Leuven, Belgium), a 2K15 ultracentrifuge (Sigma, Osterode, Germany), a Basic 20 pH meter Crison, Barcelona, Spain), a vibrating zircon ball mill (Retsch, Haan, Germany), an oven model 207 (Selecta, Barcelona, Spain), and a Classic ML analytical balance (Mettler Toledo, Columbus, OH, USA) were used throughout this research.

2.3. Preparation of IIPs

IIPs were synthesized following a three step-procedure developed by Rodríguez-Reino *et al.* [31], with minor changes. To prepare the pre-polymerization mixture, 0.053 g of CH_3ClHg,

0.097 g of phenobarbital salt, 71 µL of MA, and 25 mL of porogen (18.75 mL ACN and 6.25 mL H_2O) were mixed into a glass tube. The mixture was then stirred for 5 min and kept into a dark place overnight. Afterward, 1.13 mL of EGDMA and 55 mg of purified AIBN were added into the test tube. The mixture was again stirred for 1 min, purged with argon for 10 min, placed into the low-profile roller (33 rpm) and incubated at 60 °C for 24 h. The synthesized material was vacuum filtered, washed 3 times with a 3:1 acetonitrile/water mixture, and oven-dried at 40 °C overnight. NIPs (non-imprinted polymers) were prepared in the same manner, without adding the template ion.

The template (MeHg ion) was leached by passing 400 mL of 1.0 M thiourea solution in 1.0 M HCl through a syringe containing 0.5 g of IIPs at a flow rate of 0.5 mL min^{-1}. The polymer was then washed with ultrapure water, dried at 40 °C in an oven (12 h), and stored in sealed bottles until use in further analysis.

In accordance with previous works [31], scanning electron microscopy (SEM) characterization of IIP and NIP showed agglomeration of spherical particles of diameters lower than 10 µm. Characterization by FT-IR spectrometry (FT-IR spectra of IIPs after template removal and before template removal, and NIP in Figure 1) shows similar characteristic peaks for all cases: 3584 cm^{-1} (stretching peak of O-H), 2986 cm^{-1} (stretching vibration of C-H_3), 1732 cm^{-1} (stretching vibration of C=O), 1456 cm^{-1} (bending vibration of C=C, C=N), 1389 cm^{-1} (bending vibration of C-H_3), 1254 cm^{-1} (bending vibration of N-H), and 1148 cm^{-1} (stretching vibration of C-O). All these bands confirmed that the IIP has been successfully formed, and the presence of prominent C=N-, N-H bands confirm that the complexing agent phenobarbital trapped into the polymeric matrix.

2.4. Ultrasound assisted extraction of mercury species from seaweed

Dehydrated edible seaweed samples were pulverized using a ball mill and dried in an oven at 70 °C for 24 h. Seaweed samples and the CRM were subjected to ultrasound assisted extraction (UAE) by weighing by triplicate 0.2 g portions of homogenized dried seaweed and adding a

volume of 10 mL of the extractant solution [0.1% (v/v) HCl, 0.12% (w/v) L-cysteine, 0.1% (v/v) mercaptoethanol] [36,37]. These suspensions were ultrasonicated at 45 kHz and room temperature for 30 min. After centrifugation (5000 rpm, 30 min), supernatants (extracts) were separated, and these extracts used in further experiments.

2.5. IIP-based solid phase extraction procedure

Sorbent particles (200 mg of IIPs) were placed between two Teflon frits into 5 mL syringes, and the sorbent was conditioned by passing volumes of 20 mL of NH_3/NH_4Cl buffer solution (pH 9.0) at a 2 mL min^{-1}. Loading stage consisted of passing 10 mL of seaweed extract (pH adjusted to 9.0) at 2 mL min^{-1} flow rate, which was followed by a cleaning step with 10 mL of pH 9.0 NH_3/NH_4Cl buffer solution also at a flow rate of 2 mL min^{-1}. The retained Hg ions were then eluted with 2 mL of a solution containing 0.8% (v/v) 2-mercaptoethanol and 20% (v/v) methanol (pH adjusted to 4.5) at a flow rate of 1.0 mL min^{-1}. The eluate was dried under N_2 gas at 40°C, and re-dissolved in 200 μL of mobile phase solution (0.4% (v/v) 2-mercaptoethanol and 10% (v/v) methanol, pH 2.0) for LC-ICP-MS analysis.. A pre-concentration factor of 50 was achieved by the previous treatment

First attempts for total Hg determination in seaweed after a microwave assisted acid digestion procedures led to undetected total Hg concentrations in the tested seaweed samples because the strong matrix effect of the acid matrix. Therefore, the same extraction procedure (section 2.4) followed by the IIP-based SPE (but re-dissolving the residues in 5-10 mL of ultrapure water after N_2 stream drying) was used for total Hg (tHg) determination by ICP-MS.

2.6. Determinations by LC-ICP-MS and ICP-MS

Operating conditions for Hg (II) and MeHg species separation and determination (reverse-phase chromatography) are listed in Table 1. Quantification of both Hg species was achieved by using calibration matched with the mobile phase covering the 0-50 μg L^{-1} and 0-20 μg L^{-1} range for Hg (II) and MeHg, respectively (chromatograms in Figure 2 show signals at a retention time of 3.5 min for MeHg, and at 4.8 min for Hg (II)).

Total Hg determination were assessed by ICP-MS following the operating conditions listed in Table 1 (^{103}Rh at 10 µg L^{-1} as an internal standard) and using aqueous calibration covering the 0-10 µg L^{-1} concentration range. Elements involved in cross-reactivity studies (Ca, As, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Na, P, Pb, and Zn) were also measured by ICP-MS by using suitable aqueous calibrations and internal standards (^{74}Ge, ^{54}Sc and ^{103}Rh, all at 10 µg L^{-1}).

3. Results and Discussion

3.1. IIP-based solid phase extraction

3.1.1. Loading conditions: extract pH and loading flow rate

The hydrogen ion amount and the charge distribution on the absorbent surface play an important role in the adsorption of target ions, especially in metal ion speciation [38]. The effect of varying the pH of the extract on the absorption process was investigated in the range from pH 5.0 to 11.0, performing the experiments in triplicate. Briefly, a volume of 10 mL of seaweed extract was spiked with 0.5 µg L^{-1} MeHg standard (pH adjusted with 0.1 M HCl or with 0.1 M NH_3OH) and passed through the syringe under non optimized conditions (loading/elution flow rate of 1.0 mL min^{-1}) and 2.0 mL of 0.8% (v/v) 2-mercaptoethanol and 20% (v/v) methanol (pH 4.5) as an eluting solution [31]. The Hg (II) concentration measured in the eluates and the analytical recovery of MeHg under several pHs are shown in Figure 3(a), and low mercury species retention was observed when the extracts were adjusted at the lowest pHs. These findings are probably because of the hindrance of H^+ ions. Moreover, the absorption of both Hg(II) and MeHg diminishes at when adjusting the extracts' pHs at the highest values (10 and 11), and a pH 9.0 was selected and used for further stduies. Previous results when pre-concentrating Hg species from seawater revealed optimum pHs within the 8.0-9.0 range [31].

Seaweed extracts (10 mL spiked with 0.5 μg L^{-1} MeHg) at pH 9.0 were loaded at several loading flow rates (0.5, 1.0, and 2 mL min^{-1}) in triplicate (non optimized elution conditions as shown above were used). Figure 3(b) shows that there were not retention differences in Hg (II) under all tested loading flow rates, but improved analytical recoveries for MeHg were obtained at higher loading flow rates. Since flow rates higher than 2.0 mL min^{-1} were not possible with the peristaltic pump used, a flow rate of 2.0 mL min^{-1} was selected.

3.1.2. Eluting conditions: eluting flow rate and volume of the eluting solution

Several elution speeds (0.5, 1.0 and 2 mL min^{-1}) and elution volumes (0.5, 1.0, 2.0, 3.0 and 4.0 mL) of a 0.8% (v/v) 2-mercaptoethanol and 20% (v/v) methanol (pH 4.5) eluting solution were evaluated. Analytical recoveries of MeHg were similar at 0.5 and 1.0 mL min^{-1} elution flow rates (Figure 3(c)), but the concentration of Hg (II) in the eluate decreased when eluting at 0.5 mL min^{-1}. The highest elution flow rate (2.0 mL min^{-1}) led to poor desorption for both MeHg and Hg(II). Therefore, 1.0 mL min^{-1} was selected as the best elution speed for further studies.

Finally, the influence of the volume of the eluting solution has been demonstrated to be quite important (Figure 3(d)) and analytical recoveries of MeHg, and also Hg(II) concentrations are increased when using volumes of the eluting solution of 2.0 mL or higher (analytical recovery for MeHg is close to 100% when using the highest volumes). A volume of the eluting solution of 2.0 mL was therefore selected.

3.2. Breakthrough volume, mass capacity and reusability

In order to find the maximum volume that can be loaded into the IIP syringes without a breakthrough of the analyte (breakthrough volume) the optimum SPE conditions were applied varying the sample volume (10, 25, 37.5, 50, and 100 mL of aqueous solutions at pH 9.0 containing 1.0 μg L^{-1} of MeHg and Hg (II)). Experiments in triplicate (Figure 4(a)) proved that no significant losses of MeHg and Hg (II) took place even when a volume of 100 mL of sample was loaded. This finding implies that the IIP could be successfully used as an SPE

sorbent for loading high volumes of extracts, and the pre-concentration factor can therefore be quite high.

Mass capacity of the IIP sorbent was calculated by loading volumes of 50 mL (5 aliquots of 10 mL) of aqueous solution (pH 9.0) containing 100 μg L^{-1} of MeHg in triplicate under the optimum conditions. After passing through the syringe, each 10 mL aliquots were collected and analysed for MeHg. No chromatographic signals were recorded for MeHg in the solutions after being loaded the fourth 10 mL aliquot. Taking into account a volume of 30 mL and the amount of IIP used, the mass capacity of the IIP was found to be 20.0±0.1 μg g^{-1}.

The reusability of the IIP absorbent was evaluated with the same set of three syringes and using aqueous standards of 1.0 μg L^{-1} of MeHg and Hg (II) (50 μg L^{-1} after pre-concentration). As shown in Figure 4(b), the analytical recovery of MeHg and Hg (II) was found between 80-100 % at least after performing 15 absorption/desorption cycles. These findings suggest that the same IIP syringe (the same IIP portion) can be reused 15 times without losing recognition/sorption properties.

3.3. Cross-reactivity

The selectivity of the prepared IIP and NIP for the target compounds (Hg (II) and MeHg) was evaluated by comparing their extraction efficiencies with those of several foreign metal ions. An aqueous 0.1% (v/v) HCl, 0.12% (w/v) L-cysteine, 0.1% (v/v) mercaptoethanol solution (pH 9.0) spiked with 0.04 μg L^{-1} of MeHg and 0.1 μg L^{-1} of Hg (II), As(III), Cd(II), Pb(II), Cr(III), and Co(II) at 2 μg L^{-1}, and Ca(II), Mg(II), Fe(III), Cu(II), Na(I), K(I), and Zn(II)) at 20 μg L^{-1}, was prepared and subjected to the optimized IIP-SPE in triplicate. After eluate evaporation to dryness, the residues were re-dissolved in 600 μL of ultrapure water and directly analysed by ICP-MS. The selectivity was studied by calculating extraction efficiencies, distribution ratios, and selectivity coefficients as shown in Table 2.

According to the results, IIP sorbent favoured the extraction of MeHg and Hg (II) ions and high extraction efficiencies (97% and 95%, respectively) were obtained (Table 2). NIP material showed a low extraction efficiency for target analytes (12% for MeHg and 10% for Hg (II)), which indicates that Hg(II) and MeHg interaction with IIP occurs through the recognition cavities in IIP and not by surface absorption (unspecific interactions in NIP). The distribution coefficients for MeHg and Hg (II) have been found to be higher (30 and 19, respectively) than those found for the foreign ions (Table 2), which implies that the imprinting process produces cavities with adequate conformations for the interactions between the target ions (Hg(II) and MeHg) and IIP particles. As a conclusion, the synthesised IIP showed excellent recognition ability and selectivity for MeHg and Hg (II) species.

3.4. Calibration and matrix effect

Many studies on analytical troubleshooting have focused on the problems which arise due to matrix effects, mainly signal suppression/enhancement in the presence of matrix concomitants. Matrix effects on MeHg and Hg (II) have been estimated by comparing the slopes of mobile phase (0.4% (v/v) 2-mercaptoethanol and 10% (v/v) methanol, pH 2.0) matched calibration curves and standard addition curves (solutions prepared from seaweed extracts spiked with variable MeHg and Hg(II) concentrations and subjected to the IIP-based SPE procedure described in section 2.5.). Matched calibrations and standard addition calibrations were prepared by covering five concentration levels within the 0-20 μg L^{-1} range for MeHg and 0-50 μg L^{-1} range for Hg (II) (regarding standard addition calibrations, the seaweed extracts were spiked with adequate MeHg and Hg(II) concentrations taking into account a pre-concentration factor of 50). The mean slopes obtained for calibrations (experiments in triplicate) were 3012±547 and 4987±166 for MeHg and Hg (II), respectively; whereas, mean slopes for the standard addition calibration were 3048±142 for MeHg and 5322±174 for Hg (II). There was not statistically significant differences between the slopes of the standard addition calibration

graphs and the slopes of aqueous standard calibration graphs ($p>0.05$), which means that the matrix effect is negligible. Therefore, determinations can be performed by using aqueous calibration and the equations (mean values and standard deviation for the slope), as well as the calibration range and correlation coefficients, are given in Table 3. The lower concentration in the calibration range is the instrumental limit of quantification (expressed as μg L^{-1}) which is discussed in the further section.

The EU has established that a correlation coefficient higher than 0.9980 is required to obtain satisfactory linearity using confirmatory methods [39]. Acceptable linearity was obtained for the aqueous calibration and standard addition curves for both Hg (II) and MeHg (correlation coefficients higher than 0.999).

3.5. Limit of detection and limit of quantification

The limit of detection (LOD) was calculated as 3 times the standard deviation (3 σ) of eleven replicate measurements of the blank sample, while the limit of quantification (LOQ) was calculated as 10 times the standard deviation (10 σ). Therefore, eleven reagent blank samples were prepared and treated as described in section 2.5, and the analytical responses were then expressed as concentrations dividing by the mean slope of the calibration graph. LOD and LOQ values referred to the mass sample (seaweed) were calculated after considering the pre-concentration factor of 50 of the IIP-based SPE process. The calculated instrumental LOD and LOQ were 0.007 and 0.02 μg L^{-1} for MeHg and Hg (II), respectively; whereas, LOQs were 0.02 and 0.07 μg L^{-1} for MeHg and Hg (II), respectively. Taking into account the extraction procedure for isolating the mercury species from the solid seaweed, the LODs and LOQ of the method (expressed as μg kg^{-1} dw, dried weight) are listed in Table 3. These LODs (Table 3) are much better than some published LODs for Hg assessment in seaweed such as 0.120 μg kg^{-1} for MeHg by pre-concentration and LC-CV-AFS [23], 1.3 μg kg^{-1} for MeHg in cyanobacteria [40], and 0.01 mg kg^{-1} dw for MeHg using an automatic Hg analyzer [41]. The LOD obtained

by Morrison *et al.* [24] was 0.435 μg kg^{-1} ww using the 1630 USEPA method, while the LOD for biota obtained by Shoham-Frider *et al.* [27] was 0.07 μg kg^{-1} dw.

On the other hand, the Regulation No 464/2018 of the European Parliament and the Council establishes the maximum residue level (MRL) for Hg in algae, prokaryotic organisms, and food products based on seaweed as 0.01 mg kg^{-1} [42], value much higher than the LOD found in the present study which demonstrates the applicability of the proposed method edible seaweed analysis.

The sensitivity of the proposed method is similar or even better than those reported by other authors when using SPE methods based on MIPs/IIPs for mercury speciation in foodstuff [21,28,43] and also for potentiometric determination of Hg based on IIP modified carbon electrodes [34,44] (Table 4).

3.6. Repeatability, reproducibility, and accuracy

Reproducibility (inter-day assay) and repeatability (intraday assay) were studied using seaweed extracts spiked with MeHg and Hg (II) at different concentration levels. Inter-day assay was performed by spiking seven seaweed extracts at three concentration levels of MeHg (0.02, 0.1, 0.4 μg L^{-1}; i.e. concentrations of 1, 5, and 20 μg L^{-1}after pre-concentration), and three Hg (II) concentration levels (0.04, 0.2, 1 μg L^{-1}; i.e., 2, 10, 50 μg L^{-1} after pre-concentration) and measuring the seven replicates of each concentration level in the same day. Intraday assay was performed by preparing seven standard addition curves in seven different days by spiking in triplicate several seaweed extract aliquots at five concentration levels of MeHg (1, 2, 5, 10 and 20 μg L^{-1} after pre-concentration) and Hg (II) (2, 5, 10, 20 and 50 μg L^{-1} after pre-concentration). As it can be observed in Table 5, good analytical recovery and precision is obtained since all analytical recoveries ranged between 89-112% for MeHg and 86-108% for Hg (II), and the relative standard deviations (RSDs) were lower than 20% (13% for MeHg and 16% for Hg (II) for all concentration levels).

In addition to the analytical recovery, accuracy of the developed method was also tested by analysing a BCR 463 (tuna fish) CRM (a CRM for total Hg and/or Hg species in seaweed is not commercially available). After BCR 463 UAE and IIP-based SPE (section 2.4 and 2.5) and ICP-MS determination, a total Hg content of 3.01±0.06 mg kg^{-1} was obtained, which is in good agreement with the certified value (2.85±0.16 mg kg^{-1}). LC-ICP-MS analysis gave a Hg(II) concentration of 0.01±0.001 mg kg^{-1}, and an MeHg concentration of 2.86±0.05 mg kg^{-1}. The found MeHg concentration is in good agreement with certified MeHg content in BCR 463 (3.01±0.06 mg kg^{-1}). In addition, the total Hg concentration as a sum of Hg(II) and MeHg concentrations after LC-ICP-MS (2.87±0.07 mg kg^{-1}) also agrees with the certified total Hg content in BCR 463 (2.85±0.16 mg kg^{-1}).

3.8. Applications

Three edible seaweed samples were subjected to the optimised IIP-based SPE after UAE extraction and before LC-ICP-MS (Hg(II) and MeHg assessment) and ICP-MS (total Hg assessment) analysis. Results are given in Table 6 and it can be observed that the results obtained by LC-ICP-MS (sum of the species) are in good agreement with the total Hg concentration levels measured directly in the pre-concentrated eluates by ICP-MS. Hg (II) is the major species in the tested seaweed sample, and the highest Hg (II) content was recorded in sea spaghetti species (0.11±0.02 mg kg^{-1} dw).

Conclusion

Ionic imprinted polymer based on the interaction between MeHg (template) and phenobarbital (complexing agent) has found to offer excellent recognition capabilities for MeHg and Hg(II). The IIP-based SPE procedure results robust since large sample volumes (seaweed extracts) can be loaded without impairment of the analytical performances. High pre-concentration factors can be therefore achieved, which implies quite sensitive determinations. The prepared material

has demonstrated a large absorption capacity and stability, and each 200 mg portions can be reused at least fifteen times (fifteen absorption/desorption cycles). The optimized IIP-based SPE combined with LC-ICP-MS allows low limits of detection, and the methodology can be successfully applied for quantifying mercury species (MeHg and Hg(II)) at very low levels in complex samples such as seaweeds.

Acknowledgment

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Figures' captions

Figure 1: FT-IR spectra of IIP (before and after template removal) and NIP

Figure 2: LC-ICP-MS chromatograms for a 5.0 μg L^{-1} Hg(II) and 2.0 μg L^{-1} MeHg aqueous standard (a), and a pre-concentrated extract from a Sea-spaghetti sample (b)

Figure 3: Influence of pH of the seaweed extract (a), loading flow rate (b), elution flow rate (c), and elution volume (d) on the IIP-based SPE of Hg(II) and MeHg

Figure 4: Effect of the sample volume (breakthrough volume of the IIP-based SPE procedure) on the analytical recovery of Hg(II) and MeHg (a), and analytical recovery of Hg(II) and MeHg after several loading/elution cycles (reusability of each IIP portion) (b)

Table 1: Operating ICP-MS conditions for total Hg determination and cross reactivity studies and operating LC-ICP-MS conditions for Hg speciation

Operating ICP-MS conditions		
Radiofrequency power		1600 W
Ar flow rate (L min^{-1})	Nebulization	0.92
	Auxiliary	1.2
	Plasma	16
O_2 flow rate (L min^{-1})		0.01[a]
Standard mode	Ca, Cu, K, Mg, Na, P	
KED collision mode;		
He flow rate	4.0 mL min^{-1} (As, Cd, Co, Cr, Fe, Hg, Pb, Zn)	
Analytes	^{75}As, ^{43}Ca, ^{111}Cd, ^{59}Co, ^{53}Cr, ^{63}Cu, ^{57}Fe, ^{39}K, ^{202}Hg, ^{26}Mg, ^{23}Na, ^{31}P, ^{208}Pb, ^{66}Zn	
Internal standards	^{74}Ge (As, Co, Cr, Fe, and Zn)	
	^{54}Sc (Ca, K, Mg, Na, and P)	
	^{103}Rh (Cd, Hg, and Pb)	
Operation LC conditions		
Column	Kinetex C-18 100 A (100×2.10 mm, 5 µm)	
Mobile phase	0.4% mercaptoethanol, 10% methanol, pH 2.0	
Flow rate	0.3 mL min^{-1}, 8.5 min	
Injection volume	50 µL	

(a) Auxiliary O_2 only when operating as LC-ICP-MS

Table 2: Extraction efficiency, distribution ratio and selectivity coefficients for the IIP and the NIP applied to the SPE of seaweed extract

Ions	Extraction efficiency (E) /%[a]		Distribution ratio (D)[b]		Selectivity coefficient (S)[c]	
	IIP	NIP	IIP	NIP	IIP	NIP
MeHg	96.8	11.7	30	0.13	_	226
Hg (II)	95.0	9.6	19	0.11	2	282
Cd(II)	78.3	0.2	3.61	0.00	8	---[d]
Pb(II)	26.0	0.2	0.35	0.00	85	---[d]
Al(III)	35.6	10.4	0.55	0.12	54	258
Cr(II)	0.9	0.1	0.01	0.00	3249	---[d]
Fe(III)	5.8	1.2	0.06	0.01	491	2394
Co(II)	0.7	0.2	0.01	0.00	4301	---[d]
Ni(II)	3.1	0.2	0.03	0.00	940	---[d]
Cu(II)	0.4	0.0	0.00	0.00	---[d]	---[d]
Zn(II)	0.5	0.0	0.00	0.00	---[d]	---[d]
As(III)	1.4	0.6	0.01	0.01	---[d]	4654
Na(I)	0.0	0.3	0.00	0.00	---[d]	---[d]
K(I)	0.0	1.7	0.00	0.02	---[d]	1770
Ca(II)	2.5	0.0	0.03	0.00	1174	---[d]
Mg(II)	1.0	0.8	0.01	0.01	3014	3627

(a) $E(\%)=\frac{A_2}{A_T} x100$

(b) $D=\frac{A_2}{A_1}$

(c) $S_{MeHg/M}=\frac{D_{MeHg}}{D_M}$

(d) Not calculated

A_1 = Analyte concentration at equilibrium

A_2 = Analyte concentration enriched by IIP/NIP SPE at equilibrium

A_T = Total analyte concentration

M = Hg(II), Cd(II), Pb(II), Al(III), Cr(III), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), As(III), Na(I), K(I), Ca(II), Mg(II)

Table 3: Linearity, equation of calibration and LOD/LOQ of the method

	MeHg	Hg (II)
Calibration range	0.02-20 µg L^{-1}	0.07-50 µg L^{-1}
Aqueous calibration equation (n=3)	peak area = 0 + 4987(±166) [MeHg]	peak area = 0 + 3012(±547) [Hg(II)]
Correlation coefficient	>0.999	>0.999
Limit of detection	0.007 µg kg^{-1}	0.02 µg kg^{-1}
Limit of quantification	0.02 µg kg^{-1}	0.07 µg kg^{-1}

Table 4: Comparison of characteristic performances obtained by using the proposed (IIP)-SPE method and other SPE methods and potentiometric assays based on IIPs for mercury determination/speciation

Sample	Analyte(s)	Adsorbent for SPE	Detection technique	LOD	Enrichment factor	Reference
Fish	Hg (II) and MeHg	IIP	AFS		_	[21]
Fish	MeHg	MIP	HRCS-AAS	6.6 µg kg^{-1}	1	[28]
Wine	Hg (II)	Silica gel-IIP composite	CV-AAS	0.02 µg L^{-1}	_	[32]
Fish	Hg (II)	IIP	CV-AAS	0.01 µg L^{-1}	120	[43]
Fish[a]	Hg (II)	MWCNT-IIP composite	Potentiometry	6.3×10^{-8} mol L^{-1}	_	[34]
Fish and shrimp[a]	Hg (II)	Graphene oxide-IIP composite	Potentiometry	1.95×10^{-9} mol L^{-1}		[44]
Seaweed	Hg (II) and MeHg	IIP-SPE	LC-ICPMS	0.02 µg kg^{-1} for Hg (II) and 0.007 µg kg^{-1} for MeHg	50	This work

(a) This procedure does not imply a SPE stage

AFS: atomic fluorescence spectrometry, CV-AAS: cold vapour atomic absorption spectrometry, HRCS-AAS: high resolution continuum source - atomic absorption spectrometry, LC-ICP-MS: liquid chromatography - inductively coupled plasma - mass spectrometry

Table 5: Inter-day and intraday analytical recovery and precision (RSD).

	MeHg			Hg(II)		
	Concentration / µg L^{-1}	Analytical recovery / %	RSD / %	Concentration / µg L^{-1}	Analytical recovery / %	RSD / %
Inter-day	1	112±8	8	2	86±10	12
	5	97±13	13	10	108±18	16
	20	98±10	10	50	102±8	8
Intraday	1	94±7	6	2	98±8	8
	2	92±9	9	5	100±7	7
	5	89±7	9	10	102±10	10
	10	89±8	9	20	102±4	4
	20	95±6	6	50	97±5	4

Table 6: Mercury species concentration in BCR-463 and in commercial edible seaweed samples

Sample	Hg (II) / mg kg^{-1a}	MeHg / mg kg^{-1a}	tHg, mg kg^{-1b}	tHg / mg kg^{-1c}
Wakame	0.06±0.01	0.01±0.002	0.07±0.01	0.07±0.01
Sea-spaghetti	0.11±0.02	0.06±0.02	0.17±0.03	0.19±0.02
Hijiki	0.06±0.01	0.01±0.002	0.07±0.01	0.06±0.003

(a) Hg(II) and MeHg concentrations after IIP-based SPE and LC-ICP-MS determination

(b) Total Hg expressed as the sum of Hg (II) and MeHg concentrations after IIP-based SPE and LC-ICP-MS determination

(c) Total Hg after IIP-based SPE and ICP-MS determination

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Dear Editor,

I am sending to you the revised version of our manuscript TAL-D-20-02796 ‘Mercury speciation in edible seaweed by high performance liquid chromatography - inductively coupled plasma mass spectrometry after ionic imprinted polymer-solid phase extraction’. Reviewers’ comments have been taken into account and all queries have been answered. Two copies of the manuscript [clean copy and manuscript with Track Changes (red underline) have been uploaded.

Waiting your news

Best regards,

Dr. Antonio Moreda-Piñeiro (corresponding author)

The research summaries results regarding the selective and interference-free speciation of mercury (methylmercury and inorganic mercury) in edible seaweeds. Despite seaweed pre-concentrate essential and toxic elements, the levels of mercury in seaweed are very low, and the presence of this toxic element in this foodstuff is usually ignored. However, the assessment of mercury (and mercury species) must be controlled on the basis of several food safety regulations. The current research proposes the use of an ionic imprinted polymer-based solid phase extraction procedure for selectively pre-concentrating mercury species (methylmercury and inorganic mercury) from seaweed extracts. The developed procedure is robust and the high pre-concentration factor allows the determination of total mercury after applying atomic spectrometric techniques, and also mercury speciation when using hyphenated techniques such as HPLC-ICP-MS.

Figure 1

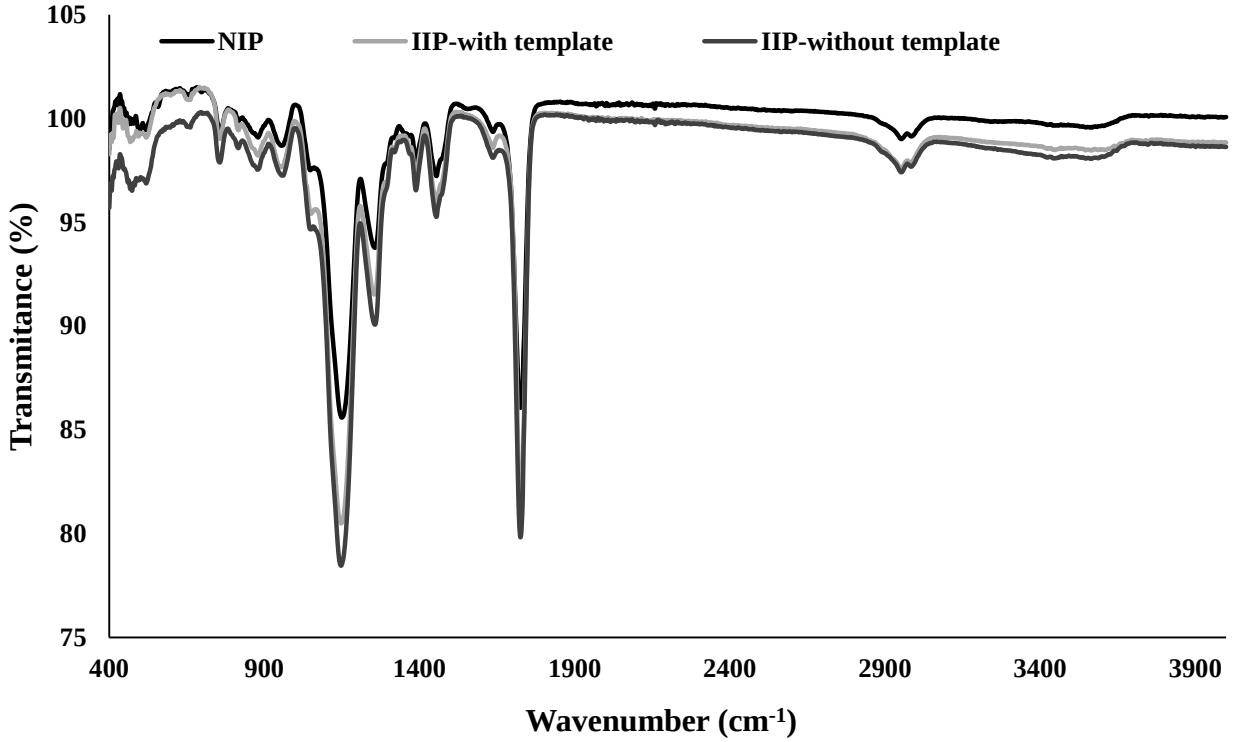

NIP
IIP-with template
IIP-without template
Transmitance (%)
105
100
95
90
85
80
75
400
900
1400
1900
2400
2900
3400
3900
Wavenumber (cm-1)

Figure 2

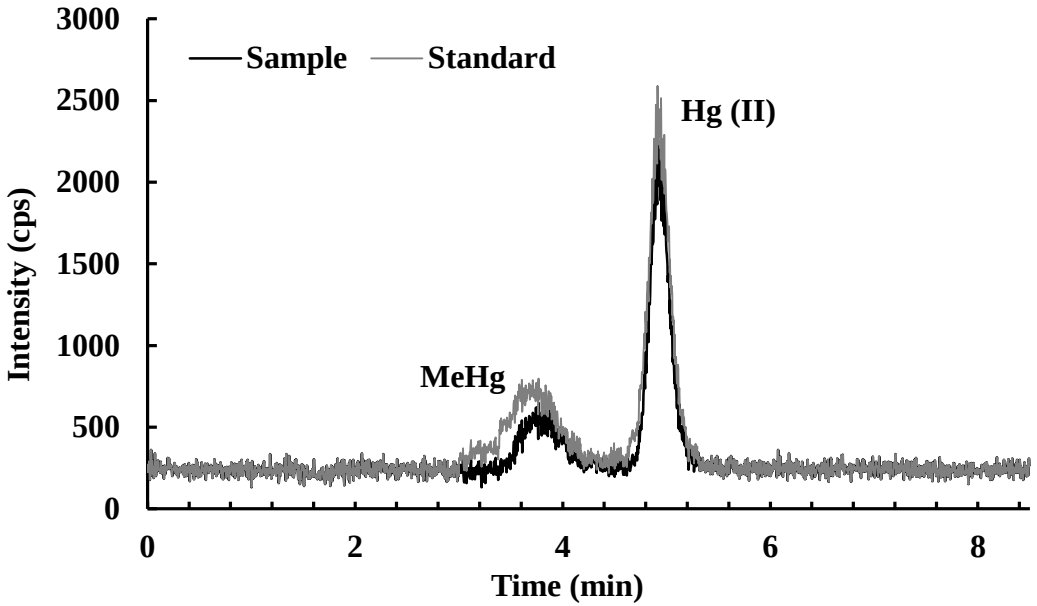
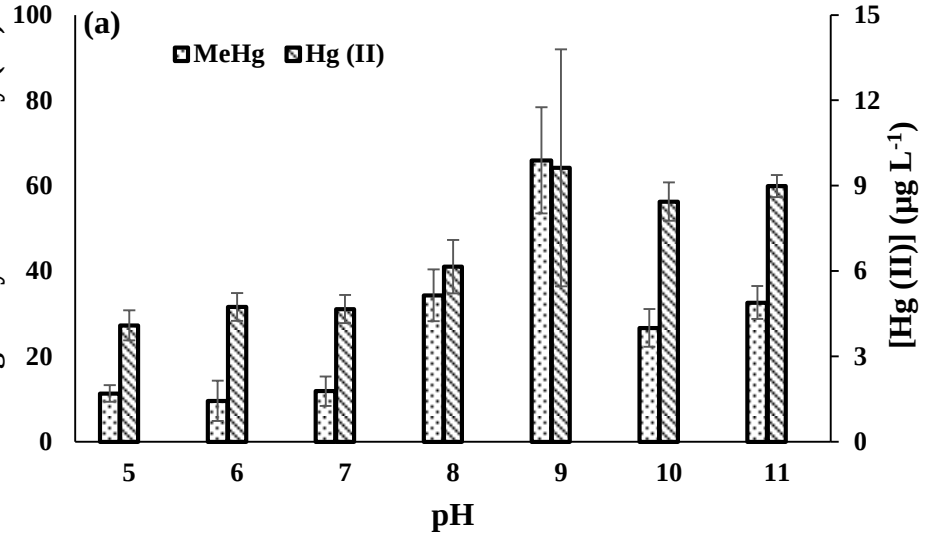

3000
2500
2000
1500
1000
500
0
Intensity (cps)
Sample
Standard
Hg (II)
MeHg
0
2
4
6
8
Time (min)

Figure 3


(a)
MeHg
Hg (II)
100
80
60
40
20
0
15
12
9
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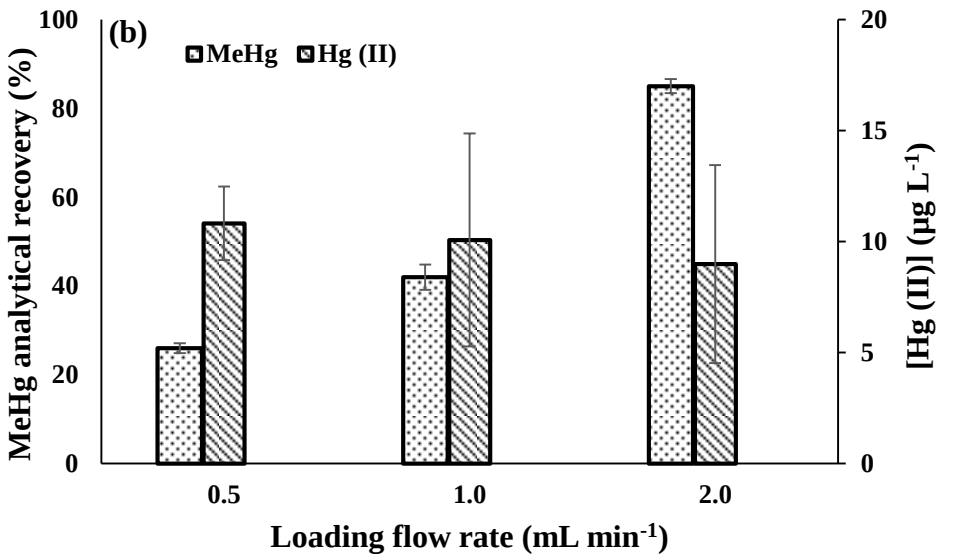
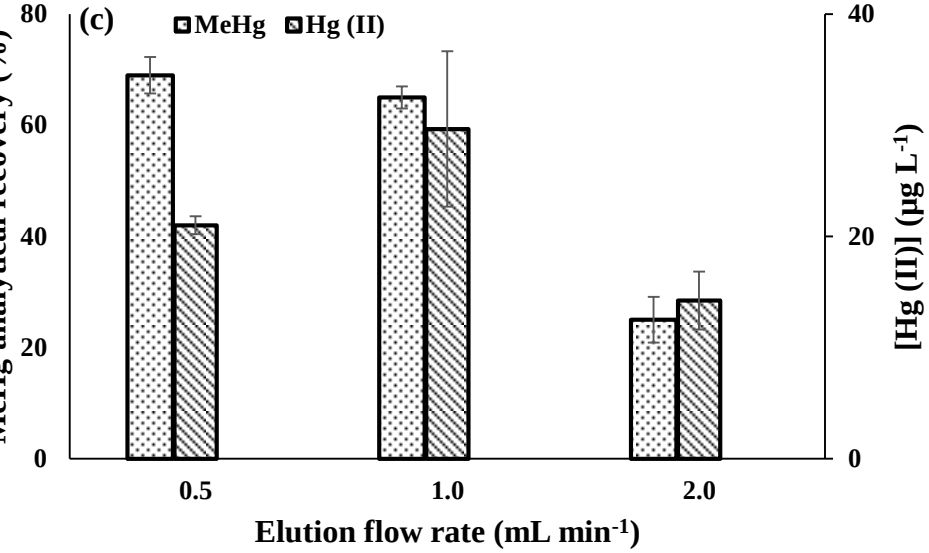
3
0
5
6
7
8
9
10
11
pH
[Hg (II)] (μg L-1)


(b)
MeHg
Hg (II)
MeHg analytical recovery (%)
100
80
60
40
20
0
20
15
10
5
0
0.5
1.0
2.0
Loading flow rate (mL min-1)
[Hg (II)] (μg L-1)


(c)
MeHg
Hg (II)
80
60
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20
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40
20
0
0.5
1.0
2.0
Elution flow rate (mL min-1)
[Hg (II)] (μg L-1)

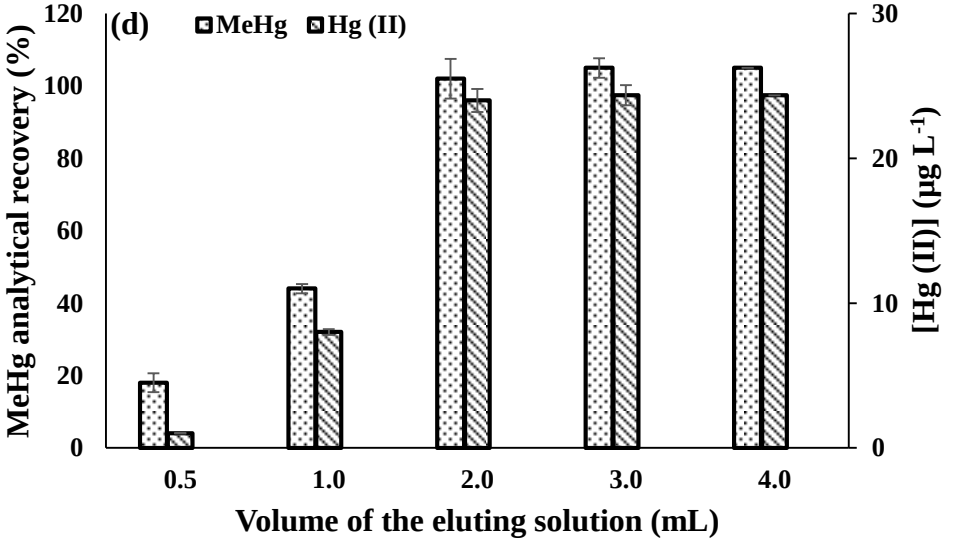
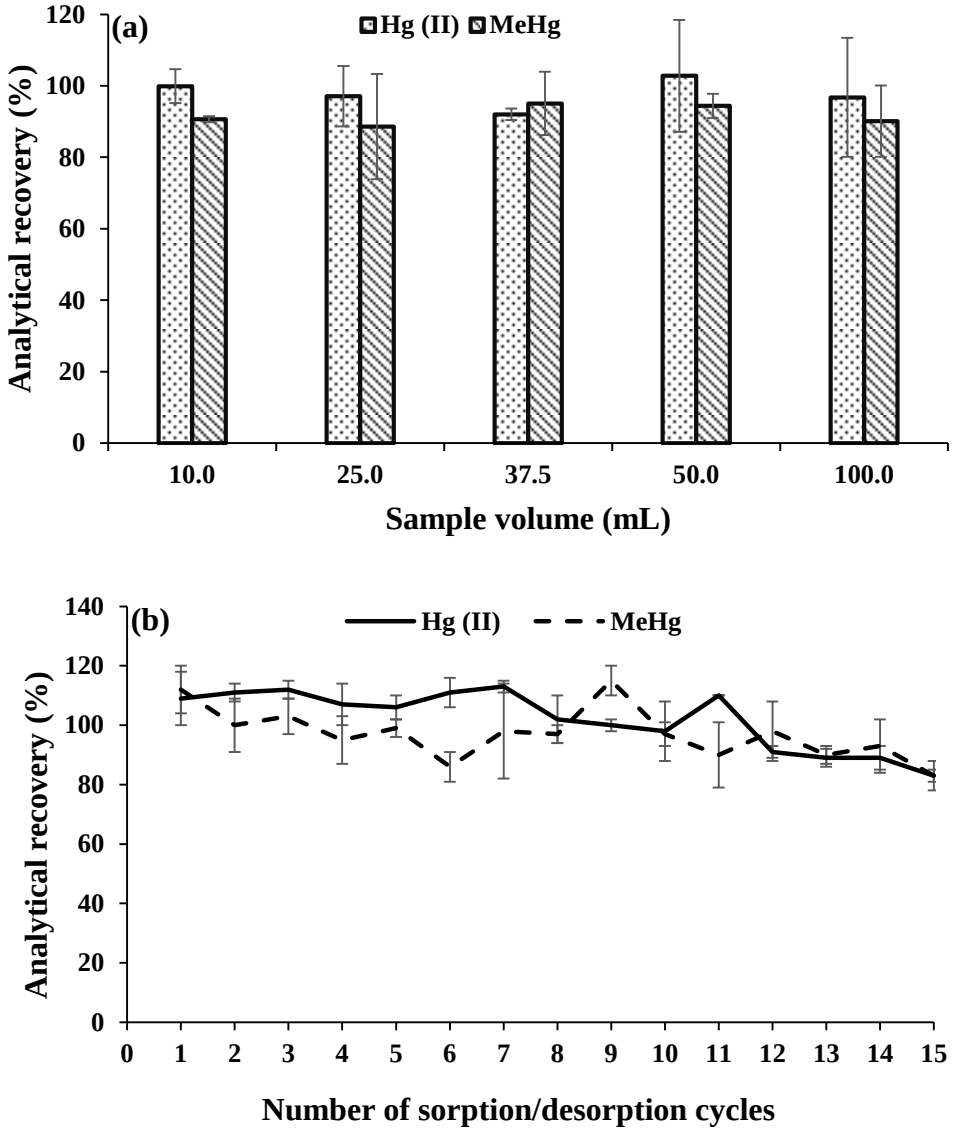

(d)
MeHg
Hg (II)
MeHg analytical recovery (%)
120
100
80
60
40
20
0
30
20
10
0
0.5
1.0
2.0
3.0
4.0
Volume of the eluting solution (mL)
[Hg (II)] (μg L-1)

Figure 4


(a)
Hg (II)
MeHg
Analytical recovery (%)
120
100
80
60
40
20
0
10.0
25.0
37.5
50.0
100.0
Sample volume (mL)
(b)
Hg (II)
MeHg
Analytical recovery (%)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Number of sorption/desorption cycles