EVALUATION OF MONOLITHIC COLUMN FOR INORGANIC MERCURY AND METHYLMERCURY DETERMINATION IN FISH SAMPLE ANALYSIS

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ABSTRACT

In the present work, the applicability of a monolithic column was evaluated for mercury speciation analysis in certified and real fish samples. The influence of the flow and composition of the mobile phase (2-mercaptoethanol concentration and methanol content) on the retention times of iHg and MeHg was evaluated. The optimal separation conditions were achieved for a mobile phase containing 5 % methanol and 0.02 % mercaptoethanol in acetate buffer (pH 5.0) at a flow rate of 1.6 ml min^{-1}. Different extraction conditions were studied to analyze a certified reference material with the optimized methodology and the best results (93 % recovery) were obtained for microwave-assisted extraction with a mixture of HCl 6 M + NaCl 0.5 M. Finally, the proposed method was satisfactorily applied to real fish samples collected from different markets in the central region of Chile, presenting levels ranging from 0.73 – 3.4 mg kg^{-1}.

Keywords:
Mercury speciation
Monolithic column
Fish samples

1. INTRODUCTION

Mercury (Hg) is a contaminant widely distributed in the environment and is considered as one of the most toxic metals for biological species and mammals [1,2]. In general, the toxicity of mercury is extensively documented and depends on its concentration and chemical forms, of which organic compounds, such as methylmercury (MeHg), are more toxic [1]. For human beings, the exposure to mercury species, especially methylmercury, can adversely affect the nervous, cardiovascular and immune systems. The general population can be exposed to mercury in several ways, especially, via ingestion of contaminated seafood [3-5]. The FDA (Food and Drug Administration) has set a maximum allowable level of 1 mg kg^{-1} of MeHg in seafood products, while the European Food Safety Authority (EFSA) proposed a maximum allowable level of 0.5 mg kg^{-1} in all types of fish, except predators, for which the maximum limit is 1.0 mg kg^{-1} of MeHg (Commission Regulation 466/2001). Considering the variability of fish consumption among countries, a tolerable daily index (TDI) has been proposed with the aim of minimizing the impact of exposure. The USEPA and the NAS/NRC have set a value of 0.1 µg (Hg)/kg/day, while recent recommendations from the EFSA/WHO based on epidemiological studies set maximum levels at 4 µg/kg per week and 1.3 µg/kg per week, for inorganic mercury and MeHg, respectively. However, there is evidence that exposure in populations with high levels of seafood consumption can be considerably higher than these values [1].

In recent years, a large number of analytical methodologies have been proposed for mercury speciation analysis [6-8]. In general, this analysis is conducted by using a chromatographic separation technique coupled with a selective detection method [9-11]. One common and simple approach considers liquid chromatographic separation of mercury species followed by on-line UV-oxidation and detection by cold vapor (CV)-atomic fluorescence spectrometer (HPLC-UV-CV-AFS). This system has been applied satisfactorily to determine mercury species in different media, such as fish tissue [10], soils [12], human hair [13], urine and blood [14]. For chromatographic separation, a reversed phase-based C-18 stationary phase and aqueous buffer-organic modifier mixed mobile phases are commonly used. In addition, thiol reagents, such as mercaptoethanol [15], diethylditiocarbamate [16] or L-cysteine [17], are added to the mobile phase to improve the retention of ionic mercury species. These approaches have shown adequate analytical performance, and the mercury species can be separated in isocratic mode [6].

Monolithic supports were developed in the 90's and, since then, have been shown to be efficient stationary phases for different applications (e.g., separation of biomolecules, organic acids and inorganic anions) [18-20]. Some of the advantages of monolithic columns over particle-packed columns are fast separation, short analysis time and low column pressure drop [21]. Despite these advantages, the potential use of monolithic phases for elemental speciation analysis is scarcely reported. Although recent reviews demonstrate the applicability of these columns for elemental speciation analysis (e.g., arsenic, chromium) [22] and for metal-biomolecules analysis [23], these analyses have not been extended to mercury speciation so far. In this work, we evaluate a monolithic silica-based column for inorganic mercury and methylmercury in fish samples.

2. EXPERIMENTAL

2.1. Reagents and standards

All chemicals used in this study were analytical grade. Reagents and standard solutions were prepared using an ultrapure water system (Millipore, Bedford, MA, USA).

A Certipur® nitrate mercury (II) standard (1000 mg L^{-1}, in HNO_3 2 mol L^{-1}) was obtained from Merck (Darmstadt, Germany). A methylmercury chloride standard (CH_3HgCl, 1000 µg/mL in H_2O) was purchased from LGC standards (Manchester, USA). Stock solutions of these reagents were stored at 4 °C in the dark. Working standards were obtained by diluting with water weekly for solutions of 10 mg (Sn) L^{-1} and daily for 10-100 µg (Sn) L^{-1}.

Fuming hydrochloric acid 37 % and NaCl (p.a. EMSURE®) were obtained from Merck (Darmstadt, Germany), and 2-mercaptoethanol (99 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 2.0 % (w/v) solution was prepared with tin (II) chloride ($SnCl_2x2H_2O$, 98 %) purchased from Lobachimie (Mumbai, India). Prior to use, this solution was purged with nitrogen for 20 minutes to eliminate mercury impurities.

A mixture of 6 M HCl and 0.5 M NaCl was selected as the most suitable extraction agent. Glassware was rinsed with deionized water, decontaminated overnight in a 20 % (v/v) nitric acid solution and then rinsed again with deionized water.

2.2. Instrumentation

An atomic fluorescence spectrometer (Millennium Merlin, Model 10.025, PS Analytical, Orpington, Kent, England) was used to determine the Hg concentrations by cold vapor-atomic fluorescence spectrometry (CV-AFS).

The chromatographic separation of Hg species was performed using a Jasco HPLC PU-2089S Plus chromatograph (Easton, MD, USA) equipped with quaternary pumps, degassers, an injector with a 20 µL loop and an RP-column. The mobile phase was pumped with a flow rate of 1.6 ml/min. Post-column oxidation of MeHg to Hg^{2+} was achieved by UV irradiation (Integrated UV-cracker/Heating/Cooling unit Model 10.820, P.S. Analytical, Ltd., Orpington, UK). The reductant was added at a flow rate of 5 ml/min to convert Hg^{2+} to Hg^0 with the addition of HCl 0.1 M at a flow rate of 10 ml/min. Finally, the detection of mercury species was performed by the PSA Millennium Merlin atomic fluorescence spectrometer. The HPLC and CV-AFS conditions are shown in Table 1.

The digestions and extractions were performed in a closed-vessel microwave digestion system Model START D (Milestone, Sorisole, Italy).

2.3. Sample collection and treatment

Different frozen, canned and fresh fish samples were collected from different local stores throughout Valparaiso city. These samples were thawed, crushed, homogenized and frozen to -20 °C overnight, freeze-dried, and stored at 4 °C in the dark until analysis.

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Table 1: HPLC-CV-AFS optimized conditions for determination of inorganic mercury and methylmercury.

HPLC System	
RP-C18 column	Chromolith® RP-18e column (100 mm × 4.6 mm)
Mobile phase	95 % Phase A + 5 % Phase B **Phase A**: Buffer pH 4.8 (Sodium acetate/acetic acid, 0.1 M) + 0.01 % 2-mercaptoethanol. **Phase B**: Methanol
Flow rate	1.6 ml min^{-1}
Injection Volume	20 µl
CV-AFS detection system	
HCl concentration	1.5 M
HCl flow	10 ml min^{-1}
$SnCl_2$ concentration	2 % w/v in HCl 1.5 M
$SnCl_2$ flow	5 ml min^{-1}
Argon flow	300 ml min^{-1}

2.4. Analysis of certified and real fish samples

For the total mercury determination, 0.2 g of the freeze-dried samples was directly introduced to microwave vessels with 4.0 ml of HNO_3 and 1.0 ml of H_2O_2. The microwave vessel was closed, heated to 180 °C for 20 minutes and cooled to room temperature over 10 minutes. The digested solution was transferred to a 25 ml volumetric flask and the sample was brought to volume with deionized water.

For mercury speciation, 0.2 g of freeze-dried samples was introduced directly to microwave vessels together with 10 ml of the extractant solution. The suspension was heated to 55 °C for 25 minutes and cooled to room temperature for 10 minutes. The supernatant was transferred to a 10 ml volumetric flask and was brought to volume with an acetate-acetic acid buffer (pH 4.8, 0.1 M) containing 2-mercaptoethanol (0.02 % v/v). This solution was filtered with a PVDF-filter (Sterlitech, USA, 0.22 µm) and injected in the HPLC-UV-CV-AFS system.

2.5. Evaluation of the figures of merit

Analytical figures of merit, such as the detection limit (LOD), quantification limit (LOQ) and precision (relative standard deviation, % RSD), were evaluated according to IUPAC recommendations [24].

The accuracy of the developed methodology was assessed by the determination of total mercury and methylmercury concentrations in a certified reference material (Tuna Fish, ERM-CE 464).

To evaluate the precision, the RSD (%) was evaluated from replicates obtained from standards containing a mobile phase and an appropriate amount of analyte, with mercury species contents varying from 20 to 80 µg L^{-1}.

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic separation

In this study, the separation method evaluated considers the complexation of cationic mercury species with 2-mercaptoethanol, producing more hydrophobic compounds. These species can be retained on a C-18 stationary phase, where the content of organic modifier (i.e., methanol) and the flow rate of the mobile phase can control the retention times of the mercury species. Then, to propose one reproducible and efficient separation method for simultaneous determination of methylmercury (MeHg) and inorganic mercury (iHg), the effects of experimental factors were studied and optimized.

In Figure 1, the effect of methanol content in the mobile phase on the retention times (RTs) of MeHg and iHg are presented. As expected in reversed-phase separation, the retention times of both species decreased when the methanol content increased. Additionally, significant differences in the RTs are observed with methanol content lower than 5 %., while for major contents (> 10 %) both compounds are not retained and eluted in the dead time. Then, the highest resolution is reached for 1 % of methanol. However, this condition produces a relatively high retention time and a slight band broadening, which was probably due to longitudinal diffusion.

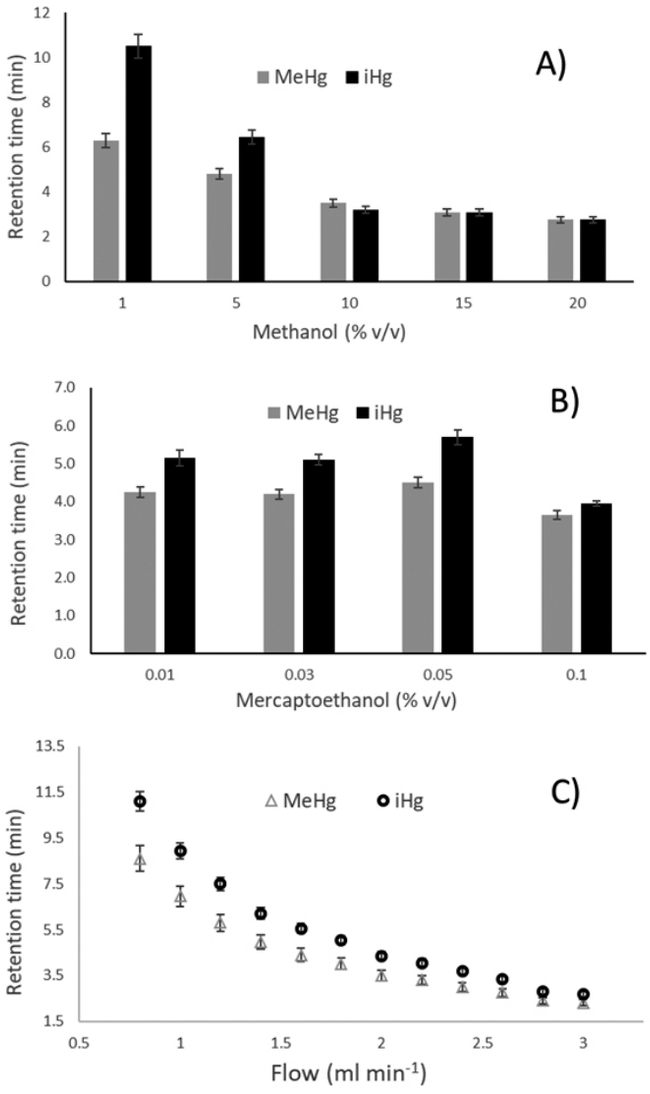

Figure 1. Influence of A) methanol content, B) mercaptoethanol concentration and C) flow rate on methylmercury (MeHg) and inorganic mercury (iHg) retention times. The experiments A and B were realized at 1.0 ml/min.

The influence of the 2-mercaptoethanol concentration in the mobile phase on the retention times of both mercury species is presented in Figure 1-B. For MeHg, no significant influence is observed, while for iHg a slight increase in RT is observed, especially between 0.01 and 0.05 % mercaptoethanol, which could be attributed to the different stoichiometry of the two complex species. While one mole of MeHg requires only one mole of 2-mercaptoethanol, one mole of iHg requires at least two equivalent moles. However, the greatest differences in RT are observed for 0.05 % of this complexing compound, and this condition was considered for the following experiments.

To adjust the analysis time with an adequate separation resolution, the effect of the flow rate was evaluated. The results are presented in Figure 1-C. As can be observed, the retention time of both species decreases with increasing mobile phase flow, and the best separation is reached when the flow is lower than 2.5 ml min^{-1}. In addition, the peak width decreases from 1.5 to 0.36 min when the flow is increased to 0.8 and 3.0 ml min^{-1}. These results are consistent with the small effect of mass transfer on peak broadening for high flow in a monolithic column [19]. Additionally, the resolution reaches an optimal value of 1.5 for a flow between 1.4 and 1.8 ml min^{-1}. Therefore, the best compromise between analysis time and resolution was reached for 1.6 ml min^{-1}, and this value was employed for subsequent experiments.

Table 2. Comparison of reported analytical method and proposed method.

Chemical species	Technique	Analytical column	Separation conditions	Analysis time (min)	REF
Hg^{2+},CH_3Hg^+, $C_2H_5Hg^+$, $PhHg^+$	HPLC–CV-AFS	HyPurity RP/C18 (250 × 4.6mm, 5µm)	Gradient elution. Phase A: Cysteine 5 mmol L-1 in water Phase B: Cysteine 5 mmol L^{-1} in metanol. Flow rate: 1.0 mL min^{-1}	7[A]	[25]
Hg^{2+}, CH_3Hg^+	HPLC-CV-AFS	Eclipse XDB C8 (150 x 4.6 mm, 5 µm)	Isocratic elution. Phase: 75% methanol + 1,5 mM ammonium pyrrolidine dithiocarbamate. Flow rate: 1.0 mL $min^{-1.}$	13	[26]
Hg^{2+}, CH_3Hg^+	HPLC-CV-AFS	Eclipse XDB C8 (150 x 4.6 mm, 5 µm)	Isocratic elution. 75 % methanol, 1.5 mM Ammonium pyrrolidine dithiocarbamate. Flow rate: 1.0 mL $min^{-1.}$	10	[27]
Hg^{2+}, CH_3Hg^+	HPLC-ICP–MS	Zorbax Eclipse Plus C-18 (100 x 4.6 mm, 3.5 µm)	Isocratic elution. Phase: 55 % methanol, 0,1% mercaptoethanol, 60 mmol L^{-1} ammonium acetate (pH 4.0). Flow rate: 0.45 mL min^{-1}	4	[28]
Hg^{2+},CH_3Hg^+, $C_2H_5Hg^+$, $PhHg^+$	HPLC-CV-AFS	Hypersil BDS C18 (125 x 2 mm, 3 µm)	Isocratic elution. Phase: 7 % metanol + 0.05% 2-mercaptoethanol at pH 5 in acetate buffer. Flow rate: 0.15 mL min^{-1}	20[A]	[15]
Hg^{2+}, CH_3Hg^+	HPLC-CV-AFS	Hypersil ODS C18 (250 x 4.6mm, 5 µm)	Isocratic elution. Phase: 80% Methanol + 0.0015 mol L^{-1} APDC and 0.01 mol L^{-1} NH_4CH_3OO (pH 5.5). Flow rate: 1.5 mL $min^{-1.}$	10	[29]
Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$	HPLC-CV-AFS	Venusil MP-C18 (150 x 4.6 mm, 5µm)	Isocratic elution. Phase: 3% acetonitrile + 2-mercaptoethanol 0.1% in 60 mM ammonium acetate-acetic acid (pH 4.5). Flow rate: 2.0 mL min^{-1}	7[A]	[30]
Hg^{2+}, CH_3Hg^+	HPLC-CV-AFS	Hypersil ODS RP/C18 (250 x 4.6mm, 5 µm)	Isocratic elution. Phase: 80% metanol + 0.0015 mol L^{-1} APDC and 0.01 mol L^{-1} NH_4CH_3OO (pH 5.5). Flow rate: 1.5 mL min^{-1}	8[A]	[31]
Hg^{2+}, CH_3Hg^+	HPLC-CV-AFS	Chromolith® RP18-e (100 x 4.6 mm)	Isocratic elution. Phase: 5 % methanol + 0.01 % 2-mercaptoethanol in acetate buffer (pH 4.8). Flow: 1.6 mL min^{-1}	6	This work

[A]: Time required for elution of iHg and $MeHg^+$. APDC: Ammonium pyrrolidine dithiocarbamate. HPLC: High performance liquid chromatography. CV: Cold vapour. AFS: Atomic fluorescence spectroscopy.

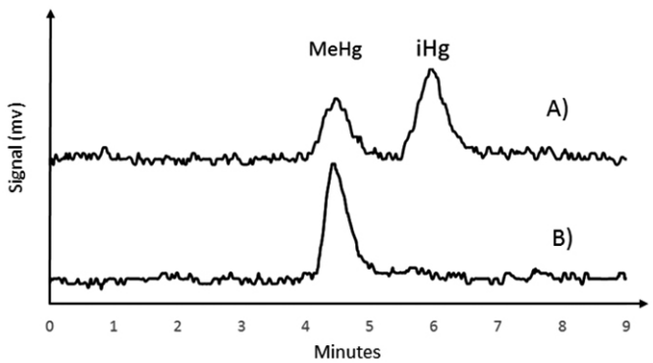

Figure 2. Typical chromatogram obtained for A) Standard solution (10 µg Hg l^{-1}) and B) Certified reference material extract (Tuna Fish, ERM-CE 464) analyzed using optimal conditions (see Table 1).

A typical chromatogram obtained in optimal conditions for a standard solution and for real sample extract is presented in Figure 2. As expected, a good separation and an adequate resolution are observed for synthetic and real samples for both species.

In Table 3, the chromatographic conditions for methods recently reported for the determination of iHg and MeHg are summarized and compared with the proposed method. As seen, our method requires a shorter analysis time compared with those in reported methods. ICP-MS detection-based method is the only method requiring the lowest time because this technique does not require the reduction and photooxidation steps that are mandatory for CV-AFS. In addition, the required methanol content is considerably lower than that in previously reported methods, which is very interesting for the economy of solvents and for the application of this separation method coupled with atomic detection techniques (e.g., ICP-MS based) where organic solvents are detrimental for routine analysis.

3.2. Evaluation of figures of merit

Linearity was established by measuring iHg and MeHg standards and reaching a limit of 200 µg l^{-1}. The coefficient of regression (R^2) was highest at 0.99 for both species. The analytical repeatability was determined by the analysis of six replicate standard solutions resulting in a coefficient of variation of 7.5 %. The limit of quantification (LOQ) values were 2.5 and 8.8 µg l^{-1} for MeHg and iHg, respectively. These values are comparable to similar methods proposed in the literature and were adequate for monitoring mercury species in seafood according to FAO and EFSA recommendations.

3.3. Application to certified and real fish samples

To efficiently extract mercury species from fish samples, the extracted methylmercury concentration from the certified fish (Tuna fish ERM-CE 464) was evaluated using three different extraction solutions: 1) HCl 6M; 2) HCl 6M + NaCl 0.5M; and 3) tetramethylammonium hydroxide (25 %). In this way, the recovery (%) obtained for each solution was 78 %, 93 %, and 76 %, respectively. Clearly, the best recovery was obtained with solution 2, with a concentration of 5.09 ± 0.14 mg kg^{-1} (Certified value: 5.50 ± 0.17 mg kg^{-1}), which was probably due to the combined effect of hydronium and chloride ions for extraction and the solubilization of methylmercury during the extraction step. Similar results were reported in previous studies, where closed-vessel microwave-assisted extraction was not considered [15]. For these reason, this method was considered for following analyses.

The results obtained for real fish samples are presented in Table 3. As expected, the methylmercury content exceeds 80 % of the total mercury for all analyzed samples. In addition, the fresh samples that were acquired in fishing coves present methylmercury levels higher than the recommended values, whereas the commercial products do not exceed these limits. These differences

could be explained by the control of industrial activities by governmental authorities, which is not the case for artisanal fisheries existing in fishing coves. The major control of fish used for consumption must be audited in order to decrease the human exposition risk, especially for children and pregnant women.

Table 3: Total mercury and methylmercury levels found in fish samples collected in supermarkets (C) and fishing coves (F) from some chilean coast sites. Concentration expresed as mean ± standard deviation (n=3).

	Total Mercury (mg kg $^{-1}$)		Methylmercury (mg kg $^{-1}$)		Codex Maximum Level[A]	RSA Chile Maximum level[B]
Fish sample	Dry	Wet	Dry	Wet		
Albacore (F)	3.8 ± 0.1	2.29 ± 0.06	3409 ± 35	3409 ± 35	1.0 mg kg $^{-1}$ of MeHg for predatory species	1.5 mg kg $^{-1}$ of MeHg for predatory species
Albacore (C)	1.69 ± 0.09	1068 ± 56	1475± 49	1475 ± 49		
Shark (F)	1.38 ± 0.04	839 ± 26	1090 ± 56	644 ± 32		
Tune (C)	0.87 ± 0.04	529 ± 25	731 ±28	439 ± 17		

[A]: Recommended values (wet weigth), currently in discussion (www.fao.org ; last revision: march 2018).
[B]: Maximum level (wet weigth), Reglamento sanitario de los alimentos (DTO 977/96), Minsal, Chile, 13-05-1997 (www.minsal.com)

4. CONCLUSIONS

An analytical method was developed to simultaneous determine inorganic mercury and methylmercury with chromatographic separation using a monolithic column. In optimal conditions, this separation procedure results were faster and required lower organic solvent consumption compared to reported methods.

Finally, the proposed method was applied to certified and real fish samples, demonstrating good analytical performance and the applicability of the proposed methodology to complex biological samples. Additionally, some fish samples contain mercury levels higher than the recommended values, and their consumption can pose risk to human populations, indicating the need for major control of fish, especially for fresh samples.

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