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Usefulness of ionic liquids as mobile phase modifiers in HPLC-CV-AFS for mercury speciation analysis in food

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Different ionic liquids (ILs) were studied in this work as mobile phase modifiers for the separation and determination of Hg^{2+}, methylmercury (CH_3Hg^+) and ethylmercury ($C_2H_5Hg^+$) species by reversed-phase high-performance liquid chromatography coupled to UV-cold vapor atomic fluorescence spectrometry (RP-HPLC-UV-CV-AFS). Several parameters influencing the chromatographic separation of Hg species, such as pH, sodium chloride concentration, organic solvent concentration, as well as chemical structure and concentration of ILs were evaluated. After a careful optimization, the separation of Hg species was achieved within 12 min using a C_{18} column and a gradient developed by mixing methanol and a solution composed of 0.4% (v/v) 1-octyl-3-methylimidazolium chloride [C_8mim]Cl, 100 mmol L^{-1} NaCl and 20 mmol L^{-1} buffer citric acid/citrate at pH 2.0. In addition, a multivariate methodology was applied to optimize the parameters involved in UV-CV-AFS detection of Hg species. The proposed method allowed the separation of inorganic and organic Hg species in a single chromatographic run. The limits of detection obtained for Hg species were in the range of 0.05–0.11 μg L^{-1}. The usefulness of the proposed method was demonstrated by performing Hg speciation analysis in highly complex samples, such as seafood, yeast and garlic, obtaining accurate and precise results in all cases.

1. Introduction

Mercury (Hg) and its different chemical species, mainly Hg^{2+}, methylmercury (CH_3Hg^+), ethylmercury ($C_2H_5Hg^+$) and phenylmercury ($C_6H_5Hg^+$), are considered very harmful pollutants, due to their high toxicity and the ability to accumulate in biological tissues and different environmental compartments.[1] Therefore, several countries have established maximum residual limits (MRLs) for total Hg and some Hg species.[2] For example, the European Commission Regulation 1881/2006/EC establishes MRLs of 0.5 mg kg^{-1} in fresh water fish and 1.0 mg kg^{-1} in seafood.[3,4] Also, the Codex Alimentarius establishes MRLs of 1.0 μg L^{-1} for total Hg in mineral water, and 0.5 μg g^{-1} and 1 μg g^{-1} for CH_3Hg^+ in non-predatory and predatory fish, respectively.[5] Likewise, the World Health Organization and the United Nations Environment Programme regulate Hg levels in blood at 10 μg L^{-1} and urine at 50 μg g^{-1} (as creatinine).[6] On the other hand, although there are not Hg MRLs specifically defined for garlic and yeast, total Hg concentrations are very low in these types of foods according to a study reported by the European Food Safety Authority (EFSA) (6–7.8 μg kg^{-1} in vegetables and 96–102 μg kg^{-1} in products for special nutritional use).[7]

Another important matter about Hg is the high dependence existing between toxicity and its different species.[1] In fact, owing to the lipophilic nature of organic Hg species, *i.e.* CH_3Hg^+, $C_2H_5Hg^+$ and $C_6H_5Hg^+$, these are more toxic than inorganic Hg.[8] Therefore, there is continuing demand for accurate and highly sensitive methods for Hg speciation analysis in food, biological and environmental samples.[9] To fulfill those needs, the coupling of instrumental separation techniques such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) with atomic spectrometry detectors has been exploited for Hg speciation analysis due to its several advantages, including automation, reproducible retention times and separation of a high number of Hg species within one single chromatographic run.[8,10] Among these separation techniques, HPLC is highly applied due to the possibility of separating elemental species that markedly differ in their chemical structure and properties, such as those of Hg. Thus, Hg speciation analysis has been performed by coupling HPLC with cold vapor generation atomic fluorescence spectrometry (CV-AFS),[11,12] inductively coupled plasma mass spectrometry (ICP-MS)[13,14] and CV atomic absorption spectrometry (CV-AAS).[15,16] Due to the high sensitivity of ICP-MS, the HPLC-ICP-MS technique has been

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applied to determine Hg species in environmental water samples with very low detection limits (1.0 and 0.3 ng L^{-1} for Hg^{2+} and CH_3Hg^+, respectively).[2] Likewise, HPLC-ICP-MS has been used for Hg speciation analysis in freshwater fish with detection limits in the order of 0.60 μg kg^{-1} for Hg^{2+}, 0.56 μg kg^{-1} for CH_3Hg^+, 1.12 μg kg^{-1} for $C_2H_5Hg^+$ and 1.68 μg kg^{-1} for $C_6H_5Hg^+$.[14] However, the relatively high costs of acquisition, operation and maintenance of HPLC-ICP-MS can still limit its implementation in some analytical laboratories dedicated to food quality control.[14] Likewise, other drawbacks of this technique are the high number of polyatomic interferences and matrix effects caused when complex samples like blood, serum or urine are analyzed.[17] A much more cost-effective technique that is becoming popular in routine analytical laboratories for sensitive Hg speciation analysis is HPLC-CV-AFS.[18] For example, the determination of Hg^{2+} and CH_3Hg^+ species in seafood samples has been performed by HPLC-CV-AFS and low detection limits were obtained (0.3 μg CH_3Hg^+ kg^{-1} and 0.4 μg Hg^{2+} kg^{-1}).[19] In a different method, the same technique was applied to marine organisms certified reference materials and limits of detection of 7.5, 7.5 and 17 μg kg^{-1} were obtained for Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$, respectively.[20]

Ion exchange (IEC) and reversed-phase (RP) are the most common principles used for HPLC separation of Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$ and $C_6H_5Hg^+$ species.[10,21] Normally, RP-HPLC separation of Hg species is performed with mobile phases consisting of a mix of buffer, organic solvents and a counter ion or chelating agent.[22] Thus, L-cysteine, ammonium or sodium pyrrolidinedithiocarbamate (APDC, SPDC) can improve the separation of Hg species due to the formation of stable covalent bonds with mercapto functional groups. This derivatization avoids polar interactions between cationic Hg species and free silanol groups of the stationary phases, however special care is needed (*i.e.* a right pH, ionic strength, *etc.*) to assure the chemical stability of the chelating reagents throughout analysis and the formation of the complexes.[23] Also, tetraalkylammonium salts have been used as ion pairing reagents for the separation of Hg species by RP-HPLC.[24] The retention mechanism could involve the initial formation of anionic complexes between the cationic Hg species and halides (*e.g.* chloride), followed by the formation of the ion pairs with tetraalkyammonium cations.[25] However, the use of organic solvents in the mobile phases of RP-HPLC causes some drawbacks in detectors such as ICP-MS (*e.g.* plasma destabilization, signal drift and carbon deposition on the sampler and skimmer cones).[14] In order to solve these problems, alternative ion-pairing reagents must be assayed for Hg species separation.

Ionic liquids (ILs) are defined as salts with melting points at or close to room temperature and their physicochemical properties depend on the chemical nature and size of the constituting cations and anions.[26,27] The use of ILs in RP-HPLC as mobile phase modifiers can significantly reduce peak tailing by suppressing the activity of silanol groups, thus shortening the retention time of polar analytes.[28] Furthermore, cations and anions of ILs structures can be proposed as effective tools to form ion pairs with different elemental species.[29] Previous works done in our laboratory have confirmed the aforementioned properties of some ILs, such as 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl) and 1-octyl-3-methylimidazolium chloride ([C_8mim]Cl), for the speciation analysis of Se and As in complex samples, including wine, beer, yeast and garlic.[30,31] Despite it can be considered that ILs offer a fresh approach for looking at novel intermolecular interactions and retention processes, there are still no reports on their application in RP-HPLC to separate Hg species.

In this work, the potential of some ILs as mobile phase modifiers for RP-HPLC separation of Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$ species is explored. The effects of different alkyl methyl imidazolium ILs and a phosphonium IL on RP-HPLC separation and CV-AFS detection are discussed in detail. Also, the influence of different variables such as pH, ionic strength and methanol concentration in the mobile phase is presented along with multivariate optimization results of CV-AFS detection conditions. The high potential of ILs for the separation and determination of Hg species is demonstrated in this work by analyzing different complex matrix samples, such as seafood, yeast and garlic.

2. Experimental

2.1 Instrumentation

The separation of Hg species was performed with an HPLC instrument that included a YL9101 vacuum degasser, a YL9110 quaternary solvent delivery pump (YL Instrument Co., Ltd., Seoul, South Korea), a Rheodyne valve with a 100 μL loop injector (Cotati, CA, USA) and a Hypersil GOLD aQ C_{18} from Thermo Scientific (Massachusetts, USA). Detection of Hg species was performed with a Rayleigh AF-640A atomic fluorescence spectrometer (Beijing Rayleigh analytical Instrument Corp., Beijing, China) that included a Hg hollow cathode lamp. An on-line UV photo-oxidation unit was connected between the HPLC and AFS instruments for decomposition of organic Hg species. This consisted of a polytetrafluoroethylene (PTFE) tube (0.5 m length, 0.8 mm I.D. and 1.2 mm O.D.) wrapped around a 20 W Hg UV lamp. Instrumental conditions of HPLC-CV-AFS hyphenated technique are shown in Table 1. An ultrasound bath (40 kHz and 600 W) with temperature control (Test Lab, Buenos Aires, Argentina) was used to degas the mobile phases and solvents before chromatography. The pH measurements were performed with a Horiba F-51 pH meter (Kyoto, Japan). A freeze-dryer Virtis freeze mobile (New York, USA) Model 6 Lyophilizer 12 L and a grinder Ultracomb (Buenos Aires, Argentina) model MO-8100A were used to dry and homogenize the samples, respectively. Extraction of Hg from the samples was performed using a LUGUIMAC LC-15 centrifuge (Buenos Aires, Argentina) and a Vicking vortex (Buenos Aires, Argentina). An Ohaus Pionner PA214 (Melrose, USA) balance was used.

2.2 Reagents and solutions

A 1000 mg L^{-1} stock Hg^{2+} standard solution was prepared dissolving 135.4 mg of $HgCl_2$ 99.9% (Merck, Darmstadt, Germany) in 100 mL of 0.1 mol L^{-1} HNO_3 solution (Merck). Lower concentrations were prepared by diluting the stock solution

Table 1 HPLC-UV-CV-AFS instrumental conditions

HPLC	
Column	Thermo Scientific – Hypersil GOLD aQ – C_{18} column (150 mm × 4.6 mm i.d.)
Mobile phase	(A) 100% (v/v) methanol
	(B) 0.4% (v/v) [C_8mim]Cl; pH = 2.0; 0.02 mol L^{-1} citric acid/citrate buffer; 0.1 mol L^{-1} NaCl
Mobile phase flow rate	1.0 mL min^{-1}
Gradient	0–12 min 5–100% (A); 12–17 min 100% (A)
Injection volume	100 μL
Column temperature	25 °C
UV-CV-AFS	
Lamp and wavelength	Hg high intensity hollow cathode lamp, 253.7 nm
Main current	40 mA
Auxiliary current	0 mA
Photomultiplier detector voltage	−270 V
Atomization temperature	100 °C
Reductant	0.1% (w/v) $NaBH_4$ in 0.05% (w/v) NaOH at 6 mL min^{-1}
Carrier	3% (v/v) HNO_3 at 6 mL min^{-1}
Post-column reductant	0.75% (w/v) $K_2S_2O_8$ in 10% (v/v) HCl at 1.5 mL min^{-1}
Carrier gas	Argon at 400 mL min^{-1}
UV lamp	Hg lamp, 20 W
Reaction coil	PTFE tube, 0.5 m length, 0.8 mm I.D., 1.2 mm O.D

with ultrapure water. Individual stock solutions of organic Hg species at 1000 mg Hg L^{-1} were prepared by weighting 126.4 mg of CH_3HgCl 99% (Sigma-Aldrich, St. Louis, MO, USA) and 132.2 mg of C_2H_5HgCl 99% (Merck), followed by dissolution with methanol (Merck) up to a final volume of 100 mL. These solutions were stored away from light at 4 °C to prevent decomposition of Hg species. All working standard solutions were prepared daily. Under the aforementioned conditions, the Hg species were fully soluble.[32]

The reducing agent was $NaBH_4$ (Fluka) prepared at 0.05% (w/v) NaOH (Aldrich) and the carrier agent was HNO_3 (Merck).[33] Tygon type tubes (Gilson, Villiers Le-Bell, France) were used to carry the reagents. All bottles used to store the samples, standard solutions and mobile phases along with glassware were washed in 5% (v/v) HNO_3 (Merck) for 24 h and later rinsed with ultrapure water (18 MΩ cm).

Several ILs including, 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl), 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl), 1-octyl-3-methylimidazolium chloride ([C_8mim]Cl) and 1-dodecyl-3-methylimidazolium bromide ([C_{12}mim]Br) were synthesized according to a method proposed by Baltazar *et al.*[34] Acceptable purity for the ILs (about 90% or higher) can be achieved according to that procedure. The structures and properties of these ILs are shown in Table 2. Characterization of synthesized ILs was performed based on infrared spectra and spectral purity was confirmed in all cases. Tributyl(methyl) phosphonium methylsulphate ([$P_{4,4,4,1}$]CH_3SO_4) (95%) was purchased from Sigma-Aldrich. Stock solutions of [C_4mim]Cl, [C_6mim]Cl and [C_8mim]Cl at 5% (v/v), [C_{12}mim]Br and [$P_{4,4,4,1}$]CH_3SO_4 at 5% (w/v) were prepared in ultrapure water and assayed as mobile phase modifiers. Citric acid (Sigma-Aldrich), acetic acid (Sigma-Aldrich) and sodium dibasic phosphate (JT Baker, Pennsylvania, USA) were used as buffers for pH adjustment. Total Hg determinations were performed with concentrated HNO_3 (Merck) and HCl (Merck).

2.3 Collection and treatment of food samples

Seafood, garlic and yeast samples were purchased at local supermarkets (Mendoza, Argentina). Different fresh seafood samples were analyzed in this work, including silverside, chilean jack mackerel, praw and sea bream. Also, different canned fish samples such as hake, mackerel, mussel and tuna were studied in this work. Before analysis, soft tissues of seafood, garlic and yeast were homogenized, lyophilized during 48 h and then stored at −18 °C.

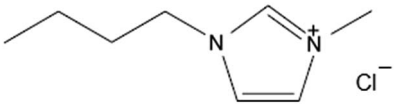
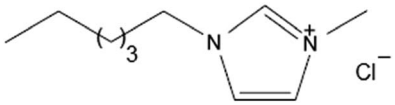
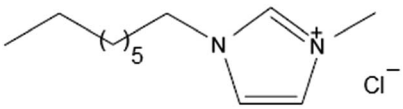
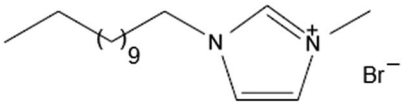
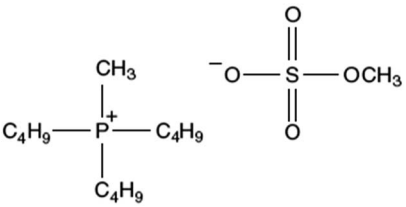
2.4 Total Hg determination

Total Hg determination was performed by CV-AFS technique. A wet digestion procedure was applied based on a reported procedure with some modifications.[35] Basically, 1.0 g of each sample was added with 5.0 mL HNO_3 and 5.0 mL HCl and the mixture was left overnight at room temperature. Afterwards, the suspension was heated at 110 °C on a hotplate for 4 hours using a cooling condenser under constant reflux to avoid Hg losses by volatilization. Clear digestion solutions were obtained when this procedure was applied to the different samples; however, a filtration was performed in order to separate fine particles that could still remain in the solutions. Finally, the sample solutions were adjusted to 5% (v/v) HNO_3 and diluted to 50 mL in a volumetric flask with ultrapure water. The conditions for CV-AFS determination were as follows: 0.15% (w/v) $NaBH_4$ (in 0.05% (w/v) NaOH) as reducing agent, 5% (v/v) HNO_3 as carrier at 12 mL min^{-1} flow rate. Argon flow rate was 600 mL min^{-1} and atomization temperature was 200 °C. Other instrumental parameters were as mentioned in Table 1. Three replicate determinations were performed per each analyzed sample.

2.5 Mercury speciation analysis by HPLC-UV-CV-AFS

Acid extraction of Hg species from seafoods, garlic and yeast samples was performed following a procedure reported

Table 2 Chemical structure and properties of the ILs used in this work[49,50]

Chemical formula and name[a]	Structure	Properties (25 °C)	
		Density (g mL^{-1})	Viscosity (mPa s)
[C_4mim]Cl, 1-butyl-3-methylimidazolium chloride		1.08	147
[C_6mim]Cl, 1-hexyl-3-methylimidazolium chloride		1.03	716
[C_8mim]Cl, 1-octyl-3-methylimidazolium chloride		1.01	337
[C_{12}mim]Br, 1-dodecyl-3-methylimidazolium bromide		N.R.[b]	N.R.[b]
[$P_{4,4,4,1}$]CH_3SO_4, tributyl(methyl)methylsulfatephosphonium		1.067	409

[a] All ILs are soluble in water. [b] Not reported.

elsewhere.[36] Basically, 300 mg of each sample was added with 5 mL of a 5 mol L^{-1} HCl solution in a centrifuge tube and the mixture sonicated for 10 min using an ultrasonic bath. The Hg extraction efficiencies obtained with these procedures were: 82–87% for seafoods, 80% for garlic and 51% for yeast. After the extraction, the suspension was centrifuged at 3000 rpm for 15 min. The extract was then transferred to a new centrifuge tube. Before HPLC analysis, the pH of the extracts was adjusted to 2.0 with a 5 mol L^{-1} NaOH solution and the addition of 400 μL of a 0.25 mol L^{-1} citric acid/citrate solution to buffer the samples extracts. The samples extracts were filtered with 0.20 μm regenerated cellulose filters and diluted with ultrapure water at different volume ratios depending on the type of sample (1 : 5 for fish and 1 : 10 for garlic and yeast samples) before the injection into HPLC. The samples extracts were stored at 8 °C for no more than two days in case these were not injected immediately into HPLC. No retention of Hg species occurred on regenerated cellulose during the filtration process (98–102% recovered). The separated organic Hg species were on-line photo-oxidized in a UV digestion unit coupled to the AFS instrument by mixing on-line the HPLC eluent with a 0.75% (w/v) $K_2S_2O_8$ solution (Table 1). The separation of Hg species was performed under gradient conditions. The composition of mobile phases A and B, along with a description of the gradient applied, are mentioned in Table 1. Quantification was performed based on the peak areas ($n = 3$) obtained by HPLC-UV-CV-AFS. Other experimental conditions were as mentioned in Table 1.

3. Results and discussion

3.1 Preliminary studies on CV-AFS detection of Hg species

The effect of different variables on the detection of Hg species by the UV-CV-AFS technique had to be assayed before performing the HPLC separation studies. This was due to the lack of information in the literature about the effect of different ILs on CV generation. All experiments described in Section 3.1 were performed under a flow injection mode, *i.e.* without the HPLC column (injector and pumps only), in order to rapidly evaluate the effect of the experimental.

3.1.1 Influence of UV radiation on the oxidation of organic Hg species. In the present work, oxidation of organic Hg species was performed with 1.0% (w/v) $K_2S_2O_8$ solution in 10% (v/v) HCl at 1.5 mL min^{-1} flow rate.[33] The UV-CV-AFS instrumental conditions applied during the evaluation of all factors mentioned in Section 3.1 were as follows: 0.1% (w/v) $NaBH_4$ (in 0.05% (w/v) NaOH), 5% (v/v) HNO_3 and reagents flow rate of 6.0 mL min^{-1}. Argon flow rate was 600 mL min^{-1} and atomization temperature was 200 °C. Fluorescence signal of Hg^{2+} species was the same with or without UV treatment. On the other hand, when no UV photo-oxidation was applied, the signals for CH_3Hg^+ and $C_2H_5Hg^+$ were 8% and 38% of the signal obtained

for Hg^{2+}, respectively. However, with UV photo-oxidation, the signals of both organic Hg species were the highest and similar to that obtained for Hg^{2+}. Thus, conversion efficiencies of 98 and 103% were obtained for CH_3Hg^+ and $C_2H_5Hg^+$, respectively. According to these results, UV photo-oxidation was applied in this work.

3.1.2 Influence of ILs. The effect of the ILs on Hg signal was evaluated by individually injecting each Hg species into the CV-AFS instrument. The Hg signals were evaluated in solutions having the same composition than the mobile phases and containing 0.1% to 0.5% (v/v) of each IL at 1.0 mL min^{-1}. Five different ILs were tested including four containing alkyl methyl imidazolium cations ([C_4mim]Cl, [C_6mim]Cl, [C_8mim]Cl and [C_{12}mim]Br) and [$P_{4,4,4,1}$]CH_3SO_4. Concentration of Hg species in these experiments was 50 μg Hg L^{-1}. The results are shown in Fig. 1a and b. Both for 0.1% and 0.5% (v/v) of each IL, a decrease in Hg^{2+} signal was observed from 100% without IL to 23% with [C_{12}mim]Br. Therefore, a correlation between the alkyl chain length of imidazolium cations and the intensity of Hg^{2+} signal was confirmed. The IL/Hg molar ratio was varied between 2.3×10^4 for [C_4mim]Cl and 1.2×10^4 for [C_{12}mim]Br. On the other hand, when 0.1% [$P_{4,4,4,1}$]CH_3SO_4 was selected, the signal for Hg^{2+} species was higher than that observed when no IL was in the analyte solutions. Signals for organic Hg species showed

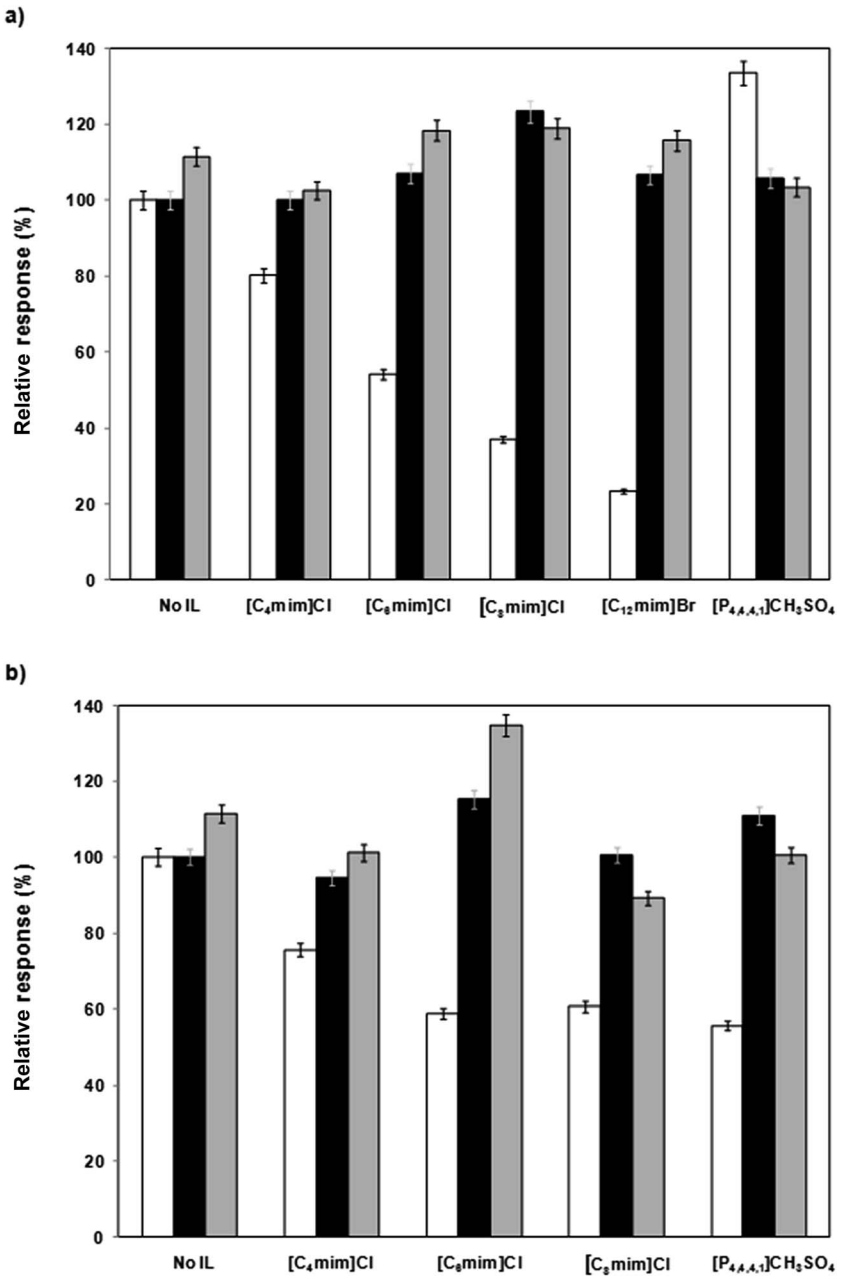


Fig. 1 UV-CV-AFS relative response of Hg species with respect to Hg^{2+} in mobile phases containing the different ILs evaluated in this work (n = 3). (a) 0.1% (v/v) and (b) 0.5% (v/v) IL concentration. (□) Hg^{2+}, (■) CH_3Hg^+, (■) $C_2H_5Hg^+$. Concentration of each Hg species = 50 μg Hg L^{-1}.

moderate change when the ILs were present, even though in some cases such as [C_8mim]Cl the signal was increased up to 120%.

For 0.5% (v/v) of imidazolium ILs (Fig. 1b), the decrease of Hg signal was not as significant as for 0.1% (v/v). In fact, the most significant decrease was around 60% for [C_8mim]Cl while [C_{12}mim]Br could not be assayed in these experiments at 0.5% (v/v) due to the foaming effect caused in the gas–liquid separator of the instrument. Interestingly, Hg signal was also decreased to 60% by [$P_{4,4,4,1}$]CH_3SO_4. The explanation to this behaviour cannot be related with the effect observed at 0.1% (v/v). In the case of CH_3Hg^+ and $C_2H_5Hg^+$, the effect was similar to that observed at 0.1% (v/v) IL. Based on these preliminary experiments, 0.1% (v/v) [C_8mim]Cl was chosen for other experiments described in Section 3.1. A possible explanation to the effect of ILs on Hg signal obtained in CV-AFS requires further studies to establish the reaction mechanisms involved in Hg vapor generation, such as those performed using isotopic markers.[37]

3.1.3 Effect of methanol. The effect of methanol on Hg signals was evaluated for concentrations of 5, 25, 50 and 70% (v/v) to establish if this solvent could be used in the mobile phase and a possible effect on CV-AFS detection of Hg. The results showed that Hg^{2+} signal was not affected by methanol up to 50% (v/v), but an increase in the sensitivity of 150% was observed for 70% (v/v) concentration. For organic Hg species, the signals were decreased when the methanol concentration was increased up to 25% (v/v) (42% for CH_3Hg^+ and 60% for $C_2H_5Hg^+$ with respect to Hg^{2+} signal). Based on these results, a methanol concentration of 5% (v/v) was chosen to develop other preliminary experiments described in Section 3.1 because no significant deterioration of the CV-AFS sensitivity was observed for all Hg species, especially the organic ones. It has to be mentioned that further experiments are needed to determine the accurate mechanism explaining the effect of methanol on Hg detection by the CV-AFS technique.

3.1.4 Sensitivity enhancement by sodium chloride. Since NaCl was another compound to be included in the mobile phase, its influence on Hg fluorescence signal was evaluated in the range of 0–0.1 mol L^{-1}. The results showed that signals were improved for all Hg species; *i.e.*, up to 200% for Hg^{2+} and 150% for CH_3Hg^+ and $C_2H_5Hg^+$. The same effect has been reported in other works without giving a deep discussion about it.[25,38] It could be related to different factors and further studies on the effect of salts on the generation and releasing of Hg^0 should be carried out in order to clarify these observations.

3.2 Chromatographic separation of Hg species by IL-RP-HPLC

3.2.1 Effect of pH. The CV-AFS instrumental conditions mentioned in Section 3.1 were applied for the evaluation of all parameters corresponding to RP-HPLC separation of Hg species. However, the studies described in Section 3.2 were performed with the HPLC column as part of the instrumental setup. The effect of pH on the separation of Hg species was studied with [C_8mim]Cl (0.2% (v/v)) as this IL has a mid size alkyl chain among all ILs included in this work. A 0.2% (v/v) [C_8mim]Cl concentration was used in this part of the study to set a value within the range of 0.1 to 0.5% (v/v), *i.e.* the range were preliminary studies were performed (see Section 3.1). Different buffers (0.02 mol L^{-1}) were used to adjust the pH at 2.0 (citric/citrate), 3.0 (citric/citrate), 5.0 (acetic/acetate) and 7.0 (monophosphate/diphosphate). The RP-HPLC separation was studied under isocratic conditions with the mobile phase at 1 mL min^{-1}. The separation of organic Hg species was achieved at pH 2.0 (Fig. 2a), with narrow peaks and the highest AFS signal. However, Hg^{2+} species was not eluted from the column even after 45 min. On the other hand, broader peaks were observed at pH 3.0 (Fig. 2b), while none of the species were eluted from the column at pH values of 5.0 and 7.0. Ultimately, pH 2.0 was chosen because full separation of CH_3Hg^+ and $C_2H_5Hg^+$ species was obtained.

3.2.2 Formation of chlorocomplexes. The effect of Cl^- anion on the separation, peak shape and detection of Hg species was evaluated in this work. The need to carry out these studies was mainly due to the possible formation of chlorocomplexes between Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$ species and

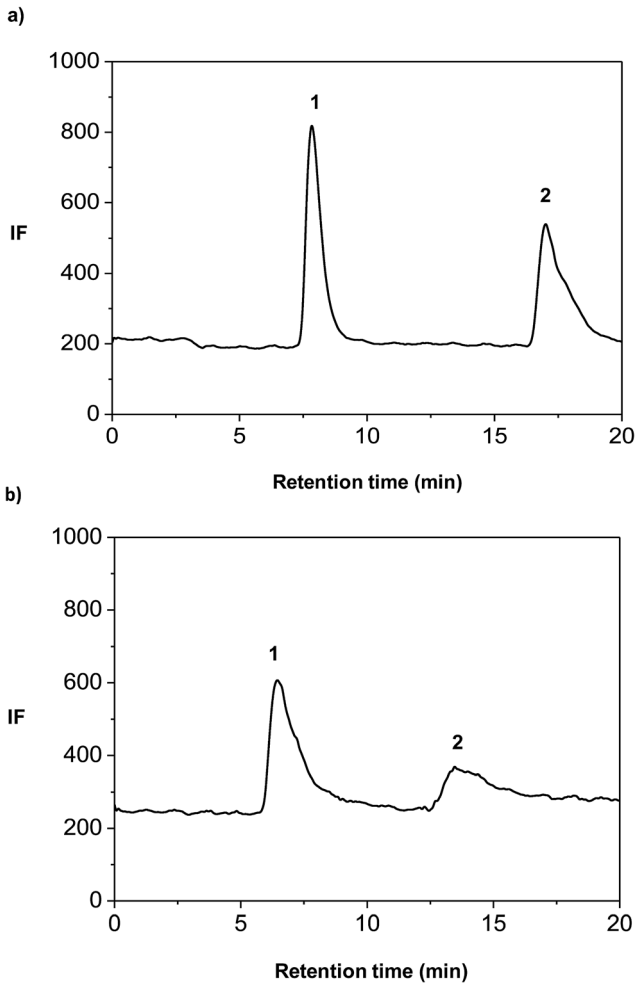


Fig. 2 Effect of pH on the separation of Hg species. (a) pH = 2.0 and (b) pH = 3.0. IF: fluorescence intensity. (1) CH_3Hg^+ and (2) $C_2H_5Hg^+$. Concentration of each Hg species = 50 μg Hg L^{-1}.

Cl^- anion, which has been demonstrated in early works done on 1960's and 1970's. For example, Barbieri and Bjerrum reported solubility measurements in 1 M $NaCl/NaClO_4$ solutions suggesting the formation of negatively charged C_2H_5Hg–chloride complexes.[39] Also, Rizzardi *et al.* found evidences of the formation of $C_2H_5HgCl_2^-$ and $C_2H_5HgCl_3^-$ during ion exchange experiments, while Bertazzi *et al.* demonstrated that $RHgCl_n^{1-n}$ ($R = CH_3$ and C_2H_5, $n = 1, 2, 3$) chlorocomplexes are formed in aqueous solutions.[40–42] Later on, Tajima *et al.* reported the extraction of ion pairs formed between Hg^{2+} halides and tetra-*n*-butylammonium halide salts[42] and this was exploited in other works on Hg speciation analysis.[28,43]

Therefore, it is highlighted in this work the main role played by Cl^- anion for the separation of Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$ species, which could occur *via* the formation of negatively charged chlorocomplexes followed by the ion pairing reaction with the cation of ILs. The following equations can be written in relation to the mechanism of separation proposed in this work:

$$\text{(I)}\ Hg^{2+} + 4Cl^- \rightleftarrows HgCl_4^{2-}$$

$$\text{(II)}\ 2[C_8mim]^+ + HgCl_4^{2-} \rightleftarrows [C_8mim]_2HgCl_4$$

and:

$$\text{(I)}\ RHg^+ + Cl^- \rightleftarrows RHgCl$$

$$\text{(II)}\ RHgCl + Cl^- \rightleftarrows RHgCl_2^-$$

$$\text{(III)}\ [C_8mim]^+ + RHgCl_2^- \rightleftarrows [C_8mim]RHgCl_2$$

where $R = CH_3$ or C_2H_5.

The effect of Cl^- (NaCl) was investigated in the concentration range of 0.01–0.25 mol L^{-1}. The results showed that the increase of NaCl concentration yielded higher sensitivity for the detection of Hg species by CV-AFS. This phenomenon was already described in Section 3.1.4. For all these experiments, the methanol concentration had to be increased from 5 to 50% (v/v), after the elution of the organic Hg species, in order to elute Hg^{2+} species from the column. However, under these conditions the retention time for Hg^{2+} was 23 min and a broad peak was observed. Likewise, the effect of NaCl on the separation of Hg species was characterized by a much better elution of Hg^{2+} species (narrower peaks) as compared to the case when no NaCl was added to the mobile phase. Finally, a concentration of 0.1 mol L^{-1} NaCl was chosen because of the highest sensitivity and separation performance achieved.

The stronger retention of Hg^{2+} on the stationary phase compared to CH_3Hg^+ and $C_2H_5Hg^+$ species can be explained based on the aforementioned separation mechanism. Thus, the $[C_8mim]_2HgCl_4$ ion pair can be expected to have a lower polar character than $[C_8mim]CH_3HgCl_2$ and $[C_8mim]C_2H_5HgCl_2$ and hence, it was strongly interacting with the C_{18} stationary phase.

3.2.3 Effect of methanol concentration in the mobile phase. With the aim of performing an isocratic elution of all Hg species from the HPLC column, the effect of methanol was evaluated at 25, 50 and 70% (v/v) in the mobile phase. Other variables were set as follows: 0.02 mol L^{-1} citric/citrate buffer, 0.2% (v/v) $[C_8mim]Cl$ and 0.1 mol L^{-1} NaCl. When methanol was 25% (v/v), CH_3Hg^+ and $C_2H_5Hg^+$ were eluted at 4.2 and 6.3 min, respectively, *i.e.* with shorter retention times than those observed in previous conditions. However, Hg^{2+} species was not eluted under these isocratic conditions. On the other hand, when 50% (v/v) methanol was tested, CH_3Hg^+ and $C_2H_5Hg^+$ species were co-eluted at 5 min, while Hg^{2+} species was at 19.6 min. Similarly, the organic Hg species were co-eluted at 4 min for 70% (v/v) methanol, while Hg^{2+} species did it at 15 min. Therefore, for next experiments, the concentration of methanol in the mobile phase was set at 25% (v/v) in order to determine if a change in the type and concentration of IL could improve the elution of Hg^{2+} while preserving the separation of CH_3Hg^+ and $C_2H_5Hg^+$ species. In this case, an increase in methanol concentration did not affect the sensitivity of CV-AFS.

3.2.4 Comparative evaluation of different ILs on the separation of Hg species. Four imidazolium ILs having alkyl groups with different carbon chain lengths (C_4–C_{12}) and one phosphonium IL were evaluated in this work. Other variables in these experiments were fixed as follows: 0.02 mol L^{-1} citric/citrate buffer, 0.2% (v/v) IL, 0.1 mol L^{-1} NaCl and 25% (v/v) methanol. The results showed (Fig. 3a–d) a direct correlation between the retention times of CH_3Hg^+ and $C_2H_5Hg^+$ species and the alkyl chain length of the substituting groups of imidazolium ILs. In general, the longer the alkyl chain, the higher were the retention times. With $[C_4mim]Cl$, the organic Hg species were not separated and co-eluted at 4 min. However, these Hg species were completely separated with $[C_6mim]Cl$ and $[C_8mim]Cl$, even though the sensitivity was higher with $[C_8mim]Cl$. The retention of Hg species was totally modified by $[C_{12}mim]Br$ with respect to $[C_8mim]Cl$ IL, *i.e.* higher retention times were observed for both organic Hg species (5.4 min for CH_3Hg^+ and 8.4 min for $C_2H_5Hg^+$). Also, the sensitivity was significantly decreased with $[C_{12}mim]Br$ due to significant formation of foam inside the gas–liquid separator of the AFS instrument, which made it difficult to transport Hg^0 to the atomizer. It is worthy to mention that Hg^{2+} was not eluted with none of the imidazolium ILs assayed in these experiments and under the conditions indicated in this section.

On the other hand, no peaks were observed for none of the Hg species when $[P_{4,4,4,1}]CH_3SO_4$ was used in the mobile phase, even after a 30 min run. Thus, the interaction between Hg species (as chlorocomplexes)[33,38] and the $[P_{4,4,4,1}]^+$ cation was very strong and the formed ion pairs were permanently retained on the C_{18} stationary phase.[28] In fact, it has been already reported the strong ion pairing effect obtained with phosphonium type ILs, especially with Hg chlorocomplexes.[32] For this reason, the use of $[P_{4,4,4,1}]CH_3SO_4$ IL was discarded in this work.

Considering the above-mentioned results, the possibility of separating the Hg species under isocratic conditions and using different ILs concentrations in the mobile phase had to be evaluated. The IL chosen for this experiment was $[C_8mim]Cl$ at concentrations ranging 0.1–0.6% (v/v). Higher concentrations were not tested due to deterioration of the sensitivity, particularly for Hg^{2+} species (Section 3.1.2). The results showed no significant differences in the retention times of the organic Hg species within this concentration range while

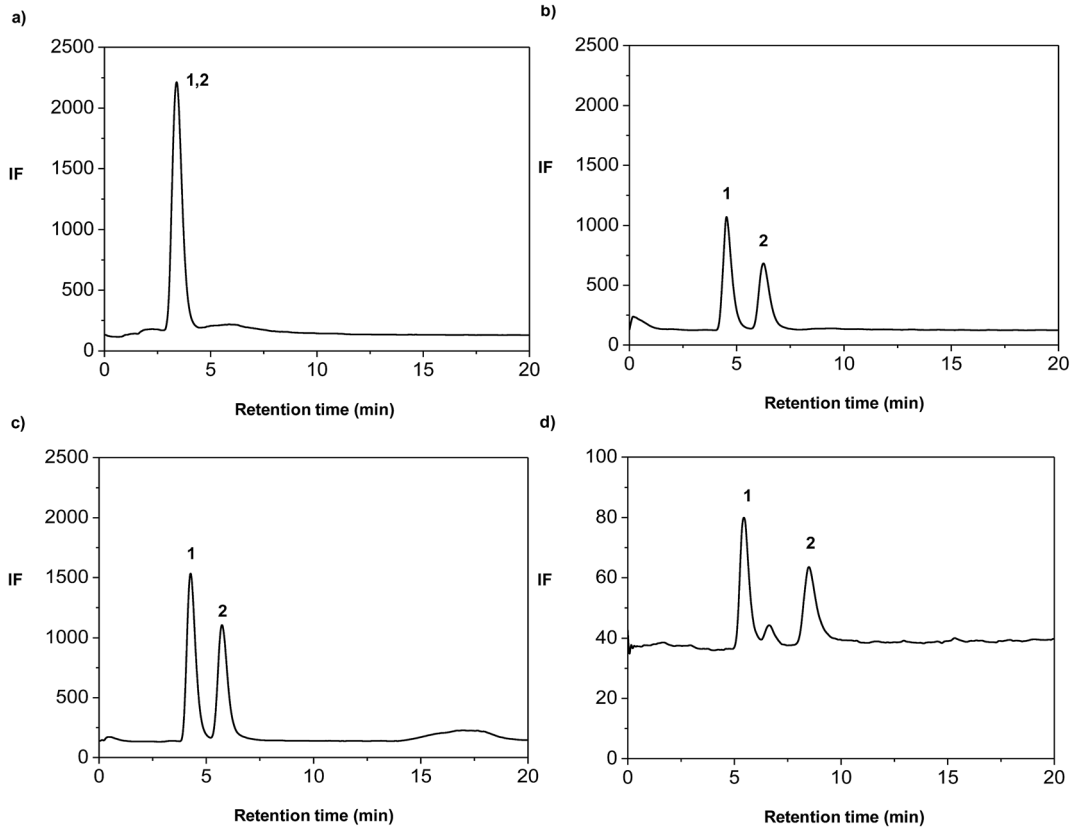


Fig. 3 Effect of IL type on the separation of Hg species. (a) $[C_4mim]Cl$, (b) $[C_6mim]Cl$, (c) $[C_8mim]Cl$, (d) $[C_{12}mim]Br$. (1) CH_3Hg^+, (2) $C_2H_5Hg^+$. Concentration of each Hg species = 50 μg Hg L^{-1}.

Hg^{2+} was permanently retained on the stationary phase. In order to elute all Hg species from the column, the application of isocratic conditions was discarded, and a gradient with methanol was proposed. In this case, a concentration of 0.4% (v/v) $[C_8mim]Cl$ was chosen to assure the formation of ionic pairs with Hg species and considering that the concentration of the IL was going to be reduced during the gradient program.

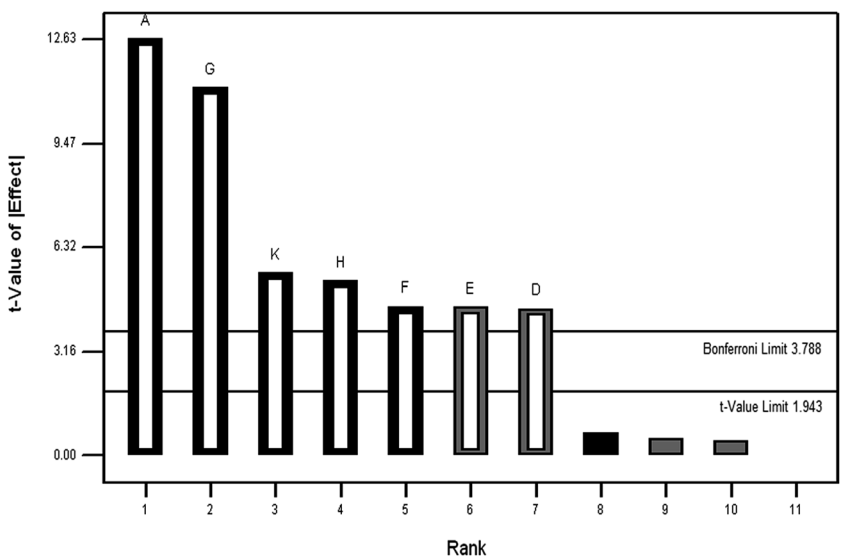


Fig. 4 Pareto graph of Hg^{2+} species to determine the significant effects of variables in the fluorescence signal. The plot is similar for the other Hg species. (■) Positive significant effect. (■) Negative significant effect. (A) Concentration of $K_2S_2O_8$, (D) concentration of HNO_3, (E) reducing and carrier agents flow rate, (F) argon flow rate, (G) atomization temperature, (H) dummy 1, (K) dummy 4.

Initially, a gradient was applied by increasing the methanol concentration from 25 to 70% (v/v) within the first 8 min of chromatography, keeping it constant at 70% (v/v) until Hg^{2+} was eluted from the column. Under these conditions, CH_3Hg^+ and $C_2H_5Hg^+$ species were eluted within 8 min, while the retention time for Hg^{2+} was 21 min. In order to reduce the total time required for the separation of Hg species, a linear gradient was developed, starting at 0% (v/v) methanol and increasing its concentration up to 100% (v/v) within 15 min. The sensitivity of CV-AFS to Hg species was not modified by these methanol concentrations. Thus, all Hg species were eluted and resolved within 12 min (6.1, 7.4 and 11.5 for CH_3Hg^+, $C_2H_5Hg^+$ and Hg^{2+}, respectively).

3.3 Multivariate optimization of UV-CV-AFS detection conditions

Once the final RP-HPLC conditions for best separation of Hg species were obtained by an univariant method, a multivariate method was applied for the optimization of CV-AFS sensitivity. The study was developed measuring the peak area obtained after the injection of each Hg species at 50 μg L^{-1} into the HPLC instrument (without column) and under the same conditions of the mobile phase (composition and flow rate) where each Hg species was eluted from the column (see Table 1). Design Expert® 7.0 (Stat-Ease Inc., Minneapolis, USA) software was used to process all results. An experimental Plackett Burman design with 3 central points was applied for the determination of the main variables affecting CV-AFS sensitivity. The analyzed factors and intervals were $NaBH_4$ concentration (0.1–0.5% w/v), HNO_3 concentration (3.0–7.0% v/v), $K_2S_2O_8$ concentration (0.5–1.5% w/v), $K_2S_2O_8$ flow rate (1.5–3.0 mL min^{-1}), reducing and carrier agents flow rate (6.0–9.0 mL min^{-1}), atomization temperature (100–300 °C) and argon flow rate (400–800 mL min^{-1}). Thus, 15 experiments were performed.

A Pareto graph was employed to choose significant effects for each Hg species. After the analysis, the factors with significant effects were $K_2S_2O_8$ concentration, reducing and carrier agents flow rate, argon flow rate and atomization temperature for all the Hg species. The selected variables from Pareto graph were evaluated by analysis of variance (ANOVA) obtaining significant models for all the responses. Large adjusted *R*-square, of 0.981 for Hg^{2+}, 0.967 for CH_3Hg^+ and 0.986 for $C_2H_5Hg^+$ were

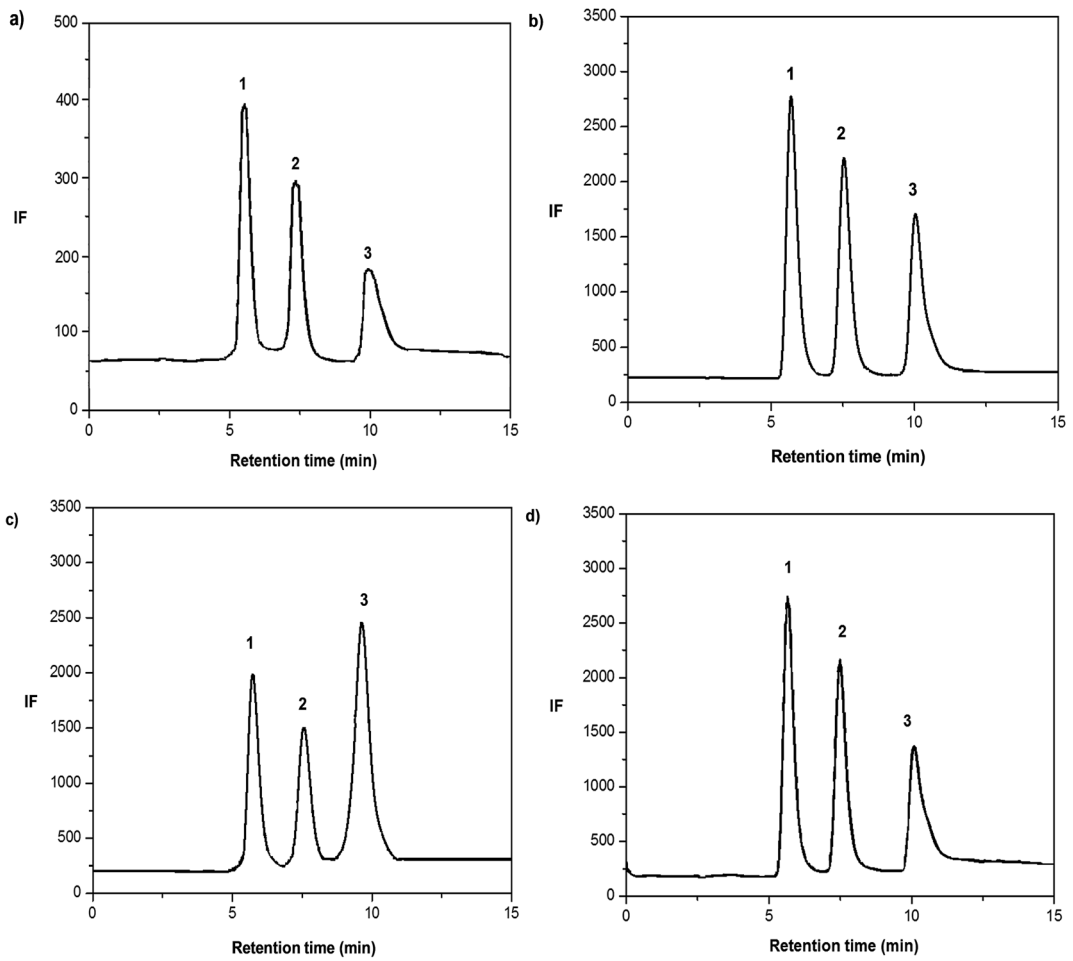


Fig. 5 Chromatograms obtained after Hg speciation analysis of (a) 10 μg Hg L^{-1} standard species mix, (b) 50 μg Hg L^{-1} standard species mix, (c) yeast sample and (d) canned mussels extracts spiked at 50 μg Hg L^{-1}. (1) CH_3Hg^+, (2) $C_2H_5Hg^+$, (3) Hg^{2+}. Eluents: (A) 100% (v/v) methanol (B) 0.5% (v/v) [C_8mim]Cl; pH = 2.0; 0.02 mol L^{-1} citric acid/citrate buffer; 0.1 mol L^{-1} NaCl. Gradient: 0–12 min 5–100% (A); 12–17 min 100% (A).

obtained. With these four factors, an optimization step with a response surface model (RSM) was performed. A Box-Behnken design was applied to determine the values where sensitivity of the Hg species would had to be higher. A total of 27 experiments were performed including 3 central points. The fixed factors (not significant) were established as follows: 0.1% (w/v) $NaBH_4$ concentration, 3% (v/v) HNO_3 concentration and 1.5 mL min^{-1} $K_2S_2O_8$ flow rate. RSMs were plotted for each Hg species and cubic models were obtained with transformed responses of natural logarithm (ln) in all species. All models were highly significant and the lack of fit non-significant. The statistical parameters were acceptable for all the Hg species. Large adjusted *R*-square models were obtained (0.96–0.98) indicating a good fit of the obtained model to the results obtained in the experiments. The variation coefficient values (0.32–0.50) indicated a low standard deviation and hence, high reproducibility and precision were obtained. Once a model was chosen for each Hg species response and evaluated by an ANOVA test, a desirability function was used to optimize the variables. All the factors were optimized in range and the responses were the highest according to the optimization criteria. The adjusted optimum conditions given by the model were as follows: 0.75% (w/v) $K_2S_2O_8$, 1.5 mL min^{-1} reducing and carrier agents flow rate, 400 mL min^{-1} argon flow rate and 100 °C atomization temperature. While conditions before multivariate optimization were: 1% (w/v) of $K_2S_2O_8$, 6 mL min^{-1} for reducing and carrier agents flow rate, 600 mL min^{-1} for argon flow rate and 200 °C atomization temperature. Afterwards, an experimental confirmation of these optimal conditions was performed. The peak areas corresponding to each Hg species were within an acceptable error range, with relative error (RE%) values in the range of 1.44% and 2.56%. Therefore, the model accurately predicted the responses for all the Hg species (Fig. 4).

Typical chromatograms obtained under the optimized conditions are shown in Fig. 5a and b. A 2-fold increase in sensitivity was obtained for organic Hg species after performing the multivariate optimization compared to the initial CV-AFS conditions mentioned earlier in this work. Likewise, a 0.5-fold increase was observed for Hg^{2+} species.

Table 3 Chromatographic parameters and analytical performance of the proposed method for Hg speciation analysis (95% confidence level, $n = 3$)

Parameter	CH_3Hg^+	$C_2H_5Hg^+$	Hg^{2+}
Lineal range (μg L^{-1})	0.17–1000	0.21–1000	0.38–1000
r^2	0.99	0.99	0.99
RSD (%) retention time	0.6	0.5	0.5
RSD (%) peak area	1.0	2.0	2.0
LOD (μg L^{-1})	5×10^{-2}	6×10^{-2}	0.1
R^a	—	2.3	2.8
k'^b	0.15	0.50	0.99
N^c	140	153	441
α^d	—	2.81	2.51

[a] Resolution $= 2(t_{R2} - t_{R1})/(w_2 + w_1)$. [b] Capacity factor $= (t_R - t_0)/t_0$. [c] Number of theoretical plates $= 16(t_R/w)^2$. [d] Selectivity $= k_n/k_{n-1}$.

3.4 Analytical performance and chromatographic parameters

Chromatograms obtained after injection of aqueous standards mix and samples spiked with Hg species are shown in Fig. 5a–d. The resolutions for Hg species were calculated as 2.3 between $C_2H_5Hg^+$ and CH_3Hg^+ and 2.8 between $C_2H_5Hg^+$ and Hg^{2+} (Table 3). The total separation time was less than 12 min.

Table 4 Comparison of different methods for Hg speciation analysis using RP-HPLC-CV-AFS

Sample	Hg species	Mobile phase	Elution mode	Elution time (min)	LOD (μg L^{-1})	Ref.
Fish sample	Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$	1 g L^{-1} L-cysteine, 0.06 mol L^{-1}, NH_4Ac in water	Isocratic	N.R.	0.1, 0.05, 0.07	51
Fish and mollusks	Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$, $PhHg^+$	(A) 3% (v/v) CH_3CN, 60 mmol L^{-1} NH_4Ac (pH 6.9), 0.02%, 2-mercaptoethanol; (B) 30% (v/v) CH_3CN, 60 mmol L^{-1} NH_4Ac (pH 6.9), 0.02%, 2-mercaptoethanol	Gradient 0–11 min: 100% (A); 11–12 min: 100% (A) → 100% (B); 12–30 min: 100% (B); 30–31 min: 100%, (B) → 100% (A)	30	0.5, 0.2, 0.2, 0.2	11
Tuna fish and seafood	Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$	Acetonitrile : water (65 : 35); 0.1 mol L^{-1} acetic/acetate buffer pH = 5.5; 1.5×10^{-3} mol L^{-1} APDC[a]	Isocratic	20	0.2, 0.1, 0.2	45
Fish muscle tissue and water	Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$, $PhHg^+$	Acetonitrile : water (10 : 90) pH = 6.8; 0.12% (w/v) L-cysteine	Isocratic	9	0.7, 1.1, 0.8, 0.9	43
Fresh fish, canned fish, garlic and yeast	Hg^{2+}, CH_3Hg^+, $C_2H_5Hg^+$	(A) 0.02 mol L^{-1} citric/citrate buffer pH = 2.0; 0.1 mol L^{-1} NaCl; 0.4% (v/v) $[C_8mim]Cl$ (B) methanol	Gradient 0–12 min: 5–100% (B); 12–15 min: 100% (B)	12	0.1, 0.05, 0.06	This work

[a] APDC: ammonium pyrrolidine dithiocarbamate.

Table 5 Results obtained after Hg speciation analysis in the food samples studied in this work (95% confidence level, $n = 3$)

Sample	Total Hg (μg g^{-1})	R^a (%)	Hg^{2+} Added μg L^{-1}	Hg^{2+} Found μg L^{-1}	R^a (%)	CH_3Hg^+ Added μg L^{-1}	CH_3Hg^+ Found μg L^{-1}	R^a (%)	$C_2H_5Hg^+$ Added μg L^{-1}	$C_2H_5Hg^+$ Found μg L^{-1}	R^a (%)
Canned hake (*Merluccius gayi*)	0.41 ± 0.02	96	—	<LOD	—	—	6.5 ± 0.3 (0.22)[b]	—	—	4.4 ± 0.4 (0.13)[b]	—
			10	9.6 ± 0.4	96	10	16.3 ± 0.5	98	10	13.9 ± 0.6	95
			50	49 ± 1	99	50	54 ± 1	95	50	53 ± 1	97
Canned mackerel (*Scomber scombrus*)	0.71 ± 0.02	95	—	4.2 ± 0.5 (0.14)[b]	—	—	7.9 ± 0.8 (0.26)[b]	—	—	5.8 ± 0.5 (0.19)[b]	—
			10	14.1 ± 0.3	99	10	17.5 ± 0.5	96	10	15.6 ± 0.7	98
			50	53 ± 0.4	98	50	56 ± 1	96	50	52 ± 2	93
Canned mussel (*Mytilidae edulis*)	0.47 ± 0.03	99	—	2.9 ± 0.4 (0.09)[b]	—	—	5.2 ± 0.2 (0.17)[b]	—	—	4.6 ± 0.8 (0.15)[b]	—
			10	12.6 ± 0.2	97	10	15.0 ± 0.4	95	10	14.2 ± 0.5	96
			50	50 ± 1	94	50	54 ± 1	97	50	52 ± 1	94
Canned tuna (*Thunnus atlanticus*)	0.55 ± 0.03	98	—	3.4 ± 0.8 (0.11)[b]	—	—	6.0 ± 0.9 (0.20)[b]	—	—	5.2 ± 0.2 (0.17)[b]	—
			10	13.1 ± 0.7	97	10	15.9 ± 0.4	99	10	14.8 ± 0.2	96
			50	53 ± 1	99	50	54 ± 1	97	50	53 ± 1	96
Fresh silverside (*Odontesthes incisa*)	0.28 ± 0.01	99	—	<LOD	—	—	6.9 ± 0.5 (0.23)[b]	—	—	<LOD	—
			10	9.6 ± 0.6	96	10	16.5 ± 0.3	96	10	9.5 ± 0.2	95
			50	47 ± 1	95	50	55 ± 1	97	50	48 ± 1	95
Fresh chilean jack mackrerel (*Trachurus murphyi*)	0.34 ± 0.02	99	—	<LOD	—	—	4.6 ± 0.7 (0.15)[b]	—	—	4.0 ± 0.8 (0.13)[b]	—
			10	9.7 ± 0.7	97	10	14.2 ± 0.4	96	10	13.5 ± 0.3	95
			50	50 ± 1	100	50	54 ± 1	98	50	53 ± 1	97
Fresh praw (*Pleoticus muelleri*)	0.72 ± 0.02	98	—	3.9 ± 0.2 (0.13)[b]	—	—	7.5 ± 0.6 (0.25)[b]	—	—	6.3 ± 0.3 (0.21)[b]	—
			10	13.6 ± 0.2	97	10	17.3 ± 0.6	98	10	16.2 ± 0.4	99
			50	53 ± 1	97	50	56 ± 1	96	50	56 ± 1	99
Fresh sea bream (*Pagellus bogaraveo*)	0.49 ± 0.03	98	—	<LOD	—	—	6.8 ± 0.8 (0.23)[b]	—	—	5.4 ± 0.4 (0.18)[b]	—
			10	9.8 ± 0.4	98	10	16.4 ± 0.6	96	10	15.4 ± 0.5	100
			50	49 ± 1	97	50	56 ± 1	98	50	55 ± 1	98
Garlic (*Allium sativum*)	0.25 ± 0.01	98	—	4.2 ± 0.7 (0.14)[b]	—	—	2.0 ± 0.7 (0.06)[b]	—	—	<LOD	—
			10	14.1 ± 0.5	99	10	11.5 ± 0.6	95	10	9.7 ± 0.4	97
			50	54 ± 1	99	50	51 ± 1	97	50	49 ± 1	99
Yeast (*Saccharomyces cerevisiae*)	0.43 ± 0.02	98	—	4.0 ± 0.3 (0.13)[b]	—	—	1.7 ± 0.6 (0.05)[b]	—	—	1.2 ± 0.2 (0.04)[b]	—
			10	13.6 ± 0.4	96	10	11.4 ± 0.5	97	10	11.0 ± 0.3	98
			50	52 ± 1	97	50	50 ± 1	97	50	50 ± 2	98

[a] Recovery = (found − base)/added × 100. [b] Concentration of Hg species expressed as μg Hg g^{-1} of sample.

Complete and baseline separation of Hg species was achieved even in the complex samples analyzed in this work. The limits of detection (LODs) were calculated based on the signal at the intercept and three times the standard deviation about regression of the calibration curves. The LODs obtained after injection of aqueous standards of Hg species into RP-HPLC-UV-CV-AFS were 0.05, 0.06 and 0.11 μg L^{-1} for CH_3Hg^+, $C_2H_5Hg^+$ and Hg^{2+}, respectively. Additionally, the method detection limits (MDLs) (3σ) for CH_3Hg^+, $C_2H_5Hg^+$ and Hg^{2+} in seafood were 7.6 ng Hg g^{-1}, 10.8 ng Hg g^{-1} and 18.2 ng Hg g^{-1}, respectively. The MDLs for CH_3Hg^+, $C_2H_5Hg^+$ and Hg^{2+} in garlic and yeast were 15.2 ng Hg g^{-1}, 21.6 ng Hg g^{-1} and 36.4 ng Hg g^{-1} for CH_3Hg^+, $C_2H_5Hg^+$ and Hg^{2+}, respectively. Relative standard deviations (RSDs) for peak areas were between 1.01 and 2.03% and for retention times were between 0.53 and 0.63%. The linear ranges of calibration curves were 0.2–1000 μg L^{-1} for CH_3Hg^+ and $C_2H_5Hg^+$ and 0.4–1000 μg L^{-1} for Hg^{2+}. A comparison of the LODs obtained by the proposed method with those reported in other works on Hg speciation analysis can be found in Table 4. The LODs obtained with the proposed method are comparable to those reported in the literature.

In RP-HPLC, the most common agents for Hg speciation analysis have been chelating agents such as 2-mercaptoethanol, L-cystein, EDTA or APDC and ion-pair agents such as tetraalkylammonium salts that require the formation of halide complexes with Hg species.[18] The ILs evaluated in this work can be considered effective alternatives to tetralkylmmonium salts for ion-pairs formation. The analytical figures of merit obtained with these ILs were similar or better than those reported after the application of other methodologies using CV-AFS detection and having short chromatographic runs.[26,44–47] On the other hand, the LODs obtained with the proposed methodology (0.05–0.11 μg L^{-1}) are comparable to those observed with other detection systems such as ICP-MS (0.014–0.042 μg L^{-1}),[14] CV-AAS (0.6 μg L^{-1})[46] and DAD (0.32–1.91 μg L^{-1}).[47]

3.5 Determination of Hg species in foods

Mean concentration values ($n = 3$) for total Hg in several seafood, garlic and yeast samples were obtained by CV-AFS analysis (Table 5). Total Hg concentrations in seafood were lower than the maximum permissible limit established in these types of samples (1 mg kg^{-1}).[4] These concentrations were higher than those found in garlic and yeast samples because Hg can be accumulated in seafood throughout food chain.[48] Also, a recovery study was performed by spiking the different samples at 20 μg Hg L^{-1} before the digestion procedure. The recovery values were in the range of 94.6–99.2%.

Since a partial lack of chromatographic resolution and loss of sensitivity were observed for Hg species when undiluted samples extracts were injected into the HPLC column, 5-fold and 10-fold dilutions of the extracts were assayed to overcome possible matrix effects. A 5-fold for fish sample extracts and 10-fold dilution for garlic and yeast were necessary to obtain symmetric peaks and best resolution, being comparable to those obtained with Hg aqueous standards. Afterwards, the proposed RP-HPLC-UV-CV-AFS method was applied for Hg speciation analysis by injecting the diluted samples extracts as such or spiked at 10 μg L^{-1} and 50 μg L^{-1} of each Hg species. Table 5 shows the concentrations found for the Hg species expressed as μg Hg g^{-1} of dried sample. Concentrations of Hg species in canned seafood were in the ranges of <LOD–0.14 μg g^{-1}, 0.17–0.26 μg g^{-1} and 0.13–0.19 μg g^{-1} for Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$, respectively. In the case of fresh seafood, the concentrations of Hg^{2+} species ranged from <LOD to 0.13 μg g^{-1}, CH_3Hg^+ concentrations were from 0.15 to 0.25 μg g^{-1} and $C_2H_5Hg^+$ concentrations were from <LOD to 0.21 μg g^{-1}. For garlic, Hg^{2+} concentration was 0.14 μg g^{-1}, CH_3Hg^+ was 0.016 μg g^{-1} and $C_2H_5Hg^+$ was <LOD. For yeast, the concentrations were 0.13 μg g^{-1}, 0.05 μg g^{-1} and 0.04 μg g^{-1} for Hg^{2+}, CH_3Hg^+ and $C_2H_5Hg^+$, respectively. In general, it was observed that organic Hg species were predominant over Hg^{2+} species in seafood samples, while the opposite happened in garlic and yeast samples. These findings confirmed what has been reported by other authors about organic Hg species as the major species found in seafood. In fact, CH_3Hg^+ is the main species accumulated and biomagnified in the aquatic food chain due to its high lipophilic character and represents 90% of total Hg.[10] Finally, acceptable recovery values were obtained in the different samples analyzed in this work (93–100%).

4. Conclusions

An exploration of the effects of ILs on RP-HPLC separation of Hg species and their detection by CV-AFS was carefully performed in this work. The possible mechanisms involved in the proposed method for the separation of Hg species by ILs could include the formation of anionic chlorocomplexes, their ion pairing reaction with ILs cations and the retention of the formed ion pairs on the C_{18} stationary phase. However, another mechanism involving the interaction of Hg chlorocomplexes with the cations of ILs already retained in the column could not be discarded. The ILs assayed in this work can be considered effective modifiers to be added to the mobile phases of RP-HPLC in order to obtain excellent separation of most common Hg species in different types of samples.

Finally, the application of the method for Hg speciation analysis in real samples containing highly complex matrices, such as seafood, garlic and yeast was demonstrated. This characteristic, along with the possibility of using a low cost detector such as AFS, turn the proposed method into a valuable tool for Hg speciation analysis in most routine analytical laboratories.

Conflicts of interest

There are no conflicts of interest to declare.

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