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# Ionic liquid-assisted separation and determination of selenium species in food and beverage samples by liquid chromatography coupled to hydride generation atomic fluorescence spectrometry

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ABSTRACT

Different ionic liquids (ILs) were assayed as mobile phase modifiers for the separation and determination of selenite [Se(IV)], selenate [Se(VI)], selenomethionine (SeMet) and Se-methylselenocysteine (SeMeSeCys) by reversed-phase high-performance liquid chromatography coupled to hydride generation atomic fluorescence spectrometry (RP-HPLC-HG-AFS). The use of several ILs: 1-butyl-3-methylimidazolium chloride, 1-hexyl-3-methylimidazolium chloride ([$C_6$mim]Cl), 1-octyl-3-methylimidazolium chloride, 1-dodecyl-3-methylimidazolium bromide, 1-hexadecyl-3-methylimidazolium bromide and tributyl(methyl)phosphonium methylsulphate was evaluated. Also, the effect of pH, buffer type and IL concentration on the separation of Se species was studied. Complete separation was attained within 12 min using a $C_8$ column and a gradient performed with a mobile phase containing 0.1% (v/v) [$C_6$mim]Cl at pH 6.0. The proposed method allows the separation of inorganic and organic Se species in a single chromatographic run, adding further benefits over already reported methods based on RP-HPLC. In addition, the influence of ILs on the AFS signals of each Se species was evaluated and a multivariate methodology was used for optimization of AFS sensitivity. The limits of detection were 0.92, 0.86, 1.41 and 1.19 μg $L^{-1}$ for Se(IV), Se(VI), SeMet and SeMeSeCys, respectively. The method was successfully applied for speciation analysis of Se in complex samples, such as wine, beer, yeast and garlic.



## 1. Introduction

Selenium (Se) is a trace element that can be beneficial or harmful to humans depending on its concentration and chemical speciation [1]. It can be found in 25 human proteins and it is crucial for several enzymatic processes and biological functions [2]. In foods, Se can not only be present as selenoproteins but also as different selenoaminoacids [3]. Therefore, speciation analysis of Se is necessary to fully determine the possible benefits or risks linked to this element in food consumed by humans. However, fundamental analytical principles must be fulfilled in order to perform accurate speciation analysis, such as efficient extraction of Se species from food solid matrices while preserving chemical speciation, availability of highly sensitive detectors for quantification at trace or ultratrace levels and a selective determination. Several methods have been reported in the literature for extraction of Se species from solid matrices [4], being enzymatic-assisted extraction under mild conditions (37 °C and pH 7.5), one of the most common methods used for food complex matrices [5]. For the separation of Se species, high-performance liquid chromatography (HPLC) and gas chromatography (GC) [4] coupled to sensitive detectors such as inductively coupled plasma mass spectrometry (ICP-MS) or atomic fluorescence spectrometry (AFS) are the most common techniques [6,7]. Moreover, when HPLC is used for speciation analysis, reversed-phase (RP) and anion exchange chromatography (AEC) are normally applied [7].

The separation of Se species by RP is performed following the formation of ion-pairs and their interaction with non polar stationary phases ($C_8$ or $C_{18}$). The most commonly used ion-pairing agents have been perfluorocarboxylic acids such as trifluoroacetic acid (TFA) [8] and heptafluorobutanoic acid (HFBA) [9]. Likewise, tetra-alkylammonium salts [10] and alkylsulfonic salts [11] have

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been used. However, poor separation of inorganic Se species has been obtained when these ion-pairing agents are individually used and a limited resolution with a mix of different ion-pairing agents [12]. Therefore, since Se speciation analysis is highly important to ensure food quality and to evaluate the impact on health due to Se consumption, it is crucial to search for more efficient ion-pairing agents that allow full chromatographic separation of both organic and inorganic Se species in food samples.

Ionic liquids (ILs) are liquid salts at room temperature with multiple applications in analytical chemistry, including extraction of inorganic and organic analytes, stationary phases for GC separation, functionalization of columns and mobile phase additives in HPLC [13]. In the last case, and depending the cation and anion of the IL, a direct interaction with stationary phases used in RP-HPLC or with species might be expected [14]. Thus, some advantages of using ILs in RP-HPLC are: 1) improved peak shape and resolution, 2) suppression of silanol groups and 3) shorter retention times for some analytes [14]. In fact, a recent work published by our group has demonstrated the utility of ILs to separate As species [15], but imidazolium-type ILs have already been applied in RP-HPLC-ICP-MS for Se speciation [16,17]. However, in the last case, a mixture of two ILs was needed for full separation and acceptable resolution of Se species. On the other hand, AFS technique has not been widely used as a detector along with ILs, remaining unknown the effect of these solvents on Se fluorescence signal.

In this work, a complete study on the effects of several imidazolium- and phosphonium-type ILs on the separation and determination of Se(IV), Se(VI), SeMet and SeMeSeCys species in RP-HPLC-HG-AFS was performed. The possible mechanisms involved in the separation of Se species, when ILs are used as mobile phase additives, were evaluated. Thus, the influence of the alkyl chain length of different ILs containing the imidazolium cation: 1-butyl-3-methylimidazolium chloride ([$C_4$mim]Cl), 1-hexyl-3-methylimidazolium chloride ([$C_6$mim]Cl), 1-octyl-3-methylimidazolium chloride ([$C_8$mim]Cl), 1-dodecyl-3-methylimidazolium bromide ([$C_{12}$mim]Br) and 1-hexadecyl-3-methylimidazolium bromide ([$C_{16}$mim]Br) on RP-HPLC separation was studied. Tributyl(methyl)phosphonium methylsulphate ([$P_{4,4,4,1}$]$CH_3SO_4$) was included in this study for comparative purpose. Furthermore, the effects of ILs on hydride generation conditions applied for Se detection by AFS were investigated following a multivariate methodology. The method developed in this work allowed the separation of both inorganic and organic Se species within the same chromatographic run. Finally, the potential of the proposed method was demonstrated by performing Se speciation analysis in several food samples with highly complex matrices.

## 2. Experimental

### 2.1. Instrumentation

The separation of Se species was performed with an HPLC instrument composed of 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 an Agilent Zorbax SB-$C_8$ column from Agilent technologies (Santa Clara, CA, USA). A Rayleigh AF-640A atomic fluorescence spectrometer (Beijing Rayleigh analytical Instrument Corp., Beijing, China) was used for Se detection. Instrumental conditions of HPLC-HG-AFS are shown in Table 1. An ultrasound bath (40 kHz and 600 W) with temperature control (Test Lab, Buenos Aires, Argentina) was employed to degas the mobile phases and solvents before chromatography. A Horiba F–51 pH metre (Kyoto, Japan) was used for pH measurements in mobile phases, standard solutions and samples.

### 2.2. Reagents and solutions

Stock standard solutions (1000 mg Se $L^{-1}$) of inorganic Se(IV) and Se(VI) were prepared from sodium selenite ($Na_2SeO_3$) (99%) (Sigma-Aldrich, St Louis, USA) and anhydrous sodium selenate ($Na_2SeO_4$) (98%) (Sigma-Aldrich), respectively, in ultrapure water (18 MΩ cm) obtained from a Milli-Q system (Millipore, Academic, USA)] and acidified at 0.1 mol $L^{-1}$ HCl (Merck, Darmstadt, Germany). Organic species Se-L-methionine ($C_5H_{11}NO_2Se$) (98%) (Sigma-Aldrich) and Se-(Methyl)selenocysteine hydrochloride (95%) ($C_4H_9NO_2Se$·HCl) (Sigma-Aldrich) stock standard solutions (1000 mg Se $L^{-1}$) were prepared with ultrapure water and stored at 4 °C in amber-coloured HDPE bottles. Working solutions were prepared by serial dilution of stock solutions.

Potassium iodide (Aldrich) was applied as reducing agent after UV-photo-oxidation of Se species. Sodium borohydride ($NaBH_4$) (Fluka) (reducing agent) dissolved in 0.05% (w/v) NaOH (Aldrich) and HCl (Merck) (carrier agent) were used for hydride generation. Tygon type tubes (Gilson, Villiers Le-Bell, France) were used to carry the reagents. All bottles used for storing the samples, standard solutions and mobile phases along with glassware were washed in 10% (v/v) $HNO_3$ (Merck) for 24 h and later rinsed with ultrapure water (18 MΩ cm).

Several ILs including, [$C_4$mim]Cl, [$C_6$mim]Cl, [$C_8$mim]Cl, [$C_{12}$mim]Br and [$C_{16}$mim]Br were synthesized according to a method proposed by Baltasar et al. [18]. Characterization of ILs was performed by comparison of infrared spectra with available databases (Solvent Innovation GmbH, Köln, Germany). [$P_{4,4,4,1}$]$CH_3SO_4$ was purchased from Sigma-Aldrich. Stock solutions of [$C_4$mim]Cl, [$C_6$mim]Cl and [$C_8$mim]Cl at 5% (v/v) and [$C_{12}$mim]Br, [$C_{16}$mim]Br and [$P_{4,4,4,1}$]$CH_3SO_4$ at 5% (w/v) were prepared in ultrapured water and assayed as mobile phase additive. Citric acid (Sigma-Aldrich), acetic acid (Sigma-Aldrich), sodium dibasic phosphate (JT Baker) and 2-Amino-2-hydroxymethyl-propane-1,3-diol (JT Baker) were used to prepare different buffers. Nitric acid 65% (w/v) (Merck) was used for sample digestion during total Se determinations. Enzymatic hydrolysis was performed with Protease XIV (Sigma-Aldrich).

### 2.3. Collection of food and beverage samples

Beer, wine and yeast samples were purchased at local supermarkets (Mendoza, Argentina). Selenium-enriched garlic was produced at the San Carlos experimental station (Mendoza, Argentina) of the Instituto Nacional de Tecnología Agropecuaria (INTA) in the season between April 2014 and December 2014. The garlic clone “Rubi INTA” was used in this work and fortified with a 169 g Se $L^{-1}$ aqueous solution. Fortification of garlic with Se was performed at four doses: 0, 5, 10 and 15 kg Se $ha^{-1}$, following a random plot design and three replicates. For plantation, 10-l pots filled with peat and 10% soil were used. The plants were watered daily and fertilized with commercially available 15N:30P:15 K when it was needed. Once the harvesting was performed, the bulbs were dried to commercial moisture, isolated from leaves and roots, and stored at −18° C. For lyophilization and homogenization, a freezer dryer Virtis freeze mobile (New York, USA) Model 6 Lyophilizer 12L and a grinder Ultracomb (Buenos Aires, Argentina) model MO-8100A were used, respectively.

### 2.4. Total Se determination

Total Se concentration was obtained by HG-AFS after sample digestion. Beer (5.0 mL), wine (5.0 mL), yeast (1.0 g) and garlic (1.0 g) were digested with 5 mL of $HNO_3$, 5 mL of $H_2O_2$ or 5 mL HCl depending on the complexity of the samples. Prior to the determination, Se(VI) was reduced to Se(IV) with 3 mL of 6.0 mol $L^{-1}$

**Table 1**
Instrumental and chromatographic separation conditions.

| HPLC | |
|---|---|
| Column | Agilent Zorbax SB-$C_8$ column (150 mm × 4.6 mm i.d.) |
| Mobile phase | A: 0.1% (v/v) [$C_6$mim]Cl; pH = 6.0; $5.0 \times 10^{-3}$ mol $L^{-1}$ phosphate buffer; 5% (v/v) methanol<br>B: 0.1% (v/v) [$C_6$mim]Cl; pH = 6.0; 0.05 mol $L^{-1}$ phosphate buffer; 5% (v/v) methanol |
| Gradient | 0–7 min A at 0.5 mL $min^{-1}$; 7–8 min A-B and 0.5 – 1.0 mL $min^{-1}$; 8–12 min B at 1.0 mL $min^{-1}$<br>7 min: 100% A, 0% B; 7,2 min: 80% A, 20%B; 7,4 min: 60% A, 40% B; 7,6 min: 40% A; 60% B; 7,8 min: 20% A, 80% B: 8 min: 0% A, 100% B. |
| Injection Volume | 100 μL |
| Column temperature | 25 °C |
| Chromatographic mode | Gradient |
| HG-AFS | |
| Carrier gas and flow rate | Ar, 800 mL $min^{-1}$ |
| Atomization temperature | 400 °C |
| Lamp and wavelength | Se high intensity hollow cathode lamp, 196 nm |
| Main current | 40 mA |
| Auxiliary current | 0 mA |
| Photomultiplier detector voltage | −270 V |
| Reductant | 1.5% (w/v) $NaBH_4$ in 0.05% (w/v) NaOH |
| Carrier | 12.0% (v/v) HCl |
| Reductant and carrier flow rates | 6.0 mL $min^{-1}$ |
| Reductant (post-column treatment) | 0.5% (w/v) KI in 0.2% (w/v) NaOH |
| Reductant (post-column treatment) flow rate | 1.5 mL $min^{-1}$ |

HCl, while heating at 100 °C by 7 min. Finally, the samples were filtered, adjusted to 20% (v/v) HCl and diluted to 50 mL in a volumetric flask with ultrapure water. The conditions for HG-AFS determinations were as follows: 0.7% (w/v) $NaBH_4$ (in 0.05% (w/v) NaOH), 5% (v/v) HCl and reagents flow rate at 12 mL $min^{-1}$. Argon flow rate was 800 mL $min^{-1}$ and atomization temperature was 300 °C. Other instrumental parameters are listed in Table 1.

### 2.5. Selenium speciation analysis

A volume of 1 mL of wine or beer sample was mixed with diluted NaOH in order to adjust the pH to 6.0. Then, 500 μL of a 0.5 mol $L^{-1}$ $H_2PO_4^-/HPO_4^{2-}$ buffer solution was added and the volume was made up to 5 mL with ultrapure water in a volumetric flask. Extraction of Se species was performed from 0.2 g of garlic or yeast samples with 5 mL of Tris buffer solution (0.05 mol $L^{-1}$ at pH 7.5) and 0.02 g of Protease XIV for enzymatic hydrolysis (85% extraction). The solution was kept at 50 °C and it was permanently stirred for 24 h. All samples solutions were filtered with 0.22 μm regenerated cellulose filters before injection into HPLC.

Separation of Se species was performed under gradient conditions by changing mobile phase flow rate and buffer concentration during the chromatography. Composition of mobile phases A and B and chromatographic analysis were established as mentioned in Table 1. Quantification was performed based on the peak areas obtained by RP-HPLC-HG-AFS. Other experimental conditions were as listed in Table 1.

## 3. Results and discussion

### 3.1. Effect of ILs on HG-AFS response

Initially, the potential effect of the ILs used as mobile phase additives on HG-AFS sensitivity was studied in this work. The Se species were individually injected into the HPLC system without a column being connected and pumped directly into the HG-AFS instrument at 1.0 mL $min^{-1}$. Different mobile phases containing a buffer (pH = 2.0, 0.02 mol $L^{-1}$ citric/citrate), 5% (v/v) methanol and each IL at two concentrations [0.1% (v/v) and 0.5% (v/v)] were evaluated. Also, UV-digestion with 0.1% (w/v) KI (in 0.2% (w/v) NaOH) was applied as reported elsewhere [15]. The conditions used for HG-AFS were as follows: 1.4% (w/v) $NaBH_4$ (in 0.05% (w/v) NaOH), 8.5% (v/v) HCl and reagents flow rate of 5.4 mL $min^{-1}$. Argon flow rate was 800 mL $min^{-1}$ and atomization temperature was 300 °C. In this part of the study, [$C_4$mim]Cl, [$C_6$mim]Cl, [$C_8$mim]Cl, [$C_{12}$mim]Br, [$C_{16}$mim]Br and [$P_{4,4,4,1}$]$CH_3SO_4$ ILs were evaluated. However, [$C_{12}$mim]Br at 0.5% (v/v) and [$C_{16}$mim]Br could not be included in this study due to intense foaming effect caused in the gas-liquid separator of the HG-AFS instrument. The results are shown in Fig. 1a [0.1% (v/v) IL] and Fig. 1b. [0.5% (v/v) IL]. The analytical signal obtained for each Se species was different depending on the cation of each IL and concentration. Thus, no major changes (12%) were observed on the Se(IV) signal when imidazolium-type ILs were assayed. On the other hand, the reduction of the Se(VI) signal varied between 26% to 76% when IL concentration was 0.1% (v/v) and around 91% when IL concentration was 0.5% (v/v). For SeMet, the signal decreased by 60% when IL concentration was 0.1% (v/v), while a decrease between 83% and 93% was observed at 0.5% (v/v) IL. The signals obtained for SeMeSeCys decreased upon each IL and concentration, observing variations between 40% and 60%. The decrease in HG-AFS sensitivity could be related with possible interfering effects occurred during the reduction of Se(IV) by $I^-$ or changes caused in the kinetics of hydride generation reaction. On the other hand, signals were not significantly modified when the concentration of [$P_{4,4,4,1}$]$CH_3SO_4$ was changed, but organic Se species showed less sensitivity than inorganic ones.

### 3.2. Separation of Se species by IL-RP-HPLC

#### 3.2.1. Effect of pH

The pH of samples and mobile phases plays an important role in the separation of Se species because their dissociation equilibriums are strongly dependant on pH changes. The Se species included in this study have several ionisable groups and p $K_a$ values ranging between 1.92 and 9.0. Therefore, the effect of pH on the separation of Se species was evaluated for values between 2.0 and 7.5. Also, high stability of stationary phase is reported by column manufacturer in that range. Furthermore, it has to be mentioned that the choice of the $C_8$ column was because this stationary phase is normally used for Se speciation analysis [19]. Several buffers

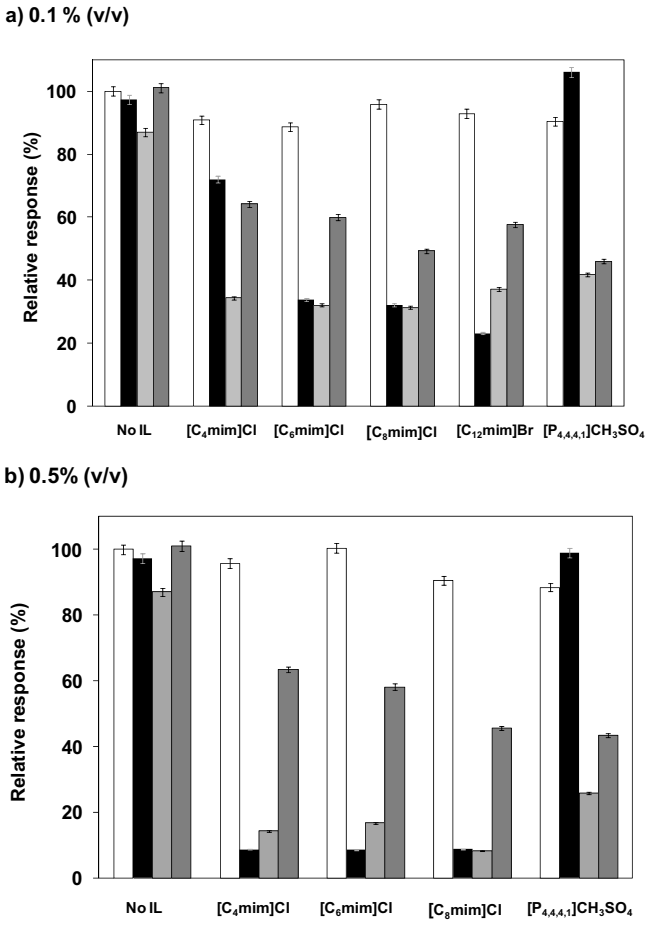


**Fig. 1.** HG-AFS relative response (n = 3) of Se species with respect to Se(IV) in the presence of different ILs concentrations in the mobile phase. a) 0.1% (v/v) and b) 0.5% (v/v) IL. (□) Se(IV), (■) Se(VI), (■) SeMet, (■) SeMeSeCys. $C_{Se\ species}$ = 200 μg Se $L^{-1}$.

at 0.02 mol $L^{-1}$ concentration were employed to adjust the pH at 2.0, 3.5, 6.0 and 7.5. For this evaluation, [$C_8$mim]Cl IL was chosen because it was considered to have a mid-length alkyl chain among all imidazolium ILs studied in this work. Experiments were performed in isocratic mode with a mobile phase flow rate of 1 mL $min^{-1}$. Other conditions were as mentioned in Table 1.

The separation of Se species was not significantly influenced within the pH interval under study, which might be attributed to complete formation of anionic Se species suitable for interaction with the IL cation. Thus, two peaks were observed, the first corresponding to unresolved organic Se species followed by another peak for Se(IV). Furthermore, since none of the ILs assayed in this work had exchangeable protons, the influence of pH on their dissociations can be considered negligible. Therefore, full dissociation of ILs can be expected at any pH value and hence, IL cations might be readily available for ion pairs formation with anionic Se species. However, under the above-mentioned chromatographic conditions, the Se species were not fully separated. Individual injection of the Se species at pH 6.0 showed that SeMet and SeMetSeCys were eluted at 3.0 min, while Se(IV) was observed at 4.5 min. On the other hand, Se(VI) was not eluted from the column. It must be considered that under the working conditions (pH = 6.0), Se(VI) exist as the fully-deprotonated $SeO_4^{2-}$ ($pK_a$ = 1.9) species, while Se(IV) occurs as $HSeO_3^-$ species ($pK_{a1}$ = 2.46 and $pK_{a2}$ = 7.31) [12]. Therefore, a stronger interaction can be expected for Se(VI) with the IL cation, which in turns makes more feasible the formation ion pairs and its retention on the $C_8$ stationary phase. A similar behaviour was observed by Chen et al. using [$C_4$mim]Cl at 0.4% (v/v) [16]. Finally, a pH of 6.0 was chosen as a good compromise between $C_8$ stationary phase stability and Se species dissociation required for ion pairing reaction with IL cation.

### 3.2.2. Buffer concentration and mobile phase flow rate

The effect of citric acid/citrate buffer was evaluated at two concentrations: $5.0 \times 10^{-3}$ mol $L^{-1}$ and 0.05 mol $L^{-1}$. The results showed that for a buffer concentration of $5.0 \times 10^{-3}$ mol $L^{-1}$, SeMet and SeMeSeCys were partially resolved, while Se(IV) was eluted at high retention time and Se(VI) was not eluted. On the other hand, when the buffer concentration was 0.05 mol $L^{-1}$, SeMet, SeMeSeCys and Se(IV) species were not separated and Se(VI) was eluted within 9 min. Based on these results, a buffer concentration gradient was developed in order to obtain fast and improved separation of Se species. The gradient started with a buffer concentration of $5.0 \times 10^{-3}$ mol $L^{-1}$ during 6 min and mobile phase flow rate of 0.5 mL $min^{-1}$. This initial step improved the separation of SeMeSeCys and SeMet species. Afterwards, the gradient program continued with a second step where buffer concentration was linearly increased up to 0.05 mol $L^{-1}$ in 1 min and was kept constant until the end of chromatography. Also, the mobile phase flow rate was increased up to 1.0 mL $min^{-1}$ to speed up the elution of Se(VI) species. The application of this gradient program improved peaks resolution and the elution of Se(VI) species in shorter times. However, baseline resolution of the organic Se species was not completely achieved. The gradient conditions were adopted for further experiments.

### 3.2.3. Effect of the type of IL on Se species separation

Possible changes on the separation of Se species with the type of IL were evaluated. For this study, the concentration of each IL was fixed at 0.1% (v/v) to avoid a significant deleterious effect on HG-AFS sensitivity (Fig. 1). Fig. 2(a–d) shows the chromatograms obtained when [$C_4$mim]Cl, [$C_6$mim]Cl, [$C_{12}$mim]Br and [$P_{4,4,4,1}$]$CH_3SO_4$ were used as mobile phase modifiers. [$C_8$mim]Cl has not been included in this figure but its effect on the separation was discussed earlier in Section 3.2.2. [$C_{16}$mim]Cl could not be evaluated due to the intense foaming effect caused by this IL inside the gas-liquid separator of AFS instrument.

The results showed changes in retention times and chromatographic resolution of Se species depending on the alkyl chain length of imidazolium-type ILs (Fig. 2a–c). The first Se species eluted with any of the ILs assayed in this work was SeMeSeCyst, which was observed at 5 min, thus indicating that this species was weakly retained in the column. On the other hand, the longer the alkyl chain, the stronger was the retention of Se(VI). For example, in the case of [$C_{12}$mim]Br a retention time of 14 min was observed for Se(VI), while 10 min were registered with [$C_4$mim]Cl. This behaviour could be due to the balance of hydrophobicity and ionic strength. The retention of Se(VI) was similar between [$P_{4,4,4,1}$]$CH_3SO_4$ and [$C_{12}$mim]Br.

Regarding the resolution of Se species, different results were obtained depending on each IL. For [$C_8$mim]Cl and [$C_{12}$mim]Br, poor resolution was observed for SeMeSeCys and SeMet, but they were fully separated with [$C_4$mim]Cl. However, SeMet and Se(IV) remained unresolved with [$C_{12}$mim]Br. Likewise, SeMeSeCys and SeMet were not resolved with [$P_{4,4,4,1}$]$CH_3SO_4$ (Fig. 2d). On the other hand, the best resolution was obtained with [$C_6$mim]Cl. These results showed the special role played by the type of IL on the separation of Se species.

Despite [$C_6$mim]Cl provided acceptable separation of Se species, baseline resolution of peaks for SeMet and Se(IV) was not achieved. Therefore, a new gradient was developed consisting of a first stage where buffer concentration was kept constant at $5.0 \times 10^{-3}$ mol $L^{-1}$ during 7 min at a flow rate of 0.5 mL $min^{-1}$, followed by a second

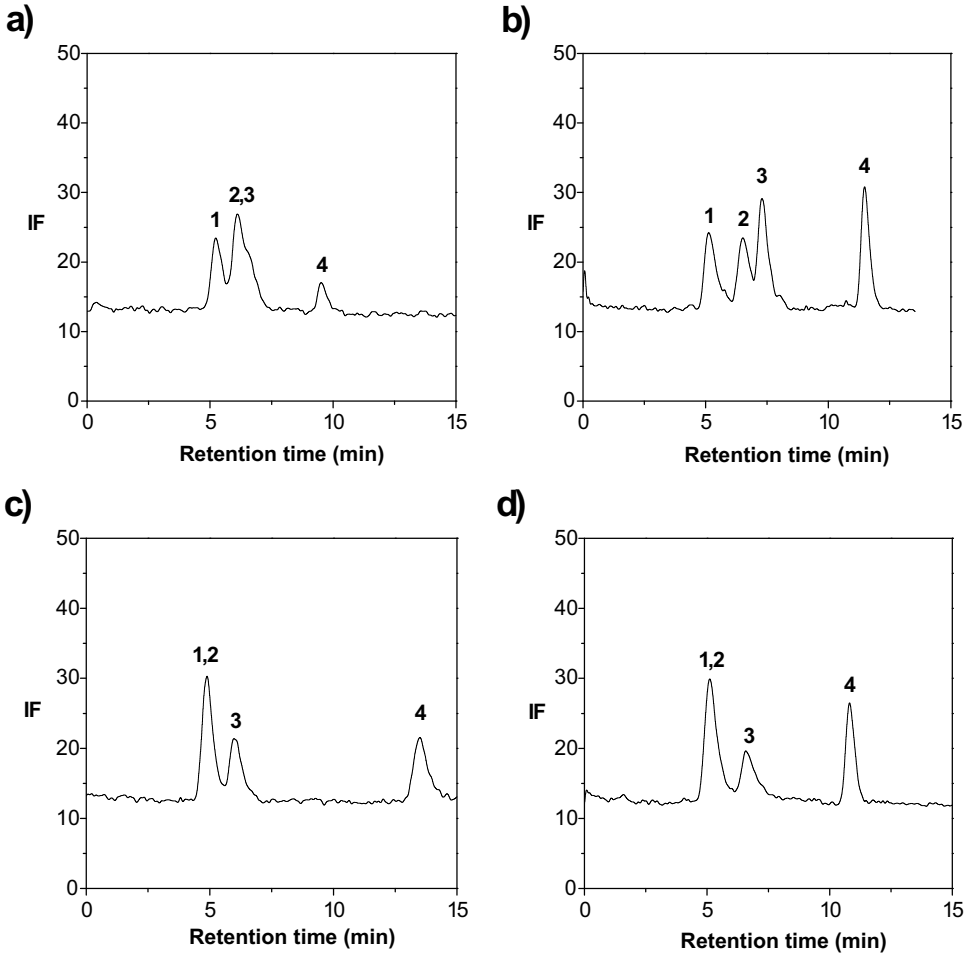


**Fig. 2.** Effect of ILs on the separation of Se species. a) [$C_4$mim]Cl, b) [$C_6$mim]Cl, c) [$C_{12}$mim]Br and d) [$P_{4,4,4,1}$]$CH_3SO_4$. 1: SeMeSeCys, 2: SeMet, 3: Se(IV), 4: Se(VI). $C_{Se\ species}$ = 200 μg Se $L^{-1}$.

stage where buffer concentration and flow rate were increased up to 0.05 mol $L^{-1}$ and 1.0 mL $min^{-1}$, respectively, within 1 min. Under these new conditions, full resolution of the four Se species studied in this work was obtained. The potential effects of ILs on HPLC separations had been discussed by some authors previously [14,16]. It has been pointed out that ILs could interact with the stationary phase and other components of the mobile phase (e.g. buffer) to form a new pseudo-stationary phase. In our work, interaction of IL cations with the non-polar alkyl chain of this stationary phase might be responsible for the modifications observed in the separation of Se species. However, since ILs are complex entities, several mechanisms might be expected in chromatography. Therefore, a mix of different roles can be attributed to ILs including, hydrophobic partitioning, ion-exchange and ion-pairing. The extension of these interactions might depend on the chosen IL, its chemical structure and concentration.

Previous experiments done in this work were performed with each IL at 0.1% (v/v). Therefore, in order to evaluate the effect of IL concentration on the separation of Se species, two levels were tested: 0.05% (v/v) and 0.2% (v/v). Higher concentrations were not assayed due to deleterious effect on HG-AFS sensitivity caused by ILs. The results showed poor resolution of SeMet and Se(IV) species when ILs concentration was 0.05% (v/v), while at 0.2% (v/v) the resolution was similar to that achieved at 0.1% (v/v). However, a sensitivity loss was obtained when IL concentration was 0.2% (v/v), for both organic (30%) and inorganic (20%) Se species. According to these results, 0.1% (v/v) [$C_6$mim]Cl was selected as optimal condition for Se species separation.

### 3.3. Multivariate optimization of AFS detection conditions

Once the conditions for chromatographic separation were optimized by an univariant method, a multivariate method was developed to achieve the highest sensitivity possible for all Se species detected by HG-AFS. The study was developed with peak area (PA) information of each species. Design Expert® 7.0 (Stat-Ease Inc., Minneapolis, USA) software was used to process all results. An experimental Plackett Burman design was applied for the determination of the main variables affecting HG-AFS sensitivity. The analyzed factors and intervals were $NaBH_4$ concentration (1.0–2.0% w/v), HCl concentration (8.0–16.0% v/v), KI concentration (0.1–0.5% w/v), KI flow rate (1.5-3.0 mL $min^{-1}$), reducing and carrier agents flow rate (5.0-7.0 mL $min^{-1}$), atomization temperature (300–450 °C) and argon flow rate (700–900 mL $min^{-1}$). The selected ranges for each factor were chosen according to previous experiments [15]. The evaluation was performed measuring the peak area for each Se species at 200 μg $L^{-1}$ after injection into HPLC (no column) and under the best conditions obtained for separation, i.e. mobile phase flow rate of 0.5 mL $min^{-1}$ for organic species and 1.0 mL $min^{-1}$ for inorganic species. Thus, 12 experiments were performed.

A Pareto graph was employed to choose significant effects for each Se species. After the analysis, the factors with significant

effects were HCl concentration, KI concentration, KI flow rate and atomization temperature for all Se species. The selected variables from Pareto graph were evaluated by analysis of variance (ANOVA) obtaining significant models for all the responses and large adjusted R-square (0.970 for SeMeSeCys, 0.957 for Se(VI), 0.980 for SeMet and 0,925 for Se(IV)) which indicates a good relationship between the experimental data and the fitted model. 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 Se species would had to be higher. A total of 27 experiments were performed including 3 central points. The experimental ranges for the significant factors were the same than the screening stage and the fixed factors (not significant) were established as follows: 1.5% (w/v) $NaBH_4$ concentration, 6.0 mL $min^{-1}$ flow rate for reducing and carrier agents and 800 mL $min^{-1}$ argon flow rate. RSMs were plotted for each Se species and quadratic models were obtained with transformed responses in the case of Se(IV), an inverse function ($X^{-1}$) for Se(VI) and an inverse square root ($X^{-1/2}$) for SeMeSeCys and SeMet. This approach was used to adjust the statistical parameters and obtain the appropriated model diagnostic plots as normal probability and residuals vs. predicted. All models were highly significant and the lack of fit non-significant. The statistical parameters were acceptable for all Se species. Large adjusted R-square were obtained (0.94–0.98) indicating a good fit of the obtained model to the results obtained in the experiments. The variation coefficient values (0.77–5.93) indicated a low standard deviation and hence, high reproducibility and precision are obtained. The model coefficients were calculated by backward multiple regression and validated by ANOVA.

Once a model was chosen for each Se 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 maximum as optimization criteria. The adjusted optimum conditions given by the model were as follows: 12% (v/v) HCl, 0.5% (w/v) KI at a flow rate of 1.5 mL $min^{-1}$ and 400 °C atomization temperature. Afterwards, an experimental confirmation of these optimal conditions was performed. The peak areas corresponding to each Se 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 Se species.

A typical chromatogram obtained under the optimized conditions is shown in Fig. 3a. A 4-fold increase in sensitivity was obtained for organic Se species after performing the multivariate optimization compared to the initial HG-AFS conditions mentioned earlier in this work. Likewise, a 1.5-fold increase was observed for inorganic Se species. Based on these findings, a significant effect on UV-digestion or reduction processes of organic Se species is shown, which produces a remarkable effect on the sensitivity of HG-AFS technique.

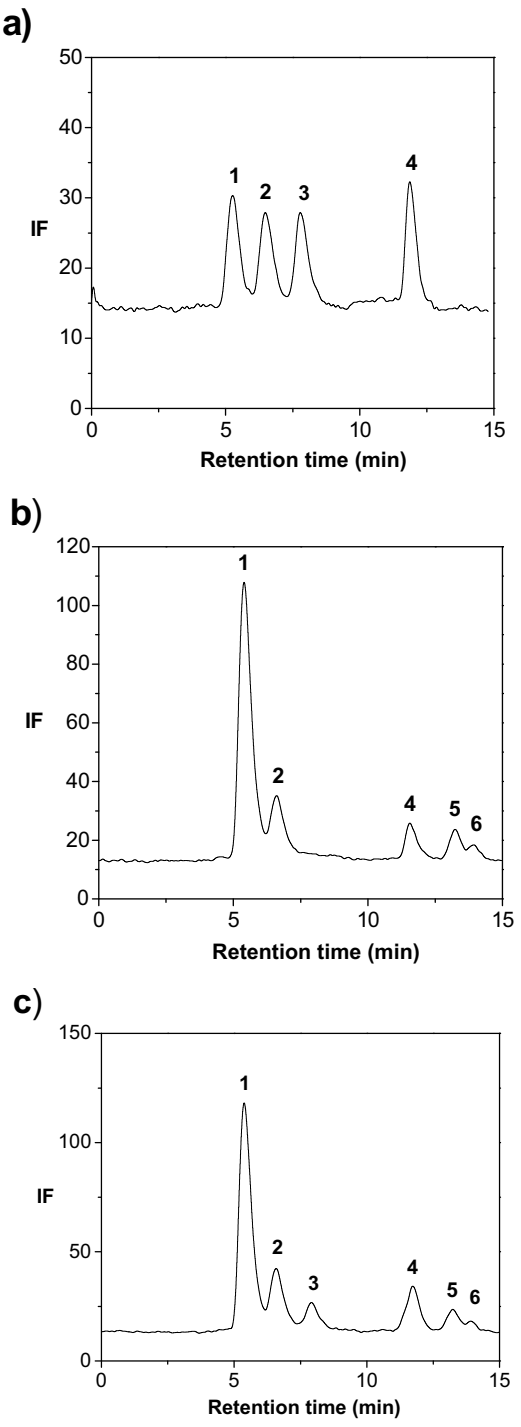


**Fig. 3.** IL-RP-HPLC-HG-AFS chromatograms obtained after Se speciation analysis of a) 200 μg Se $L^{-1}$ standard species mix, b) garlic sample and c) garlic sample extract spiked at 50 μg Se $L^{-1}$ with each Se species. 1: SeMeSeCys, 2: SeMet, 3: Se(IV), 4: Se(VI), 5 and 6: unidentified Se species.

### 3.4. Analytical performance and chromatographic parameters

The analytical figures of merit are reported in Table 2. The LODs were calculated based on the signal at the intercept and three times the standard deviation about regression of the calibration curve. Values in the range of 0.86–1.4 μg Se $L^{-1}$ were obtained. The calibration curves showed a satisfactory linearity for each Se species from LODs up to at least the upper limit evaluated in this work, i.e. 2000 μg $L^{-1}$. The correlation coefficients were between 0.9944 and 0.9995. Table 2 also shows different parameters that characterize the chromatographic separation at the optimal conditions. Thus, reproducible retention times were observed throughout 12 h. Relative standard deviations (RSD) of peak areas were calculated for six replicate measurements of a standard mixture containing all Se species at 200 μg Se $L^{-1}$. The results were in the range of 1.2%–1.4%. The resolutions of IL-RP-HPLC separation of the Se species were calculated as 0.98 between SeMeSeCys and SeMet, 1.0 between SeMet and Se(IV), and 2.93 between Se(IV) and Se(VI). The total separation time was less than 12 min. Furthermore, no significant differences were found between the retention times of Se species in aqueous standards and samples, indicating that there were no matrix effects.

**Table 2**
Chromatographic parameters and analytical performance of the proposed method for Se species determination.

| Parameter | SeMeSeCys | SeMet | Se(IV) | Se(VI) |
|---|---|---|---|---|
| Lineal range ($\mu g\,L^{-1}$) | 2.0–2000 | 2.0–2000 | 1.0–2000 | 1.0–2000 |
| $r^2$ | 0.9985 | 0.9995 | 0.9992 | 0.9944 |
| RSD (%) retention time | 2.1 | 0.9 | 2.4 | 0.9 |
| RSD (%) peak area | 1.32 | 1.41 | 1.22 | 1.26 |
| LOD ($\mu g\,L^{-1}$) | 1.19 | 1.41 | 0.92 | 0.86 |
| R[a] | – | 0.98 | 1.00 | 2.93 |
| $k'$[b] | 0.11 | 0.37 | 0.65 | 1.5 |
| N[c] | 253.09 | 481.02 | 473.59 | 1234.87 |
| $\alpha$[d] | – | 3.36 | 1.75 | 2.31 |

[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 = $k_n/k_{n-1}$.

### 3.5. Comparison between ILs and common ion pairing reagents

Separation of both organic and inorganic Se species by RP-HPLC is challenging and it normally requires the use of ion-pairing agents to make feasible the interaction of anionic Se species with non polar stationary phases, such as $C_8$ or $C_{18}$. However, although the separation of organic Se species can be obtained by RP-HPLC using one ion-pairing reagent, inorganic species are not separated when a similar approach is followed [8]. Therefore, more elaborated separation methods that include the simultaneous application of several ion-pairing reagents must be developed in order to separate Se(IV) and Se(VI) species. For example, Thosaikman et al. [12] separated inorganic and organic Se species, but two ion pairing agents had to be used: 1-butanesulfonic acid (BA) and trifluoroacetic acid (TFA). Moreover, another disadvantage is caused by some ion pairing agents (e.g perfluorocarboxilic acid) that must be used at a low working pH, typically in the range of 2.0–2.5, which can affect the stability of stationary phases. On the contrary, ILs have demonstrated to be excellent alternatives to be used in RP-HPLC for efficient separation of many analytes at pHs where stationary phases are highly stable [16,17].

In the proposed method, the correct selection of experimental conditions such as 0.1% (v/v) [$C_6$mim]Cl in the mobile phase yielded excellent separation for both inorganic and organic Se species within 12 min. A comparison of the proposed method with others reported in the literature is shown in Table 3. LODs obtained in the present work were similar or better than those reported in other methods that used highly sensitive detectors such as ICP-MS. Moreover, the proposed method has been demonstrated to be useful for Se speciation analysis in highly complex matrix samples such as wine, yeast, beer and garlic. Other related methods have required a mixture of two or more ILs at high concentrations, which might represent a real disadvantage to implement them in routine analytical laboratories due to the relatively high costs of some ILs [15,16]. On the other hand, the proposed method requires only one IL which is used at low concentrations.

### 3.6. Determination of Se species in food and beverages samples

Total Se was determined in food samples by HG-AFS technique and concentration results are reported in Table 4. A recovery study was performed by spiking each sample at 20 $\mu g$ Se $L^{-1}$ before determination. The recovery values were satisfactory and in the range of 95.8% to 103%.

Afterwards, the proposed method was applied for Se speciation analysis in several food samples (Table 5). Initially, undiluted wine and beer samples spiked with each Se species at 50 $\mu g$ Se $L^{-1}$ were injected into HPLC to test their separation in the presence of the

**Table 3**
Comparison of different methods for Se speciation analysis using RP-HPLC based on classic ion pairing reagents and ILs.

| Sample | Se Species | Mobile phase | Elution Mode | Detector | Column Temperature (°C) | Elution time (min) | LOD ($\mu g\,L^{-1}$) | [Refs.] |
|---|---|---|---|---|---|---|---|---|
| Yeast and clover | Se(IV), Se(VI), SeMet, SeMeSeCys, $SeCys_2$, SeEt | 0.4% (v/v) [$C_4$mmim]$BF_4$ + 0.4% (v/v) [$C_4$mim]Cl, pH = 6.0 | Isocratic | ICP-MS | 25 | 8 | 0.27–5.37 | [16] |
| Pork, chicken, rice and wheat | Se(IV), Se(VI), SeMet, $SeCys_2$ | A:0.2% (v/v) [$C_6$mim]$BF_4$, 5% (v/v) methanol, pH = 6.0<br>B: 0.4% (v/v) [$C_4$mim]$BF_4$, 5% (v/v) methanol, pH = 6.0 | Gradient | ICP-MS | 35 | 13 | 0.90–1.57 | [17] |
| Se-enriched pakchoi | Se(IV), Se(VI), SeMet, SeMeSeCys $SeCys_2$ | pH = 4.5<br>$4.0 \times 10^{-3}$ mol $L^{-1}$ TFA[a]<br>$8.0 \times 10^{-3}$ mol $L^{-1}$ BA[b] | Isocratic | ICP-MS | N.R.[c] | 13 | 0.85–4.68 | [12] |
| Food suplemments | Se(IV), Se(VI), SeMet, SeMeSeCys, $SeCys_2$ | A: $2.0 \times 10^{-3}$ mol $L^{-1}$ sodium 1-pentanesulfonate, $5.0 \times 10^{-3}$ mol $L^{-1}$ citric acid in 3% (v/v) methanol (pH = 2.70)<br>B: $5.0 \times 10^{-3}$ mol $L^{-1}$ $NaH_2PO_4$, $5.0 \times 10^{-3}$ mol $L^{-1}$ citric acid in 3% (v/v) methanol (pH = 2.77) | Gradient | ICP-MS | N.R.[c] | 8 | 0.04–0.07 | [11] |
| Wine, beer, yeast and Se-enriched garlic | Se(IV), Se(VI), SeMet, SeMeSeCys | A: 0.1% (v/v) [$C_6$mim]Cl; pH = 6.0<br>$5.0 \times 10^{-3}$ mol $L^{-1}$ phosphate buffer; 5% (v/v) methanol<br>B: 0.1% (v/v) [$C_6$mim]Cl; pH = 6.0<br>0.05 mol $L^{-1}$ phosphate buffer; 5% (v/v) methanol | Gradient | HG-AFS | 25 | 12 | 0.86–1.41 | This work |

[a] TFA: trifluoroacetic acid.
[b] BA: 1-butanesulfonic acid.
[c] Not reported.

**Table 4**
Total Se concentration found in the samples studied in this work (95% confidence level, n = 3).

| Sample | Total Se ($\mu g\,g^{-1}$) | Recovery (%)[a] |
|---|---|---|
| Garlic 1 | 71.4 ± 2.7 | 96.4 |
| Garlic 2 | 37.8 ± 0.9 | 95.8 |
| Garlic 3 | 47.9 ± 0.9 | 99.6 |
| Garlic 4 | 29.8 ± 1.5 | 101 |
| Yeast 1 | 0.17 ± 0.03 | 96.7 |
| Yeast 2 | 0.39 ± 0.07 | 103 |
| Wine 1 | 15.9 ± 0.4[b] | 98.7 |
| Wine 2 | 8.9 ± 0.2[b] | 97.8 |
| Beer 1 | 9.8 ± 0.4[b] | 101 |
| Beer 2 | 12.6 ± 0.6[b] | 96.8 |

[a] Recovery = [Found − base]/added × 100.
[b] $\mu g\,L^{1}$.

different samples matrices. A partial loss of chromatographic resolution was observed with respect to an aqueous standards mix of Se species (Fig. 3a). In order to avoid column overload, due to the injection of major organic and inorganic compounds occurring in the samples into the column, 5-fold and 10-fold dilutions (with ultrapure water) were evaluated. Interestingly, a minimal dilution (5-fold) yielded symmetric peaks and optimal resolution, with Se species being eluted at similar retention times than those for aqueous standards. Therefore, a 5-fold dilution was applied to wine and beer samples before injection into HPLC-HG-AFS. On the other hand, a typical enzymatic extraction was performed for garlic and yeast samples and the extracts were injected directly into the HPLC column without additional dilutions. No retention time shifts were observed. Furthermore, experiments were done in order to determine a permanent retention of Se species inside the column and lack of analytical recovery. No significant differences were found between Se concentration in the samples (or in garlic and yeast extracts) and the total Se accounted considering all Se species observed in the chromatograms after HPLC-HG-AFS analysis.

A typical chromatogram obtained after Se speciation analysis in garlic extracts is shown in Fig. 3b. SeMet, SeMeSeCys and Se(VI) were confirmed by retention times matching with Se species standard mix (Fig. 3c). Likewise, two unknown Se-containing peaks eluting at 13.5 and 14.0 min were detected in garlic extracts. Although, they could not be identified due to unavailability of Se species standards, the separation of these Se species was also feasible, demonstrating the potential of the proposed method for the determination of a higher number of species than just only those evaluated in this work. These two unknown Se species could be some of the several reported in garlic, which include, selenocystin ($Secys_2$), glutamylmethylselenocysteine (γ-Glu-MeSeCys), selenohomolantionine and selenohomocysteine [20,21] However, it has to be considered that several published works have also reported selenium species without identification. In fact, in some cases, up to 4 unidentified Se species have been reported by HPLC-ICP-MS in Se-enriched *Brassica* plants [12].

A previous study has demonstrated that the types of Se species found in enriched garlic depends on different factors, for example, the chemical form of Se used for plant treatments [20]. In the present work, SeMeSeCys was the major species and accounted for 60% of total Se found in the samples. This result is in agreement with previous studies done on Se-enriched *Allium* plants, including garlic, onion and leek [20]. Fig. 3c shows the chromatogram of a garlic sample spiked at 50 μg Se $L^{-1}$ of each species. Also, additions at 500 μg Se $L^{-1}$ were made to garlic extracts only for those Se species found at the highest concentrations (SeMet and SeMeSeCys), in order to accurately confirm their identity in the presence of the large size peaks observed in the chromatograms. Concentrations of Se species in garlic samples were between 97.9 ± 1.1–158.9 ± 1.9 $\mu g\,L^{-1}$ for Se(VI), 476.8 ± 9.0–819.9 ± 10.9 $\mu g\,L^{-1}$ for SeMet and 954.0 ± 13.1–2899 ± 38.0 $\mu g\,L^{-1}$ for SeMeSeCys.

Speciation analysis of Se in wines, beers and yeasts only evidenced inorganic Se species [Se(IV) and Se(VI)] and their concentrations were in agreement with those reported in other works [19,22,23]. For wine, concentrations of Se(IV) were between <LOD and 8.0 ± 0.1 $\mu g\,L^{-1}$, while Se(VI) was between <LOD and 6.9 ± 0.1 $\mu g\,L^{-1}$. For beer, concentrations of Se(IV) were between 8.4 ± 0.1 and 11.6 ± 0.2 $\mu g\,L^{-1}$. In the case of yeast, concentrations of Se(IV) were between 9.6 ± 0.2 and 11.5 ± 0.2 $\mu g\,L^{-1}$ and Se(VI) was between <LOD and 10.9 ± 0.2 $\mu g\,L^{-1}$. Finally, a recovery study was performed on the samples with additions at 50 μg Se $L^{-1}$ and 500 μg Se $L^{-1}$ for each Se species. Acceptable recovery values were obtained in the different samples (95.0%–105%).

**Table 5**
Results obtained after Se speciation analysis in food and beverages studied in this work (95% confidence level, n = 3).

| Sample | Se(IV) | | | Se(VI) | | | SeMet | | | SeMeSeCys | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Added ($\mu g\,L^{-1}$) | Found ($\mu g\,L^{-1}$) | R(%)[a] | Added ($\mu g\,L^{-1}$) | Found ($\mu g\,L^{-1}$) | R(%)[a] | Added ($\mu g\,L^{-1}$) | Found ($\mu g\,L^{-1}$) | R(%)[a] | Added ($\mu g\,L^{-1}$) | Found ($\mu g\,L^{-1}$) | R(%)[a] |
| Garlic 1 | – | <LOD | – | – | 97.9 ± 1.1 | – | – | 819.9 ± 10.9 | – | – | 2899 ± 38 | – |
| | 50 | 51.5 ± 0.7 | 103 | 50 | 146.1 ± 2.4 | 96.4 | 500 | 1300 ± 21 | 96.0 | 500 | 3378 ± 39 | 95.8 |
| Garlic 2 | – | <LOD | – | – | 100.6 ± 1.2 | – | – | 602.8 ± 8.7 | – | – | 1310 ± 23 | – |
| | 50 | 47.5 ± 0.6 | 95.0 | 50 | 148.5 ± 2.1 | 95.8 | 500 | 1100 ± 20 | 99.4 | 500 | 1798 ± 28 | 97.6 |
| Garlic 3 | – | <LOD | – | – | 102.2 ± 1.3 | – | – | 638.6 ± 8.9 | – | – | 1806 ± 28 | – |
| | 50 | 52.5 ± 0.8 | 105 | 50 | 149.8 ± 2.4 | 95.2 | 500 | 1130 ± 17 | 98.3 | 500 | 2295 ± 35 | 97.7 |
| Garlic 4 | – | <LOD | – | – | 158.9 ± 1.9 | – | – | 476.8 ± 9.0 | – | – | 954 ± 13.1 | – |
| | 50 | 49.2 ± 0.6 | 98.4 | 50 | 210.8 ± 2.8 | 104 | 500 | 970.2 ± 18.5 | 98.7 | 500 | 1444 ± 22 | 98.0 |
| Yeast 1 | – | 9.6 ± 0.2 | – | – | <LOD | – | – | <LOD | – | – | <LOD | – |
| | 50 | 60.2 ± 0.6 | 101 | 50 | 49.6 ± 0.8 | 99.2 | 50 | 51.2 ± 0.8 | 102 | 50 | 49.4 ± 0.6 | 98.8 |
| Yeast 2 | – | 11.5 ± 0.2 | – | – | 10.9 ± 0.2 | – | – | <LOD | – | – | <LOD | – |
| | 50 | 63.1 ± 0.6 | 103 | 50 | 62.3 ± 0.8 | 103 | 50 | 47.8 ± 0.7 | 95.6 | 50 | 51.8 ± 0.7 | 104 |
| Wine 1 | – | 8.0 ± 0.1 | – | – | 6.9 ± 0.1 | – | – | <LOD | – | – | <LOD | – |
| | 50 | 57.6 ± 0.7 | 99.2 | 50 | 57.8 ± 0.8 | 102 | 50 | 51.9 ± 0.7 | 104 | 50 | 47.9 ± 0.7 | 95.8 |
| Wine 2 | – | <LOD | – | – | <LOD | – | – | <LOD | – | – | <LOD | – |
| | 50 | 51.2 ± 0.7 | 102 | 50 | 49.9 ± 0.7 | 99.8 | 50 | 49.6 ± 0.7 | 99.2 | 50 | 52.5 ± 0.6 | 105 |
| Beer 1 | – | 8.4 ± 0.1 | – | – | <LOD | – | – | <LOD | – | – | <LOD | – |
| | 50 | 57.1 ± 0.8 | 97.4 | 50 | 51.6 ± 0.8 | 103 | 50 | 48.4 ± 0.6 | 96.8 | 50 | 49.0 ± 0.8 | 98.0 |
| Beer 2 | – | 11.6 ± 0.2 | – | – | <LOD | – | – | <LOD | – | – | <LOD | – |
| | 50 | 60.8 ± 1.1 | 98.4 | 50 | 49.4 ± 0.6 | 98.8 | 50 | 51.2 ± 0.7 | 102 | 50 | 48.6 ± 0.7 | 97.2 |

[a] R(%): Recovery = [Found − base]/added × 100.

## 4. Conclusions

A novel method for Se speciation analysis in food samples has been developed in this work. A comprehensive study of the effects of several ILs used as mobile phase additives on Se species separation and determination by RP-HPLC-HG-AFS has been performed. The separation mechanisms involved when ILs are used in RP-HPLC could be attributed to different processes, i.e. interaction between anionic Se species and IL molecules retained on the stationary phase, but also hydrophobic partitioning of the ion pairs formed between ILs and Se species with the $C_8$ stationary phase. Moreover, the correct choice of the IL and its concentration were critical factors to attain complete separation. Thus, retention times and resolution of Se species were demonstrated to be highly dependent on the length of alkyl chains in imidazolium-type ILs.

The limits of detection obtained with the proposed method were comparable or even better than those reported in the literature using RP-HPLC coupled to sensitive detectors such as ICP-MS. Furthermore, multivariate optimization allowed a significant enhancement in HG-AFS sensitivity for all Se species, even in the presence of ILs. Therefore, this tool can be considered an important allied for method development when hyphenated techniques are applied for elemental speciation analysis. Finally, improved separation of both organic and inorganic Se species was achieved when ILs were added to the mobile phase, which in turns allowed the successful application of the method for Se speciation analysis in highly complex samples such as wines, beers, yeast and garlic.

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