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Ionic liquids improved reversed-phase HPLC on-line coupled with ICP-MS for selenium speciation

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ABSTRACT

Room-temperature ionic liquids (RTILs) improved reversed-phase high performance liquid chromatography (RP-HPLC) on-line combined with inductively coupled plasma mass spectrometry (ICP-MS) was developed for selenium speciation. The different parameters affecting the retention behaviors of six target selenium species especially the effect of RTILs as mobile phase additives have been studied, it was found that the mobile phase consisting of 0.4% (v/v) 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 0.4% (v/v) 1-butyl-2,3-dimethylimidazolium tetrafluroborate ([BMMIM]BF_4) and 99.2% (v/v) water has effectively improved the peak profile and six target selenium species including Na_2SeO_3 (Se(IV)), Na_2SeO_4 (Se(VI)), L-selenocystine ($SeCys_2$), D,L-selenomethionine (SeMet), Se-methylseleno-L-cysteine (MeSeCys), seleno-D,L-ethionine (SeEt) were separated in 8 min. In order to validate the accuracy of the method, a Certified Reference Material of SELM-1 yeast sample was analyzed and the results obtained were in good agreement with the certified values. The developed method was also successfully applied to the speciation of selenium in Se-enriched yeasts and clover. For fresh Se-enriched yeast cells, it was found that the spiked $SeCys_2$ in living yeast cells could be transformed into SeMet. Compared with other ion-pair RP-HPLC–ICP-MS approaches for selenium speciation, the proposed method possessed the advantages including ability to regulate the retention time of the target selenium species by selecting the suitable RTILs and their concentration, simplicity, rapidness and low injection volume, thus providing wide potential applications for elemental speciation in biological systems.

1. Introduction

The trace mineral selenium is an essential nutrient of fundamental importance to organism [1,2], while it has a very narrow range of concentration between selenium deficiency and toxicity. The biological effects, toxicity and bioavailability of selenium are highly dependent on its chemical forms. In living system, selenium is mainly present as organoselenium compounds: selenoamino acids and selenium proteins, which would take part in the metabolism of living organism. Now there are many selenium nutritional supplements for human beings, such as yeast, clover, medicinal plants [3,4] and so on. Therefore, for a better understanding and exploration of the bioavailability of selenium to living organism, speciation of selenium is much more important and significant than its total concentration determination [5].

Elemental speciation analysis is usually achieved by hyphenation techniques in which a high efficient separation technique is employed to separate different species of interested element and a sensitive detection technique is used to determine the target elemental species at low levels. Hyphenated techniques for the speciation of selenium have been well documented in recent reviews [6,7]. Inductively coupled plasma mass spectrometry (ICP-MS) is the most widely employed elemental-specific detection technique because it offers high sensitivity and good methodology when reference compounds are available, and relatively easy of interfacing with commonly used separation techniques. Of all separation techniques, HPLC is the most widely used technique [8–12] for selenium speciation because it is easily interfaced to ICP-MS through conventional nebulizers, has various separation modes, requires no derivatization to create volatile compounds which is required sometimes in gas chromatography (GC) analysis, and exhibits good reproducibility of which electrophoresis (CE) analysis is often lacking.

For various HPLC separation modes, ion-exchange HPLC both in cation-exchange mode and anion-exchange mode have been applied for selenium speciation [13], while size-exclusion chromatography (SEC) was suitable for the separation of biochemical macromolecules such as various seleno-proteins [9]. Ion-pair reversed-phase (RP)-HPLC with appropriate ion-pair reagents added in the mobile phase was well suited for simultaneous separation of anionic, cationic and neutral molecules [8,9,11,14–19], resulting in most extensive application for the study of selenium compounds. The commonly used ion-pair reagents are

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perfluorocarboxylic acid such as trifluoroacetic acid (TFA) [14,15] and heptafluorobutanoic acid (HFBA) [8,9,16,17]. TFA as ion-pair reagent would result in a good separation improvement with compromise between resolution and extended retention time. However, some selenium species (inorganic selenium and selenoamino acids) cannot be separated [15]. Compared with TFA, HFBA as ion-pair reagent has much stronger ion-pairing power, resulting in a better chromatographic resolution, and an associated extended separation time, but Se(IV) and Se(VI) cannot be separated well yet. Tetra-alkylammonium salts [18] and alkylsulfonic salts [19] were also used as mobile phase additives to improve the chromatographic separation of selenium species. However, there are two problems associated with this technique. Firstly, the use of organic additives would cause carbon deposition on the surface of sampling cone and therefore worsen the detection limits of ICP-MS. Secondly, adoption of gradient elution would induce a severe matrix variation which leads to sensitivity drift in ICP-MS.

Room-temperature ionic liquids (RTILs), due to their unique properties of negligible vapor pressure, good thermal stability and solubility for inorganic and organic compounds [20,21], and the possibility of designing them to give a range of distinctive properties simply by synthesis, are receiving much attention as environmentally benign solvents in heterogeneous catalysis, synthesis, electrochemistry, sensors, battery applications, and analytical chemistry [22]. RTILs have also been used as novel separating reagents in various separation methods, including GC [23], HPLC [24–27], and CE [28]. Existing completely as cations and anions, RTILs could give original selectivity or solubility properties, and recent reviews on ionic liquids in different separation techniques have been published [29,30]. RTILs have a dual nature: they completely dissociate as hydrophobic cations and anions in the mobile phase, both species can participate in the retention mechanism to affect the chromatographic results, and the effect of the two oppositely charged ions may be synergistic or antagonist [26,29,30]. RTILs as mobile phase additives in HPLC are clearly superior to the classic additives in terms of efficiency [24,31,32], peak shape improvement [33], and outstanding silanol-suppressing potency [25], consequently especially suitable for basic polar compounds separation. RTILs were also used as mobile phase additives in HPLC for the separation of amino acids [34–36], the shape of peaks including asymmetry and peak tailing could be improved. However, to the best of our knowledge, there is no report on the application of RTILs as mobile phase additives in HPLC for trace elements or speciation analysis.

The goal of this study is to develop a new method of RTILs improved RP-HPLC–ICP-MS for the speciation of inorganic and organic selenium in biological samples. The effect of alkyl-methylimidazolium class RTILs as mobile phase additives on the separation of target selenium species including Se(IV), Se(VI), selenocystine ($SeCys_2$), selenomethionine (SeMet), Se-methylseleno-L-cysteine (MeSeCys) and selenoethionine (SeEt) were investigated, and the optimized HPLC separation conditions were established. The developed method was applied to the speciation of selenium in a Certified Reference Material of SELM-1 yeast sample to validate the accuracy of the method, and also in real samples of selenium-enriched yeast and the Se-enriched clover, and the selenium species transformation in the fresh yeast cell was also investigated.

2. Experimental

2.1. Apparatus

An Agilent 7500a ICP-MS (Tokyo, Japan) was interfaced to HPLC through a Babington nebulizer as an on-line detector. Optimization of the ICP-MS instrument (i.e., sensitivity, resolution, oxide, and doubly charged ion formation) was performed with conventional pneumatic nebulization method (PN-ICP-MS) prior to connection with HPLC. After optimization, the RF power was fixed as 1150 W, and the flow rate of plasma gas and carrier gas were 15 L min^{-1} and 1.1 L min^{-1}, respectively. The scanning mode was peak-hopping with the dwell time was 100 ms per isotope. ^{77}Se, ^{78}Se and ^{82}Se were simultaneously monitored, and ^{82}Se was used for quantitative analysis. For chromatographic separation, an HPLC system equipped with two LC-10AD high-pressure pumps, CTO-10A column oven and SPD-10AV UV spectrometry detector (Shimadzu, Japan) were utilized. To combine HPLC with ICP-MS, the HPLC outlet (1.0 mL min^{-1}) was connected to the Babington nebulizer situated in a spray chamber via a minimum length piece of Teflon tubing (i.d. 0.5 mm, length 30 cm) with a finger-tight PEEK fitting. The chromatographic column used was C_{18} column (shim-pack CLC, 5 μm, 4.6 mm × 150 mm, Japan). The mobile phase consisting of 0.4% (v/v) [BMIM]Cl, 0.4% (v/v) $[BMMIM]BF_4$, and 99.2% high purity deionized water (pH = 6) was used for isocratic elution at a flow rate of 1.0 mL min^{-1}. The sample was directly injected into the HPLC after preconditioning the separation C_{18} column with the mobile phase for 20 min, and the elution behaviors of various target selenium species were examined by on-line ICP-MS detection. The peak area obtained by HPLC–ICP-MS was used for quantification in this work.

2.2. Standard solutions and reagents

RTILs: 1-butyl-3-methylimidazolium tetrafluroborate ($[BMIM]BF_4$, 97%), 1-ethyl-3-methylimidazolium tetrafluroborate, ($[EMIM]BF_4$, 97%), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl, 97%) and 1-butyl-2, 3-dimethylimidazolium tetrafluroborate, ($[BMMIM]BF_4$, 97%) were purchased from Hangzhou Chemer Chemical Co., Ltd. (Hangzhou, China) and employed as the mobile phase additives in this experiment. Stock solutions (5% (v/v)) of each RTILs were prepared by dissolving corresponding RTILs in high purity deionized water.

Stock solutions (1.0 mg mL^{-1}) of Se(IV) and Se(VI) were prepared by dissolving a certain amount of Na_2SeO_3 (97%, Wako, Japan) and Na_2SeO_4 (97%, Wako, Japan) in high purity deionized water, respectively. A stock solution (0.1 mg mL^{-1} as Se) of selenocystine ($SeCys_2$) was prepared by dissolving L-selenocystine (98%, Acros Organics, USA) in 0.1 mol L^{-1} HCl. Stock solutions (0.1 mg mL^{-1} as Se) of selenomethionine (SeMet), Se-methylseleno-L-cysteine (MeSeCys) and selenoethionine (SeEt) were prepared by dissolving D,L-selenomethionine (99+%, Acros Organics, USA), Se-methylseleno-L-cysteine (98%, Acros Organics, USA) and seleno-D,L-ethionine (Toronto Research Chemicals Inc., Canada) in high purity deionized water, respectively.

Selenium-enriched yeast 1 and 2 were provided by College of Food Sciences and Technology, Huazhong Agricultural University (Wuhan, China). Selenium-enriched yeast 3 was the living yeast cells (The strain: *Saccharomyces cerevisiae* BY4742 MATα his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0) co-incubated with Se(IV). Selenium-enriched clover (*Trifolium repens* L.) was planted and watered with Se(IV) and Se(VI) for a month in our lab.

All reagents used were of specpure or at least of analytical reagent grade. High purity deionized water obtained by a Milli-Q system (18.2 MΩ cm, Millipore, Molsheim, France) was used throughout this work. All laboratory wares were cleaned by soaking in 10% high purity nitric acid for 48 h.

2.3. Sample preparation

2.3.1. Cultivation and preparation of Se-enriched yeast 3

The yeast cell solution (10^9 colony forming units/mL) was firstly pre-cultured for 24 h at 30 °C. Then 50 μL of yeast cell solution was

transferred into 5 mL of fresh yeast peptone dextrose (YPD) and cultured for another 24 h at 30 °C. After that, the pre-cultured yeast cells were co-incubated with 5 mmol L^{-1} Se(IV) for 24 h to obtain the selenized yeast cells.

2.3.2. Cultivation and preparation of Se-enriched clover

The clover was planted in a pot with a regular supplementation of selenium. A solution containing 1 μg mL^{-1} Se(IV) and 1 μg mL^{-1} Se(VI) was watered for one month growth. Then, the plant was removed and washed by water to exclude contamination from the surface. Samples were cut into small pieces, and dried at 80 °C in an oven for one week [37]. Then the samples were ground with pestle and mortar to a homogenized powder.

2.3.3. Extraction of selenocompounds

For Se-enriched yeast 3, the yeast cells were washed by high purity deionized water for 3 times to exclude the culture medium, and were wet weighed prior to hot water extraction.

Hot water extraction was employed for extracting different selenium species from both yeast and clover. As briefed in the previous report [38], 5 mL of high purity deionized water was added to a 25 mg sample in a 15-mL centrifuge tube and followed by a hot water bath at 50 °C for 24 h. Then the extracted samples were centrifuged and filtrated, and the filtrations were made up to 10 mL with high purity deionized water.

Enzymatic extraction was applied to extract different selenium species from Se-enriched yeast 3 as well. After the yeast cells were washed by 0.05 mol L^{-1} EDTA, 2 mL 0.05 mol L^{-1} EDTA and 60 μL β-mercaptoethanol were added into the sample. The mixture was shaken for 30 min at 30 °C in darkness before the sample was washed by 1 mol L^{-1} sorbitol. After that, 1 mL 40 mg mL^{-1} snailase, 1 mL 40 mg mL^{-1} cellulase, and 60 μL β-mercaptoethanol were added into the sample. The mixture was shaken for 30 min at 30 °C in darkness again. The sample was washed by 1 mol L^{-1} sorbitol twice and wet weighed. After 1.5 mL high purity deionized water was added into the wet weighed sample, the sample was ultrasonicated at 320 W, 20 times a circle, 4 s per time, 5 s intervals for each time. Finally the extracted samples were centrifuged at 12,000 rps for 10 min and the upper aqueous phase was kept for further analysis [39].

2.3.4. Sample preparation for total selenium

For total selenium analysis, 0.05 g samples were weighed into the different crucibles. 4 mL concentrated HNO_3 (65%) was added and the samples were put on the ECH-1 temperature control heating panel (Sineo microwave chemistry technology Co. Ltd., Shanghai, China) at 100 °C for digestion. The digest was filtrated and transferred into 50 mL flask and diluted to the calibration with high purity deionized water prior to PN-ICP-MS detection.

3. Results and discussion

3.1. Effect of RTILs on the retention behaviors of various selenium species

3.1.1. Effect of different kinds of RTILs and their concentrations

To study the influence of RTILs as mobile phase additives on the retention behaviors of inorganic selenium and selenoamino acid species, different concentrations of RTILs were investigated, and the results are shown in Table 1. As could be seen, addition of 0.1% (v/v) [BMIM]BF_4 had no obvious effect on the retention behaviors of four seleno amino acids species, but resulted in an obvious decrease of retention time (t_R) and retention factor (k) for Se(IV) and Se(VI). With the increase of [BMIM]BF_4 concentration from 0.1% to 0.5% (v/v), the k and t_R for MeSeCys, SeMet, SeEt, Se(IV) and Se(VI) were decreased, especially for two inorganic selenium species whose t_R and k were decreased from 5.12 min to 4.15 min and 1.48 to 1.01 for Se(IV), 15.97 min to 8.68 min and 6.73 to 3.20 for Se(VI), respectively. Although the addition of [BMIM]BF_4 in mobile phase resulted in the retention behavior change for most of selenium species, the six target selenium species still could not be baseline separated in the whole examined [BMIM]BF_4 concentration range. Other RTILs as the mobile phase additive also showed a similar concentration effect on t_R or k for the target selenium species, and no baseline separation of six target selenium species was obtained except [BMIM]Cl. Although using 0.4% (v/v) [BMIM]Cl as mobile phase additive could provide a baseline separation of six target selenium species, the retention time for Se(VI) was longer than 20 min. Then the effect of different kinds of RTILs (0.4% (v/v) of [BMIM]BF_4, [EMIM]BF_4, [BMIM]Cl, and [BMMIM]BF_4) on the t_R and k of six target selenium species was investigated. The experimental results in Table 2 indicated that different RTILs had an obvious effect on the t_R and k of two inorganic selenium species, and this effect was highly dependent on the anionic part of RTILs. The retention behaviors were quite different for Se(IV) and Se(VI) when the anionic parts of the RTILs were BF_4^- and Cl^-, respectively. Compared to Cl^-, more amount of chaotropic anion BF_4^- was adsorbed on the stationary phase [26] and had more competitive adsorption to the target anions of Se(IV) and Se(VI).

3.1.2. Effect of mixed RTILs on the retention behaviors of target selenium species

Based on the above experimental results, it could be concluded that the use of single RTIL as mobile phase additive could not meet the requirement of baseline separation of six target selenium species in a relatively short time. To make full use of these properties of RTILs, a mixture of [BMIM]Cl and [BMMIM]BF_4 was investigated as the mobile phase additives to achieve a baseline separation of all target selenium species in a short time, especially to avoid overlapping of peaks between inorganic selenium and selenoamino acids by adjusting a suitable ratio and ion strength. Fig. 1 shows the effect of the ratio of [BMMIM]BF_4 to [BMIM]Cl on the retention behaviors of six target selenium species with the total RTILs concentration fixed at 1.0% (v/v). As could be seen, the ratio of [BMMIM]BF_4 to [BMIM]Cl had no obvious effect on the separation of four studied selenoamino acids. However, the k for Se(VI) was dramatically decreased when the ratio of [BMMIM]BF_4 to [BMIM]Cl

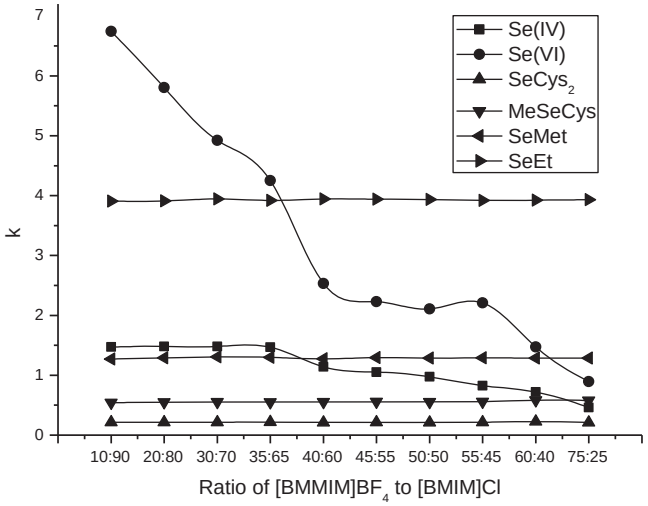

Fig. 1. The effect of different ratios of [BMIM]Cl to [BMMIM]BF_4 as mobile phase additive on retention behaviors of selenium species (mobile phase: total concentration of [BMMIM]BF_4 and [BMIM]Cl: 1.0% (v/v) (pH = 6), flow rate: 0.7 mL min^{-1}, column temperature: 25 °C, $C_{Se(IV)}$ = 500 ng mL^{-1}, $C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 100 ng mL^{-1}).

Table 1
The effect of concentration of $[BMIM]BF_4$ as mobile phase on retention time and retention factor of target selenium species[a].

$[BMIM]BF_4$	0% (v/v)		0.1% (v/v)		0.2% (v/v)		0.3% (v/v)		0.4% (v/v)		0.5% (v/v)	
	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k
Se(IV)	5.50	1.66	5.12	1.48	4.50	1.24	4.41	1.20	4.25	1.06	4.15	1.01
Se(VI)	17.0	7.25	16.0	6.73	12.1	4.84	11.4	4.49	9.73	3.71	8.68	3.20
$SeCys_2$	2.69	0.30	2.67	0.29	2.65	0.28	2.65	0.28	2.66	0.29	2.66	0.29
SeMet	4.99	1.41	5.01	1.42	4.30	1.08	4.33	1.09	4.30	1.08	4.30	1.08
MeSeCys	3.42	0.65	3.42	0.65	3.20	0.55	3.20	0.55	3.17	0.53	3.17	0.53
SeEt	9.11	3.41	9.07	3.39	8.88	3.30	8.35	3.04	8.13	2.93	8.13	2.93

[a] Flow rate: 0.7 mL min^{-1}.

Table 2
The effect of types of RTILs as mobile phase additives on retention time and retention factor of target selenium species.

Mobile phase[a]	$[BMIM]BF_4$		$[EMIM]BF_4$		[BMIM]Cl		$[BMMIM]BF_4$	
	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k
Se(IV)	4.25	1.06	3.71	0.80	7.98	2.86	4.72	1.28
Se(VI)	9.73	3.71	4.91	1.38	21.8	9.53	8.25	2.99
$SeCys_2$	2.66	0.29	2.62	0.27	2.62	0.27	2.64	0.28
SeMet	4.30	1.08	4.84	1.34	4.87	1.36	4.83	1.34
MeSeCys	3.17	0.53	3.29	0.59	3.32	0.61	3.35	0.62
SeEt	8.13	2.93	10.3	4.00	10.4	4.05	10.4	4.01

[a] Concentration of RTILs in mobile phase: 0.4% (v/v), flow rate: 0.7 mL min^{-1}.

was varied from 10:90 to 40:60, then kept almost unchanged in the range of 40:60–55:45, and was decreased again when the ratio of $[BMMIM]BF_4$ to [BMIM]Cl was higher than 55:45, while the k for Se(IV) was slowly decreased with the ratio of $[BMMIM]BF_4$ to [BMIM]Cl varying in the range of 35:65–75:25. To get the baseline separation of six target selenium species, the ratio of $[BMMIM]BF_4$ to [BMIM]Cl of 50:50 was applied for further experiments. Fig. 2 demonstrates the effect of total concentration of $[BMMIM]BF_4$ and [BMIM]Cl (50:50) on the retention behaviors of six selenium species. As could be seen, the k for four selenoamino acids was kept constant with the total concentration of $[BMMIM]BF_4$ and [BMIM]Cl ranging from 0.2% (v/v) to 1.0% (v/v). The k for Se(VI) was decreased with the total RTILs concentration increasing from 0.2% (v/v) to 0.7% (v/v), and then kept constant with the further increase of RTILs concentration to 1.0% (v/v). For Se(IV), the k was decreased firstly then kept unchanged when the total RTILs concentration was higher than 0.4% (v/v). Based on the above experimental results, the 50:50 mixture of 0.4% (v/v) [BMIM]Cl and 0.4% (v/v) $[BMMIM]BF_4$ aqueous solution was used as the mobile phase additive for HPLC separation of six target selenium species.

3.2. Effect of mobile phase pH on the retention behaviors of various selenium species

Since the component and concentration of the mobile phase were optimized, the effect of mobile phase pH on the retention behavior of six target selenium species was also investigated in the pH range of 3–8. It was found that six target selenium species could be separated at pH of 5–7. Fig. 3 shows the chromatograms of six target selenium species at typical mobile phase pH of 3, 6 and 8. As could be seen, change of pH has less influence on the separation of selenoamino acids because they always existed as amphoteric in pH range of 3–8. However, at low pH, the chromatographic peaks of Se(IV) and Se(VI) were very weak, even could not be detectable at pH < 4. At higher pH, for example, pH 8, Se(IV) and Se(VI) were eluted rapidly, which led to overlapping with the chromatographic

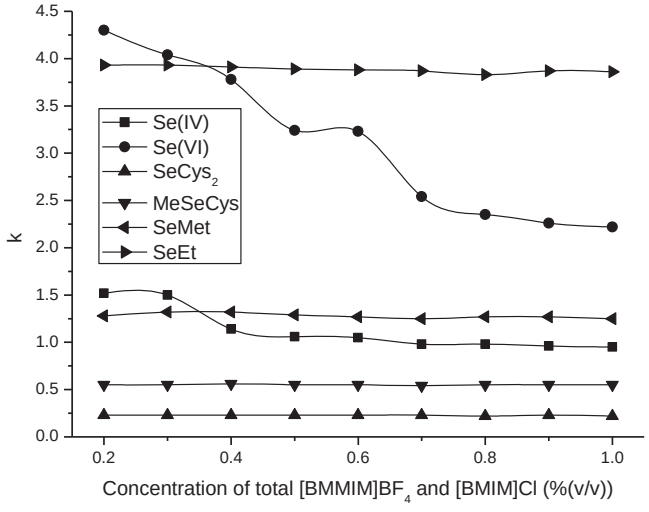

Fig. 2. The effect of total concentration of [BMIM]Cl and $[BMMIM]BF_4$ as mobile phase additive on retention behaviors of selenium species (mobile phase: [BMIM]Cl:$[BMMIM]BF_4$ = 1:1 (pH = 6), flow rate: 0.7 mL min^{-1}, column temperature: 25 °C, $C_{Se(IV)}$ = 500 ng mL^{-1}, $C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 100 ng mL^{-1}).

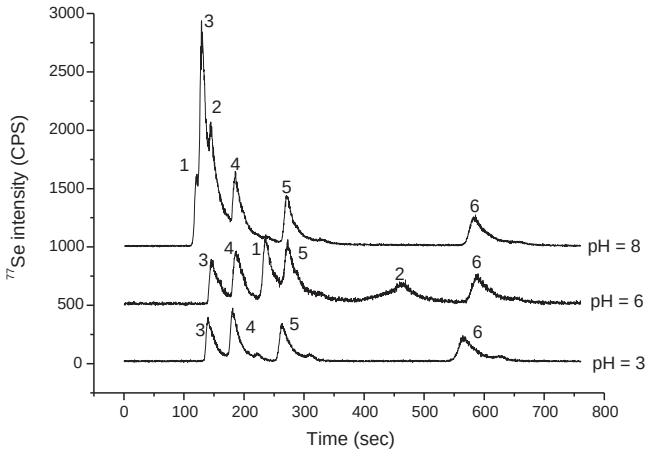

Fig. 3. The effect of pH on retention behaviors of selenium species (mobile phase: 0.4% (v/v) $[BMMIM]BF_4$ + 0.4% (v/v) [BMIM]Cl, flow rate: 0.7 mL min^{-1}, column temperature: 25 °C, $C_{Se(IV)}$ = 500 ng mL^{-1}, $C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 100 ng mL^{-1}, 1: Se(IV), 2: Se(VI), 3: $SeCys_2$, 4: MeSeCys, 5: SeMet, 6: SeEt).

peak of $SeCys_2$. Hence, the mobile phase pH 6 was selected for the subsequent experiments.

3.3. Effect of concentration of methanol added in mobile phase on the retention behaviors of various selenium species

Methanol (less than 10% (v/v)) was usually added into the mobile phase in HPLC–ICP-MS to improve the separation of various selenium species and increase the detection sensitivity of selenium. Methanol additive could decrease the adsorption of ion-pairing reagents on the column, and selectively reduce the retention time of the ionic compound containing an opposite charge to the ion-pairing reagents [40]. The effect of methanol on the retention behaviors of six selenium species was examined with the methanol concentration varying in the range of 0–5% (v/v) in the mobile phase. It was found that methanol addition had almost no effect on the retention behaviors of four studied selenoamino acids, but it reduced the *k* for the two inorganic selenium species, especially for Se(VI). With the addition of methanol, the chromatographic peak of Se(VI) became sharp and the retention time reduced drastically, but Se(VI) and MeSeCys could not be baseline separated, and the detection sensitivity of ICP-MS for selenium species was not obviously improved. Therefore, no methanol addition in the mobile phase was employed in further experiments.

3.4. Effect of flow rate and column temperature on the retention behaviors of various selenium species

The influence of flow rate of the mobile phase on the retention behaviors of six selenium species was investigated with the mobile phase flow rate varying in the range of 0.5–1.1 mL min^{-1}. It was found that the retention time for all six target selenium species was reduced with the increase of the mobile phase flow rate. As the flow rate was increased to 1.0 mL min^{-1}, all six selenium species could be separated in 8 min. Thus, 1.0 mL min^{-1} was selected as the optimum flow rate of mobile phase.

The effect of column temperature on the retention behaviors of six selenium species was also investigated with the column temperature varying in the range of 15–50 °C. It was found that the column temperature had no obvious influence on the retention behaviors of six selenium species in the whole tested temperature range. Therefore, the room temperature of 25 °C was adopted as the column temperature in the following experiments.

3.5. Comparison of RTILs with commonly used ion-pair reagents

As mentioned previously, perfluorocarboxylic acid was usually used as mobile phase additives for HPLC separation of different selenium species. TFA as ion-pair reagent results in a good separation improvement with compromise between resolution and extended retention time. However, some inorganic selenium and selenoamino acids usually cannot be separated [15]. HFBA as ion-pair reagent gives rise to an improvement of the chromatographic resolution and a good separation of various selenium species, but a long separation time and a broadened chromatographic peak are encountered [15,17]. Besides, using perfluorocarboxylic acid as ion-pair reagents might lead to a possible degradation of the silica-based columns because of the low pH (2–2.5) of the mobile phase. Tetra-alkylammonium hydroxide and alkylsulfonic acid as mobile phase additive could improve the chromatographic separation of selenium species [19], however, there were some problems associated with the subsequent ICP-MS detection. With the use of 0.4% (v/v) [BMIM]Cl and 0.4% (v/v) [BMMIM]BF_4 as mobile phase additives and an isocratic elution as described above, a good separation of all target selenium species was obtained in 8 min, and no ICP-MS sensitivity shift was found in this work.

A comparison of t_R for the six target selenium species obtained by employing RTILs as mobile phase additive with that obtained by applying commonly used ion-pair reagents in RP-HPLC is listed in Table 3. It could be seen that the target selenium species could be separated by RP-HPLC in the shortest time with RTILs using as mobile phase additives. Compared with other ion-pair reagents, adopting RTILs as mobile phase additives have some obvious advantages. (1) Because of their variety, many different kinds of the RTILs could be selected; (2) the dual nature of RTILs is beneficial for HPLC separation of polar analytes; (3) both cationic and anionic parts can affect the retention mechanism synergically or antagonistically. The anions could be adsorbed on the C_{18} stationary phase and form the ion-pair compounds with cationic solute in the mobile phase based on the Hofmeister series. The hydrophobicity of the imidazolium cation would be responsible for the association with the C_{18} stationary phase to decrease its affinity for the cationic solutes and enhance the exchange kinetics [26]. The retention mechanism for employing RTILs as the mobile phase additive is a mixed mode which involves ion-pairing, ion-exchange, and hydrophobic partitioning. By selecting appropriate kinds of RTILs, adjusting their ratio and concentration, the optimized HPLC separation conditions could be obtained. Besides, RTILs have no influence on the mobile phase pH unlike acids and amines, which is essential when C_{18} stationary phase was used, and the diluted RTILs solution can be easily removed from C_{18} column without memory effect.

3.6. Analytical performance

Fig. 4 displays the chromatograms of six selenium species in standard aqueous solution and in spiked yeast cells, respectively, obtained by RTILs improved RP-HPLC–ICP-MS under the optimized conditions. The resolutions for RTILs improved RP-HPLC separation of the target selenium species were calculated as 2.47 between $SeCys_2$ and MeSeCys, 1.79 between MeSeCys and Se(IV), 1.30 between Se(IV) and SeMet, 2.78 between SeMet and Se(VI), and 2.15 between Se(VI) and SeEt, respectively. Besides Se(IV) and SeMet were well separated, all the other selenium species were baseline separated with the total separation time less than 8 min. The separation for target selenium species was also good in yeast cells matrix, although the retention time of Se(VI) was reduced from 5.12 to 4.92 min. The analytical performance of this method was evaluated and the results are listed in Table 4. In accordance with International Conference on Harmonisation (ICH) recommendations, the limits of detection (LODs, 3σ) for six selenium species were in the range of 0.27–5.37 ng mL^{-1}. And the relative standard deviations (RSDs) were 0.8–4.6% ($c_{Se(IV)}$ = 100 ng mL^{-1},

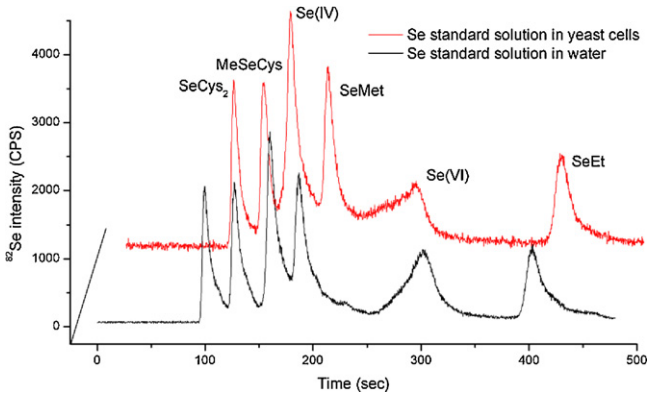

Fig. 4. HPLC–ICP-MS chromatogram for the speciation of different selenium species under the optimized conditions (mobile phase: 0.4% (v/v) [BMMIM]BF_4 + 0.4% (v/v) [BMIM]Cl (pH = 6), flow rate: 1.0 mL min^{-1}, column temperature: 25 °C, $C_{Se(IV)}$ = 500 ng mL^{-1}, $C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 100 ng mL^{-1}).

Table 3
Comparison of retention time (t_R) for the target selenium species obtained by employing different mobile phase additives (RP-HPLC–ICP-MS)[a].

Mobile phase additives	t_R (min)						Ref.
	Se(IV)	Se(VI)	$SeCys_2$	MeSeCys	SeMet	SeEt	
0.4% [BMMIM]BF_4 + 0.4% [BMIM]Cl	2.67	5.12	1.69	2.15	3.12	6.76	This work
0.1% TFA	1.0	1.17	1.5	2.3	5.7	14.5	[14]
0.1% HFBA	0.97	1.16	3.10	4.23	12.96	38.01	[15]
2.5 mM sodium 1-butanesulfonate + 8 mM TMAH[b]	3.38	3.10	3.85	–	7.50	16.13	[19]

[a] Flow rate: 1.0 mL min^{-1}.
[b] Tetramethylammonium.

Table 4
Analytical performance for different selenium species obtained by RTILs improved RP-HPLC–ICP-MS.

	t_R (min)	LOD (ng mL^{-1})	RSD[a] (%)	Linear range (ng mL^{-1})	Linear equation	Correlation coefficient (r^2)
$SeCys_2$	1.69 ± 0.02	0.87	3.7	5–10,000	$y = 55.222x + 101.375$	0.99934
MeSeCys	2.15 ± 0.01	0.90	0.8	5–10,000	$y = 90.167x + 335.009$	0.99993
SeMet	3.12 ± 0.01	0.27	1.7	5–10,000	$y = 76.583x + 191.035$	0.99992
SeEt	6.76 ± 0.02	0.81	1.9	5–10,000	$y = 101.88x + 217.006$	0.99995
Se(IV)	2.67 ± 0.04	5.37	4.6	20–50,000	$y = 28.895x + 70.284$	0.99697
Se(VI)	5.12 ± 0.02	1.79	2.0	10–10,000	$y = 91.561x + 263.21$	0.99969

[a] $C_{Se(IV)}$ = 100 ng mL^{-1}, $C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 50 ng mL^{-1}, $n = 7$.

$C_{Se(VI),SeCys2,SeMet,MeSeCys,SeEt}$ = 50 ng mL^{-1}, $n = 7$). The linear range for Se(IV) and Se(VI) was 20–50,000 ng mL^{-1} (r^2 = 0.99697) and 10–10,000 ng mL^{-1} (r^2 = 0.99969), respectively, while the linear range for other four selenoamino acids species were 5–10,000 ng mL^{-1} ($r^2 > 0.999$). A comparison of LODs obtained by this method with those obtained by several other approaches for selenium speciation is present in Table 5. As could be seen, the LODs for selenium species obtained by this method are comparable with the LODs reported in the literature [12,18,41–45].

3.7. Sample analysis

The method was validated by the speciation of selenium in a certified reference material of SELM-1 yeast which was purchased from NRCC (Ottawa, Canada). The extraction procedure employed for the speciation of selenium in SELM-1 was based on the method recommended by McSheehy et al. [46]. The analytical result was 3323 ± 39 μg SeMet g^{-1} for SeMet, which was in good coincidence with the certified value of 3389 ± 173 μg SeMet g^{-1}. Total selenium in SELM-1 was determined by PN-ICP-MS, and the result was 2012 ± 55 μg Se g^{-1}, which was also in a good agreement with the certified value of 2059 ± 64 μg Se g^{-1}.

For Se-enriched yeast 1, 2, 3, and Se-enriched clover after water extraction and enzymatic extraction (chromatograms not shown), SeMet and $SeCys_2$ are the main water soluble small molecule selenium species found in Se-enriched yeast and Se-enriched clover. The concentration of various selenium species in Se-enriched yeast 1, 2, and Se-enriched clover along with the recoveries for the spiked samples are given in Table 6. As could be seen, recoveries ranging from 94.9% to 116.4% were obtained. And the water extraction efficiency was calculated as ca. 20% for Se-enriched yeast and Se-enriched clover.

The analytical results for Se-enriched yeast 3 (the fresh Se-enriched yeast cells) with water extraction and enzymatic extraction are listed in Table 7. It was interesting to find that the recovery for $SeCys_2$ in enzymatic extracted samples was poor (only 19.8%) when 500 ng mL^{-1} $SeCys_2$ was spiked into the Se-enriched yeast 3, meanwhile the concentration of SeMet was sharply increased from 244.4 to 526.9 ng mL^{-1}. To explain this phenomenon with enzymatic extraction, further investigation was carried out by spiking different amounts of $SeCys_2$ into the Se-enriched yeast 3. As could be seen from Table 7, when the spiking concentration of $SeCys_2$ was 500 and 1666 ng mL^{-1}, the recovery for $SeCys_2$ was very low (19.8% and 22.4%), meanwhile the determined value of SeMet was dramatically increased to 526.9 and 1372.6 ng mL^{-1}, respectively; when the spiking concentration of $SeCys_2$ was increased to 3333 ng mL^{-1}, the concentration of SeMet reached to a maximum and remained constant with further increasing spiking concentration of $SeCys_2$ to 8333 ng mL^{-1}. Meanwhile, the concentration of $SeCys_2$ began to rise sharply after the

Table 5
Comparison of LODs for selenium speciation.

Samples	Analytes	Analytical technique	LODs (ng mL^{-1})	Refs.
Yeast, clover	$SeCys_2$, MeSeCys, Se(IV), SeMet, Se(VI), SeEt	RTILs improved RPLC–ICP-MS	0.27–5.37	This work
Human urine	TMSe[a], SeCys, Se(IV), SeUr[b], SeMet, SeEt	HPLC–ICP-MS	0.7–2	[18]
Wheat-based food	Se(VI), SeMet	Methane mixed HPLC–ICP-MS	0.35–0.49[c]	[41]
Cow milk	$SeCys_2$, Se(IV), SeMet, Se(VI)	LC–UV–HG-AFS[d]	0.4–1	[12]
Water	Se(IV), Se(VI)	CE–ICP-AES[e]	2.1–2.3	[42]
Human urine	MeSeCys, SeCys, Se(IV), SeMet	HPAEC[f]–AFS	1–5	[43]
Water	Se(IV), Se(VI)	HPLC–UV/Nano-TiO_2-ICP-MS	0.03–0.06	[44]
Yeast, wheat flour	$SeCys_2$, Se(IV), SeMet, Se(VI)	AEC–ICP-MSSEC[g]–ICP-MSRPLC–ESI MS[h]	1.5–2.03.2–28.050.0–150.2	[45]

[a] Trimethylselenonium.
[b] Selenourea.
[c] ng kg^{-1}.
[d] Hydride generation-atomic fluorescence spectrometry.
[e] Capillary electrophoresis–inductively coupled plasma-atomic emission spectrometry.
[f] High-performance anion-exchange chromatography.
[g] Size exclusion chromatography.
[h] Electrospray mass spectrometry.

Table 6
Analytical results (mean ± s.d., $n = 3$) for the speciation of selenium in real samples.

	$SeCys_2$				SeMet				Extraction efficiency [b]	Total Se (μg Se g^{-1})
	Added (ng Se mL^{-1})	Found (ng Se mL^{-1})	Recovery	Original conc.[a] (μg Se g^{-1})	Added (ng Se mL^{-1})	Found (ng Se mL^{-1})	Recovery	Original conc.[a] (μg Se g^{-1})		
Yeast 1	0	177.6 ± 5.2	–	50.8 ± 2.3	0	478.0 ± 4.5	–	85.5 ± 2.0	22.2%	616.5
	500	706.7 ± 6.7	116.4%		500	953.6 ± 22	94.9%			
Yeast 2	0	293.1 ± 6.3	–	74.4 ± 2.5	0	1299.2 ± 11	–	206.9 ± 4.4	18.2%	1545.1
	500	802.8 ± 6.3	103.3%		500	1739.4 ± 30	95.4%			
Clover	0	81.7 ± 1.6	–	44.1 ± 1.4	0	157.7 ± 1.1	–	53.2 ± 0.9	21.6%	451.3
	500	585.9 ± 8.4	105.2%		500	655.0 ± 6.0	98.3%			

[a] The original concentration = determined concentration (μg L^{-1}) × weighed amount of sample (mg)/dilution volume (10 mL).
[b] The extraction efficiency = the sum content of each selenium species in extracted samples obtained by HPLC–ICP-MS/the total selenium content in digested samples determined by PN-ICP-MS × 100%.

Table 7
Analytical results ($n = 3$) for the speciation of selenium in Se-enriched yeast 3 with water extraction and enzymatic extraction.

Yeast 3	Added (ng Se mL^{-1})	$SeCys_2$			SeMet		
		Found (ng Se mL^{-1})	Recovery	Original conc.[a] (μg Se g^{-1})	Found (ng Se mL^{-1})	Recovery	Original conc.[a] (μg Se g^{-1})
Water extraction	0	419.3	–	0.712	366.5	–	0.458
	500 SeMet	406.7	–		937.5	114.2%	
	500 $SeCys_2$	911.3	98.4%		381.2	–	
Enzymatic extraction	0	389.4	–	0.661	244.4	–	0.306
	500 SeMet	375.8	–		784.4	108.0%	
	500 $SeCys_2$	488.4	19.8%		526.9	–	
	1666 $SeCys_2$	762.0	22.4%		1372.6	–	
	3333 $SeCys_2$	2597.3	66.2%		2012.0	–	
	8333 $SeCys_2$	7913.1	90.3%		1816.1	–	

[a] The original concentration = determined concentration (μg L^{-1}) × weighed amount of sample (mg)/dilution volume (10 mL).

concentration of SeMet reached the maximum, and then linearly increased with the increase of spiking concentration of $SeCys_2$. Selenium in the form of SeMet seemed to be a stable and safe storage mode for selenium in living organism [47], when $SeCys_2$ was added into the yeast cells, there was a transformation [48] from $SeCys_2$ to SeMet, as demonstrated in Fig. 5. And this transformation was due to the snailase which was employed for enzymatic extraction. Unlike protease such as trypsin, snailase, a high performance biological agent to dissolve yeast cell wall, cannot cut proteins into peptides or amino acids, in other words the proteins in yeast cell remained active during enzymatic extraction. For the other samples, this phenomenon was not observed with spiking

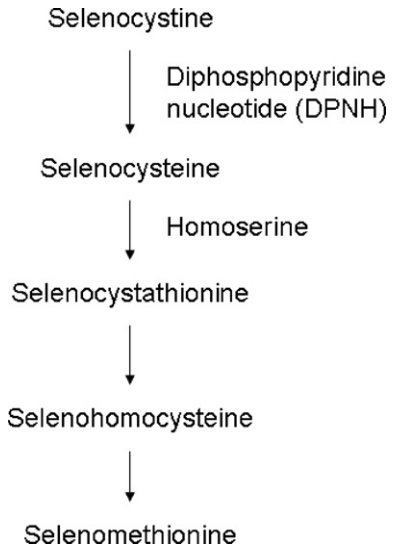

Fig. 5. A schematic diagram of transformation from $SeCys_2$ to SeMet.

$SeCys_2$ because the required enzymes in the transformation process had already lost their activity during the process of drying samples with high temperature. For extraction of different selenium species from fresh Se-enriched yeast cells samples by water extraction, the required enzymes would lose activity during water bath heating at 50 °C, hence, $SeCys_2$ could not be transformed to SeMet.

4. Conclusions

A novel approach was developed using RTILs as mobile phase additives in RP-HPLC–ICP-MS for reliable speciation of inorganic selenium and selenoamino acids in biological samples. Under the optimized conditions, all the studied selenium species could be conveniently well separated in 8 min. Compared with ion-pair reagents (such as perfluorocarboxylic acid, tetra-alkylammonium hydroxide and alkylsulfonic acid), RTILs as mobile phase additives for RP-HPLC separation of different selenium species have some attractive features. (1) The variety of RTILs results in an adjustment of the retention behavior of target selenium species and an achievement of a good separation in a relatively short time. (2) RTILs have no influence on the mobile phase pH unlike acids and amines and no obvious memory effect was observed, which is essential for C_{18} stationary phase. Besides, this method had other advantages such as simplicity, rapidness (separated in 8 min), isocratic elution and only a small injection volume (10 μL) was required. But the retention time of $SeCys_2$ (1.69 min) is only a bit longer than the dead time of 1.59 min, which needs to be solved in future work. The developed method was applied to the speciation of selenium in Se-enriched yeast and Se-enriched clover, it was found that the main water soluble selenium species in Se-enriched yeast and Se-enriched clover were SeMet and $SeCys_2$. In the fresh Se-enriched yeast cells, the added $SeCys_2$ in the living yeast cells could be transformed to SeMet through multi-steps under the functions of various enzymes, indicating that selenium was stored in the form of SeMet in the biological organism.

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