On-line pre-reduction of Se(VI) by thiourea for selenium speciation by hydride generation

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Abstract

In this study, thiourea (TU) was novelly developed as a reduction reagent for on-line pre-reduction of selenium(VI) before conventional hydride generation (HG) by KBH$_4$/NaOH–HCl. After TU on-line pre-reduction, the HG efficiency of Se(VI) has been greatly improved and because even higher than that of the same amount of Se(IV) obtained in the conventional HG system. The possible pre-reduction mechanism is discussed. The detection limit (DL) of selenate reaches 10 pg mL$^{-1}$ when using on-line TU pre-reduction followed by HG atomic fluorescence detection. When TU pre-reduction followed by HG is used as an interface between ion-pair high performance liquid chromatography and atomic fluorescence spectrometry, selenocystine, selenomethionine, selenite and selenate can be measured simultaneously and quantitatively. The DLs of these are 0.06, 0.08, 0.05 and 0.04 ng mL$^{-1}$, respectively, and the relative standard deviations of 9 duplicate runs for all the 4 species are less than 5%. Furthermore, it was successfully applied to Se speciation analysis of cultured garlic samples, and validated by determination of total selenium and selenium species in certified reference material NIST 1946.

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Keywords: Selenium; Thiourea; Hydride generation; Speciation; High performance liquid chromatography; Atomic fluorescence spectrometry

1. Introduction

It is well known that Se is essential to normal functioning of organisms in a very narrow concentration range. Below this range, Se deficiency occurs and, above it, Se becomes toxic [1]. Moreover, it has been recognized that the bioavailability and/or toxicity of Se are dependent on the species, e.g., selenite and selenate have different absorbing and metabolic pathways in rats [2]. Thus, Se speciation has become a very interesting research subject in recent years [3–5]. The coupling of a powerful separation tool such as high performance liquid chromatography (HPLC) with an element-specific detector has become very popular for this purpose. One of the popular detectors for Se determination is inductively coupled plasma mass spectrometry (ICP-MS) [6,7]. However, the detection limit of Se by conventional argon based ICP-MS is not as low as desired. This is not only because of its relatively high first ionization potential leading to lower ionization efficiency in the plasma, but the severe interferences of $^{40}$Ar$^{36}$Ar, $^{40}$Ar$^{38}$Ar and $^{40}$Ar$^{40}$Ar to the major isotopes of $^{76}$Se (natural abundance 9.37%), $^{78}$Se (23.77%) and $^{80}$Se (49.61%), respectively. $^{40}$Ar$^{37}$Cl and $^{81}$Br$^{1}$H polyatomic spectral interferences to $^{77}$Se (7.63%) and $^{82}$Se (8.73%) also make the determination of Se very difficult when a sample contains even traces of chloride or bromide. Atomic fluorescence spectrometry (AFS) is a well-established and sensitive alternative detection technique for Se speciation [8–13]. Not only the sensitivity for Se can be greatly improved by hydride generation (HG) sample introduction [14,15], but also the possible interferences from the matrix are eliminated simultaneously. Prior to HG, however, Se(VI) must be converted into Se(IV) for readily forming volatile species with borohydride. Among the pre-reduction methods, 6 mol L$^{-1}$ HCl [16], K$_2$S$_2$O$_8$–NaOH/HCl [17,18] and HBr/KBrO$_3$ [19–22] are the pre-conversion media frequently used before HG by borohydride. On the other hand, K$_2$S$_2$O$_8$–NaOH/HCl can be used to convert all Se species into selenite, followed by...
reduction to selenite by HCl; bromine involved in HBr/KBrO₃ medium was proved to play a key role in the conversion of Se (VI) to Se(IV), but it has also been known to be a potential interference to HG [23]. Recently, ultraviolet (UV) radiation has been used in Se speciation analysis [24]. Guo et al. [25] used low molecular weight organic acids to convert inorganic Se(IV) to volatile Se species under UV irradiation; Wang et al. [26,27] pre-reduced Se(VI) by a new design UV/TiO₂ photocatalysis reduction device, followed by borohydride and/or electrochemical vapor generation, respectively.

In this study, thiourea (TU) was applied for on-line pre-reduction of Se(VI) in acidic medium under the assistance of hot water bath heating. Complete conversion of selenate into Se volatile species through TU pre-reduction followed by KBH₄/NaOH–HCl was achieved, as compared with the same amount of Se(IV). The possible pre-reduction mechanism was also discussed based on newly designed flow injection experiments and Raman scattering and X-ray diffraction studies. Furthermore, TU pre-reduction followed by HG as an on-line interface between ion-pair HPLC (IP-HPLC) and AFS was successfully applied to Se pre-reduction of Se(VI) in Se speciation analysis.

2. Experimental

2.1. Instrumentation

A Shimadzu LC-2010A equipped with a quaternary gradient pump unit, an UV–Vis detector (190–700 nm), an autosampler (0.1–100 μL) and the column oven (273–333 K) was controlled by Shimadzu Class-VP 6.1 chromatography workstation. All separations were performed on a Shim-pack VP-ODS column (150 mm in length × 4.6 mm I.D., 5 μm particle size) modified by tetrabutyl ammonium hydroxide (TBAH). A Shimadzu guard column (21 mm in length × 2 mm I.D., 5 μm particle size) was used to scavenge too strongly retained compounds present in sample matrix. A Model AFS-610A non-dispersive atomic fluorescence spectrometer (Beijing Braic Analytical Instruments Co., Beijing China) equipped with a high-intensity Se hollow cathode lamp (General Research Institute for Non-ferrous Metals, Beijing China) and a sunlight-blinded photomultiplier tube (PMT, Hamamatsu, Japan) was employed throughout Se detection; signal acquisition and processing were done by HWH software Version 1.0 [28]. A Master C/L peristaltic pump (Cole-Parmer, USA) and a LEAD-1 peristaltic pump (Longer Precision Pump Co., China) were used to introduce reagents. An ICP-MS equipped with dynamic reaction cell (DRC ICP-MS) (Perkin-Elmer ELAN Model DRC II, Sciex, Canada) was also used in this study. Raman and X-ray diffraction studies were performed using a Raman spectrometer (LabRam I from Dilor) with excitation at 632.8 nm by a He–Ne laser, and XRD (D/Max-RC) instrument, respectively. The schematic diagram of the proposed Se speciation system is shown in Fig. 1 and the optimum operating conditions are listed in Table 1, respectively.

In order to get efficient separation of Se volatile species after HG, a new type of gas–liquid separator was designed and illustrated in Fig. 1. The volatile Se species and the liquid mixture were carried by argon gas to gas–liquid separator at tangent direction. Because of centrifugal effect, the liquid waste fell down along gas–liquid separator wall and spilled out from the U-shape tube spontaneously, while the volatile Se species spun to Ar–H₂ flame atomizer. This design allowed an annular gas way in a small space and the pressure in gas–liquid separator was balanced to atmospheric pressure, greatly reducing the pulse effect from the peristaltic pumps. Both of which resulted in a smooth baseline without any decrease of signal intensity.

2.2. Reagents

All reagents used were of analytical grade or higher. Tetramethyl ammonium hydroxide (TMAH), tetrabutyl ammonium hydroxide (TBAH), oxalic acid, ammonium hydroxide (NH₄OH), thiourea (TU), potassium borohydride (KBH₄) and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co. Ltd. (SCRC, Shanghai China). The HPLC-grade acetonitrile was obtained from Tedia (Fairfield, USA). Se stock standard solutions of sodium selenite and sodium selenate (Beijing Chemical Reagent Co. Ltd., China).
seleno-DL-methionine (SeMet) and seleno-DL-cystine (SeCys) (Sigma, St. Louis, MO, USA) were respectively prepared by dissolving the corresponding solid compound in double distilled water (DDW, 18.2 MΩ). All solutions were stored at 277 K and protected from light. Working standard solutions were made just before use by appropriate dilution of the stock standard solutions. Frozen fish tissue, NIST 1946, from the National Institute of Standards and Technology, USA was used as a CRM.

The mobile phases used in the IP-HPLC separation of different Se species were prepared by dissolving appropriate amount of TBAH, oxalic acid and acetonitrile in DDW. The pH of the mobile phase was adjusted by oxalic acid solution and/or 10% NH₄OH. All mobile phases were filtered through 0.45 μm membrane and treated ultrasonically during 10 min before use.

### 2.3. Sample preparation

An appropriate amount of NIST 1946 and/or the cultured garlic samples that were frozen by liquid N₂ and ground, were respectively transferred into a 10-mL polystyrene centrifuge tube. After addition of 5 mL of DDW containing 5% TMAH (DDW-TMAH), the tube was shaken at 70 °C for 12 h and finally centrifuged for 30 min at 3000 rpm. The supernatant was decanted and filtered through a 0.45 μm membrane filter and the residue on the filter was then washed twice by 2 mL DDW-TMAH each time. The filtrate was finally transferred into a 10-mL volumetric flask and diluted to the mark by DDW so as to get a water-TMAH soluble extract of NIST 1946 and/or the cultured garlic samples. This was stored in a polyethylene bottle at 4 °C for further use.

For the determination of total Se in NIST 1946, the cultured garlic samples and their corresponding extracted residues, both HG AFS and DRC ICP-MS were used. The apparatus used to mineralize the samples was described previously [29], and concentrated HNO₃ and H₂O₂ (30%) were used for the digestion. After thorough mineralization, an appropriate amount of concentrated HCl was added to reduce Se(VI) to Se(IV) at 100 °C for 30 min before transfer into a 25-mL volumetric flask and dilution with DDW to the mark.

### 2.4. Se speciation analysis

After equilibration with the mobile phase A as listed in Table 1, the obtained extract was injected through the injection valve (100 μL) onto the column. The mobile phase A was switched to the mobile phase B, 5 min after the sample injection as listed in Table 1. The eluate was first mixed with KBH₄/NaOH mixture to form the cold solution merged with KBH₄/NaOH mixture to form the volatile Se species, which was carried by argon to the atomizer via gas–liquid separator and then detected by AFS (Fig. 1). After one run was completed, the mobile phase A was used to re-equilibrate the column for 10 min before the second injection was carried out.

### 3. Results and discussion

#### 3.1. On-line pre-reduction of Se(VI) with TU

It is well known that Se(VI) cannot be reduced easily by KBH₄/NaOH–HCl into H₂Se⁰ [30]. To optimize reduction conditions, effects of TU concentration and reduction temperature and time on the reduction efficiency of Se(VI) were investigated by flow injection experiments. The system shown in Fig. 1 was used, except the columns. Results showed that a small amount of TU is enough for the reduction of Se(VI). With the addition of 5% TU, one order of magnitude improvement in Se(VI) AFS intensity was obtained in comparison to using 30% HCl only (Fig. 2). The effects of reduction temperature and time were also investigated and plotted in Fig. 3. When the temperature was increased from 75 °C to 100 °C, the peak area increased a factor of two, while the reaction time, which depends on the size of reaction tube and the flow rates of TU and HCl, also demonstrated remarkable effect on the Se(VI) signal. The signal increased 1.3 times when the time increased...
However, the signal decreases again when the time is longer than 30 s, as shown in Fig. 3.

To understand the possible reduction mechanism of Se(VI) by TU, the chemical isomerism of TU in acidic medium should be taken into account. In acidic medium, there are two isoforms of TU as shown in Eq. (1); the thiol of TU can be easily oxidized into TU disulfide indicated in Eq. (2), the redox potential is 0.42 V [31]. Because the redox potentials of $\text{HSeO}_4^{2-}/\text{H}_2\text{SeO}_3^{2-}$ and $\text{H}_2\text{SeO}_3^{2-}/\text{Se}$ as shown in Eqs. (3) and (4) are respectively 1.090 V and 0.741 V [32], Se(VI) can be reduced spontaneously to Se(IV) and even to Se(0).

$$
\begin{align*}
\text{S} & = \text{C(NH}_2\text{)}_2\text{SH} \rightarrow \text{C(NH)}\text{–NH}_2 \\
\text{NH}_2\text{–C(NH)}\text{–S–C(NH)}\text{–NH}_2 + 2e^- + 2H^+ & = 2\text{NH}_2\text{–CNHz–SH} \\
\text{HSeO}_4^{2-} + 3H^+ + 2e^- & = \text{H}_2\text{SeO}_3 + \text{H}_2\text{O} \\
\text{H}_2\text{SeO}_3 + 4H^+ + 4e^- & = \text{Se} + 3\text{H}_2\text{O} \\
\text{Se(IV)} + 6\text{NH}_2\text{–C(NH)}\text{–SH} & = [\text{NH}_2\text{–C(NH)}\text{–S–Se–S–(NH)}\text{–C–NH}_2] \\
& + 2[\text{NH}_2\text{–C(NH)}\text{–S–S–(NH)}\text{–C–NH}_2]
\end{align*}
$$

A batch experiment showed that addition of TU into an acid solution of selenate caused formation of an incarnadine precipitate after hot-water bath heating. The precipitate is apparently the same as the product obtained when TU was directly added into an acidic selenite solution. The product shown in Eq. (5) was observed by Marcucci et al. [33]. The Raman spectrum of the isolated product showed that there are strong peaks in the region of 200–300 cm$^{-1}$ [34], suggesting that such a (S-Se-S)-structure exists in the product. The product could be further reduced into dark powder when the reaction time was increased. X-ray diffraction study indicated that the powder has a hexagonal structure that is the same as that of element Se, which is in agreement with the results obtained by D’Ulivo et al. [35]. This evidence also partly explained that the reduction of Se(VI) to Se(0), which is favoured by thermodynamics and in agreement with the batch experiments performed, is avoided by selecting a reaction time short enough (less than 30 s). When the on-line pre-reduction conditions were optimized as shown in Table 1, the vapor generation efficiency of Se(VI) was greatly improved and the detection limit (3$\sigma$) of Se(VI) reaches 0.01 ng mL$^{-1}$ under flow injection mode.

### 3.2. Chromatographic separation of Se species and analytical performance of the proposed system

Similar effects of TU that depress the signals of Se(IV), SeMet and SeCys were also observed in the batch experiments as described by D’Ulivo et al. [33]. Thus, sequential on-line introduction of TU was adopted to avoid the contact of TU and the above mentioned Se species. TU was introduced after Se(IV), SeMet and SeCys were eluted out of the column. Different from Se(IV), that was reduced by KBH$_4$/NaOH–HCl into H$_2$Se, SeMet and SeCys were reduced into volatile alkylselenide [36] and then determined by AFS.

The most common Se species of selenite, selenate, SeMet and SeCys could be quantitatively separated by an isocratic elution with the mobile phase A in IP-HPLC system. After the separation of SeMet, SeCys and selenite within 10 min, selenite was still retained on the column as shown in Fig. 4(a). In order
to shorten the retention time of selenate, mobile phase B was used 5 min after the elution with mobile phase A. More effective separation was obtained as shown in Fig. 4(b). The sensitivities of Se species, however, slightly decreased because of the higher concentration of acetonitrile and pH value of the mobile phase B. The detection limit, linearity and precision are listed in Table 2. It should be noted that the detection limit of selenate is much lower compared with those previously reported[37,38]. Such an improvement is a remarkable merit of the on-line pre-reduction of Se(VI) by TU proposed in this study.

3.3. Se speciation analysis of the water–TMAH extractable fraction of cultured garlic samples and validation of the proposed method by NIST 1946

The proposed system was applied to Se speciation analysis of the water–TMAH extractable fractions of CRM NIST 1946 and the cultured garlic samples, which were cultured with selenite and/or selenate for 1 month. Typical chromatograms of Se species in the water–TMAH extractable fractions of the garlic sample and CRM NIST 1946 are shown in Fig. 4c and d, respectively, and the results are listed in Table 3. The water–TMAH extractable fraction of the garlic sample cultured with selenite mainly contained SeMet and selenite, while in the garlic sample cultured with selenate 4 Se species, SeMet, selenite, selenate and an unknown one, were determined. The relative standard deviation (RSD) was lower than 5% (n=9) for all the species determined, and the recoveries were between 93.2% and 105% as listed in Table 2. Although there are no Se-species certified values for NIST 1946, SeCys, selenite and selenate were still found in its water–TMAH extractable fraction. The amount of Se species determined and Se found in the residue was well in agreement with the total Se certified value, not only confirming the reliability of this proposed method but also offering new Se-species information for CRM NIST 1946. Moreover, the same samples were analyzed by IP-HPLC-DRC.

Table 2
Merits of ion-pair HPLC separation and on-line TU reduction HG AFS system

<table>
<thead>
<tr>
<th>SeCys</th>
<th>SeMet</th>
<th>Selenite</th>
<th>Selenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity R² (0.01–1.00 μg mL⁻¹)</td>
<td>Y=65338X−2218; 0.999</td>
<td>Y=36144X−4164; 0.997</td>
<td>Y=68279X−5797; 0.998</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>2.7</td>
<td>4.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Detection limit (μg L⁻¹)a</td>
<td>0.06</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>RSD (%)b</td>
<td>2.3</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Recovery (%)c</td>
<td>105.3</td>
<td>96.4</td>
<td>96.0</td>
</tr>
</tbody>
</table>

a Calculated as 3σ of the background signal and expressed in μg L⁻¹ as Se.
b Calculated as the nine chromatographic runs of a standard solution containing 100 μg L⁻¹ each of the investigated species.
c Obtained by spiking 1.0 μg mL⁻¹ SeCys, 0.5 μg mL⁻¹ SeMet, 0.5 μg mL⁻¹ selenite and 0.5 μg mL⁻¹ selenate in the water–TMAH soluble extract of the garlic sample cultured with selenite.

Fig. 4. Typical chromatograms of Se speciation (1, SeCys; 2, SeMet; 3, Se(IV); 4, Se(VI); 5, unknown): (a) isocratic elution, 1–35 min 100% mobile phase A; (b) 1–5 min 100% mobile phase A, 5–25 min 100% mobile phase B; (c) Se speciation in the water–TMAH extractable fraction of sodium selenate cultured garlic under the isocratic elution of 0–5 min by mobile phase A and then mobile phase B from 5 to 35 min; (d) Se speciation in the water–TMAH extractable fraction of CRM NIST 1946 by the elution program as (c).
Table 3
Determination of total Se and Se species in cultured garlic samples and NIST 1946 by the proposed method and IP-HPLC-DRC ICP-MS (μg g⁻¹ Se, n=9)

<table>
<thead>
<tr>
<th>Sample</th>
<th>SeCys</th>
<th>SeMet</th>
<th>Se(IV)</th>
<th>Se(VI)</th>
<th>Unknown</th>
<th>Total extractable Se</th>
<th>Se in the residue</th>
<th>Se in the extract and the residue</th>
<th>Total Se</th>
<th>Certified value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIST 1946</td>
<td>0.325±0.021</td>
<td>ND</td>
<td>0.0875±0.011</td>
<td>ND</td>
<td>0.0327±0.061</td>
<td>0.445±0.029</td>
<td>0.0816±0.020</td>
<td>0.527±0.049</td>
<td>0.487±0.032</td>
<td>0.491±0.043</td>
</tr>
<tr>
<td>Sample 1d</td>
<td>ND</td>
<td>3.46±0.3</td>
<td>1.36±0.11</td>
<td>12.2±0.24</td>
<td>6.62±0.23</td>
<td>23.6±0.65</td>
<td>60.1±0.34</td>
<td>83.7±0.99</td>
<td>83.7±0.12</td>
<td></td>
</tr>
<tr>
<td>Sample 2e</td>
<td>ND</td>
<td>3.52±0.21</td>
<td>1.25±0.36</td>
<td>12.1±0.27</td>
<td>6.38±0.18</td>
<td>23.2±0.67</td>
<td>58.9±0.17</td>
<td>82.1±0.84</td>
<td>81.5±0.28</td>
<td></td>
</tr>
</tbody>
</table>

IP-HPLC-DRC ICP-MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>SeCys</th>
<th>SeMet</th>
<th>Se(IV)</th>
<th>Se(VI)</th>
<th>Unknown</th>
<th>Total extractable Se</th>
<th>Se in the residue</th>
<th>Se in the extract and the residue</th>
<th>Total Se</th>
<th>Certified value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIST 1946</td>
<td>0.341±0.001</td>
<td>ND</td>
<td>0.0814±0.009</td>
<td>ND</td>
<td>0.0201±0.011</td>
<td>0.442±0.034</td>
<td>0.0818±0.021</td>
<td>0.524±0.045</td>
<td>0.521±0.023</td>
<td>0.491±0.043</td>
</tr>
<tr>
<td>Sample 1d</td>
<td>ND</td>
<td>1.33±0.17</td>
<td>4.05±0.40</td>
<td>ND</td>
<td>5.38±0.63</td>
<td>20.0±0.07</td>
<td>25.4±0.69</td>
<td>25.3±0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 2e</td>
<td>ND</td>
<td>3.52±0.21</td>
<td>1.25±0.36</td>
<td>12.1±0.27</td>
<td>6.38±0.18</td>
<td>23.2±0.67</td>
<td>58.9±0.17</td>
<td>82.1±0.84</td>
<td>81.5±0.28</td>
<td></td>
</tr>
</tbody>
</table>

**Proposed method**

- NIST 1946: 0.325±0.021 ND
- Sample 1d: 3.46±0.3 ND
- Sample 2e: 3.52±0.21 ND

**IP-HPLC-DRC ICP-MS**

- NIST 1946: 0.341±0.001 ND
- Sample 1d: 1.33±0.17 ND
- Sample 2e: 3.52±0.21 ND

**References**


