Direct amino acid analysis method for speciation of selenoamino acids using high-performance anion-exchange chromatography coupled with integrated pulsed amperometric detection

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Abstract

Speciation analysis of selenomethylcysteine (SeMeCys), selenomethionine (SeMet) and selenocystine (SeCys) has been performed using a direct amino acid analysis method with high-performance anion-exchange chromatography (HPAEC) coupled with integrated pulsed amperometric detection (IPAD). Three selenoamino acids could be baseline-separated from 19 amino acids using gradient elution conditions for amino acids and determined under new six-potential waveform. Detection limits for SeMeCys, SeMet and SeCys were 0.25, 1 and 20 µg/L (25 µL injection, 10 times of the baseline noise), respectively. The relative standard deviations (RSDs) of 200 µg/L SeMeCys, SeMet and SeCys were 3.1, 4.1 and 2.8%, respectively (n=9, 25 µL injection). The proposed method has been applied for determination of selenoamino acids in extracts of garlic and selenious yeast granule samples. No selenoamino acids were found in garlic. Both SeMet and SeCys were detected in selenious yeast tablet with the content of 45 and 129 µg Se/g, respectively. Selenoamino acids standards were spiked in garlic and yeast granule samples and the recovery ranged from 90 to 106%.

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1. Introduction

Selenium plays dual role for human beings, beneficial or harmful, just correlating to the ingestion dose and species. Appropriate amount and speciation of selenium benefit people while too low or excess amount of selenium and Se(IV) does great harm to human health. The mechanism of how this element interacts in human body is still puzzling. In some areas, people lack selenium supplementation from food and water due to its absence in local geological components. So some food products, such as selenium-enriched garlic and selenious yeast, have been adopted as popular diet for selenium supplementation. The speculation and identification of selenium in those foods are being paid more attention.

For selenium compounds analysis, various separation and detection methods have been adopted. Among the separation methods, ion-exchange liquid chromatography is seldom used due to the poor separation of most selenium compounds except selenite and selenate. Cavalli and Cardellicchio had adopted AminoPac PA1 anion-exchange column to separate selenoamino acids and amino acids simultaneously [1]. However, the peaks could not be baseline separated and the peak shape was not sharp and symmetrical. Reverse-phase liquid chromatography separation of selenoamino acids is tedious because pre-derivatization is obligatory. Ion-pair reverse-phase liquid chromatography is the most effective separation method and has been widely adopted. The detectors, such as hydride generation-atomic fluorescence spectrometer (HGAFS) [2–4], hydride generation-atomic absorption spectrometer (HGAAS) [5–7], inductively coupled plasma mass spectrometer (ICP–MS) [8–12] and electrospray ionization mass spectrometer (ESI–MS) [13,14], have been adopted to detect selenium species. Selenoamino acids were oxidized, and then reduced to SeH2 before entering AFS and AAS for detection. ICP–MS is a very sensitive and selective detector for selenium species analysis but the application is limited by the high apparatus and operation maintenance cost. ESI–MS detector could obtain more information about the structure of selenium compounds, which is especially useful to
find unknown selenium compounds deducing from the segments but suffers from complex matrix interferences. The detection limits of these detectors are in the level of several or several decades μg/L. As a sensitive and selective technique among the different electrochemical detection (ED) techniques, integrated pulsed amperometry detection (IPAD) has been widely and effectively adopted in the determination of amino acids since the new six-potential IPAD waveform was introduced by Clarke et al. [15–18]. The introduction of this new IPAD waveform made the direct determination of amino acids possible and no derivatization step was needed thus eliminating complexity of the instrument and interferences of matrix.

For electrochemical detection of selenoamino acids, few papers could be traced in literature. Welch et al. [19] incorporated a cyclic potential sweep into the triple-step potential waveform. It proceeded through the formation and subsequent removal of the surface oxide with simultaneous current integration as well as the rapid decay of analytical sensitivity. Cavalli and Cardellinichio [1] introduced quadruple-potential IPAD waveform for direct detection of selenoamino acids and amino acids. It used +0.35 V potential to oxidize NH3 group and applied positive potential (+0.9 V) to clean the Au surface. Judging from the tailing peaks of selenoamino acids and amino acids, it seemed that the applied potential waveform was not very effective. The detection limits lie in the range of 450–620 μg/L. More recently, the cleaning and activation steps from the quadruple-potential waveform were included in a new six-potential IPAD waveform for direct detection of amino acids and amino sugars on gold electrodes after an anion-exchange separation. This waveform minimizes baseline shift during the gradient and improves linearity and signal-to-noise ratio. Electrode fouling is relatively rare, and the detection response remains stable for many weeks of continuous use.

In this paper, three selenoamino acids were separated from 19 amino acids using NaOH and NaOAc gradient elution on AminoPac PA10 anion-exchange column based on the acidity and polarity of different compounds. Selenoamino acids were oxidized using new six-potential IPAD waveform. The parameters affecting the separation and detection of selenoamino compounds, the performance and application of this method are discussed in the paper.

2. Experimental

2.1. Instrument

The ICS-2500 module ion chromatography system ( Dionex, Sunnyvale, CA, USA) was used throughout the experiment. The system is composed of a G50 gradient pump with on-line degassing, an ED50A electrochemical detector (Au working electrode, pHAg/AgCl combined reference electrode and Ti counter electrode), an AS50 autosampler and LC30 column thermostat. The injection volume is 25 μL with 25 μL injection loop installed and fulfilled by the autosampler. AminoPac PA10 analytical column (500 mm x 2 mm), AminoPac PA10 guard column (50 mm x 2 mm) and the ED50A detection cell were put into LC30 column thermostat to keep the temperature constant at 30 °C.

The data were acquired and processed using Chromelon 6.5 workstation. The gradient separation condition and detection waveform were separately listed in Tables 1 and 2, which are the same as that used in direct amino acids analysis method [18].

2.2. Reagents and standards

All chemicals were of reagent grade except where specified. Amino acids for standard reference material (SRM 2389 NIST, Gaithersburg, MD, USA) were used to prepare the standard solution. Selenocystine (SeCys), selenomethionine (SeMet) and selenomethylcysteine (SeMeCys, 95%) were purchased from Sigma. Appropriate amount of SeCys was dissolved in 0.02 mol/L HCl and other two selenoamino acids were dissolved in water to obtain 500 mg/L standard solution (calculated as Se). Sodium azide was purchased from Sigma–Aldrich (St. Louis, MO, USA). The injected standard mixture solutions of amino acids and selenoamino acids were made by diluting SRM 2389 and corresponding aliquots of single component stock solutions.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A: H2O (%)</th>
<th>B: 250 mmol/L NaOH (%)</th>
<th>C: 1 mol/L NaOAc (%)</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>64</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>64</td>
<td>16</td>
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</tr>
<tr>
<td>12.0</td>
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<td>32</td>
<td>8</td>
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<td>16.0</td>
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<tr>
<td>24.0</td>
<td>36</td>
<td>24</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>40.0</td>
<td>36</td>
<td>24</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>40.1</td>
<td>20</td>
<td>80</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>42.1</td>
<td>20</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42.2</td>
<td>84</td>
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<tr>
<td>65.0</td>
<td>84</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Curve 5 is linear; curve 8 is one of the four available convex curves (6–9) with 50% of change at approximately 60% of a time segment and 75% change at approximately 90% of the same programmed time segment.

<table>
<thead>
<tr>
<th>Time (ms)</th>
<th>Potential (V) vs. pHAg–AgCl</th>
<th>Potential (V) vs. Ag–AgCl</th>
<th>Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.13</td>
<td>–0.20</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.13</td>
<td>–0.20</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.33</td>
<td>–0.00</td>
<td>Begin</td>
</tr>
<tr>
<td>210</td>
<td>0.33</td>
<td>–0.00</td>
<td></td>
</tr>
<tr>
<td>220</td>
<td>0.55</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>460</td>
<td>0.55</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>470</td>
<td>0.33</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>560</td>
<td>0.33</td>
<td>–0.00</td>
<td>End</td>
</tr>
<tr>
<td>570</td>
<td>–1.67</td>
<td>–2.0</td>
<td></td>
</tr>
<tr>
<td>580</td>
<td>–1.67</td>
<td>–2.0</td>
<td></td>
</tr>
<tr>
<td>590</td>
<td>0.93</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>0.13</td>
<td>–0.2</td>
<td></td>
</tr>
</tbody>
</table>
solutions of selenoamino acids with an aqueous diluent containing 20 mg/L sodium azide. All standards were stored in a refrigerator at 4 °C.

To prepare the gradient mobile phases, 18.3 MΩ cm water, granular sodium hydroxide (GR, Beijing Chemical Factory, China) and anhydrous sodium acetate (Dionex) were used. Mobile phase I was 18.3 MΩ cm water vacuum filtered through a 0.22 μm nylon filter. Mobile phase II, a 250 mmol/L sodium hydroxide solution, was prepared by diluting an aliquot of 50% (m/m) sodium hydroxide, which was prepared by dissolving granular solid sodium hydroxide in 18.3 MΩ cm water. The resulting 50% (m/m) solution was kept undisturbed for 24 h to allow the sodium carbonate to settle at the bottom prior to the last dilution step to 250 mmol/L. Mobile phase III, a 1 mol/L sodium acetate solution, was prepared by dissolving an aliquot of anhydrous sodium acetate in 18.3 MΩ cm water and filtering the solution through a 0.22 μm nylon filter. Once the mobile phase was prepared, it was kept under nitrogen to prevent entry of atmospheric carbon dioxide.

2.3. Sample preparation

According to literature [8], the fresh garlic sample (0.15 g) was accurately weighed into 15 mL centrifuge tube. Four millilitres of water was added and boiled for 1 h. The extractant was stored in refrigerator for about 30 min and then centrifuged at 3000 rpm. The supernatant solution was pipetted and pH was adjusted to approximately 7.0 using 1 mol/L NH₃H₂O and then centrifuged for another 30 min. The extractant was diluted 50-fold then filtrated through 0.22 μm nylon membrane before injecting into the high-performance anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) system for selenoamino acids analysis.

Weigh 0.2000 g selenious yeast granule into 15 mL centrifuge tube. Five millilitres of water was added and boiled for 1 h. This extractant solution was left to cool to room temperature then centrifuged for 30 min. The extractant was diluted 50-fold and then filtrated through 0.22 μm nylon membrane before injecting into the HPAEC–PAD system for selenoamino acids analysis.

3. Results and discussion

The proposed method used for selenium compounds separation and detection is completely the same as used in direct amino acids analysis method. On the basis of the differences of acidity and hydrophobicity, 19 amino acids and 3 selenoamino acids could be baseline separated. In the following experiment, the analysis parameters for amino acids are not optimised; furthermore, the performance experiments are only focused on three selenoamino acids.

3.1. Separation of selenium compounds

Ion-exchange chromatography is seldom applied for the separation of selenium compounds due to poor resolution except selenite and selenate. It had been reported that Selenocystine and methyl-Selenocysteine could not be baseline separated using isocratic elution due to their similar pKₐ [8]. However, AminoPac PA10 anion-exchange column used in our experiment could separate Selenocystine and methyl-Selenocysteine very well. This column was specially designed for separation of amino acids and could tolerate wide pH range from 0 to 14. So NaOH solution could be used as eluent to separate selenoamino acids with similar pKₐ. SeMeCys and SeMet were eluted successively using NaOH gradient. SeCys must be eluted with strong NaOAc elution because there are two carboxyls in the molecular structure, which combine the resin firmly. The gradient conditions for anion-exchange separation have been shown in Table 1 and the separation chromatogram is shown in Fig. 1. The peaks are baseline separated and the peak shape is sharp and symmetrical.

3.2. Electrochemical response of selenoamino acids on Au electrode

Amino acids are detected on the basis of surface-catalyzed oxidation of the amine functionalities activated by the transient formation of surface oxide on Au electrode. The detection of selenoamino acids is also based upon the same theory. Sharp and symmetrical peak could be obtained in the present detection waveform for amino acids and three selenoamino acids. However, slight tailing could be observed for SeCys peak. Why is this compound so hard to be removed or why does this compound combine with the Au surface so tightly? The reason might be as follows. As seen from the molecular formula, selenoamino acids contain selenium element compared with conventional amino acids, which is somehow similar with sulfur-containing amino acids. It is well known that sulfur has high affinity with Au, which has been extensively used in Au electrode surface modification. Methionine, Cysteine, SeMet and SeMeCys include one sulfur or selenium atom in the molecular formula. In the proposed six-potential waveform, these compounds could be oxidized and removed from the Au surface within as short time as possible. However, selenocystine contains two selenium atoms in the molecular formula. So this compound might integrate with Au surface much stronger than the above compounds. Under the
same clean potential. SeCys oxidation product is cleaned more slowly, thus leading to the slight peak tailing. So, as we can see from the following data, the detection limit of SeCys is higher than the other selenoamino acids with symmetrical and sharp peaks.

### 3.3. Performances

The linearity was performed using 10 μg/L–5 mg/L selenoamino acids standard solutions prepared in water. All the results are listed in Table 3. For different selenium compounds, the dynamic correlation range is diverse. The lower limit of SeMeCys and SeMet is 50 μg/L and the upper limit has great diversity (SeMeCys: 2000 μg/L and SeMet: 1000 μg/L). The dynamic correlation range of SeCys is from 100 to 3000 μg/L. The correlation efficient is better than 0.999. Three selenoamino acids at 200 μg/L concentrations were injected for nine successive times. The relative standard deviation (RSD) of SeMeCys, SeMet and SeCys was 3.1, 4.1 and 2.8%, respectively. Based on 10 times of baseline noise, the detection limits for SeMeCys, SeMet and SeCys were 0.25, 1 and 20 μg/L (25 μL injection), respectively, which has been shown in Table 3. Our results are far superior to literature (450–620 μg/L), which used the same detector as we used [1]. The great difference of detection limits is partially caused by the improvement of the stationary phase and the dimension of the analytical column, but the improvement of the IPAD detection waveform might be another reason. In our waveform, the integration time lasts for 350 ms at −0.05 to 0.28 V and then cleaned at very negative potential (−2.0 V) to remove reaction products and interferences from Au surface. Activation at 0.6 V potential is included at the end of the waveform to activate the Au surface to prepare for next cycle. In the literature [1], the integration potential and time is both higher than ours (−0.10 to 0.35 V, 500 ms). The potential to remove reaction products is positive and the potential to clean the Au surface is negative. From the comparisons of detection limits, we can speculate that the clean and activation potential rather than the integration potential and time are the main reasons related with the detection sensitivity. Our waveform could remove reaction products and interferences completely at extremely negative potential, and the following activation of the Au surface was ready for next analysis.

### 3.4. Application in garlic and selenious yeast samples

Two kinds of samples, garlic and selenious yeast granule, were analyzed. Judged from the retention time, no selenoamino acids were detected in garlic samples and both SeMet and SeCys were detected in selenious yeast granule with the content of 45 and 129 μg Se/g, respectively. It was further verified by spiking selenoamino acids standard into the sample solution (Fig. 2). The recovery of three selenoamino acids spiked into the sample is between 90 and 106% (Table 4). It can also be seen from Fig. 2 that several amino acids were also detected in the selected selenious yeast granule sample.

### 4. Conclusion

Direct amino acid analysis method with high-performance anion-exchange chromatography with integrated pulsed amperometric detection has been successfully applied in speciation analysis of selenomethylcysteine, selenomethionine and selenocystine. Using the high efficient AminoPac PA10 column, three selenoamino acids could be well separated from other amino acids and sharp and symmetrical peak shape were obtained. The detection limits for SeMeCys, SeMet and SeCys were from seven...
eral to several decades μg/L, which were far lower than the same IPAD detector using different potential waveform, indicating advantages of our method. Moreover, the detection limits were similar to those of element-specific detectors, such as HGAFS, HGAAAS and ICPMS. The proposed method could be used for determination of selenoamino acids in garlic, selenious yeast granule and other amino acids-containing samples.

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References