



Hydrophilic interaction liquid chromatography in the speciation analysis of selenium



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ABSTRACT

The hydrophilic interaction liquid chromatography (HILIC) coupled to mass spectrometry was employed to study retention behavior of selected selenium compounds using two different HILIC stationary phases: silica and zwitterionic. Two organic solvents – acetonitrile and methanol – were compared as a component of mobile phase. Separation parameters such as a content of organic modifier, the eluent pH and inorganic buffer concentration were investigated. Based on all observations, methanol seems to be beneficial for the separation of studied compounds. The optimal HILIC separation method involved silica column and eluent composed of 85% MeOH and CH₃COONH₄ (8 mM, pH 7) was compared to RP method in terms of time of the single run, the separation efficiency and limit of detection.

1. Introduction

Selenium is an important element from environmental and biological point of view [1,2]. Its compounds are distributed through the environment as a result of natural processes (weathering of minerals, volcanic activity and erosion of soils) and human activity (industrial and agricultural uses). Selenium has been recognized as an essential nutrient because it plays a key role in several major metabolic pathways such as thyroid hormone metabolism, antioxidant defence systems, and immune functions [3]. The deficiency of selenium has been linked to a range of serious conditions like cancer, cardiovascular and inflammatory diseases and other free radical related problems such as premature ageing [4]. However, it is known that selenium compounds are toxic at high concentrations [5]. Different species of selenium have different chemical properties, environmental effects, biological utilizations, toxicities, and nutrition values and selenium organic compounds are less toxic and more bioavailable than inorganic forms [6,7]. Selenomethionine is the dominant species in rice [8], while vegetables from *Allium* and *Brassicaceae* family such as onion, garlic or broccoli are better sources of methylselenocysteine [9]. Therefore, a proper evaluation of selenium behavior requires knowledge of not only the total amount of selenium but also the appropriate selenium species concentration.

The direct coupling of high performance liquid chromatography

(HPLC) to inductively coupled plasma mass spectrometry (ICP-MS) is the most common analytical approach for the separation of selenium species in a variety of matrices [10]. Because of the different forms of selenium present in the samples, different mechanisms of chromatographic separation are used, usually reversed-phase [11–13] and anion exchange [14–16] modes. Anion exchange is mainly used to address resolution issues of inorganic selenium species but difficulties arises with resolving organoselenium compounds, while reversed-phase mode suffers from low resolution of inorganic selenium forms.

Hydrophilic interaction liquid chromatography (HILIC) can be described as a reversed reversed-phase chromatography performed using a polar stationary phase. The mobile phase employed is highly organic in nature (> 70% solvent, typically acetonitrile) containing also a small percentage of aqueous solvent/buffer or other polar solvent [17]. The water/polar solvent forms an aqueous-rich sub-layer adsorbed to the polar surface of the stationary phase into which analytes partition. The retention mechanisms in HILIC are complex but are believed to be a combination of hydrophilic partitioning interaction and secondary electrostatic and hydrogen bonding interactions [17]. These mechanisms result in an elution order that is roughly the opposite of that in reversed phase.

The aim of this study was to examine the possible application of HILIC approach with two different columns (silica and zwitterionic) as well as acetonitrile and methanol as organic solvents for the separation

Abbreviations: ACN, acetonitrile; HILIC, hydrophilic interaction chromatography; MeOH, methanol; LOD, limit of detection; Se(IV), selenite; Se(VI), selenate; SeMet, selenomethionine; MeSeCys, methylselenocysteine

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of some selenium compounds in comparison with reversed phase mode. As a compound four selenium species were selected: selenite, selenate, selenomethionine and methyl-selenocysteine due to the fact that they are present in the plant materials supplemented with selenium compounds [9, 14]. Such material was used in this study to test the potential of optimized method in the analysis of real sample.

2. Materials and methods

2.1. Reagents

The commercial standards of sodium selenite ($\geq 98\%$), sodium selenate ($\geq 95\%$), selenomethionine ($\geq 99\%$, SeMet), Se-(methyl)selenocysteine hydrochloride ($\geq 95\%$ MeSeCys) as well as the other chemicals were purchased from Merck-Sigma (Steinheim, Germany). Methanol and acetonitrile were of HPLC grade from Merck (Darmstadt, Germany). Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) with the electrical resistivity of $18 \text{ M}\Omega\text{-cm}$ was used in all experiments. Stock solutions of all analyzed selenium species as well as their diluted mixtures were prepared in water. To avoid the selenomethionine oxidation the addition of β -mercaptoethanol to the standard solution was made (2% v/v) [18].

2.2. Instrumentation

Chromatographic analysis was carried out using the Shimadzu LC system consisted of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC and 8030 Mass spectrometer (Shimadzu, Japan). A MS system was equipped with electrospray ionization source (ESI) operated in negative or in positive-ion mode according to species being determined. ESI conditions were following: capillary voltage 4.5 kV, temperature 400°C , the source gas flow 3 L/min, drying gas flow 10 L/min. Fragmentation spectra of all standards of selenium compounds were recorded and used to find characteristic MRM ion pairs, that were used for analysis (Table 1S.).

Chromatographic columns used in the study were as follow: SeQuant™ ZIC-HILIC column ($100 \times 2.1 \text{ mm}$, $3.0 \mu\text{m}$) from Merck (Darmstadt, Germany) and Atlantis HILIC ($100 \times 2.1 \text{ mm}$, $3.0 \mu\text{m}$) from Waters (Dublin, Ireland) at 30°C . The mobile phase was a mixture of organic solvent (acetonitrile or methanol) and 8 mM formic acid at pH 2.8 or 8 mM ammonium acetate solution (pH 4.5 and 7) or water, depending on the optimization step. The mobile phase was delivered at 0.2 mL/min in isocratic mode. The analytes were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra.

2.3. Calculation of $\log P_{o/w}$ of selenium compounds

The theoretical values of pK_a as well as $\log P_{o/w}$ were taken from database available on <http://www.hmdb.ca/> (values calculated using ChemAxon).

2.4. Plant growth and natural samples

The onions (*Allium cepa*) were used in the experiment to evaluate the potential of HILIC chromatography in the analysis of selenium in natural samples. The onion bulbs were hydroponically grown in black containers at room temperature in aerated 1/8 Knopp nutrient solution according to the method described by Wierzbička [19]. Cultivation and supplementation of onions was based on procedure described by Wróbel et al. [20]. After obtaining the roots of 3 cm long, onions were divided into three groups (12 pieces in each). One group was supplemented with Na_2SeO_3 and the second one with Na_2SeO_4 (both in concentration of 5 mg/L equals to Se). The third group was the control group and it was left in the nutrient solution. The onions were cultivated in such conditions for 8 days. After this time fresh plants were

rinsed with water and then divided into roots, bulbs and leaves. For this study onion leaves were taken as a natural sample.

2.5. Extraction procedure

The extraction procedure of selenium was based on the method described by Montes-Bayon [21] with some small changes. 0.4 g of onion leaves was taken and placed in 10 mL tube. Then 5 mL of ammonium acetate buffer (25 mM, pH 5.6) was added. The tubes were closed tightly and placed in mechanical rotator for 20 h in room temperature. After that time the supernatant was centrifuged for 20 min (2000 rpm) and then filtrated using $0.45 \mu\text{m}$ PTFE filters.

3. Results and discussion

3.1. Effect of mobile phase composition

Studies on HILIC chromatography showed that the selection of mobile phase composition has a great impact on the retention mechanism. It is known that water from the mobile phase is retained on the surface of the HILIC column [22,23], thus the concentration of water is higher near the stationary phase than in the mobile phase, providing the evidence for partition mechanism. The elution strength of mobile phase increases with increasing content of water component in the eluent. Under such conditions, the polarity difference between the aqueous layer and the mobile phase is significant and the polar analytes are strongly retained in the water layer. As the water content increases, the polarity difference between the eluent and the water layer decreases. This entails an increase in the eluent's competitiveness with respect to the aqueous layer and a reduction in the retention time of the analytes.

Another factor that affects the elution strength of the eluent is the type of organic solvent. In HILIC, the elution strength of organic solvents increases in the order of the increasing solvent polarity and the ability to participate in proton-donor/proton-acceptor interaction. Acetonitrile, considered as a weak solvent, is mostly used as an eluent component in HILIC, but methanol (strong eluent) is also used [23]. According to Buszewski et al. [24] methanol is adsorbed near the stationary phase via hydrogen bonding with residual silanols, while acetonitrile can react with silanols via dipole-dipole interactions. The amount of adsorbed ACN molecules is even four times higher than for methanol. In our study ACN as well as MeOH was used as an organic component of the mobile phase. The obtained retention factors were plotted against the ACN or MeOH content in the range of 35–95% (v/v). Results for silica and ZIC column are shown in Figs. 1 and 1S, respectively. With the increase of ACN content in the eluent, the increase in the retention factors for all of the analytes is observed. This trend is maintained for all columns used in the study. Replacing ACN with MeOH does not change the elution order, however the retention factors significantly decrease when ZIC column is used. For silica column U-shape curves are obtained for inorganic form of selenium. Bare silica column contains only non-reacted residual silanols, which are weak acids and become deprotonated at higher pH of mobile phase. Thus, electrostatic interactions between negatively charged silanols groups and charged analytes should be considered [25, 26]. Silica column showed dual retention mechanism: HILIC with MeOH content higher than 80% and reversed-phase with lower content of organic modifier. The pK_a value of bare silica is around 4.5 [27], so at pH equals to this value, 50% of silanol groups are deprotonated. In our study the pH was increased from 2.8 to 7. The lowest value of the eluent pH (2.8) is below the pK_a of silica, so the stationary phase is not dissociated. At pH 7 the silica surface is negatively charged and can interact with positively charged analytes. On the other hand, the pK_a values of our analytes are in range from 1.46 to 3.8. They are also negatively charged at pH 4.5, thus the electrostatic repulsion between them and silica surface can be observed. From the pH 2.8 to 7 the decrease of the retention of selenium

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