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Determination of selenium species in plant leaves by HPLC–UV–HG-AFS

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Abstract

The purpose of this work was the development of a method for the determination of Se compounds in leaves of plants. Water-soluble Se compounds were extracted from samples by water. Enzymatic hydrolysis with the non-specific enzyme protease XIV was used for the release of Se compounds bound to proteins. Separation of Se species was made by ion exchange chromatography, using an anion exchange column for Se^{IV}, Se^{VI} and selenomethionine (SeMet), and a cation exchange column for selenomethylselenocysteine (SeMeSeCys) and selenocystine ($SeCys_2$). Columns were connected "on line" to a hydride generation atomic fluorescence spectrometer (HG-AFS) using a UV lamp between the separation and detection system. The repeatability of the results obtained by the developed method was under 15% (R.S.D.) for all Se species; the detection limit was 2–10 ng Se/g of supernatant. The accuracy was checked by comparison with some literature data for reference materials since there were no suitable certified reference materials available. The method was used for the determination of Se compounds in chicory (*Cichorium intybus* L.) leaves from plants which were cultivated aeroponically with elevated concentrations of Na_2SeO_4 for different periods. Se accumulated efficiently in chicory leaves; up to 480 μg/g after 41 days of exposure, mostly (64%) as Se^{VI}, i.e. in the form of Se added. Beside inorganic Se, in the extracts from enzyme hydrolysis we also found SeMet (4.2–8.4%) and SeMeSeCys (<DL−0.7%). Some unidentified peaks were also observed in the chromatograms of plant extracts.

Keywords: Plants; Se compounds; HPLC–UV–HG-AFS

1. Introduction

The most important source of the essential element selenium for humans and animals is food. Beside the total content of selenium, the chemical form in which selenium is present is also most important due to the differences in bioavailability and toxicity of the different forms. Plants are mostly poor sources of selenium. But plants that are capable of accumulating higher amounts of selenium during cultivation and transforming it to an appropriate chemical form are one of the potential sources for enhancement of Se daily intake [1,2].

Accurate determination of selenium compounds in plant samples is not straightforward. There are several reasons for this. Important ones are the low concentration of Se in samples, low extraction efficiencies of Se compounds from the sample matrix and the instability of Se compounds during the whole process from sampling to measurement [3]. Neutral conditions should be used due to the instability of Se compounds in acidic and basic conditions. Water extractions were used when water-soluble compounds were investigated [4–7], and enzymatic hydrolysis, mostly with the nonspecific enzyme protease XIV, for Se compounds bound to proteins [4,8–10]. For further separation of selenium species, ion exchange or ion pairing reverse phase high performance liquid chromatography (HPLC) have been used most often [11]. A very sensitive detection system is needed for the detection of Se in the low concentration range (few ng/g) in the eluent, for which inductively coupled plasma mass spectrometry is mainly used [12]. Hydride generation atomic

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fluorescence spectrometry (HG-AFS) could also be used. Since only Se^{IV} forms volatile H_2Se, other Se species should first be transformed; thus, organic Se compounds should be broken down and rapid reduction of Se^{VI} should take place. For this purpose, an additional decomposition unit, using UV or microwave radiation, and/or additional reagents such as HBr–$KBrO_3$ or HCl should be inserted between the HPLC and HG-AFS systems [9,13–16]. The advantage of using HCl as a reagent is that the same reagent is used for conversion to Se^{IV} and for hydride generation, and likewise the influence of interferences in the hydride generation step is decreased in this way [17].

The aim of this study was the development of a method for the determination of Se compounds in plant leaves using HPLC separation and an HG-AFS detection system. Since we wanted to obtain quantitative results and not merely qualitative ones, special attenition was paid to the assessment of some important analytical parameters such as accuracy and precision. The developed method was applied for the identification of Se species in chicory leaves. Chicory grows easily and is usually cultivated for the production of inulin from its leaves or roots. The leaves are also used as a salad throughout the year. Chicory consumption is very popular in Slovenia and in the northern parts of Italy and is resistant to low temperature, down to −12 °C. In our experiment, chicory (*Cichorium intybus* L.) was treated with Se enriched nutrient solution for various time periods in an aeroponic cultivation system, where changes in the distribution of Se species in the leaves with time was of interest.

2. Experimental

2.1. Samples

Chicory (*Cichorium intybus* L. cultivar Anivip) leaves from different groups of plants were used: a control group (six plants) and exposed groups. Exposure lasted for 5 days (four plants), 10 days (four plants) or 41 days (six plants). Plants were grown from seeds and when they were 90 days old, they were transferred to an aeroponic system. Aeroponics is a form of hydroponic plant cultivation in which the plant roots are suspended in a closed chamber and misted with a complete nutrient solution. Aeroponics requires no solid or aggregate growing medium and allows for easy access to roots. The chamber and misting system provide complete control of temperature, nutrient level, pH, humidity, misting frequency and duration, and oxygen availability. In our case, the roots were sprayed every 15 min with Resh nutrient solution containing the macro- and microelements essential for growth of leafy vegetables (4.17 g Fe-EDTA, 0.0393 g $CuSO_4{\cdot}5H_2O$, 0.0075 g MoO_3, 0.203 g $MnSO_4$, 0.0044 g $ZnSO_4{\cdot}7H_2O$, 0.286 g H_3BO_3, 1.0 g Na_2SO_4, 40.5 g $MgSO_4{\cdot}7H_2O$, 22.0 g KH_2PO_4, 32.8 g K_2SO_4, 82.1 g $Ca(NO_3)_2$, 14.2 g NH_4NO_3 in 100 L of water) and enriched in Se in the form of Na_2SeO_4 at a concentration of 7 μg Se/mL. Leaves were lyophilised at −50 °C and 0.050 mbar (Freeze-dryer CHRIST ALPHA 1-4, LOC-1) and milled in a planetary micro mill (FRITSCH, Pulverisette 7, Idar-Oberstein, Germany; speed 6, time 6 min).

2.2. Chemicals

MilliQ water (Millipore Corporation, Bedford, MA, USA) was used in the whole process and the following chemicals: 65% HNO_3 (Merck, Darmstadt, Germany, suprapur), 96% H_2SO_4 (Merck, suprapur), 30% HCl (Merck, suprapur), 36% HCl (Merck, p.a.), 30% H_2O_2 (Merck, p.a.), V_2O_5 (Merck, p.a.), 40% HF (Merck, suprapur), NaCl (Merck, suprapur), tris(hidroxymethyl)aminomethane hydrochloride (Tris–HCl, Sigma–Aldrich, Taufkirchen, Germany, ultrapur), dithiotreitol (DTT, Sigma–Aldrich, >99%), saccharose (Merck, p.a.), phenylmethylsulphonyl fluoride (PMSF, Merck, p.a.), mercaptoethanol (Sigma–Aldrich, >98%), NaN_3 (Sigma–Aldrich, >99%), NaOH (Merck, puriss p.a.), $NaBH_4$ (Fluka Chemie, Steinheim, Germany, purum p.a.), $(NH_4)_2HPO_4$ (Fluka Chemie, puriss p.a), $NH_4H_2PO_4$ (Fluka Chemie, puriss p.a.), pyridine (Fluka Chemie, puriss p.a.), HCOOH (RDH, extra pure), diammoniumhydrogen citrate (Fluka Chemie, puriss p.a.), citric acid (Fluka Chemie, puriss p.a.), CH_3COONH_4 (Merck, practopur), 90% CH_3COOH (Merck, suprapur), protease XIV (Sigma–Aldrich, 4 U/mg).

For preparation of Se solutions Na_2SeO_3 (Sigma–Aldrich, >98%), Na_2SeO_4 (Sigma–Aldrich, SigmaUltra), selenomethionine (SeMet, Fluka Chemie, >99%), selenocystine ($SeCys_2$, Fluka Chemie, >98%) and selenomethylselenocysteine (SeMeSeCys, Fluka Chemie, >98%) were used.

2.3. Instrumentation

The HPLC system consisted of a Varian ProStar 210 pump (Varian, Mitchell Drive, Walnut Creek, CA, USA), a Rheodyne 7725i injector (Rheodyne, Cotati, USA) and Hamilton PRP X100 and X200 columns (Hamilton, Reno, NV). The HG-AFS system was constructed of a peristaltic pump (Ismatec, MCP 380, Glattbrugg, Switzerland), a gas liquid separator (A-type, PS Analytical, Orpington, UK); a gas dryer (Nafion dryer, Perma Pure Products, Toms River, NY) and an AFS detector (Excalibur, PS Analytical, Orpington, UK) with a Se boosted discharge lamp (Super Lamp, Photron, Narre Warren, Vic., Australia). A UV unit (PS Analytical, Orpington, UK) with a 12 m coil of fluorinated ethylene propylene (FEP) around a 78 W lamp was also used.

2.4. Procedures

2.4.1. Extraction

Water extraction: 10 g of water was added to 0.2 g sample in a 15 mL centrifuge tube and the mixture was shaken for 5 min at room temperature.

Enzyme hydrolysis: 10 g of solution containing 80 mg of the enzyme protease XIV was added to 0.2 g sample in a

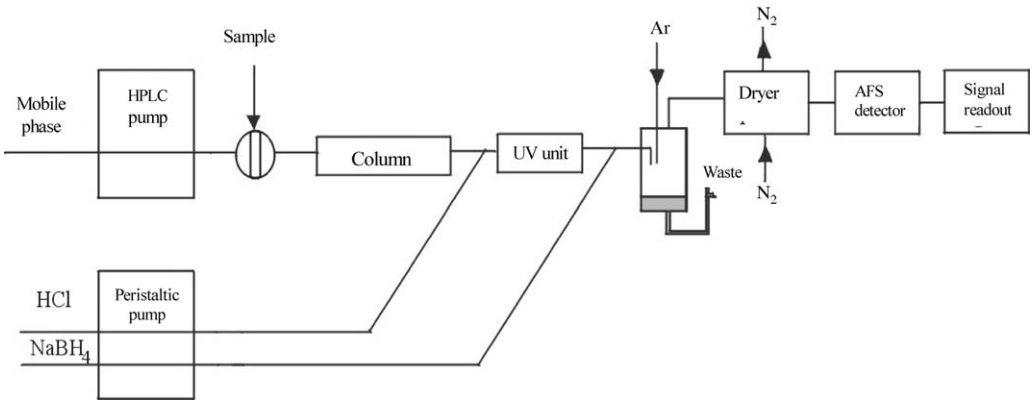

Fig. 1. Scheme of the HPLC–UV–HG-AFS system.

15 mL centrifuge tube and the mixture was shaken for 24 h at 37 °C.

After extraction the mixture was centrifuged for 30 min at 14,000 rpm/min (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany). Supernatants were separated from sediment and filtered through a 0.22 μm Millex GV filter (Millipore Corporation). Supernatants and sediments were stored at −20 °C until analyses for total Se and Se speciation were carried out.

2.4.2. Determination of Se compounds

An HPLC–UV–HG-AFS system was used for the determination of Se compounds. The scheme of the system used is shown in Fig. 1. HPLC conditions are summarised in Table 1. The eluent from the column was mixed with concentrated HCl (flow rate 3 mL/min) and then passed through the UV unit. 1.2% $NaBH_4$ in 0.1 mol/L NaOH (flow rate 3 mL/min) was added after the UV unit. Argon as carrier gas (260 mL/min) transferred H_2Se from the gas liquid separator through the dryer into the AFS detector. The dryer gas was nitrogen with a flow rate 3 L/min. The AFS unit was equipped with a Se boosted discharge lamp (primary current 20 mA, boosted current 25 mA).

2.4.3. Total Se determination

The procedure used for the determination of total Se in lyophilised plant samples and sediments after extraction is described in detail elsewhere [18]. 0.2 g of sample was weighted in a Teflon tube and 0.5 mL concentrated H_2SO_4 and 1.5 mL concentrated HNO_3 were addded and heated for 60 min at 130 °C in the screw capped tube in an aluminium block. After cooling 2 mL of 30% H_2O_2 was added and the tube was reheated for 10 min at 115 °C, then 0.1 mL of 40% HF was added and heated for 10 min at 115 °C, and again after the addition of 2 mL of H_2O_2 the solution was kept at 115 °C for 10 min. After cooling the addition of 0.1 mL of V_2O_5 in H_2SO_4 followed and the solution was heated at 115 °C for 20 min. Reduction to Se^{IV} was made by 2.5 mL concentrated HCl for 10 min at 100 °C. The solutions were then diluted, depending on the foreseen selenium concentration in the samples, and detection of Se was performed by countinuous HG-AFS.

A detailed description of the procedure used for the determination of total selenium in supernatants and chromatographic fractions is given in reference [19]. 0.5 mL of concentrated HNO_3 was added to 1 g of fraction or 0.5 g of supernatant in a Teflon tube. Heating in the screw capped tube for 10 min on a hot electric plate followed. After cooling addition of 0.5 mL of H_2O_2 and heating for 5 min followed and was repeated three times. The next step was reduction with 1 mL concentrated HCl for 10 min at 100 °C. After dilution to 15 g, Se was determined by countinuous HG-AFS.

3. Results and discussion

3.1. Optimisation of detection by UV–HG-AFS

The basic principle of hydride generation is formation of H_2Se by the reaction between Se^{IV} and $NaBH_4$ in acidic

Table 1
HPLC conditions

Parameter	Anion exchange	Cation exchange	Size exclusion
Column	Hamilton PRP X100 (250 mm × 4.1 mm × 10 μm)	Hamilton PRP X200 (250 mm × 4.1 mm × 10 μm)	Superdex 75 HR 10/30 (300 mm × 10 mm) and Superdex Peptide HR 10/30 (300 mm × 10 mm)
Mobile phase	40 mmol/L phosphate buffer (pH 6.0) 0.5 mL/min	10 mmol/L pyridine (pH 1.5) 1 mL/min	10 mmol/L Tris–HCl (pH 8.0, 0.1% NaN_3) 0.75 mL/min
Injection volume	100 μL	100 μL	100–200 μL

medium. Chatterjee and Irgolic reported that 11 Se compounds had the ability to form volatile Se species with $NaBH_4$, the only exception was SeO_4^{2-} which did not react at all [20]. However, the efficiences of formation of H_2Se were very low with respect to Se^{IV} and inappropriate for on-line conection to the HPLC separation unit. Therefore, Se compounds have to be transformed to the Se^{IV} oxidation state, so organic Se compounds have first to be destroyed, the products of decomposition and any inorganic SeO_4^{2-} present have then to be reduced. So, it was necessary to introduce one or more intermediate units and also add one or more reagents between the column and the HG-AFS system. Despite the fact that the mechanisms of photooxidation and photoreduction in a UV unit have not yet been explained, this unit was chosen because of its simplicity and efficiency and the fact that the reactions occur simultaneously. Some important factors that influence the efficiency of oxidation/reduction are the residence time of Se species in the UV unit, the power of the UV lamp and the material of the coil surrounding the lamp [9,16]. A 75 W lamp with 12 m coil made of FEP teflon was used so that the residence time was 2 min. According to this characteristic and the fact that the reduction of SeO_4^{2-} is complete in less than 10 min at 100 °C using 6 mol/L HCl in a batch system [19], concentrated HCl with a flow rate of at least 3 mL/min had to be used in the on-line system. Lower flow rates led to lower efficiencies, especially of reduction, due to the greater dilution of HCl. Fig. 2 shows efficiences of transformation of Se species calculated relative to Se^{IV} under different conditions in the UV–HG-AFS system. The highest efficiences were obtained with the use of the UV unit and concentrated HCl, and were 80% for selenomethionine (SeMet), 90% for Se^{VI} and 100% for selenocystine ($SeCys_2$).

Another factor that influences the efficiency of the UV–HG-AFS detection system is the composition of the carrier or mobile phase in the HPLC–UV–HG-AFS system. Several mobile phases potentially useful in size exclusion or ion exchange chromatography were tested. Results for Se compounds are shown in Fig. 3 relative to Se^{IV}. The use of Tris–HCl was the most problematic because it affected all Se species including Se^{IV} and no response was obtained for

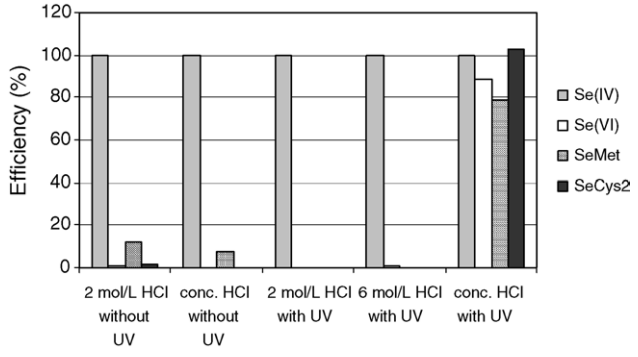

Fig. 2. Efficiencies of transformation into H_2Se of Se^{VI}, SeMet and $SeCys_2$ with respect to Se^{IV} under different conditions in the HG-AFS system (see Section 2 for details).

30 and 50 mmol/L Tris–HCl (Fig. 3). Tris–HCl buffer with as low a concentration as possible (10 mmol/L) should be used. The same rule was valid for buffers containing pyridine. Though pyridine does not affect Se^{IV}, pyridine absorbs UV light and therefore the power of UV light available for photooxidation and photoreduction of Se compounds was diminished.

3.2. Optimisation of chromatographic separation

Because selenoaminoacids and inorganic forms of Se have ionic properties over a wide pH range, ion-exchange chromatography could be used for their separation. Both anionic and cationic exchange columns were applied in our case. Hamilton PRP X100 and Hamilton PRP X200 columns were chosen because they are very robust and could be used over a wide pH range. It is known that organic solvents have a negative impact on hydride generation [13,17], therefore their use in mobile phases should be limited to a minimum.

For anion exchange chromatography, phosphate buffer was selected as the mobile phase as it is frequently used in connection with hydride generation techniques [16,21–25]. The concentration, pH and flow rate of mobile phase were optimised. Good separation of five Se species was obtained with 40 mmol/L phosphate buffer. At a concentration lower than 20 mmol/L retention times of the eluting species were prolonged too much, and higher concentrations led to overlapping of the peaks. Optimistion of the pH of phosphate buffer was made in the range 4.0–8.0. The retention time of selenoaminoacids increased with increasing pH. At pH 8.0 SeMet was retained for more than 60 min. Inorganic Se acids behaved in a contrary fashion, retention times decreasing with increasing pH. Retention of these acids on the anionic column was controlled by protonation of selenooxoanions and phosphate anions. According to the pK values, the major anion present in the pH range 4.0–6.0 was $H_2PO_4^-$, competing with $HSeO_3^-$, and between 6.0 and 8.0 $H_2PO_4^-$ was in equilibrium with HPO_4^{2-}, competing with $HSeO_3^-$ and SeO_4^{2-}. These all anions competed for ammonium groups of the stationary phase. At the selected pH, $HSeO_3^-$ and SeO_4^{2-} were present and SeO_4^{2-} eluted later because of its stronger electrostatic interaction with the stationary phase. Due to ionic interactions on the column, we predicted that Se^{IV} would elute after the selenoaminoacids. The longer retention of SeMet could be explained by interaction of the hydrophobic part of the molecule with the polymeric base of the column. The best separation of $SeCys_2$, Se^{IV}, SeMet and Se^{VI} was obtained at pH 6.0.

When selenomethylselenocysteine was added to a mixture of SeMet, $SeCys_2$, Se^{IV} and Se^{VI}, it eluted between $SeCys_2$ and Se^{IV} but the peaks were not well separated at a flow rate of 1 mL/min. Better separation was obtained at 0.5 mL/min. Fig. 4 shows the separation of five Se compounds on a Hamilton PRP X-100 anion exchange column with 40 mmol/L phosphate buffer (pH 6.0) at a flow rate of 0.5 mL/min using the UV–HG-AFS detection system.

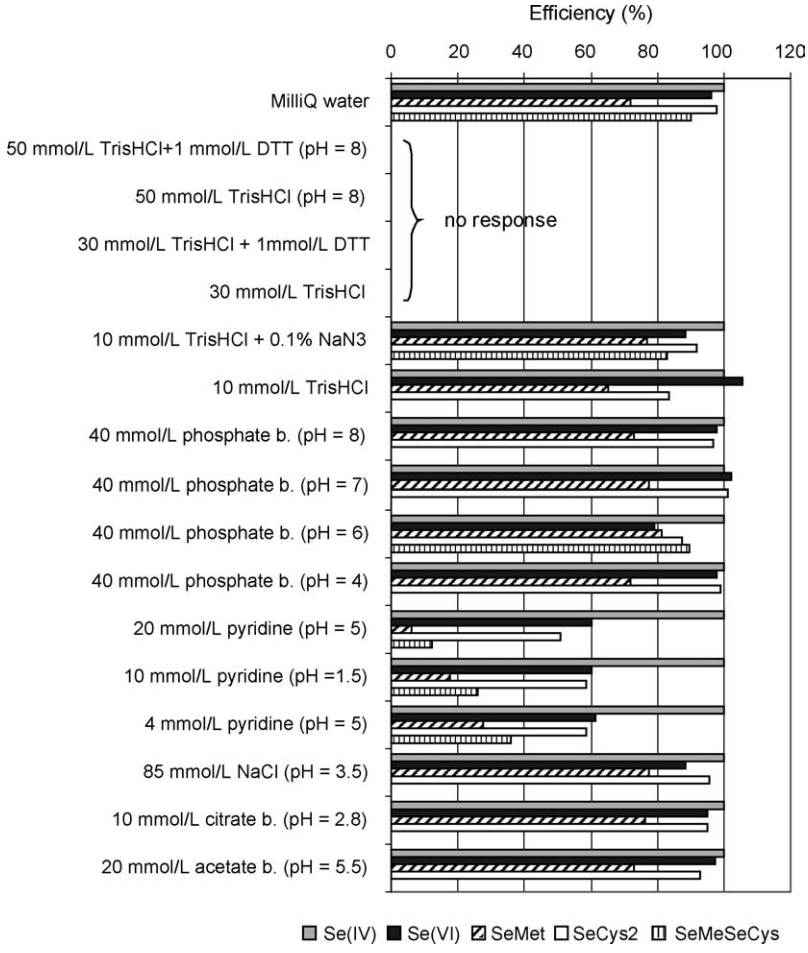

Fig. 3. Efficiencies of FI-UV–HG-AFS for Se^{VI}, SeMet, $SeCys_2$ and SeMeSeCys with respect to Se^{IV} in different mobile phases.

As $SeCys_2$ was eluted in the void volume of the anion exchange column, separation on the cation exchange column was also used. Twenty millimole per litre pyridine solution is an often mentioned mobile phase in the literature [26–29].

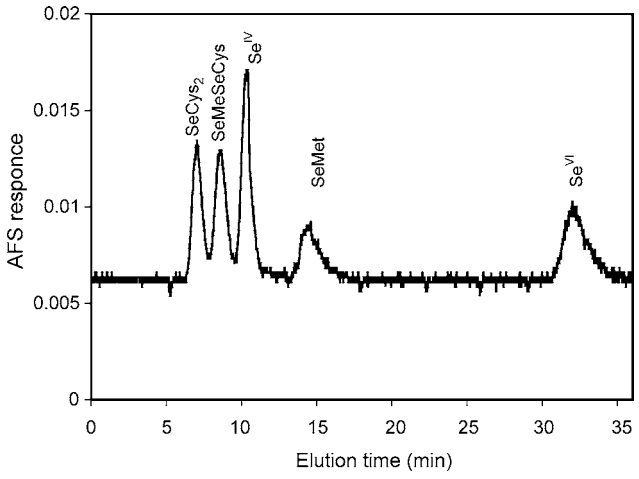

Fig. 4. Chromatogram of a mixture of Se species with mass fraction of Se around 100 ng/g each on an anionic column.

It turned out that it was not suitable for our detection system because the efficiency of the FI-UV–HG-AFS system with this eluent was low, especially for SeMet (Fig. 3). So we decided to study the influence of pH on elution time at two lower concentrations of pyridine, 4 and 10 mmol/L. Anions eluted in the void volume of the cationic column. The retention times of selenoaminoacids increased with decreasing pH, which was especially noticable for $SeCys_2$. The explanation for this was the strong binding of $SeCys_2$ on the stationary phase at pH 1.5 where it was present in a doubly protonated form. Inorganic Se acids behaved oppositely, elution times decreasing with decreasing pH. Separation at the lowest pH of 1.5 turned out to be the best at both concentrations. Ten millimole per litre pyridine was chosen since elution times were shorter, with the exception of SeMet. Fig. 5 shows separation of five Se compounds on a Hamilton PRP X200 cation exchange column with 10 mmol/L pyridine (pH 1.5) at a flow rate of 1 mL/min using the UV–HG-AFS detection system.

According to the above results for separation of Se compounds on the two columns (Figs. 4 and 5), an anionic column was chosen for the determination of Se^{IV}, Se^{VI} and SeMet, and a cationic column for $SeCys_2$ and SeMeSeCys.

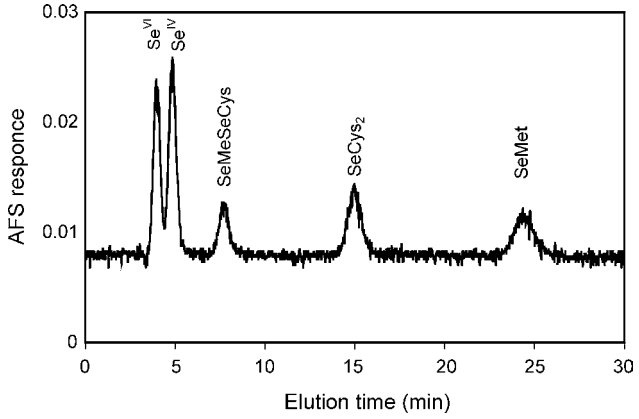

Fig. 5. Chromatogram of a mixture of Se species with mass fraction of Se around 100 ng/g each, except SeMet around 500 ng/g, on a cationic column.

3.3. Optimisation of the extraction step

Extraction efficiency depends upon the nature of the sample and the extraction conditions. But beside the maximum efficiency, the stability of Se species during the procedure should be taken into account when choosing the extraction conditions. Previous studies of the extraction of Se species were mainly done on yeast [23,24,30]. In our study, two different types of extraction were used for Se species from plant samples. The first was simple water extraction for water-soluble Se species, and the second was enzymatic hydrolysis to release Se species bound to proteins. Optimisation of both procedures was made. Extraction efficiency was calculated by comparison with the total concentration of Se in the sample. The range of variation of parameters and the working conditions used in water extraction are shown in Table 2.

The pH range of solvents studied was between 6.0 and 8.0, which is close to neutral conditions. A higher efficiency than in water was obtained with 0.1 mol/L Tris–HCl buffer. Various additions to that buffer such as saccharose, PMSF, mercaptoethanol and DTT had no influence on the extraction efficiency. Problems with Tris–HCl buffer arose when extracts were concentrated before chromatographic separation. A higher concentration of Tris–HCl after lyophilisation negatively influenced the separation of Se species on the columns.

The optimal ratio between the mass of the sample and the mass of solvent was 1:50. At a ratio of 1:10 a lower extraction efficiency was obtained because there was not enough solvent to extract all the Se. At a ratio of 1:75 the efficiency was the same as at 1:50, but in view of further determination steps the use of more concentrated extracts was preferred.

Time and type of shaking had no influence on extraction efficiency. So in regard to the stability of Se species the shortest time of shaking was chosen. Longer times from 6 to 24 h were used in the literature and sometimes heating was also applied during shaking [4,13,31]. We presumed that ultrasound would fragment plant cells and Se would be released more efficiently from cells and their components. But the same results were obtained by 5 min mechanical shaking and 5 min in an ultrasound bath, $69 \pm 2\%$ and $70 \pm 1\%$, respectively. Even more rigorous conditions for rupturing cells by treatment of the sample and sediment with liquid nitrogen did not increase the proportion of extracted Se.

Most of the water-soluble Se (60–70%) was extracted in the first step, in the second step around 10% and in the third one a negligible quantity. Of the 10% of Se extracted in the second step most was the consequence of the poor separation of supernatant and sediment after the first extraction. Only 8–8.5 g of supernatant was obtained from 10 g of water used. The rest of the water remained in the sediment after the first extraction which was then combined with fresh solvent in the second step. The same effect was reported by Francesconi for the extraction of As from algae [32].

These simple extraction conditions, MilliQ water as extracting solvent and single step extraction, were also used with enzymatic hydrolysis. Only the mass of enzyme was optimised. Twenty, 40 or 80 mg of the enzyme protease XIV in MilliQ water was added to 200 mg of lyophilised plant sample. Solutions were then incubated for 24 h at 37 °C. The efficiency increased from $67 \pm 6\%$ in water alone to an average of $76 \pm 3\%$ in the presence of enzyme, irrespective of its mass (Table 3). But from the same table, we can see that with increasing amount of enzyme the distribution of Se species in the extracts changed. A higher amount of SeMet

Table 2
Range of variation of parameters in optimisation and working conditions used in water extraction of lyophilised plant sample

Parameter	Range of variation	Working conditions used
Type of solvent (pH 6.0–8.0)	Water, 10 and 100 mmol/L Tris–HCl, 20 mmol/L phosphate buffer	Water
Different additions to Tris–HCl	PMSF, mercaptoethanol, saccharose, DTT	No additions
Mass ratio sample: solvent	1:10–1:75	1:50
Time of shaking	5 min–6 h	5 min
Type of shaking	Ultrasound bath, mechanical shaking	Mechanical shaking
Number of treatments with liquid N_2	0–2	0
Number of extractions	1–3	1
Extraction efficiency at working conditions[a]		$67 \pm 6\%$ ($n = 22$)

[a] Extraction efficiency calculated according to the total concentration of Se in the sample.

Table 3
Influence of mass of enzyme on water-soluble selenium content of chicory sample and distribution of Se species in extract

m_{enzyme} (mg)	Water-soluble Se[a] (%)	Se species[a,b] (%)				
		SeMet	A[c]	Se^{IV}	Se^{VI}	Sum
0	67 ± 6	0.012 ± 0.002	0.022 ± 0.003	0.05 ± 0.01	63 ± 6	63 ± 6
20	76 ± 1	6.5 ± 0.8	1.0 ± 0.2	0.10 ± 0.02	57 ± 4	65 ± 5
40	77 ± 3	5.6 ± 0.8	1.8 ± 0.3	0.10 ± 0.02	60 ± 5	68 ± 6
80	75 ± 1	8.1 ± 0.5	2.3 ± 0.3	0.14 ± 0.05	60 ± 3	71 ± 4

[a] Results expresed as average ± standard deviation ($n = 4$).
[b] % Se in this species with respect to total Se content in sample.
[c] Unknown species at t_e 6 min on anion exchange column, calculated on the basis of Se^{IV}.

was extracted and also a small increase in unknown species A was observed with increasing mass of enzyme, hence the use of 80 mg of enzyme was chosen. The sum of all identified species represents 85–95% of the water-soluble selenium. The necessity of a second step of enzymatic hydrolysis was also checked and was not confirmed, since only 10% of SeMet and unknown A was obtained relative to the amount extracted in the first step.

3.4. Stability of Se species

One of the possible reactions affecting the stability of supernatants during storage is oxidation of Se species. A solution of SeMet with a mass fraction of Se around 1 μg/g was oxidised in a test experiment with a 2% solution of H_2O_2 overnight at room temperature. Several oxidation products were observed (Fig. 6), mostly at different elution times on both anionic and cationic columns from standard solutions of Se species. Different oxidation products were noticed at different conditions of oxidation. After heating with H_2O_2 at 80 °C for 50 min, Se^{IV} was the main product (Fig. 6). Under both conditions the original SeMet was not observed. The same was reported by Uden et al. [4] and Gammalgaard et al. [33].

Another important factor was the stability of Se species during extraction and storage of supernatants after enzyme hydrolysis. Eighty milligrams of protease XIV was added to 10 g of a solution of SeMet with a mass fraction of 1 μg/g. The solution was incubated for 24 h at 37 °C and stored at −20 °C untill analysed. The same solution without the enzyme was stable for at least 2 weeks at −20 and 4 °C. The chromatograms on Fig. 7 showed two additional products beside SeMet after the addition of enzyme. These results differ from those of Dumont et al. who reported that transformations of SeMet were the consequence of the sample matrix and not the activity of the enzyme [13].

Products of oxidation and of enzyme activity on SeMet were not the same with the exception of the unknown peak A. For further characterisation of observed unknown products HPLC ES-MS should be used. Regarding the experimental data on the stability of Se species, we can conclude that extracts should be stored at −20 °C before analysis and analysed as soon as possible.

3.5. Analytical parameters

Table 4 summarizes some analytical parameters of the determination of Se species. The efficiency of elution of a single Se species from the column (η_{HPLC}) was determined by collection of the eluent after separation and determination of total Se in it by the procedure described in reference [19]. If η_{HPLC} is multiplied by the efficiency of the FI–UV–HG-AFS system ($\eta_{FI-UV-HG-AFS}$) comparable results are obtained for off-line and on-line ($\eta_{HPLC-UV-HG-AFS}$) connection of the systems. The detection limits (DL) of our method are comparable with those in the literature for HPLC–ICP-

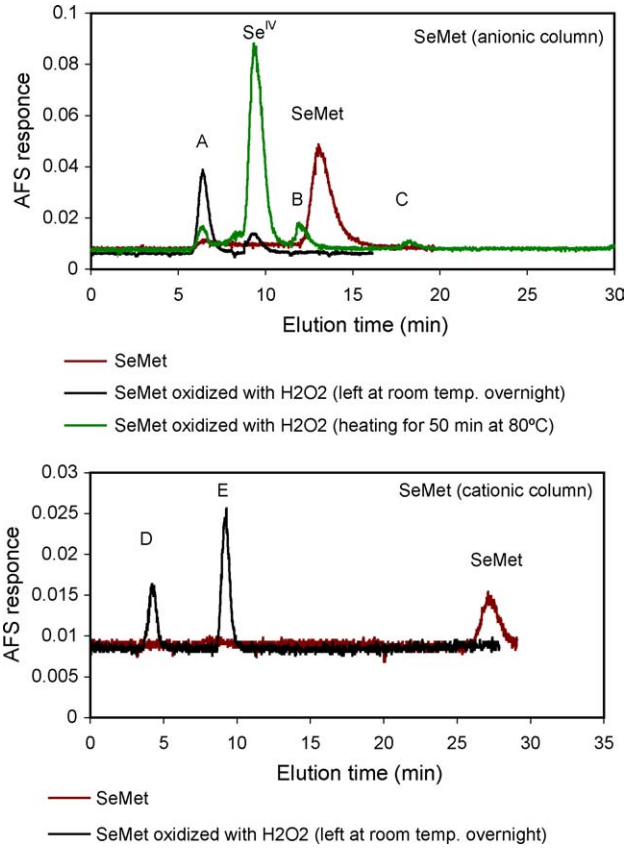

Fig. 6. Chromatogram of oxidation products of a solution of SeMet (A–E: unknown species).

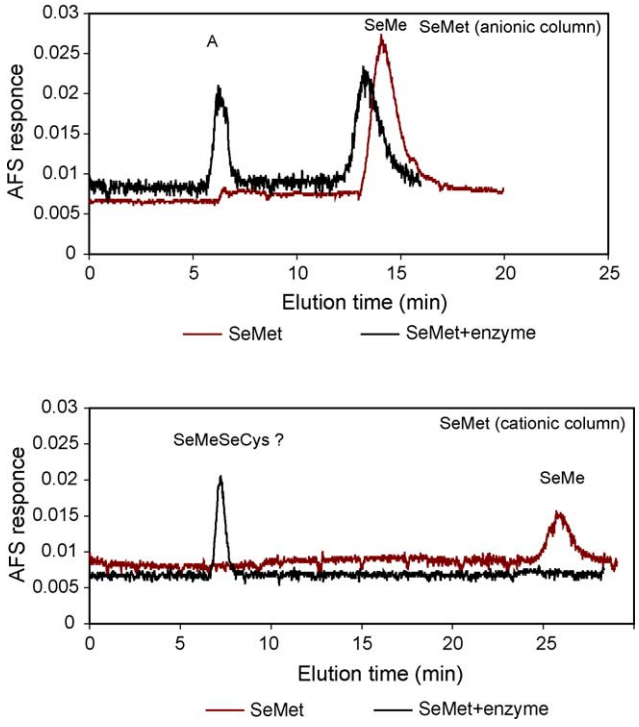

Fig. 7. Chromatograms of SeMet after the addition of enzyme (A: unknown species).

MS and HPLC–UV–HG-AFS systems [16,22,24,25,34,35].

Since there are no reference materials of biological origin with data for Se species, our results were compared with literature data for SeMet obtained in accuracy testing of the optimised method. Informative values for SeMet obtained in an interlaboratory comparison were reported for a yeast candidate reference material from the SeAs project [36]. The reference material Durum wheat flour RM 8436 was also used [37] and the agreement of the results was very good (Table 5).

3.6. Application of the method

Selenium accumulated very efficiently in the leaves of exposed chicory plants. The mass fraction of Se in leaves increased with the time of exposure (Table 6). Despite the high Se content there was no sign of poisoning such as a garlic smell, red spots on the roots, black spots on the leaves and drying of the leaves [38,39]. Se was accumulated mainly in the water-soluble form (64%), irrespective of the time of exposure. Water soluble forms were first separated by size exclusion chromatography on Superdex 75 and Superdex peptide columns. The majority of water-soluble Se, more than 95%, eluted in the low molecular fraction, MW under 1000 (data not shown).

For furher identification of the Se species ion exchange columns coupled with an UV–HG-AFS detection system were used. The results for Se species in water extracts of chicory leaves are collected in Table 7. Se^{VI} represents almost 100% of all Se in the water extract. Therefore, we can conclude that the main form acummulated was Se^{VI}, and thus, the same form as the form of added Se. Other species identified were Se^{IV}, SeMet and SeMeSeCys, as well as some unknown peaks observed with t_e 25 min (G) and in the void volume (A) on the anion exchange column. Unknown peak G was not the same as the products of oxidation or enzyme activity on SeMet, $SeCys_2$ or SeMeSeCys (data not shown for the last two). The standard addition method was also used for identification.

Table 5
Comparison of values obtained for SeMet with informative values

Samples	Our result for SeMet (μg Se/g)	Informative value for SeMet (μg Se/g)
Yeast	810 ± 30 (n = 8)	874 ± 105 [36]
Durum wheat flour RM 8436	0.61 ± 0.07 (n = 2)	0.59 ± 0.04 [37]

Table 6
Effect of time of exposure to elevated Se concentrations in nutrient solution on selenium content in chicory leaves and the distribution between water-soluble and solid residue

Time of exposure (days)	Se content in leaves[a] (μg/g)	Water-soluble Se[a] (%)	Se in solid residue[a] (%)
0	0.057 ± 0.002	51 ± 5	55 ± 4
5	88 ± 1	64 ± 6	29 ± 4
10	131 ± 12	–	–
41	480 ± 10	64 ± 2	32 ± 3

[a] Average ± standard deviation (n = 3).

Table 4
Analytical parameters of the determination of Se species

Parameter	Anionic exchange			Cationic exchange	
	Se^{IV}	Se^{VI}	SeMet	$SeCys_2$	SeMeSeCys
η_{HPLC} (%)	100 ± 6	99 ± 2	72 ± 3	61 ± 6	60 ± 2
$\eta_{FI\text{-}UV\text{–}HG\text{-}AFS}$ (%)	100 ± 3	80 ± 3	82 ± 1	91 ± 3	30 ± 1
$\eta_{HPLC\text{–}UV\text{–}HG\text{-}AFS}$ (%)	100 ± 8	80 ± 8	64 ± 3	70 ± 3	22 ± 2
Linearity (ng/g)	DL-200 (R^2 = 0.99)	DL-200 (R^2 = 0.99)	DL-200 (R^2 = 0.99)	DL-200 (R^2 = 0.99)	DL-200 (R^2 = 0.99)
Repeatability[a] (%)	8	11	14	14	15
DL (ng/g)	2.3	5.7	9	4.3	6

η: efficiency expressed as average ± standard deviation (n = 4), DL: detection limit.
[a] Repeatability within 1 week for Se species with mass fraction 100 ng Se/g.

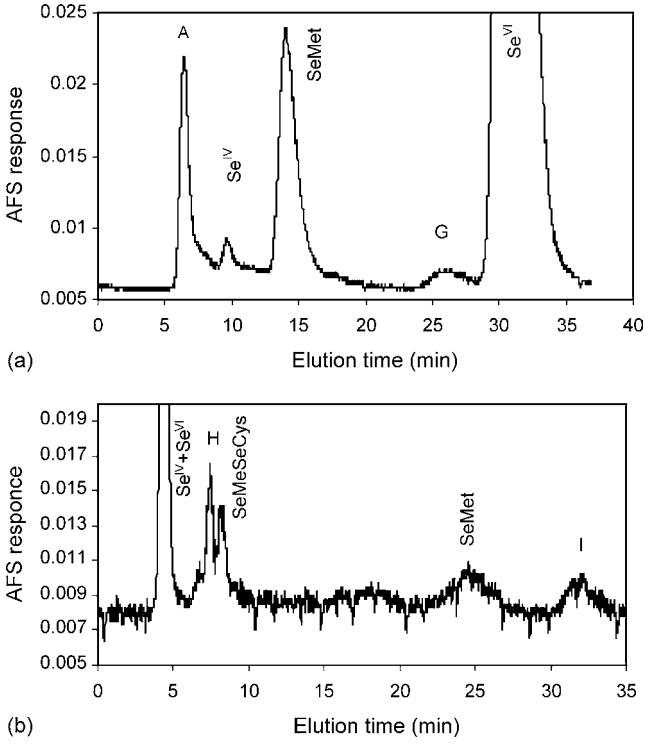

Fig. 8. Chromatograms from anion (a) and cation (b) exchange column of chicory (41 days exposed) leaves supernatant after enzymatic hydrolysis (A, G–I: unknown Se species).

Enzymatic hydrolysis was used for the determination of Se species bound to proteins. Anion and cation exchange chromatograms of the chicory cultivar Anivip exposed 41 days are presented in Fig. 8. In comparison with the chromatogram of the water extract, an increase of the SeMet peak is most obvious. The first peak from the anion column in the position of $SeCys_2$ (A) also increased from 0.01% to 2.3%. The presence of $SeCys_2$ was not confirmed on the cation exchange column. A rather high unknown peak eluted from the cation column just before SeMeSeCys (unknown H).

The results of determination of Se species in enzymatic hydrolysis extracts of chicory exposed for different time periods are summarised in Table 8. Beside four known compounds, one or two unknown peaks were observed on each column, the largest unknown A representing approximately 1–2% of the total Se in the chicory leaves. The percentage was estimated with respect to Se^{IV}. Unknown peaks did not elute at the same elution time as products of oxidation or enzymatic hydrolysis of SeMet, $SeCys_2$ or SeMeSeCys (data not shown for the last two). The amount of SeMet increased with increasing time of exposure. SeMeSeCys was detected only in extracts of the plant exposed for 41 days. The sum of Se in the identified peaks in extracts was between 73 and 77% (Table 8). Compared with the total Se in the same extracts, it could be said that we identified and quantified around 90% of the Se present in enzymatic hydrolysis extracts.

Despite the high Se concentrations, no toxic effects were observed on the plants. There were probably some protective mechanisms, similar to those of Se acummulating plants. One possibility was storage of Se^{VI} in vacuoles as this is the form found in extracts. Because SeMeSeCys was determined in the extract of the chicory cultivar Anivip exposed 41 days, the other possibility is the formation of the non-protein aminoacid SeMeSeCys and the dipeptide γ-glutamyl-SeMeSeCys [39,40].

Literature date on Se species in vegetables are very rare, and therefore, comparison with our results is very difficult; nevertheless data for the green parts of plants are compared in Table 9. We could conclude that if selenium was added in

Table 7
Se species in water extracts of chicory leaves

Time of exposure (days)	SeMet		SeMeSeCys		Se^{IV}		Se^{VI}	
	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)
0	<DL	–	<DL	–	<DL	–	<DL	–
5	tr	–	<DL	–	0.024 ± 0.004	0.032 ± 0.05	54 ± 5	62 ± 6
10	<DL	–	<DL	–	0.06 ± 0.01	0.05 ± 0.01	70 ± 7	53 ± 5
41	0.044 ± 0.008	0.012 ± 0.002	tr	–	0.22 ± 0.04	0.05 ± 0.01	313 ± 30	63 ± 6

<DL: under detection limit, tr: traces, %: fraction relative to total Se in sample.

Table 8
Se species in enzymatic hydrolysis extracts of chicory samples (in μg Se/g sample and % Se in this Se species with respect to total Se content in sample)

Time of exposure (days)	SeMet		SeMeSeCys		Se^{IV}		Se^{VI}		Unknown species[a]	Sum%	Total Se in extract (%)
	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)			
5	3.7 ± 0.5	4.2 ± 0.6	<DL	–	0.2 ± 0.04	0.2 ± 0.04	63 ± 6	72 ± 7	A, H	76 ± 8	79 ± 5
10	4.7 ± 0.8	3.5 ± 0.6	<DL	–	0.4 ± 0.1	0.32 ± 0.08	96 ± 9	73 ± 7	A, H	77 ± 8	89 ± 5
41	40.1 ± 0.4	8.1 ± 0.5	3.4 ± 0.5	0.7 ± 0.1	0.9 ± 0.2	0.14 ± 0.05	313 ± 30	63 ± 6	A,G, H, I	71 ± 4	75 ± 1

<DL: under detection limit.
[a] Unknown Se species with t_e: A and G with 6 and 25 min on anion column; H and I with 7 and 33 min on cation column.

Table 9
Comparison of results of this work with literature data for the leaves of cultivated plants exposed to selenium

Plant	Se addition	TotalSe[a] (μg/g)	Se species (%)[b]					Notes	Literature
			SeMet	$SeCys_2$	SeMeSeCys	SeO_4^{2-}	SeO_3^{2-}		
Chicory (*Cichorium intybus* L.)	Na_2SeO_4 (7 mg/L) 5 days	88	4	<DL	<DL	72	0.2		This study
	Na_2SeO_4 (7 mg/L) 10 days	131	4	<DL	<DL	73	0.3		
	Na_2SeO_4 (7 mg/L) 41 days	480	8	<DL	0.7	63	0.2		
Lupine (*Lupinus albus*)	Na_2SeO_4 (1 mg/L) 14 days	631	–	–	–	95	–		[8]
	Na_2SeO_3 (1 mg/L) 14 days	50	–	20	–	–	3		
Sunflower (*Helianthus annuus*)	Na_2SeO_4 (1 mg/L) 14 days	73	20	–	–	35	–		
	Na_2SeO_3 (1 mg/L) 14 days	12	15	–	–	–	3		
Onion (*Allium cepa*)	Na_2SeO_4 (5 mg/L) 8 days	601	0.2	0.1	2	Majority	–		[41]
	Na_2SeO_3 (5 mg/L) 8 days	154	0.3	0.5	4	–	–		
Green onion (*Allium fistulosum*)	Na_2SeO_3 (15 mg/L) 14 days	30	+[c]	+[c]	Much	+[c]	+[c]	Majority γ[d]	[42]
Onion (*Allium cepa*)	Na_2SeO_3 (10 mg/L) 7 days	17[e]	–	–	100	–	–		[43]
Lettuce (*Lactuca sativa*)	Na_2SeO_3 (10 mg/L) 7 days	27[e]	3	–	90	–	7		
Broccoli (*Brassica oleracea*)	Na_2SeO_3 (10 mg/L) 8 days	32[e]	–	–	94	–	2		

(–) Not detected.
[a] μg Se/g dry weight.
[b] % Se species relative to total Se in sample.
[c] Species detected but not quantified.
[d] γ-Glutamyl-SeMeSeCys.
[e] μg Se/g wet weight.

the form of Se^{VI} it was also accumulated in green parts of plant in this form.

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