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Effect of foliar application of selenium on its uptake and speciation in carrot

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ABSTRACT

Carrot (*Daucus carota*) shoots were enriched by selenium using foliar application. Solutions of sodium selenite or sodium selenate at 10 and 100 μg Se ml^{-1}, were sprayed on the carrot leaves and the selenium content and uptake rate of selenium were estimated by ICP–MS analysis. Anion and cation exchange HPLC were tailored to and applied for the separation of selenium species in proteolytic extracts of the biological tissues using detection by ICP–MS or ESI–MS/MS. Foliar application of solutions of selenite or selenate at 100 μg Se ml^{-1} resulted in a selenium concentration of up to 2 μg Se g^{-1} (dry mass) in the carrot root whereas the selenium concentration in the controls was below the limit of detection at 0.045 μg Se g^{-1} (dry mass). Selenate-enriched carrot leaves accumulated as much as 80 μg Se g^{-1} (dry mass), while the selenite-enriched leaves contained approximately 50 μg Se g^{-1} (dry mass). The speciation analyses showed that inorganic selenium was present in both roots and leaves. The predominant metabolised organic forms of selenium in the roots were selenomethionine and γ-glutamyl-selenomethyl-selenocysteine, regardless of which of the inorganic species were used for foliar application. Only selenomethionine was detected in the carrot leaves. The identity of selenomethionine contained in carrot roots and leaves was successfully confirmed by HPLC–ESI–MS/MS.

1. Introduction

Selenium (Se) is a trace element required in small amounts by humans and animals for the normal function of a number of selenium-dependent antioxidative enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR). This element, however, can also be toxic in larger doses (Rotruck, Ganther, Swanson, Hafeman, & Hoekstra, 1973). Both the beneficial and the possible toxic effects of selenium depend on the amount ingested and on its chemical forms (Fairweather-Tait, 1997; Vadhanavikit, Ip, & Ganther, 1993). The selenium content of plant based foods varies significantly between different regions of the world, depending on plant species and on the selenium content of the soil. According to a recent Danish survey, the estimated 5th percentile and mean dietary intake of Se were 23 and 43 μg day^{-1}, respectively (Larsen, Rokkjaer, & Chistensen, 2007). The selenium intake from food of plant origin amounted to a modest 1/5 of this intake. In comparison, the recommended dietary intake of Se is 40 and 50 μg day^{-1} for women and men, respectively (Alexander et al., 2005). While the dietary selenium intake is sufficient to saturate plasma GPx, it is too low to provide any possible cancer-preventive effect of Se, which may occur when supplementing selenium as selenised yeast at 200 μg day^{-1} (Clark et al., 1996).

Northern European countries are among low-selenium regions, and this is particularly the case for Scandinavian countries. In Finland the low Se intake in the population has been compensated for by using commercial fertilizers supplemented with sodium selenate (Se(VI)) since 1984 (Aro, Ekholm, Alfthan, & Varo, 1998), but agronomic fortification of crop plants *via* foliar application of selenium is rarely used.

In contrast to humans and animals, selenium is not considered essential to plants. The threshold toxic concentration of Se for plants depends, among other factors, on the vegetal species and on the form of Se supplied. In general, Se(VI) and selenite (Se(IV)) are readily transported through the plant cuticle and assimilated by the metabolic pathway for sulphur (Terry, Zayed, Desouza, & Tarun, 2000). However, Se(IV) is capable of being transformed to seleno-amino acids *via* this pathway more readily than Se(VI). The substitution of methionine and cysteine amino acids by their Se analogues, however, carries the risk of modification of the protein structure, which might affect plant functions (Anderson & Scarf, 1983). Therefore, biosynthesis of a di-peptide such as γ-glutamyl-selenomethyl-selenocysteine (γ-glu-MeSeCys) or a non-protein seleno-amino acid selenomethyl-selenocysteine (MeSeCys) in plants, has been considered as a tolerance mechanism against selenium toxicity (Terry et al., 2000). In humans, these species and also selenomethionine (SeMet) have been reported to have beneficial

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nutritional effects as well as possessing anti-carcinogenic effects at elevated intake levels (Combs & Gray, 1998).

Effective enrichment of agricultural crops with selenium *via* soil using selenium-enriched fertilizers can be challenging due to varying soil Se concentrations, soil types, soil redox potentials, soil pH, microbiological activity etc. (Hartfiel & Bahners, 1988). Furthermore, the high cost of Se fertiliser in combination with a modest incorporation rate should be considered (Makela et al., 1995). As an alternative, foliar application of Se has been used for enrichment of agricultural products (Smrkolj, Stibilj, Kreft, & Germ, 2006). With this method a Se-containing solution is applied to the surface of the plant leaves by spraying. The advantage of foliar application compared with soil fertilisation with Se is that the impact of soil chemistry and microbiology on the fate of selenium is by-passed, ensuring a higher efficacy even with low volumes of foliar applied Se solution. Furthermore, the technique paves the road towards enrichment of plants by costly stable isotopes, which are useful tools in plant physiological research.

Foliar application of Se(IV) or Se(VI) has been successfully applied to increase the Se content in many crops including potato (Poggi, Arcioni, Filippini, & Pifferi, 2000), rice (Hu, Chen, Xu, Zhang, & Pan, 2002), soybean (Yang, Chen, Hu, & Pan, 2003), cabbage, onion, garlic and radish (Slejkovec & Goessler, 2005), buckwheat and pumpkin (Smrkolj, Stibilj, Kreft, & Kápolna, 2005; Smrkolj et al., 2006). However, only few studies of the Se speciation following foliar Se application are available (Smrkolj et al., 2005; Smrkolj et al., 2006; Slejkovec & Goessler, 2005). The speciation information however, is necessary to evaluate the bioavailability of Se and its biological effects in humans consuming the agricultural products.

The aim of this work was to study the accumulation and speciation of selenium in carrots using foliar application of sodium selenite and sodium selenate and to test this at two different concentration levels. Carrot is a commonly consumed vegetable, which contributes to the dietary Se intake. To the best of our knowledge however, no data has previously been published regarding the identity and distribution of Se species in carrots after enrichment.

2. Experimental

2.1. Standards and reagents

All reagents were of analytical reagent grade. Nitric acid was sub-boil distilled in an all-quartz apparatus (Hans Kürner, Rosenheim, Germany) and ultrapure Milli-Q-water was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA). HPLC-grade methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland) and hydrogen peroxide (30%) from Romil Ltd. (Waterbeach, Cambridge, UK) were used. Individual stock solutions of MeSeCys, SeMet as well as Se(IV) and Se(VI) sodium salts were obtained from a commercial source (Sigma–Aldrich, Copenhagen, Denmark). Stock solutions of 100 μg Se ml^{-1} were obtained by dissolving the appropriate amount of the corresponding compound in Milli-Q water. γ-glu-MeSeCys was purchased from a commercial source (PharmaSe, Inc., Texas, USA). Selenohomocystine ($SeHoCys_2$) was kindly donated by Dr. Walter Gössler (University of Graz, Austria), while trimethylselenonium-ion ($TMSe^+$) was synthesised according to a literature method (Palmer, Fischer, Halvorson, & Olson, 1969). Se-allylselenocysteine (AllSeCys) and Se-propylselenocysteine (PrSeCys) were kindly donated by Dr. Howard Ganther (University of Wisconsin, WI, USA). Selenomethionine Se-oxide (SeOMet) was prepared in-house by oxidising 2 ml 10 ppm SeMet with 100 μl H_2O_2 (30%) for 24 h at room temperature.

For the chromatographic separations and for the sample preparation procedure, formic acid, pyridine, potassium dihydrogen phosphate, hydrochloric acid and a 25% ammonia solution were purchased from Merck (Darmstadt, Germany), while Protease XIV (~4 U mg^{-1} solid) was obtained from Sigma–Aldrich. Salicylic acid was purchased from Fluka Chemie GmbH (Deisenhofen, Germany). Two reference materials SRM-1567a (Wheat Flour) certified at 1.1 ± 0.2 μg Se g^{-1} and CRM-BCR-402 (White Clover) certified at 6.7 ± 0.3 μg Se g^{-1} were run in parallel with the samples for accuracy control purposes.

2.2. Plant cultivation and foliar application of Se

Carrot seeds (CV Mokum F1) were pre-germinated for 7 days in moist vermiculite prior to planting in a 3:1 mixture of soil (Pindstrup type 2, Denmark) and washed quartz sand (1.2–2.0 mm grain size). All plants were grown in a growth chamber at 20 ± 2 °C; 60% relative humidity and 250 μmol photons m^{-2} s^{-1} in a 16/8 h light/dark cycle. During the first 10 weeks of the growth period, plants were watered every third day with tap water, but during the last 8 weeks of the growth period, 5 ml fertilizer solution containing 2.9% nitrate, 1.9% ammonium, 0.8% phosphorous and 3.7% potassium was added per litre irrigation water. After 13 weeks of growth, plants were sprayed once per week during a four-week period, with Se solutions containing 5 μl surfactant per litre water (TWEEN 20, Sigma–Aldrich, Copenhagen, Denmark). Plants were divided into four groups containing 15 carrots each and were sprayed with solutions of Se(IV) or Se(VI) at two concentration levels each. Since there is no consensus in the literature regarding the concentrations to be used for Se enrichment *via* foliar application, a low and a high concentration were chosen, namely 10 μg Se ml^{-1} and 100 μg Se ml^{-1}. During each treatment 8 ml of Se solution (pH 7.0–7.5) was sprayed onto each plant group carefully observing that the solutions were evenly distributed on all leaves. In addition to the Se treated plants, control plants were grown under the same conditions, but were physically separated by plastic foil to avoid cross-contamination during spraying. The carrots were harvested one week after the last selenium enrichment.

2.3. Harvesting and sample handling

After harvesting, soil and adhered dust were removed from the plant samples (roots and leaves separately) first by soaking in deionised water, then in Milli-Q water for 30 min and then by rinsing with Milli-Q water. The plant parts were minced and freeze-dried separately (Christ Freeze Dryers, Beta 1–8, Montreal Biotech Inc., Dorval, Canada). The moisture content in leaves and roots was between 78% and 80% in all plants. Finally, the dry samples were homogenised using a commercial coffee-grinder (Braun Aromatic KSM 2, Germany).

2.4. Sample preparation for total selenium

For total selenium determination, complete digestion of the plant samples was performed with a microwave digestion system (Multiwave, PerkinElmer/Anton Paar, Graz, Austria). Approximately 0.25 g dry sample was digested using 2 ml HNO_3. The digestion proceeded as follows: in the first step the power was ramped during 5 min from 100 W to 600 W and held for 5 min. In the second step the power was increased up to 1000 W and held for 10 min. The temperature reached a maximum of 220 °C and the pressure 75 bar followed by a cool down cycle for 15 min. After digestion, the residue was diluted to 20 ml with Milli-Q water. The method of standard additions was used for the quantification of Se in the samples. All analyses were performed in triplicate. The limit of detection (LOD), based on 3 standard deviations of the blanks, was 45 ng Se g^{-1} (dry mass) for ^{80}Se.

2.5. Extraction of selenium species for chromatographic speciation studies

Proteolytic sample preparation was applied to extract the protein/peptide-bound Se and at the same time the non-protein bound species from the plant sample (Kápolna, Gergely, Dernovics, Illés, & Fodor, 2007). In all cases, about 0.5 ml of each of the proteolytic extracts was passed through a 12 kDa molecular weight cut-off filter (Whatman VectaSpin, England) using a centrifuge system for 60 min at 13400 rpm (Eppendorf Centrifuge Minispin Model 5452, Germany). The filtrates were stored at −18 °C until speciation analysis.

2.6. Instrumentation and data analysis

An Agilent 7500ce ICP–MS (Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction cell was applied for total selenium determination. Cell conditions were optimised for reduction of argon-based polyatomic interferences using H_2 as cell gas, which was varied at a flow rate between 0–5 ml min^{-1}. A commercial nebuliser (Micromist, Glass Expansion, West Melbourne, Australia) was used for the sample introduction under standard plasma conditions. A solution of yttrium at 5 ng Y ml^{-1} was continuously introduced as an internal standard. For chromatographic separations an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary HPLC pump, an auto sampler, a vacuum degasser system, and a temperature column compartment was utilised. The coupling between the HPLC column outlet and the sample introduction system of ICP–MS was achieved through a 500 mm length of 0.125 mm (i.d.) PEEK tubing. The instrumental operating conditions are given in Table 1. The HPLC separation systems included a strong cation exchange column (Ionospher 5C, Varian BV, Middelburg, The Netherlands) (100 mm × 3.0 mm × 5 μm) and a strong anion exchange column (ION-120, Transgenomic, UK) (120 mm × 4.6 mm × 5 μm) equipped with a guard column containing the same stationary phase material. The mobile phases and other chromatographic conditions are detailed in Table 1. Data were collected and evaluated using the Agilent Chemstation ICP–MS chromatographic software. The areas of the analytical peaks were calibrated against an external standard curve or by using the method of standard additions.

The verification of the identity of the selenium species was performed using HPLC–ESI–MS/MS (Quattro Micro, Waters, Milford, MA) in the positive ion mode. Nitrogen was used for nebulisation and as auxiliary gas, whereas argon was used as collision gas for molecular fragmentation. The experiment was divided into two separate time windows corresponding to detection of species specific transitions for achievement of optimum sensitivity. The multiple reaction monitoring (MRM) transitions used were m/z 312.7 → (166.7 and 129.7), and m/z 197.7 → (180.7 and 151.7) for detection and identification of γ-Glu-MeSeCys and SeMet, respectively. Separation was achieved using the cation exchange HPLC column protected with a SecurityGuard (4.0 × 2.0 mm) (Phenomenex, Torrance, CA) in combination with an Agilent 1100 liquid chromatograph (Agilent, Santa Clara, CA). The chromatographic conditions and other instrumental settings are detailed in Table 1. For presentation purposes chromatographic data were processed with Origin 7.0 (Microcal Software Inc., Northampton, MA, USA) and Microsoft® Excel 2000 (Microsoft Corporation, Redmont, Washington, USA) was applied for statistical evaluations.

Based on repeated analyses (n = 3), the uncertainty of the mean values was defined as the half-width of the 95% confidence interval. Two sample-t tests or a one-sided analysis of variance were applied to determine if there was any significant difference between means. All concentration values presented in this paper are expressed on the basis of dry mass (d.m.) of the sample.

Table 1
Instrumental operating conditions for HPLC–ICP–MS and for HPLC–ESI–MS/MS.

ICP–MS settings	
Forward power	1550 W
Plasma gas flow rate	14.5 l min^{-1}
Carrier gas flow rate	0.93 l min^{-1}
Integration time	0.1 s per isotope
Isotopes monitored	^{78}Se, ^{80}Se
Collision gas flow rate	3.5 ml min^{-1}
SCX–HPLC	
Column	Chrompack IonoSpher 5C (100 mm × 3.0 mm × 5 μm)
Mobile phases	(A): 0.75 mM pyridinium formate, 3% (v/v) MeOH, pH 3.0 (B): 10 mM pyridinium formate, 3% (v/v) MeOH, pH 3.0 0–3.5 min: 100% A 3.6–5 min: 95% A – 5% B 5.1–7 min: 80% A – 20% B 7.1–16 min: 100% A
Flow rate	1.0 ml min^{-1}
Injection volume	20 μl
Column heating	30 °C
SAX–HPLC	
Column	ION-120, Transgenomic (120 mm × 4.6 mm × 5 μm)
Mobile phases	(A): 0.1 mM salicylate, 3% (v/v) MeOH, pH 8.5 (B): 20 mM salicylate, 3% (v/v) MeOH, pH 8.5 0–4 min: 98% A – 2% B 4.1–12 min: 50% A – 50% B 12.1–16 min: 98% A – 2% B
Flow rate	1.0 ml min^{-1}
Injection volume	20 μl
ESI–MS/MS	
Auxiliary gas	N_2, 475 l min^{-1}
Nebulizer gas	N_2, 50 l min^{-1}
Collision gas	Argon, 8 psi
Needle voltage	3150 V
Cone temperature	120 °C
Nebulizer temperature	400 °C
Cone voltage	12 V
Collision energy	15 V
SCX–HPLC parameters for ESI–MS/MS detection	
Column	Chrompack IonoSpher 5C (150 × 2.0 mm; 5 μm)
Mobile phases	(A): 1.5 mM ammonium formate 5% (v/v) MeOH, pH 3.0 (B): 25 mM ammonium formate 5% (v/v) MeOH, pH 3.3 0.0–20.0 min: 100% A 20.0–25.0 min: 100% A – 100% B 25.0–30.0 min: 100% B 30.0–32.5 min: 100% B – 100% A 32.5–45.0 min: 100% A
Flow rate	0.25 ml min^{-1}
Column temperature	20 °C
Injection volume	25 μl

3. Results and discussion

3.1. Uptake of Se using foliar application

When plants are exposed to high concentrations of Se in their root medium (pot experiment or hydroponic cultivation) or exposed to excessive Se concentrations in foliar sprays, they may show symptoms of damage like stunting of growth, chlorosis,

withering and necrosis of leaves (Trelease & Beath, 1949). Such phenomena, like chlorotic and necrotic leaves were observed in this study when Se(VI) was applied on the leaves at 100 μg Se ml^{-1}, whereas no symptoms developed when the leaves were sprayed with the 10 μg Se ml^{-1} concentration. On the other hand, Se(IV) did not cause any visible damage to the leaves neither at 10 nor at the 100 μg Se ml^{-1} concentrations. This indicated that the upper practical selenium concentration for Se(VI) had been reached at 100 μg Se ml^{-1}.

The values obtained for CRM 402 White Clover at 6.8 ± 0.2 μg Se g^{-1} (n = 3) and for NIST SRM-1567a at 1.1 ± 0.1 μg Se g^{-1} (n = 3) did not differ from the certified values, which demonstrated good analytical accuracy. The results presented in Table 2 show that in comparison with the control samples the Se concentrations in the carrot leaves and roots were significantly increased for both species and concentrations of inorganic Se used for the foliar spraying. Furthermore, the results showed that the carrot leaves contained higher concentrations of Se in comparison with the corresponding roots, and that spraying with Se(VI) caused a higher Se content in leaves and roots than when spraying with Se(IV). In the present study, the Se concentration in the leaves reached 80 μg g^{-1} and 49 μg g^{-1} when Se(VI) and Se(IV), respectively, were used for foliar selenium application. This relatively high amount of Se, however, was not transported to the root as only 1.5 μg Se g^{-1} was detected in the roots of the plants sprayed with Se(IV), while those treated with Se(VI) contained a significantly higher concentration at 2.2 μg Se g^{-1} in their root tissue. According to a recent Danish food monitoring survey unenriched market carrots had an average Se content of 0.5 μg kg^{-1} (d.m.) (Larsen et al., 2007). In comparison with this value the experimental results from the present work demonstrates that the Se concentration in carrots can be increased using foliar application of selenium.

The Se uptake rates by carrots *via* foliar application were estimated by multiplying the total harvested mass of the carrot shoots and roots with their total Se contents, respectively, and these two values were summed up. We found that the Se uptake rate amounted to 39% or 41% when spraying with the 10 μg Se ml^{-1} solution as Se(IV) or Se(VI), respectively. In the case of the 100 μg Se ml^{-1} spraying solutions, the incorporation rate was 29% or 48% for Se(IV) or Se(VI), respectively. These relatively low values could be ascribed both to the possible phytovolatilisation of the absorbed Se through the leaves (Meija, Montes-Bayón, Le Duc, Terry, & Caruso, 2002), and also to the shape of the leaves.

Other researchers have used foliar application of selenium to increase the dietary Se intake from vegetables. Cabbage, radish, onion and garlic were enriched by spraying with Se(VI) at 10 and 20 mg Se m^{-2} twice during the growth period (Slejkovec & Goessler, 2005). The highest Se concentrations were detected in onion and radish leaves (37.4 and 37.1 μg g^{-1} d.m.) followed by the leaves of garlic (19.6 μg g^{-1} d.m.) and cabbage (11.9 μg g^{-1} d.m.). In the edible parts of these vegetables, the highest selenium accumulation was obtained in cabbage (12 μg g^{-1} d.m.), followed by radish (8.2 μg g^{-1} d.m.), garlic (6.6 μg g^{-1} d.m.) and onion (5.6 μg g^{-1} d.m.). On one hand these results demonstrate that Se was effectively taken up by the crops, but are on the other hand not immediately comparable with our experimental results for carrots. Factors such as amount of applied Se, leaf area and differences in plant-specific metabolism of selenium differ. Another research group treated pumpkin plants by selenium *via* foliar application of Se(VI) at 1.5 μg Se ml^{-1} (Smrkolj et al., 2005). The resulting selenium content of the seeds was on average 0.19 μg g^{-1} in controls and 1.1 μg g^{-1} (d.m.) in the exposed pumpkins. Using the same enrichment technique, buckwheat was treated with a solution containing 15 μg Se ml^{-1} as Se(VI) (Smrkolj et al., 2006). In the control plants the selenium content in all parts of the plant was less than 0.2 μg g^{-1} and in the selenium-treated buckwheat the selenium concentration was 2.7–4.7 μg g^{-1} (d.m.). Thus, data for selenium contained in buckwheat and pumpkins following foliar application of Se are comparable in magnitude with the results obtained for carrots in the present study. Foliar application of selenium is however, an even more effective way of enriching plants from the *Alliaceae* and *Cruciferaceae* families due to their genetic predisposition for accumulating this element.

Table 2
Concentration of selenium (mean ± SD, n = 3) in carrot leaves and roots enriched by foliar application of Se(IV) or Se(VI). Concentration values with different superscripts are significantly different (P < 0.05). ^{80}Se was used for ICP–MS detection.

Treatment	Se species applied	Plant parts	Total Se concentration (μg g^{-1})
10 μg Se ml^{-1}	Control	Leaf	0.1 ± 0.01
		Root	<0.045[A]
	Selenate	Leaf	5.7 ± 0.3[c]
		Root	0.5 ± 0.1[f]
	Selenite	Leaf	5.1 ± 0.2[c]
		Root	0.4 ± 0.1[f]
100 μg Se ml^{-1}	Control	Leaf	<0.045[A]
		Root	<0.045[A]
	Selenate	Leaf	80 ± 1.8[a]
		Root	2.2 ± 0.1[d]
	Selenite	Leaf	49 ± 1.0[b]
		Root	1.5 ± 0.1[e]

[A] Below the LOD.

3.2. Selenium speciation using anion and cation exchange HPLC separations

3.2.1. Tailoring chromatographic separation methods for selenium species in carrot

Speciation of Se was carried out to obtain information on the occurrence of Se metabolites and their distribution in the plants following foliar application of the inorganic selenium species. An anion exchange HPLC separation method was developed and tailored to separate seven selenium compounds of interest in less than 12 min (Fig. 1). Cation exchange HPLC separation also with ICP–MS detection (Fig. 2) was used as an alternative chromatographic system with the purpose of confirmation of the identity of selenium species assigned on the basis of anion exchange HPLC

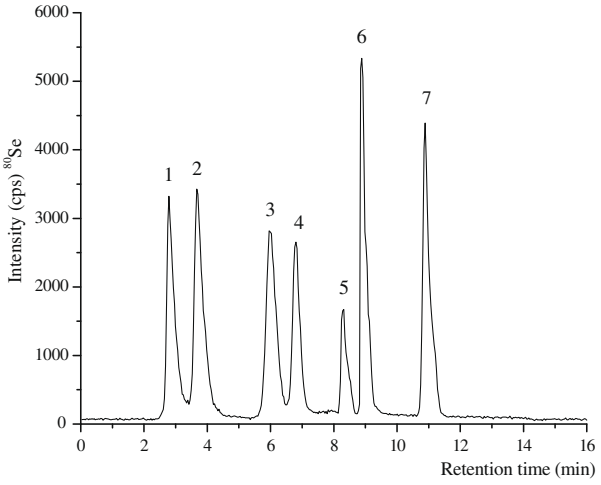

Fig. 1. Anion exchange HPLC separation with ICP–MS detection of a mixture of selenium standards at 50 ng Se ml^{-1} each, except $SeHoCys_2$ at 100 ng Se ml^{-1}. Peak: 1 = SeMet; 2 = MeSeCys; 3 = AllSeCys; 4 = $SeHoCys_2$; 5 = γ-glu-MeSeCys; 6 = Se(IV); 7 = Se(VI).

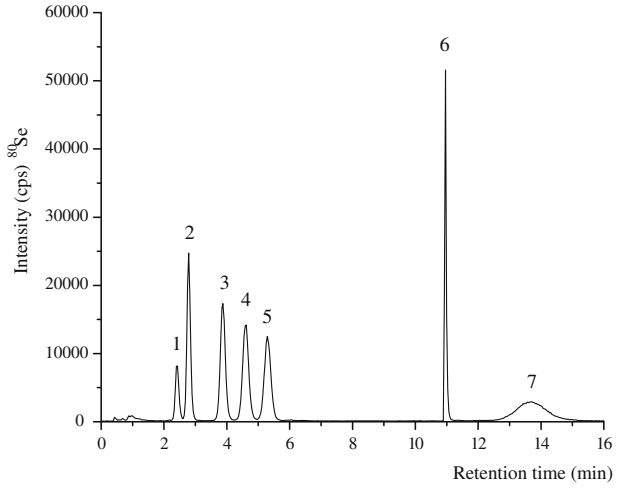

Fig. 2. Cation exchange HPLC separation with ICP–MS detection of a mixture of selenium standards at 50 ng Se ml^{-1} each. Peak identities: 1 = γ-glu-MeSeCys; 2 = MeSeCys; 3 = AllSeCys; 4 = PrSeCys; 5 = SeMet; 6 = SeOMet; 7 = TMSe$^+$.

(Larsen, Sloth, Hansen, & Moesgaard, 2003). The chromatographic conditions and settings used are detailed in Table 1.

3.2.2. Speciation analysis of plant samples by HPLC coupled with mass spectrometric detectors

The results of the speciation analyses showed that the Se species extracted from the control samples remained undetectable as they did not exceed the baseline noise (3σ) of the HPLC–ICP–MS chromatograms. Using this definition, the LOD for SeMet in the anion exchange HPLC system was estimated at 72 ng Se g^{-1} (d.m.) when selenium was detected as ^{80}Se. In contrast, the selenium species contained in the enriched carrots were detectable, but the retention time of some species was affected by the sample matrix. To counteract this undesirable effect, the samples were further diluted and co-elution with spiked authentic standards assured correct assignment of identity and quantification. The extraction efficiency, which was estimated from the Se content of the sample and that in the residue after the proteolytic digestion, was 75 ± 2% and 78 ± 3% (n = 3) for carrot leaves and roots, respectively. The chromatogram in Fig. 3 shows the anion exchange HPLC

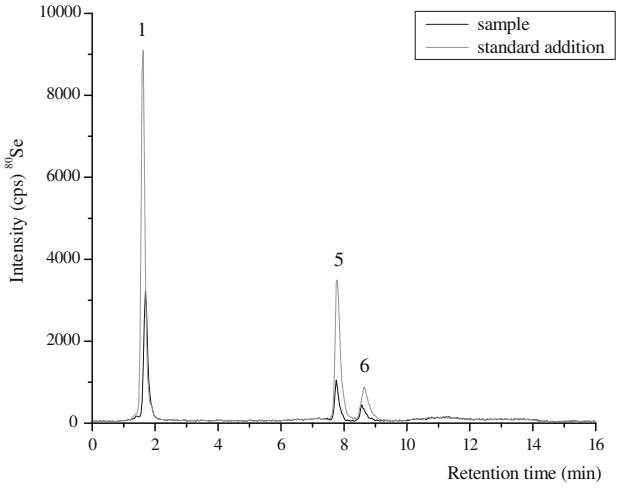

Fig. 3. Anion exchange HPLC separation with ICP–MS detection of an extract of carrot root following foliar application of selenium at 100 μg Se ml^{-1} as Se(IV). Peak: 1 = SeMet; 5 = γ-glu-MeSeCys; 6 = Se(IV). The black line corresponds to the sample and the gray line to the sample spiked with a mixture of authentic standards. Numbering of the peaks is according to the separation of standard mixtures on SAX.

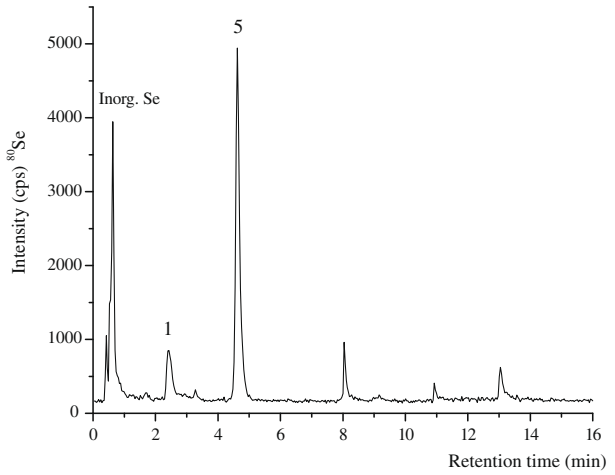

Fig. 4. Cation exchange HPLC separation with ICP–MS detection of an extract of carrot root enriched with selenium at 100 μg Se ml^{-1} as Se(IV). Peak: 1 = γ-glu-MeSeCys; 5 = SeMet. Three minor peaks eluting at retention times 8–13 min remain unidentified. Numbering of the peaks is according to the separation of standard mixtures on SCX.

separation of the Se species present in the proteolytic digest of carrot root as a result of the foliar application of 100 μg Se ml^{-1} as Se(IV). The identity of the three peaks was substantiated by the cation exchange HPLC analysis of the same sample extract as presented in Fig. 4. The two major organoselenium species found in the carrot root samples were SeMet and γ-glu-MeSeCys. Unidentified Se-containing peaks also appeared as shown in Fig. 4, but their low concentrations hampered their identification by alternative analytical techniques. The plant extract was also spiked with TMSe$^+$ but no co-elution was observed with the unknown peak detected at 13 min. In our previous study the same chromatographic system (Larsen et al., 2003) was applied for the baseline separation of TMSe$^+$ and Se-methyl selenomethionine (MeSeMet). This latter compound eluted right after TMSe$^+$. Even though in the present chromatographic separation method the highest buffer concentration exceeded that of the earlier published one, we could not exclude the presence of this species.

The quantitative data from the analyses of the carrot root samples enriched by spraying with solutions of Se(IV) or Se(VI) at 100 μg Se ml^{-1} are depicted in Fig. 5. The results show that both

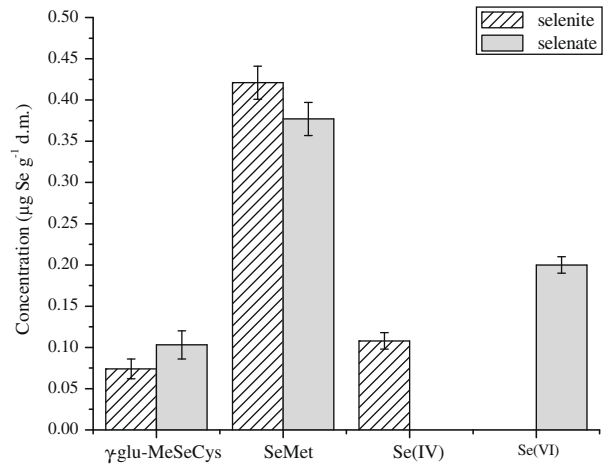

Fig. 5. Quantitative results for Se species identified in the carrot roots following foliar application of selenium at 100 μg Se ml^{-1}as Se(IV) or Se(VI). The columns and error bars represent mean ± 95% confidence interval.

inorganic Se species used for foliar spraying were metabolised by the plant to form the same two major organic Se species at the same concentrations. Even though the concentrations of SeMet and γ-glu-MeSeCys were rather low (0.4 μg Se g^{-1} and 0.1 μg Se g^{-1} d.m., respectively), it should be noted that these bioactive Se species are beneficial to human health (Kotrebai, Birringer, Tyson, Block, & Uden, 2000; Whanger, Ip, Polan, Uden, & Welbaum, 2000). In contrast, a fraction of only that inorganic selenium species, which was used for foliar spraying, remained detectable in the harvested carrot root. The remaining amount of Se(IV) and Se(VI) corresponded to 17% and 32%, respectively, of the total amount of identified species.

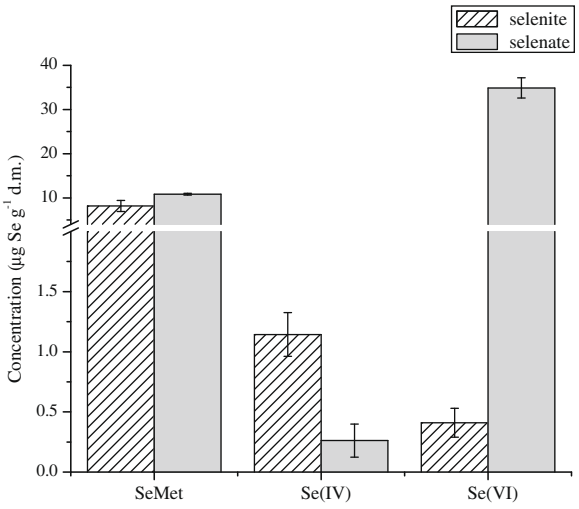

Fig. 6. Quantitative results for Se species identified in carrot leaves following foliar application of selenium as at 100 μg Se ml^{-1} Se(IV) or Se(VI). The columns and error bars represent mean ± 95% confidence interval.

The distribution of Se species in carrot leaves (Fig. 6) exhibited a different pattern compared with that in the root. Although γ-glu-MeSeCys was not detected in the leaves, SeMet was present at a significantly higher concentration (up to 10 μg Se g^{-1} d.m.) compared with that in the root (up to 0.1 μg Se g^{-1} d.m.). Furthermore, foliar application of Se(VI) caused a significant accumulation of Se(VI) in the leaves compared with that for Se(IV) when this species was applied for enrichment. Redox conversions between these two species in the leaves also seem to occur in carrot leaves. The selenium species found in carrot leaves and roots when applying the 10 μg Se ml^{-1} treatments (data not shown) largely showed the same relative distribution of Se species, only at lower concentrations.

In order to prove the assigned identity of the selenium species analysed by the two HPLC–ICP–MS systems, an attempt was made to analyse γ-glu-MeSeCys and SeMet by HPLC–ESI–MS/MS. Detections were performed in MRM mode using two pairs of parent–daughter ion transitions. Furthermore, a criterion that the relative mass spectral areas of the two transitions should match within ± 10% compared to an authentic standard was applied for proof of identity. The cation exchange chromatogram of an enzymatic digest of a carrot root shown in Fig. 7 confirms the presence of SeMet in carrot root. Even though two transitions were monitored for optimum sensitivity, the concentration of γ-glu-MeSeCys was too low for detection by HPLC–ESI–MS/MS.

3.2.3. Considerations on metabolism of selenium in carrot

Our results demonstrated that SeMet was the single most abundant organic selenium species present in carrot and that Se(VI) was the predominant form of selenium in carrot leaves when this species was used for foliar application. The concentration of SeMet amounted to 57% and 20% of all quantified selenium species in

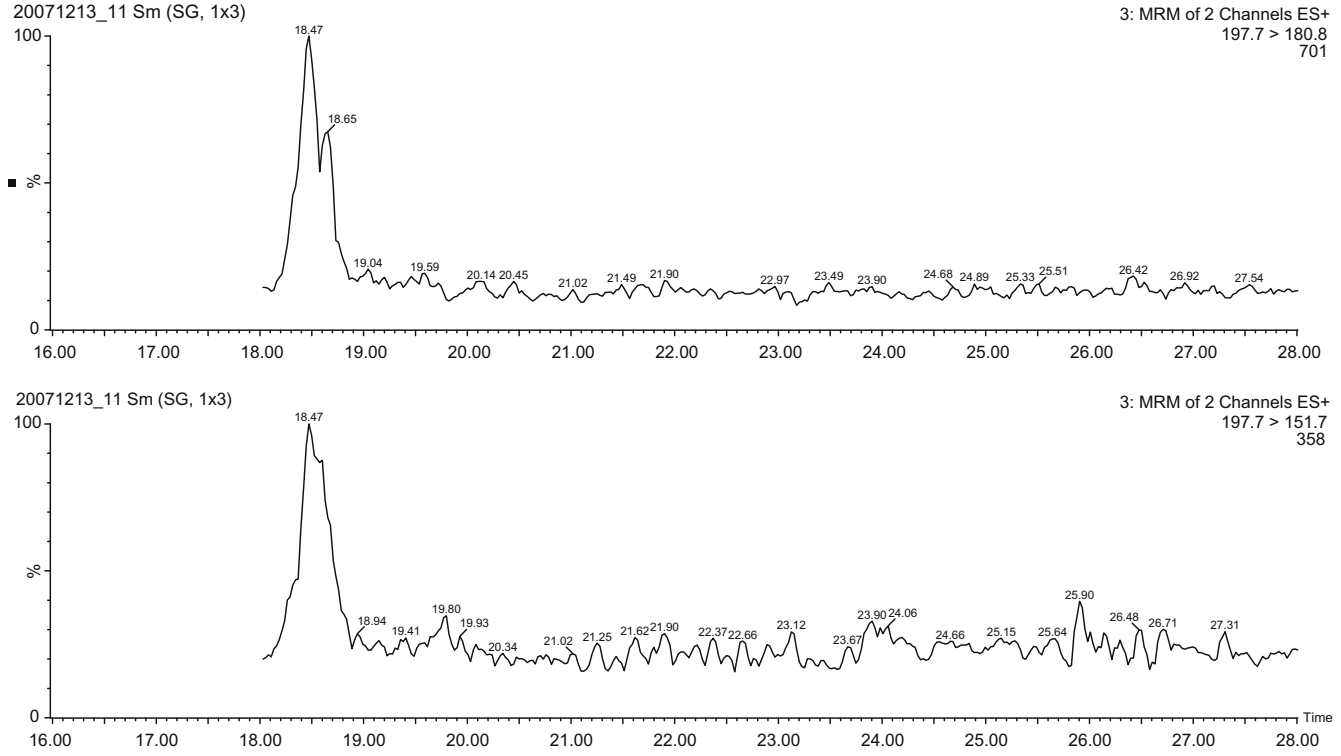

Fig. 7. Cation exchange HPLC–ESI–MS/MS chromatograms of an extract of a carrot root sample. The retention time of 18.5 min and the parent to daughter ion transitions, *m*/*z* 197.7 to 151.7 and to 180.7 correspond to SeMet.

the roots and in the leaves, respectively. This finding was in accordance with a similar study of pumpkin seeds in which SeMet was the predominant selenium species when Se(VI) was used for foliar enrichment (Smrkolj et al., 2005). Our results further demonstrated that foliar applied Se(IV) is more efficiently converted to organoselenium species than is the case for Se(VI). This difference in conversion rate may be caused by the energy-demanding first reduction step of Se(VI) to Se(IV) in the metabolic pathway of selenium in plants (Burnell, 1981; Leustek, Murillo, & Cervantes, 1994). Once Se(VI) is reduced to Se(IV), this species can be converted downstream to selenide, a part of which is metabolised to selenocysteine by cysteine synthase. Further conversions catalysed by Se-methyltransferase or by trans-sulphurylase, lead to formation of MeSeCys or SeMet, respectively (Terry et al., 2000). Our results demonstrated that carrot is able to biosynthesise γ-glu-MeSeCys and SeMet in modest amounts, and also that the findings of Se(IV) and Se(VI) in carrot are in accordance with these first steps of the biosynthetic route. In the present study however, some of the carrot leaves suffered from necrosis in response to the Se(VI) treatment at 100 μg Se ml^{-1}. Hereby the ability of the leaves' chloroplasts to reduce Se(VI) (Pilon-Smits et al., 1999) could have been impaired resulting in a possible additional accumulation of this species in the carrot leaves.

4. Conclusions

Foliar application of Se is an efficient way to enrich carrots (*Daucus carota*) by this element. The results showed that the foliar application of selenium as solutions of Se(IV) or Se(VI) was predominantly recovered in the plants' leaves and to a lesser extent in the roots. An upper tolerable concentration of sprayed Se was reached as enrichment with Se(VI) at 100 μg Se ml^{-1} caused visible damage to the plant leaves (necrotic spots). Analysis by anion exchange HPLC and by cation exchange HPLC with ICP–MS detection of proteolytic extracts of the plant's tissues demonstrated that SeMet and γ-glu-MeSeCys were the main organic Se species in the carrot roots. In contrast, in the leaves only SeMet prevailed no matter which inorganic Se species were used for the enrichment. The finding of SeMet was confirmed by HPLC–ESI–MS/MS, but the concentration of γ-glu-MeSeCys was too low for verification by this technique. The high degree of metabolic conversion of Se(IV) to organic selenium species combined with the plant's larger tolerance to this species makes sodium selenite the species of choice for foliar application of Se. Additional investigations however, are needed to find the optimum Se(IV) concentration, which is tolerable to the plant, while at the same time fully metabolised to the chemically stable and bioactive organic selenium species.

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