Anal Bioanal Chem (2006) 385: 1098–1108
DOI 10.1007/s00216-006-0535-x

ORIGINAL PAPER

**Erik H. Larsen · Ryszard Lobinski ·
Karin Burger-Meÿer · Marianne Hansen ·
Rafal Ruzik · Lena Mazurowska ·
Peter Have Rasmussen · Jens J. Sloth · Olga Scholten ·
Chris Kik**

Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate

Received: 21 March 2006 / Accepted: 5 May 2006 / Published online: 13 June 2006
© Springer-Verlag 2006

Abstract The scope of the work was to investigate the influence of selenate fertilisation and the addition of symbiotic fungi (mycorrhiza) to soil on selenium and selenium species concentrations in garlic. The selenium species were extracted from garlic cultivated in experimental plots by proteolytic enzymes, which ensured liberation of selenium species contained in peptides or proteins. Separate extractions using an aqueous solution of enzyme-deactivating hydroxylamine hydrochloride counteracted the possible degradation of labile selenium species by enzymes (such as alliinase) that occur naturally in garlic. The selenium content in garlic, which was analysed by ICP–MS, showed that addition of mycorrhiza to the natural soil increased the selenium uptake by garlic tenfold to 15 μg g^{-1} (dry mass). Fertilisation with selenate and addition of mycorrhiza strongly increased the selenium content in garlic to around one part per thousand. The parallel analysis of the sample extracts by cation exchange and reversed-phase HPLC with ICP–MS detection showed that γ-glutamyl-Se-methyl-selenocysteine amounted to 2/3, whereas methylselenocysteine, selenomethionine and selenate each amounted to a few percent of the total chromatographed selenium in all garlic samples. Se-allyl-selenocysteine and Se-propyl-selenocysteine, which are selenium analogues of biologically active sulfur-containing amino acids known to occur in garlic, were searched for but not detected in any of the extracts. The amendment of soil by mycorrhiza and/or by selenate increased the content of selenium but not the distribution of detected selenium species in garlic. Finally, the use of two-dimensional HPLC (size exclusion followed by reversed-phase) allowed the structural characterisation of γ-glutamyl-Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenomethionine in isolated chromatographic fractions by quadrupole time-of-flight mass spectrometry.

Keywords Selenium speciation · Garlic · HPLC–mass spectrometry · Selenium enrichment · Mycorrhiza

E. H. Larsen (✉) · M. Hansen · P. H. Rasmussen · J. J. Sloth
Department of Food Chemistry,
Danish Institute for Food and Veterinary Research,
2860 Søborg, Denmark
e-mail: ehl@dfvf.dk

R. Lobinski · R. Ruzik · L. Mazurowska
CNRS UMR 5034,
Hélioparc, 2, Av. Angot,
64053 Pau, France

R. Lobinski · R. Ruzik · L. Mazurowska
Department of Analytical Chemistry,
Warsaw University of Technology,
Noakowskiego 3,
00-664 Warsaw, Poland

K. Burger-Meÿer · O. Scholten · C. Kik
Plant Research International (PRI),
Wageningen University and Research Centre,
P.O. Box 16, 6700 AA Wageningen, The Netherlands

C. Kik
Centre for Genetic Resources, The Netherlands (CGN),
Wageningen University and Research Centre,
P.O. Box 16, 6700 AA Wageningen, The Netherlands

Introduction

Selenium is an essential element known to take part in human antioxidative defense and to protect against a number of diseases, including cardiovascular diseases and cancer. The Nutritional Prevention of Cancer (NPC) trial was the first human intervention trial to demonstrate the preventive potential of selenium against certain types of cancer. The study, which used 200 μg day^{-1} of selenium as selenised yeast as the intervention agent, demonstrated a marked decrease in the rate of mortality from cancers and in new cases of prostate, colorectal and lung cancers [1]. In order to substantiate these promising results, a large-scale European study has been planned in a healthy population. The Prevention of Cancer by Intervention by Selenium (PRECISE) Trial will randomise some 33000 volunteers to supplementation with 100, 200, 300 μg selenium day^{-1} as selenised yeast or placebo [2, 3]. The natural dietary intake of selenium in Northern Europe, which is on the order of 30–60 μg day^{-1} [4], is however much lower than that

expected to provide the desired cancer-preventive effect. Consequently, supplementation or enrichment of the diet with selenium is necessary in order to achieve the protective effect. Strategies must therefore be developed for provision of additional selenium intake in order for the general population to benefit from the expected prevention of disease. Such strategies may include a recommendation to the general public to use a nutritional supplement containing selenium, or to fertilise arable soils with selenium and thereby increase the selenium concentrations in vegetable and grain crops, as has been carried out successfully in Finland [5]. The degree of supplementation or enrichment with selenium and—equally important—its distribution among the naturally synthesised molecular species must be investigated in order to provide the necessary knowledge to evaluate their disease-preventive potential.

Bearing in mind the similar chemical properties of selenium and sulfur, existing knowledge about the occurrence of disease-preventive sulfur species in plant-based food is valuable when prioritising the search for and studying their naturally occurring selenium-containing counterparts. Plants from the *Allium* family, such as garlic (*Allium sativum*) and onion (*Allium cepa*) biosynthesise a range of sulfur species, which may become enriched in the plant when applying sulfate fertilisers. The sulfur species contained in garlic include the γ-glutamyl dipeptide of *S*-allylcysteine and its sulfoxide (alliin) as well as *S*-methylcysteine sulfoxide (methiin). Diallyldisulfide and its sulfoxide (allicin) are two of several sulfur species formed following the conversion of alliin by the enzyme alliinase [6, 7]. Sulfur and its species have been shown to reduce the incidence of tumours in animal models when administered via the plant material [8, 9] or as the pure compounds [10]. However, organosulfur compounds proved to have a less protective effect against cancer compared to organoselenium species. Therefore the occurrence and bioactivity of the organoselenium species have received more attention than their organosulfur analogues.

In animal experiments, selenium-enriched garlic possessed a higher tumour-preventive efficacy in comparison with selenite or nonenriched garlic [10]. Furthermore, in comparison with selenium-enriched yeast, feeding mice with selenium-enriched garlic gave a two-fold higher protective effect against mammary cancer provoked by a carcinogen [11]. This observation was associated with the existence of the predominant γ-glutamyl-Se-methyl-selenocysteine (γ-Glu-SeMeCys) in garlic, in contrast to the predominant selenium species in yeast, namely selenomethionine (SeMet). The latter compound is readily incorporated into proteins and causes tissue build-up of selenium. Selenium-enriched broccoli (a *Brassica* plant species) had a greater capacity in comparison with selenate or nonenriched broccoli to inhibit the occurrence of cellular changes that might lead to intestinal cancer tumours [12, 13]. Although selenium contained in broccoli had the least bioavailability, expressed as glutathione peroxidase activity, it possessed the greatest bioactivity. The predominant selenium species contained in broccoli were similar to those found in garlic, namely methylselenocysteine (MeSeCys) or its γ-glutamyl-dipeptide, γ-Glu-SeMeCys [14]. Garlic, however, accumulated more organo-Se than broccoli relative to the sulfur content present in both plant species [15].

Biological results from in vivo studies indicate that the naturally biosynthesised derivatives of cysteine, and in particular those of selenocysteine, possess anticarcinogenic properties without causing much build-up of selenium in the tissues [16, 17]. Among a range of selenocysteine derivatives tested in an in vitro cell culture model, MeSeCys and Se-allyl-selenocysteine (AllSeCys) showed the most promising results with respect to inhibition of cell growth and apoptosis while at the same time conserving the DNA [18]. In contrast, selenium as SeMet contained in selenised yeast [3] and in Brazil nut [19] led to increased blood and tissue selenium levels [3]. Therefore, the methylated selenocysteine derivatives occurring in some plant-based foods appear to be attractive compounds for the prevention of degenerative disease.

Conserving the selenium species originally present in the sample is crucial to obtaining accurate analytical results. Reported conversions of seleno-amino acids include oxidation [20] and degradation by naturally occurring enzymes [21]. Furthermore, the analysis of selenium species [22] for which authentic standards are unavailable restricts the qualitative information that can be gained when using HPLC–ICP–MS. Therefore, Goenante Infante et al. [23] pointed to the importance of employing molecular mass spectrometry for further characterisation of selenium species in selenised plants [24], including SeMet, SeMeCys and their γ-glutamyl derivatives in *Allium* plant species [25–29].

The aims of this investigation were to study the content of selenium and its distribution between molecular species in garlic cultivated in experimental plots. The selenium enrichment was carried out using separate or combined use of fertilisation with selenate, and addition of symbiotic fungi (mycorrhiza) to the cultivation medium. Furthermore, the study focused on clarifying whether any selenium-containing analogues of the biologically active *S*-allyl-cystein as well as *S*-propyl-cystein occurred in selenized garlic.

Experimental

Instrumentation used for selenium analysis and selenium speciation analysis

An ELAN 6100 ICP-DRC-MS instrument (Perkin–Elmer SCIEX, Concord, ON, Canada) was used for selenium determinations in garlic following microwave-assisted digestion by nitric acid, or was used as a selenium-selective detector for the HPLC system used for selenium speciation [30]. The HPLC systems used included a PE Series 200 metal-free quaternary HPLC pump (Perkin–Elmer) and a Waters 717 Plus autosampler (Waters, Milford, MA, USA), used in conjunction with a silica-based strong cation exchange HPLC column 100 mm × 3 mm (i.d.) (Ionosphere-5C, Chrompack International,

Middelburg, The Netherlands). The gradient elution program employed aqueous pyridinium formate as mobile phase. The instrumental settings are provided in detail in Table 1. For the reversed-phase HPLC, an UltiMate HPLC pump and Famos autosampler were used in combination with a PepMap C_{18} column (1 mm (i.d.) by 150 mm) packed with 3 μm stationary phase particles (LC Packings, Amsterdam, The Netherlands). The mobile phases were constantly deaerated by bubbling with a low flow of helium. Anion exchange HPLC, which was used to isolate the inorganic selenium species, employed a PRP X-100 (250 mm × 4.6 mm × 5 μm) strong anion exchange HPLC column (Hamilton, Reno, NV, USA) using gradient elution with aqueous solutions of acetic acid and triethylamine as mobile phase constituents. The chromatographic peak areas were quantified against external standard curves using TotalChrom software (Perkin–Elmer).

Structural confirmation and characterisation of selenium species

The major selenium-containing fraction contained in a proteolytic extract of a commercially available and characterised garlic sample (GarliSelect, Sabinsa Corp., Piscataway, NJ, USA) was isolated by cation exchange HPLC–ICP–MS and collected via a T-piece equipped with narrow-bore PEEK tubing using a 1:5 flow split ratio. The collected fraction was lyophilised and redissolved in 30% MeOH (v/v) and 0.1% (v/v) formic acid in water. The sample was introduced via a syringe pump at 30 μl min^{-1} into an electrospray ionisation (ESI) ion trap (IT) mass spectrometer (LCQ, Thermo-Finnigan, San Jose, CA, USA) for structural confirmation. The instrumental settings used for this and other identification work are listed in Table 1.

For identification analysis using ESI–Q–TOF–MS, 0.2 g of powdered garlic was extracted using 10 mL of water for three hours in an ultrasound bath. This treatment was repeated four times following isolation of the supernatant by centrifugation and using fresh water each time. The combined extracts were submitted to preparative size-exclusion chromatography using a HiLoad 26/60 Superdex 30 Prep column (Pharmacia, Uppsala, Sweden) with 10 mM ammonium acetate solution (pH 9.5) in water as mobile phase at 2 mL min^{-1}. The selenium-containing fractions, which were detected by ICP–MS at *m/z* 82, were lyophilised and redissolved in 100 μL 30% (v/v) methanol and submitted to preparative reversed-phase HPLC using a Sperisorb (Supelco, Bellefonte, PA, USA) HPLC column with gradient elution using 0.3% (v/v) acetic acid and methanol as mobile phases (Table 1). The two major selenium-containing chromatographic peaks were collected, lyophilised and redissolved in 100 μl of 30% (v/v) methanol with 0.1% (v/v) formic acid. The solution was introduced into the nanospray source of the Q–TOF–MS instrument at a flow rate of 3 μl min^{-1}. The voltage was 4100 V. The MS spectra were acquired in the range 50–1000 Da using a step size of 0.5 u and a dwell time of 10 ms. In the product ion scan mode, the mass range was selected according to the targeted selenospecies and the MS spectra were obtained for three Se isotopes (^{78}Se, ^{80}Se and ^{82}Se). The collision energy was 20 eV. The TOF mass analyser was calibrated using renin.

Chemicals and standard substances

Preparation of the garlic samples involved proteolysis using Protease XIV (Sigma-Aldrich, Copenhagen, Denmark) or extraction with a 0.01% v/v aqueous solution of hydroxylamine hydrochloride (Merck, Darmstedt, Germany). A certified solution of selenium at 1000 μg mL^{-1} in 2% nitric acid solution (CPI International, Santa Rosa, CA, USA) was used to construct the external calibration curves when quantifying the selenium content in the samples using gallium (^{69}Ga) as internal standard. For the selenium speciation work with HPLC–ICP–MS, mixtures of available standards were prepared in aqueous solution. Solutions of MeSeCys, AllSeCys and Se-propyl-selenocysteine (PrSeCys) were kindly donated by Dr. Howard Ganther (University of Wisconsin, WI, USA). SeMet, selenoethionine (SeEt) and selenocystine ($SeCys_2$) were obtained from a commercial source (Sigma-Aldrich, Copenhagen, Denmark). The GarliSelect sample was used as the source of γ-Glu-MeSeCys, as described in the preceeding section. Water used throughout the work was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA) and was degassed with He prior to use in order to minimise the risk of oxidation of the seleno aminoacids.

Cultivation and selenium enrichment of garlic

Initial mycorrhizal inoculum, species *Glomus intraradices*, was obtained from Biorize, Dijon, France. Seeds of leek (*Allium porrum* L.), were inoculated with mycorrhizal fungus by sowing in trays containing sterilised sand with rows in which small amounts of initial inoculum were dispersed. Five plantlets per tray were checked for the presence of mycorrhizal fungal structures in the roots three and five weeks after sowing [31]. When the roots were infected, the shoots of the leek plants were removed and the roots were used as inoculum. Cloves of garlic (*Allium sativum* L.) cultivar American Early were planted on 25 March 2004 in a turf soil in which holes were made that contained an amount of about 2 g of mycorrhizal inoculum. From 19 April onwards, the soil was enriched with selenate using a solution containing 15 g Na_2SeO_4 in 60 litres of water, which was equivalent to an added concentration of sodium selenate in the soil of 5 μg g^{-1}. Over three successive two-weekly time intervals, this concentration was gradually increased to 20 μg g^{-1} turf soil. Concrete enclosures surrounding the soil medium prevented leaching of added selenate into the natural soil.The garlic was harvested on 18 August 2004. The cultivar was grown with or without sodium selenate with and without the mycorrhizal species *Glomus intraradices* (Table 2). The bulbs were air-dried for 1.5 weeks and stored at 4 °C. Subsamples

Table 1 Settings used for mass spectrometry and for HPLC

ICP–DRC–MS					
RF power/W	1350				
Lens voltage/V	7.25				
Dwell time/ms	500				
Readings	variable				
Replicates	1				
Detected isotopes	^{78}Se; ^{80}Se				
Rejection parameter q (RPq)	0.5				
DRC gas (methane) flow rate/ ml min^{-1}	0.45				
ESI–IT–MS					
Sheath gas flow rate (arbitrary)	80				
Auxiliary gas flow rate (arbitrary)	30				
ESI spray voltage/kV	4.5				
Capillary temperature/°C	180				
Scanned mass range/Da	150–320				
Q-TOF-MS					
ESI spray voltage/kV	4.1				
Scanned mass range/Da	50–1000				
Dwell time/ms	10				
Analytical cation exchange chromatography					
Injected volume/μL	20				
Mobile phase A: 3% v/v methanol in water					
Mobile phase B: 10 mM pyridinium formate in 3% MeOH/ 97% water, pH 3.2					
Step	% A	% B	Flow rate/ml min^{-1}	Time/min	
1	96	4	1	10	Isocratic
2	86	14	2	7	Gradient
3	20	80	1	16	Isocratic
Analytical reversed-phase chromatography					
Injected volume/μL	10				
Mobile phase: 2% v/ v methanol in 0.1% formic acid in water					
Step	% A	Flow rate/μl min^{-1}	Time/min		
1	100	30	20	Isocratic	
Analytical anion exchange chromatography					
Injected volume/μL	20				
Mobile phase A: 20 mM acetic acid, 10 mM triethylamine (pH 4.7) in water					
Mobile phase B: 200 mM acetic acid, 100 mM triethylamine (pH 4.7) in water					
Step	% A	% B	Flow rate/ml min^{-1}	Time/minutes	
1	100	0	0.9	5	Isocratic
2	0	100	0.9	25	Gradient
3	0	100	0.9	10	Isocratic
Preparative reversed-phase chromatography					
Injected volume/μL	20				
Mobile phase A: 0.3% acetic acid in water					
Mobile phase B: 0.3% acetic acid in 5% MeOH in water					
Step	% A	% B	Flow rate/ml min^{-1}	Time/minutes	
1	100	0	0.9	15	Isocratic
2	0	100	0.9	5	Gradient
3	0	100	0.9	20	Isocratic

Table 2 Design of garlic cultivation. For further details see the "Experimental" section

	Mycorrhiza added	Sodium selenate added
Garlic 1	No	No
Garlic 2	Yes	No
Garlic 3	No	Yes
Garlic 4*	No	Yes
Garlic 5	Yes	Yes

*Same conditions as for sample no. 3 but grown in a separate enclosure

were lyophilised and powdered. The dry matter content was accurately determined for each sample and ranged from 30.1% to 37.8% of the wet mass.

Extraction of selenium species from garlic

Aqueous extraction

The dry garlic samples (0.2 g) were mixed with 10 mL of the hydroxylamine hydrochloride solution in a stoppered polyethylene vial. The fresh garlic cloves were peeled and the whole cloves (approximately 20 g) immediately covered with 200 mL of the same solution and homogenised using a titanium-bladed Omni-Mixer (Sorvall, Newtown, CT, USA). The samples were extracted using magnetic stirring for one hour in a water bath at 37 °C. The supernatants were isolated by centrifugation and filtered by passage through a 12 kDa cut-off filter using a cooled (4 °C) centrifuge.

Enzymatic extraction

The dry garlic samples (0.2 g) were mixed with 10 mL of water and 20 mg of proteolytic enzyme in a stoppered glass tube. The treatment was carried out for 24 hours at 37 °C using magnetic stirring. Following the enzymatic treatment, the samples were centrifuged and passed through a 0.45 µm pore size filter and then through a 12 kDa cut-off filter using a cooled (4 °C) centrifuge. The sample extracts were diluted appropriately with water prior to selenium analysis or selenium speciation analysis.

Results and discussion

Development of chromatographic methods

An existing method for the chromatographic separation of selenoamino acids by gradient elution cation-exchange HPLC [30] was adjusted in terms of buffer strength and elution programme for the separation of selenium species contained in garlic. The resulting HPLC conditions (see Table 1 for details) provided good selectivity for the five selenoamino acids, as illustrated in Fig. 2a. For confirmative qualitative analysis, a separate chromatographic

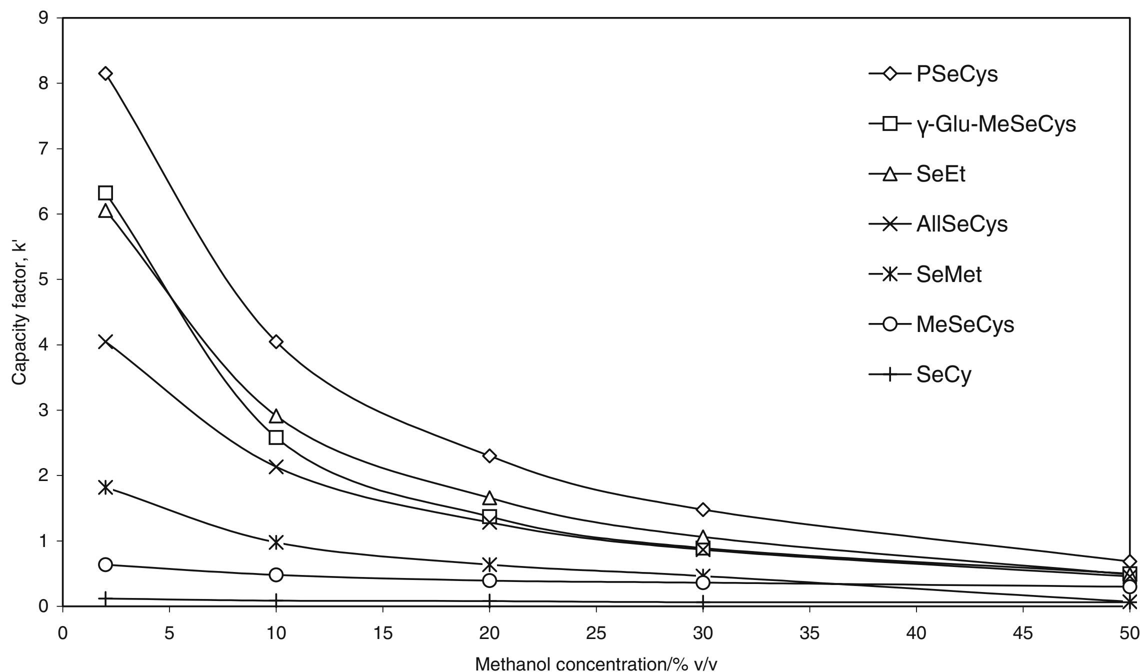

Fig. 1 Capacity factors of seven selenoamino acids in a reversed-phase HPLC system as a function of the methanol concentration of the mobile phase. Solvent: 0.1% formic acid in water. See "Experimental" section for details on the stationary phase used and for definitions of the acronyms used for the selenium species

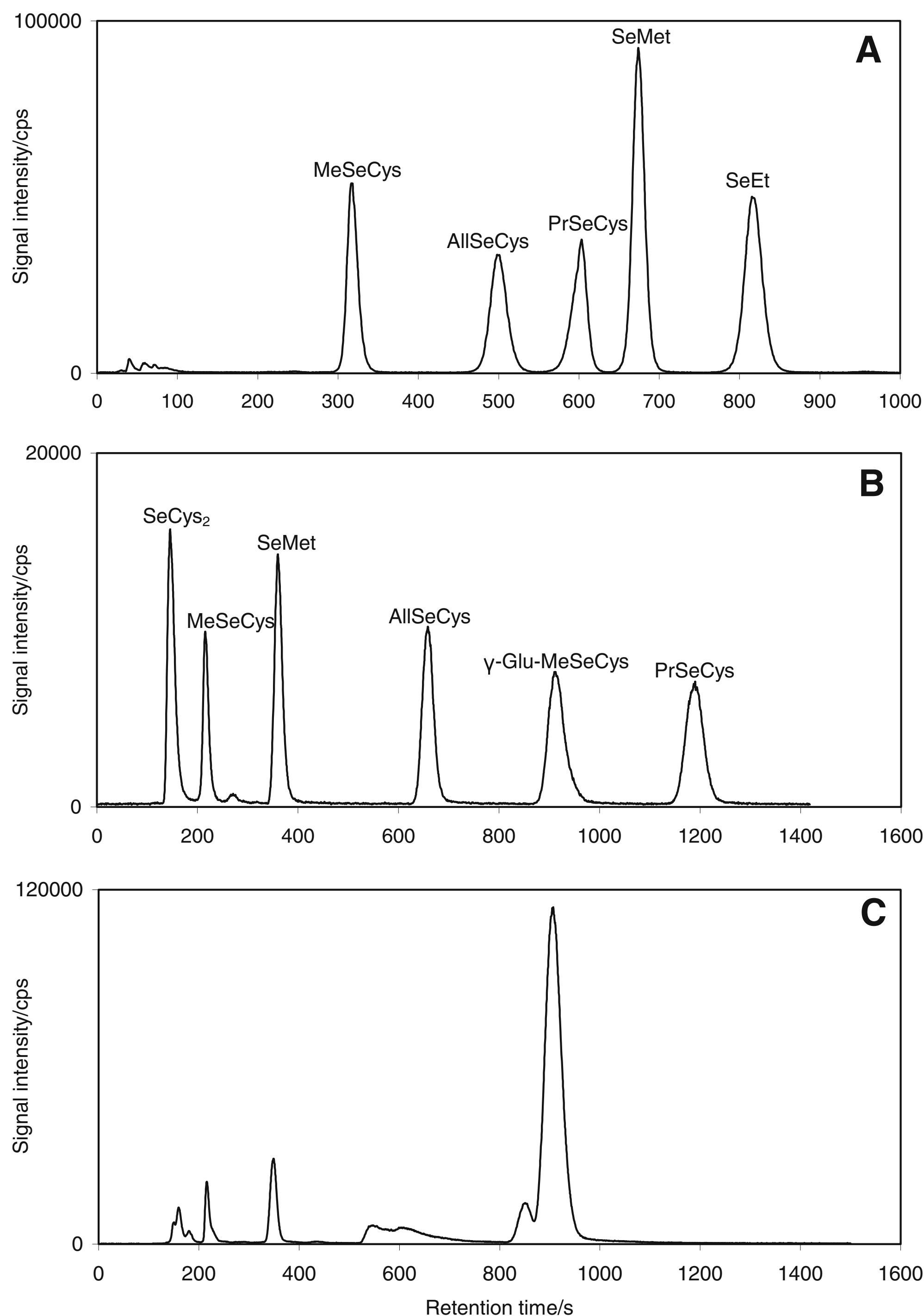

Fig. 2a–c Separation of selenoamino acids using (**a**) cation exchange HPLC–ICP–MS and (**b**) RP–HPLC–ICP–MS of standard mixtures, and (**c**) RP–HPLC–ICP–MS of an enzymatic extract of garlic sample no. 5. See "Experimental" section for definitions of the acronyms used for the selenium species

method based on reversed-phase HPLC [24] was developed and the capacity factors of six selenoamino acids were determined as a function of mobile phase composition, as shown in Fig. 1. The selectivity when using the optimum 2% v/v methanol in 0.1% v/v formic acid in water as mobile phase made possible the retention and separation of five selenoamino acids, as shown in Fig. 2. The elution of $SeCys_2$ close to the chromatographic void volume did not make the analysis of this species practical. The analysis of the garlic extracts by the RP–HPLC system (Fig. 2b), in addition to the cation exchange HPLC system, provided additional confirmation of the identities of the analytes (Fig. 2c) by retention time matching with authentic standards.

An authentic standard of γ-Glu-MeSeCys, however, was acquired by isolating it from an extract of the GarliSelect sample, and its identity was confirmed by MS^2 using ESI–IT–MS. The mass spectrum recorded in the positive mode in Fig. 3 (inset) shows signals corresponding to

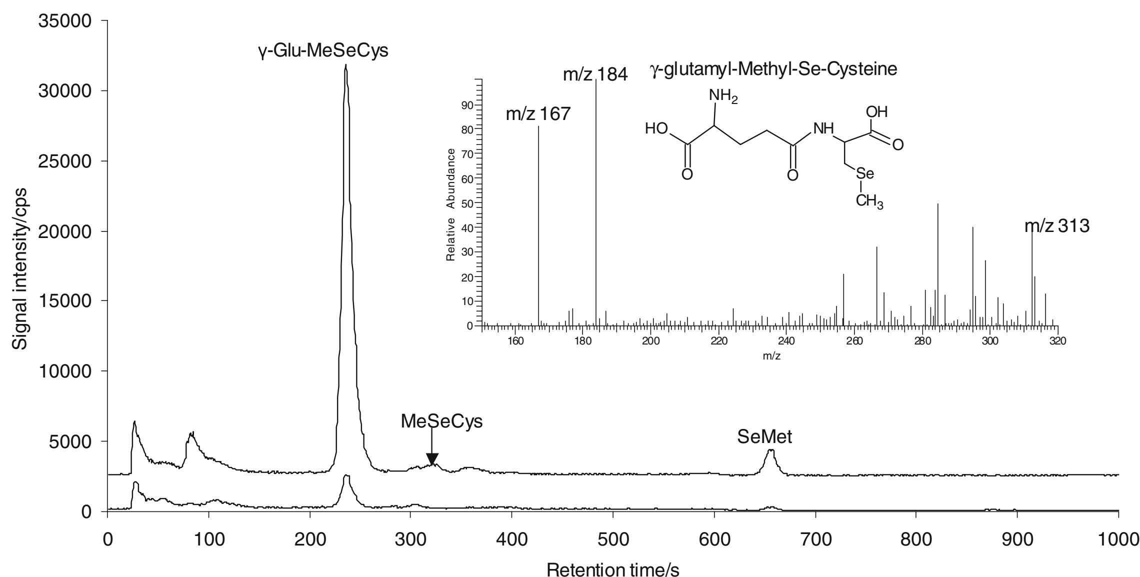

Fig. 3 Cation exchange HPLC–ICP–MS chromatograms corresponding to an enzymatic extract of garlic no. 1 (*lower trace*) and garlic no. 2 (*upper trace* +2500 cps). The ESI–IT–MS spectrum (positive ionisation mode) in the *inset* corresponds to γ-Glu-MeSeCys and shows signals for the molecular ion at *m/z* 313 and for two major fragments at *m/z* 184 and 167

the ^{80}Se isotopomer of the molecular ion at *m/z* 313 and to two signals corresponding to the fragments at *m/z* 184 and 167, which are characteristic of γ-Glu-MeSeCys [27]. Having confirmed the identity of the selenium dipeptide in this way, the isolated substance was used in mixtures along with the available standards for the RP–HPLC separations illustrated in Fig. 2b.

Selenium and selenium speciation in cultivated and enriched garlic

The results (Table 3) show that the concentration of selenium is increased from 1.5 µg g^{-1} (sample no. 1) when grown in natural soil to 15 µg g^{-1} (sample no. 2) when the garlic was cultivated in soil amended with myccorhiza. Fertilisation of the natural soil with selenate increased the selenium content in garlic strongly to several hundreds of µg g^{-1} (samples no. 3 and 4). The addition of myccorhiza in combination with selenate fertilisation (sample no. 5) had an additional but less pronounced positive effect on the selenium content. The limited set of garlic samples from the field experiments cause the quantitative results of this work to be of an explorative nature only. Further work is now in progress to further illustrate the effects of factors such as plant genotype and cultivation parameters on the selenium content of garlic samples.

The efficiency of the proteolytic extraction method prior to selenium speciation analysis of the dry samples (Table 3) was almost 100%. The aqueous extraction methods (Aqueous-1 and Aqueous-2) were applied mainly in the search for labile selenoamino acids. The cation exchange HPLC chromatograms of enzymatic extracts of garlic samples 1 and 2 in Fig. 3 show that the major compound

Table 3 Concentration of selenium in six garlic samples determined following nitric acid digestion, and total chromatographed selenium following enzymatic or aqueous extraction

	Selenium concentration/µg g^{-1} dry mass				Extraction efficiency		
	Total selenium	Chromatographed selenium					
	HNO_3 digestion	Enzymatic	Aqueous-1	Aqueous-2	Enzymatic	Aqueous-1	Aqueous-2
Garlic 1	1.5	1.1	1.2	0.9	74%	78%	60%
Garlic 2	15	14	10	1	93%	68%	5%
Garlic 3	853	812	718	249	95%	84%	29%
Garlic 4	740	683	550	213	92%	74%	29%
Garlic 5	969	968	765	394	100%	79%	41%
GarliSelect	995[B]	1057	849	n.a.	106%	85%	n.a.

[B]Manufacturer's information: 995±40 µg g^{-1}

Extraction into aqueous hydroxylamine solution was applied to the dry (Aqueous-1) and to the fresh (Aqueous-2) samples. The extraction efficiencies were estimated as the ratio between the chromatographed selenium and the total selenium content

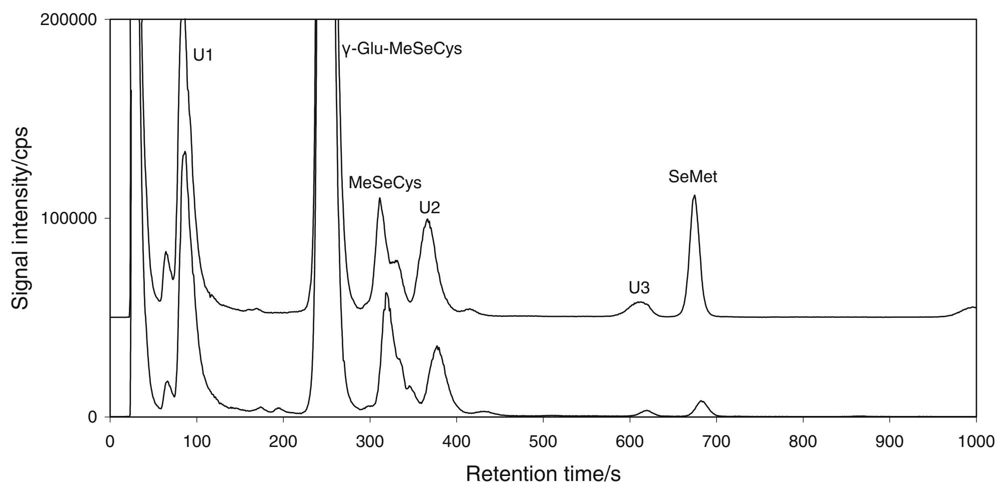

Fig. 4 Cation exchange HPLC–ICP–MS chromatograms corresponding to garlic no. 5 obtained using aqueous extraction (*lower trace*) and enzymatic extraction (*upper trace* + 50000 cps)

present is γ-Glu-MeSeCys, followed by SeMet, in addition to a small peak with the same retention time as MeSeCys. The overlaid chromatograms in Fig. 4 and the quantitative results for samples 1–5 in Table 4 illustrate the influence of the sample preparation methods used (aqueous vs enzymatic) on the selenium species detected in garlic. Besides being generally more efficient, the enzymatic extraction method (upper chromatogram in Fig. 4) liberated more SeMet that was extracted by the aqueous hydroxylamine solution. This was probably caused by the enzyme's specific ability to liberate peptide or protein-bound SeMet. Furthermore, the results for samples 1–5 in Table 4 showed that the relative amounts of γ-Glu-MeSeCys and MeSeCys were similar for both extraction metods used. This suggests that both species were readily extractable from the biological material and that the proteolytic sample pretreatment using protease XIV did not cleave the γ-peptide bond of γ-Glu-MeSeCys to form MeSeCys. Most importantly, the results show that neither the selenate enrichment nor the addition of mycorrhiza to the soil affected the relative abundance of γ-Glu-MeSeCys and MeSeCys. In contrast to the species-conserving nature of protease XIV used for sample preparation in this study, the in vitro use of fresh human saliva and of gastric enzymes liberated MeSeCys from its γ-glutamyl dipeptide contained in garlic [29].

The producer of the GarliSelect sample provided information stating that this sample contained γ-Glu-MeSeCys at 2/3 of its total selenium content, which is in accordance with the results reported in Table 4. In the absence of a certified reference material, this commercial garlic sample therefore serves the purpose of a check sample. In comparison with the cultivated garlic samples no. 1–5, GarliSelect contained a higher relative amount of selenium as MeSeCys and SeMet, which is furthermore

Table 4 Quantitive results for selenium species in six lyophilized garlic samples using cation or anion exchange HPLC–ICP–MS

	Selenium species concentration/μg Se g^{-1} dry mass							
	γ-GLU-MeSeCys		MeSeCys		SeMet		Selenite	Selenate
	Protease	Aqueous	Protease	Aqueous	Protease	Aqueous	Aqueous	Aqueous
Garlic 1	0.83	0.93	n.d.	n.d.	n.d.	n.d.	0.08	0,13
Garlic 2	9.8	9.0	0.2	n.d.	0.7	n.d.	n.d.	0.3
Garlic 3	523	486	8	8	17	2	n.d.	46
Garlic 4	476	369	8	9	16	2	n.d.	29
Garlic 5	624	473	28	36	23	4	n.d.	70
GarliSelect	646	560	69	77	58	68	n.d.	n.d.
Relative amount								
Garlic 1	75%	80%					7%	11%
Garlic 2	69%	87%	2%		5%			3%
Garlic 3	64%	68%	1%	1%	2%	0,3%		6%
Garlic 4	70%	67%	1%	2%	2%	0.4%		5%
Garlic 5	64%	62%	3%	5%	2%	1%		9%
GarliSelect	61%	66%	7%	9%	5%	8%		
Mean	67%	72%						
Stddev	5%	10%						

The relative amount of each selenium species was estimated as the ratio between the selenium species concentration and the total chromatographed selenium (for the same extraction method) reported in Table 3. n. d.: not determined

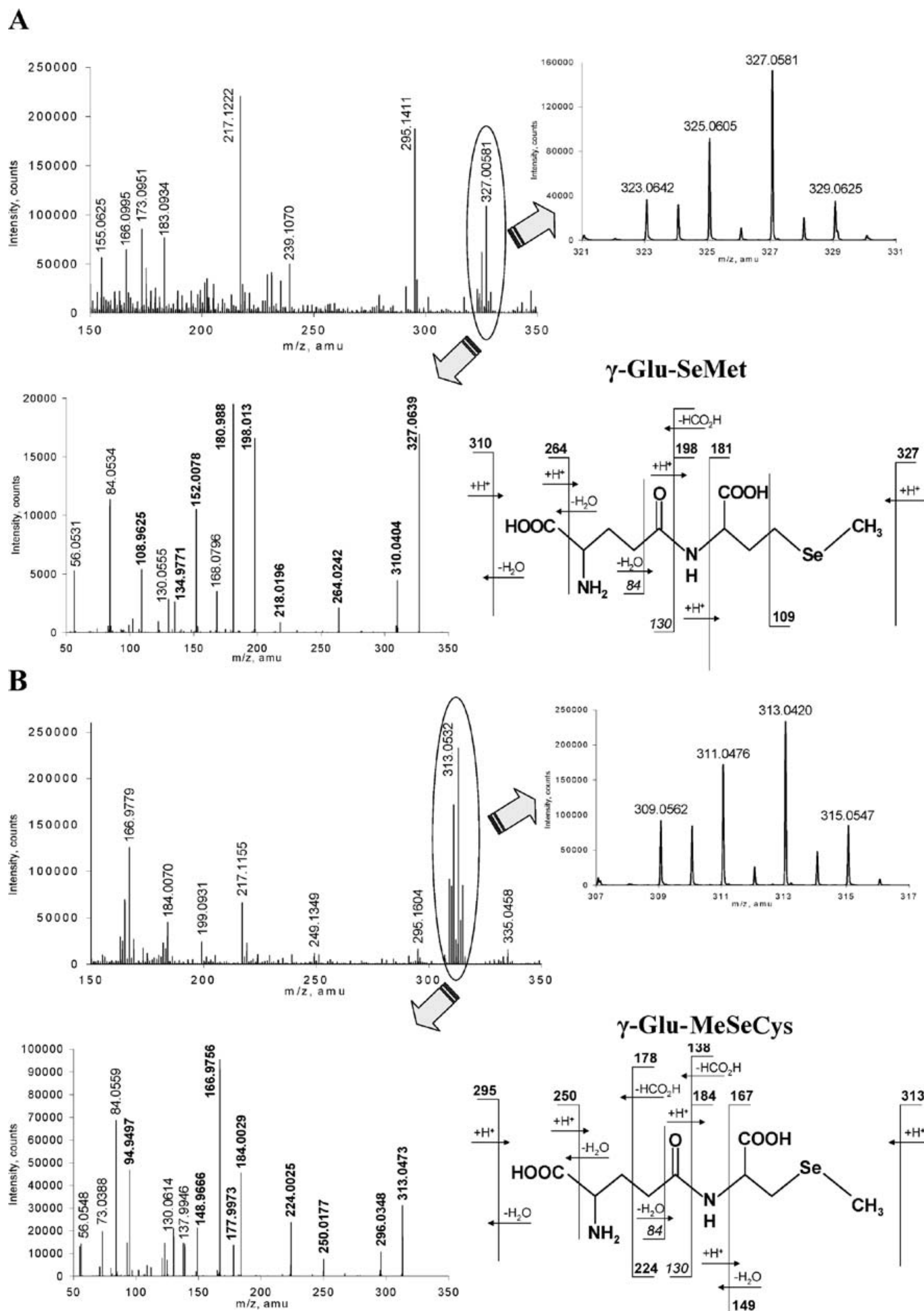

Fig. 5a, b Structural characterisation of (**a**) γ-Glu-SeMet and (**b**) γ-Glu-MeSeCys by ESI–Q–TOF–MS. The selenium isotope pattern, the fragments and the accurate masses of the molecular ions were used for identification. Prior to the introduction of the samples by a syringe pump, the selenium species were isolated from an aqueous extract by size-exclusion HPLC followed by RP–HPLC. For further details, please refer to the "Experimental" section

readily extractable into the aqueous extractant and therefore not bound to protein. The fact that GarliSelect, according to the manufacturer's information, was cultivated hydroponically might provide an explaination of these observations. Finally, the results in Table 4 show that all samples contained selenate and that selenite was undetectable in most samples. Apparently, the enzyme-catalysed reduction of selenate to selenite is slower than its further reduction to selenide prior to the plant's biosynthesis of organic selenium [32].

Structural characterisation of γ-Glu-MeSeCys and γ-Glu-SeMet by ESI–Q–TOF–MS

The chromatograms in Fig. 4 show that several unidentified peaks (U1–U3) occurred in the extracts. In order to characterise the chemical structures of these unknowns, the selenium species in an aqueous extract were isolated by two-dimensional HPLC using size-exclusion HPLC followed by RP–HPLC. The major selenium-containing fractions, which were analysed by ESI–Q–TOF MS, showed the existence of selenium-containing parent ions at *m/z* 313.0532 and 327.0058 (central masses) as neighbouring peaks, forming the isotope pattern characteristic of selenium. The molecular ions corresponding to three selenium isotopes (^{78}Se, ^{80}Se and ^{82}Se) were fragmented. The MS–MS fragments in Fig. 5a,b corresponded to γ-Glu-SeMet and to γ-Glu-MeSeCys, respectively. The measured mass of 313.0302 Da corresponding to γ-Glu-Me^{80}SeCys differed from the theoretical mass of 313.0420 Da by 38 ppm, and the measured mass for γ-Glu-^{80}SeMet of 327.0459 Da differed from the theoretical one of 327.0581 Da by 37 ppm. The accurate mass determination further confirmed the identities of the structurally characterised Se species.

Searching for AllSeCys and PrSeCys in Se-enriched garlic

One of the aims of this work was to search for selenium-containing analogues of biologically active sulfuramino acids. Comparison of the retention time of the authentic AllSeCys standard in the cation exchange HPLC system in Fig. 2a with chromatograms of proteolytic garlic extracts in Figs. 3 and 4 showed that there were no detectable peaks corresponding to this species in any of the extracts. The enzyme alliinase, for which *S*-allylcysteine is a substrate, is known to become active upon mincing the garlic cloves and to cause degradation of this sulfur species. Presumably, the same enzyme would also be able to degrade its Se-containing analogue AllSeCys, if present in garlic. Hydroxylamine, which is able to deactivate aliinase [21], was used in the aqueous extractant, but still no AllSeCys was detected (Fig. 4). This was equally the case for the dried powdered samples and for the freshly ground whole garlic cloves. For the high-selenium garlic samples (nos. 3, 4 and 5), however, a chromatographic peak indicated as U3 in Fig. 4 had the same retention time as PrSeCys. For the purpose of verification, the sample extracts were analysed by the RP–HPLC–ICP–MS system. The chromatogram for sample no. 5 in Fig. 2c show that U3 was not identical to PrSeCys, as no peak occurred at the corresponding retention time of 1200 s. This was substantiated by spiking the sample with the authentic standard substance. The RP–HPLC chromatograms did, however, confirm the presence of MeSeCys, SeMet and γ-Glu-MeSeCys in the extracts.

Conclusions

The selenium content of garlic was enhanced ten-fold by amending the natural soil with mycorrhiza. By fertilising the soil with selenate, the selenium content of the garlic was strongly enhanced. The distribution of selenium between molecular species showed almost the same relative amount present of γ-Glu-MeSeCys at 2/3 and of MeSeCys at a few percent of the total chromatographed selenium. Although searched for, AllSeCys and PrSeCys were not detected in any of the samples. The results of the work illustrate a way to reliably enhance the selenium concentration in garlic while at the same time maintaining its incorporation into biologically active derivatives of selenocysteine.

Acknowledgements The authors thank Dr. Laurent Ouerdane for his help with ESI–MS/MS experiments. The cultivation of garlic at PRI was part of SEAFOODplus, an integrated research project financially supported by the EU under FP6 (http://www.seafoodplus.org).

References

1. Clark LC, Combs GF, Turnbull BW, Slate EH, Chalkier DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL, Park HK, Sanders BB, Smith CL, Taylor JR (1996) J Am Med Assoc 276:1957–1963
2. Rayman MP (2000) Lancet 356:233–241
3. Larsen EH, Hansen M, Paulin H, Moesgaard S, Reid M, Rayman M (2004) J AOAC Int 87:225–232
4. Larsen EH, Andersen NL, Møller A, Petersen A., Mortensen GK, Petersen J (2002) Food Addit Contam 19:33–46
5. Hartikainen H (2005) J Trace Elem Med Biol 18:309–318
6. Cai X-J, Uden PC, Block E, Zhang X, Quimby B, Sullivan JJ (1994) J Agric Food Chem 42:2081–2084
7. Randle WM, Lancaster JE (2005) In: Rabinowitch HD, Currah L (eds) Allium crop science: recent advances. CABI Publications, Wallingford, UK, pp 329–356
8. Bergès R, Siess M-H, Arnault I, Auger J, Kahane R, Pinnert M-F, Vernevaut M-F, le Bon A-M (2004) Carcinogenesis 25: 1953–1959
9. Fukushima S, Takada N, Hori T, Wanabuchi H (1997) J Cell Biochem Suppl 27:100–105
10. Ip C, Lisk DJ, Stoewsand GS (1992) Nutr Cancer 17:279–286
11. Ip C, Birringer M, Block E, Kontrebai M, Tyson J, Uden PC, Lisk D (2000) J Agric Food Chem 48:2062–2070
12. Finley JW, Davis CD, Feng Y (2000) Nutr Cancer 130: 2384–2389
13. Davis CD, Zeng H, Finley JW (2002) J Nutr 132:307–309
14. Cai X-J, Block E, Uden PC, Zhang X, Quimby BD, Sullivan JJ (1995) J Agric Food Chem 43:1754–1757

15. Lawson LD (1996) In: Koch HP, Lawson LD (eds) Garlic: the science and therapeutic application of *Allium sativum* L and related species, 2nd edn. Williams & Wilkins, Baltimore, MD, pp 37–107
16. Ip C, Lisk DJ (1994) Carcinogenesis 15:573–576
17. Ip C, Lisk DJ (1994) Carcinogenesis 15:1881–1885
18. Block E, Birringer M, Jiang W, Nakahodo T, Thompson HJ, Toscano PJ, Uzar H, Zhang X, Zhu Z (2001) J Agric Food Chem 49:458–470
19. Vonderheide AP, Wrobel K, Kannamkumarath SS, B'Hymer C, Montes-Bayon M, Ponce de Leon C, Caruso JA (2002) J Agric Food Chem 50:5722–5728
20. Larsen EH, Hansen M, Fan T, Vahl M (2001) J Anal Atom Spectrom 16:1403–1408
21. Auger J, Yang W, Arnault I, Pannier F, Potin-Gautier M (2004) J Chromatogr A 1032:103–107
22. Kahakachchi C, Boakye HT, Uden PC, Tyson JF (2004) J Chromatogr A 1054:303–312
23. Goenante Infante H, Hearn R, Catterick T (2005) Anal Bioanal Chem 382:957–967
24. Goenante Infante H, O'Connor G, Rayman M, Wahlen R, Spallholz JE, Hearn R, Catterick T (2005) J Anal Atom Spectrom 20:864–870
25. Kotrebai M, Birringer M, Tyson JF, Block E, Uden PC (1999) Anal Comm 36:249–252
26. Kotrebai M, Birringer M, Tyson JF, Block E, Uden PC (2000) Analyst 125:71–78
27. McSheehy S, Yang W, Pannier F, Szpunar J, Lobinski R, Auger J, Potin-Gautier M (2000) Anal Chim Acta 421:147–153
28. Shah M, Kannamkumarath SS, Wuilloud JCA, Wuilloud RG, Caruso JA (2000) J Anal Atom Spectrom 19:381–386
29. Dumont E, Ogra Y, Vanhacke F, Suzuki KT, Cornelis R (2006) Anal Bioanal Chem 284:1196–1206
30. Larsen EH, Sloth J, Hansen M, Moesgaard S (2003) J Anal Atom Spectrom 18:310–316
31. Mosse B, Hayman DS (1971) New Phytol 70:29–34
32. Terry N, Zayed AM, de Souza MP, Tarun AS (2000) Annu Rev Plant Phys 51:401–432