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Effects of selenate supplemented fertilisation on the selenium level of cereals — identification and quantification of selenium compounds by HPLC–ICP–MS

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Abstract

Laboratory experiments were carried out to investigate whether the selenium content of different kinds of cereals grown on Austrian soil could be raised by the application of compound fertilisers containing selenium as selenate. An anion exchange chromatographic system coupled to an HP 4500 inductively coupled plasma mass spectrometer (ICP–MS) was used for the identification and quantification of selenium compounds in cereal samples. The HPLC–ICP–MS system was optimized for the separation of selenite, selenate, selenocystine, and selenomethionine using a Hamilton PRP-X100 column. Separation was obtained, with a 10 mM citrate buffer (pH 5), 2% methanol as mobile phase and a flow rate of 1.5 ml min^{-1}. Four baseline separated chromatographic peaks were obtained within 6 min. The retention behavior of a further five selenium [selenohomocystine (Sehcys), selenocystathionine (Secysta), selenoethionine (Seet), trimethylselenonium iodide (TmSe), and dimethyl(3-amino-3-carboxy-1-propyl)selenonium iodide (DmpSe)] compounds was investigated. Aqueous extraction and enzymatic hydrolysis of the biological materials were also compared with respect to the amount of selenium extracted. In the aqueous extracts only 3–9% of the total concentration of selenium was found. When the cereal samples were exposed to an enzymatic hydrolysis, recovery rates ranging from 80 to 95% were obtained. A major part of the selenate, which had been taken up by the cereals was converted to selenomethionine.

Keywords: High performance liquid chromatography; Anion exchange chromatography; Inductively coupled plasma mass spectrometry; Selenium; Selenomethionine; Agricultural samples; Cereals; Fertilisation

1. Introduction

Selenium belongs to a group of micronutrient elements required in very small amounts by animals and humans for the basic functions of life. Toxicity and essentiality have been widely discussed by many authors (e.g. Forchhammer & Boeck, 1991; Sager, 1994 a, b).

The concentration of selenium in plants and animals is strongly correlated with its concentration in soils. Plants and products derived from plants transfer the selenium taken up from the soils to humans (Girling, 1984). Se exists in soils in a number of different forms including elemental selenium, selenides, selenites, selenates, and organic selenium compounds (Berrow & Ure, 1989). On account of the existence of different selenium species in soils, total selenium concentrations does not necessarily reflect the extent to which growing plants take up selenium. Nye and Peterson found that the water-soluble Se of soils correlated better with the uptake of selenium by plants, and this could therefore be used as a measure of the potentially available soil selenium (Nye & Petersen, 1975). However, the significance of soil physical-chemical factors such as redox-behavior, pH, or microbiological activity in affecting plant selenium uptake is also considerable, e.g. the plant availability of Se increases with increasing pH values

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(Hartfiel & Bahners, 1988; Pfannhauser, 1992a). In alkaline soils (pH 7.5–8.5) selenite may be oxidized to soluble selenate ions which are readily available to plants. Whereas, in acid soils (pH 4.5–6.5) it is usually bound to iron hydroxides, and thus, is unavailable to plants. Many transfer reactions can be promoted by microbial activities, including volatilization losses.

The selenium concentration in agricultural products is very low in many areas throughout the world, and also in large areas of Middle and Northern Europe. This has been traditionally attributed to poor supply of selenium from the soil, and ultimately the underlying geology. In German agricultural soil a mean Se concentration of 0.12 mg kg^{-1} dry weight was found (Hartfiel & Bahners, 1988). The lowest Se levels were observed mainly in the south of Germany (Baden Wuerttemberg and Bavaria). Selenium levels of >0.2 mg kg^{-1} dry matter in some areas were probably caused by industrial emissions. In Norwegian farmland soils the concentration of selenium in general is very low (0.3 mg Se kg^{-1} on average; Wu & Lag, 1988). Agricultural soil in Finland has an average Se concentration of 0.2 mg kg^{-1} (Ylaeranta, 1983b).

Like in other parts of Central and Northern Europe, selenium background values (aqua regia) in air-dried agricultural soils of Eastern Austria are rather low and independent of the geological unit, due to determinations done within the ''Austrian soil monitoring program''. The average value for field soils (<2 mm, 0–20 cm) was found to be 0.23 mg kg^{-1} in Upper Austria, 0.22 mg kg^{-1} in the Marchfeld, and 0.31 mg kg^{-1} in the Burgenland. In grassland of Upper Austria (0–5 cm), the average Se concentration is 0.35 mg kg^{-1}. The selenium concentration is highest in wetlands and lowest in crystalline and clay regions (Burgenlaendische Bodenzustandsinventur, 1994; Danneberg, 1989; Oberoesterreichische Bodenzustandsinventur, 1993). The average daily selenium intake of Austrians was found to be 35.5 μg Se per day, which is lower than the recommended ''adequate and safe'' intake of 50–200 μg Se per day (Pfannhauser, 1992b). Twenty to one-hundred micrograms of Se per day have been recommended as adequate by the German Society for Nutrition (Radke, 1992).

In order to promote optimum growth and resistance to various illnesses, this led to widespread use of selenium supplementation as sodium selenite added to commercial feedstuffs for farmed animals (Oldfield, 1992) up to a total contents of 0.5 μg/g. Excess selenium is excreted, and thus leads to enhanced selenium levels in manure and in sewage sludges. Organic amendments thus cover a wide and still unpredictable source of selenium enrichment for agricultural soils, and selenium speciation in these matrices is still an interesting subject of investigation.

Enhancing the selenium levels in crops may be done either by adding organic amendments of high level selenium (sewage sludges, manure of selenium supplemented farmed animals; Cappon, 1991; Sager, 1999) or by adding selenate containing mineral fertilizers (Ylaeranta, 1983b, c). The reason for fertilisation with selenate rather than with selenite is that selenate is taken up from soil by plants up to ten times more effectively than selenite (Ylaeranta, 1983a, b). Obviously, added selenite is converted to forms that are poorly available to plants.

In the mid 1970s the average daily intake of selenium in Finland was found to be very low (20–30 μg per day; Varo & Koivistoinen, 1981). This was one of the lowest intakes reported anywhere in the world. The main reason for that was obviously the very low selenium content of Finnish cereals. During this time little grain was imported because of the high yields of grain in Finland. In 1978 and 1979, however, up to 50% imported grain, mainly from North America or Canada, was mixed into domestic grains. The selenium content of wheat and rye increased from 20 μg kg^{-1} dry matter to 100–150 μg kg^{-1}. Consequently, the total selenium intake increased to 50–60 μg per day and person, which is within the ''safe and adequate'' level, according to WHO. In 1984, Finnish authorities made a decision to increase the Se content of domestic agricultural products by adding sodium selenate to all agricultural multinutrient fertilisers (Varo & Koivistoinen, 1988). The selenium concentration of different foods, the mean Se intake of the population, as well as the average serum Se concentration of people increased from 1984 to 1988 due to enrichment of fertilisers with sodium selenate (Aro, Alfthan, & Varo, 1989; Ekholm, Ylinen, Koivistoinen, & Varo, 1990; Eurola, Elholm, Ylinen, Koivistoinen, & Varo, 1989; Eurola et al., 1990). The effects on glutathione peroxidase activities, however, remained constant (Pyykkoe, Tuimala, Kroneld, Roos, & Huuska, 1988). As the chemical form of selenium in plant products and not its total concentration determines bioavailability, high-performance liquid chromatography (HPLC) is an appropriate method for the separation, identification and quantification of selenium compounds at trace levels. Usually either ion-exchange or reversed phase ion pairing chromatography were applied for the separation of ionic selenium compounds (Dauchy, Potin-Gautier, Astruc, & Astruc, 1994; Koelbl, Kalcher, Irgolic, & Magee, 1993; Olivas, Donart, Camara, & Quevauviller, 1994; Pyrzynska, 1996). Selenium-specific detectors can considerably simplify the chromatographic process, because only the compounds containing selenium need to be separated. Additionally, even large excesses of selenium-free co-eluting substances usually do not interfere with the determination. The most sensitive selenium-specific detector, which can easily be coupled on-line to chromatographic systems is an inductively coupled plasma mass spectrometer ICP–MS; Ge, Cai, Tyson, Uden Denoyer, & Block, 1996; Goessler, Kuehnelt, Schlagenhaufen, Kalcher, Abegaz, & Irgolic, 1997; Pedersen & Larsen, 1997).

In Austria it is not allowed to add selenate to agricultural fertilisers to achieve an enrichment of selenium in plant products at the moment. The aim of this work was the identification and quantification of selenium compounds in agricultural plant samples, which were grown in pot experiments either with the addition of sodium selenate to basal multi-nutrient fertilisers or not.

2. Materials and method

2.1. Instrumentation

A HP 4500 inductively coupled plasma quadrupole mass spectrometer (Hewlett-Packard, Germany) was used as selenium-specific detector. The HPLC system consisted of a Hewlett Packard quaternary HPLC pump series 1100 (Hewlett Packard, Waldbronn, Germany) and a Rheodyne 6-port injection valve with a 50-μl injection loop. The separations were performed on a Hamilton (Reno, USA) PRP-X100 anion-exchange column (25 cm×4.1 mm i.d., spherical 10-μm styrene-divinylbenzene particles with trimethylammonium exchange sites). The mobile phase was pumped through the analytical column at a flow rate of 1.5 ml min^{-1}. The chromatographic system was connected with the Hewlett Packard HP 4500 ICP–MS with a 80-cm PEEK (polyether ether ketone) capillary tubing (1/16-in i.d.) that connected the HPLC column outlet to the inlet of the Babington nebulizer of the ICP–MS. The ion intensities at m/z 82 (^{82}Se) and 77 (^{77}Se) were monitored using the "time-resolved mode". The integration time was set to 0.3 s.

During instrumental optimization a standard solution of selenite (50 Se μg l^{-1}) in the applied mobile phase was continuously pumped to the ICP–MS at a flow rate of 1.5 ml min^{-1}. The tuning parameters for the ICP–MS were set with respect to highest signal intensities at m/z 82 and lowest background at m/z 89.

The chromatographic peaks resulting from the coupled HPLC–ICP–MS system were recorded in the graphics mode, i.e. the signal intensity in counts was recorded. The peak areas were determined using the data analysis program of the MSD ChemStation software (G1701AA, Version A.03.00, Hewlett Packard). The selenium compounds were quantified with external calibration curves established with each of the four compounds.

2.2. Reagents, standards, and mobile phases for HPLC–ICP–MS

All commercial chemicals were used without further purification. Sodium selenate (p.a., 71947) and seleno-DL-methionine (Biochemie, 84925) were purchased from Fluka, selenium dioxide (SeO_2 in diluted nitric acid, 1000 mg Se l^{-1}, Titrisol, 9915) from Merck, seleno-DL-ethionine (laboratory grade, S-3750) and seleno-DL-cystine (laboratory grade, S-1650) from Sigma. Selenohomo-cystine (Schachl, 1993), trimethylselenonium iodide (Koelbl, 1994), and selenocystathionine (Abegaz, 1997) were prepared according to literature procedures. Dimethyl(3-amino-3-carboxy-1-propyl)selenonium iodide was synthesized as described for S-methylmethionine iodide (Lewis, Johnson, & Broyer, 1971). Stock solutions were prepared with Milli-Q water (18.2 MΩ cm) from anhydrous sodium selenate (1196.4 mg to 500 ml, 1000 mg Se l^{-1}), trimethylselenonium iodide (63.6 mg to 20 ml, 1000 mg Se l^{-1}), dimethyl(3-amino-3-carboxy-1-propyl)selenonium iodide (21.4 mg to 10 ml, 500 mg Se l^{-1}), seleno-methionine (24.8 mg to 20 ml, 500 mg Se l^{-1}), selenocystine (5.3 mg to 20 ml, 125 mg Se L^{-1}), from selenocystathionine (8.5 mg to 20 ml, 125 mg Se l^{-1}), and seleno-homocystine (5.7 mg to 20 ml, 125 mg Se l^{-1}). The stock solutions were stored in the refrigerator at −20°C before use.

Solutions of the selenium compounds with concentrations in the range 2.0–20.0 μg Se l^{-1} were prepared by appropriate dilution of the stock solutions with Milli-Q water.

The mobile phase for the anion-exchange HPLC was prepared by dissolving either 2.10 (10 mM), 1.05 (5 mM), or 0.53 g (2.5 mM) citric acid monohydrate (puriss p.a., Fluka, 27490) in Milli-Q water, adjusting the pH of this solution to 5.0 by addition of NH_3 (suprapure, Merck, 5428), and filling to 1000 ml. To the citric acid buffer 0 to 5% methanol (puriss p.a., Fluka, 65543) was added.

Calibration curves for the HPLC–ICP–MS measurements of the natural samples were obtained by injecting chromatographing aliquots (50 μl) of solutions containing 2.00, 5.0, 10.0 or 20.0 μg Se l^{-1}) of selenocystine, selenite, selenomethionine and selenate.

2.3. Samples

Powdered samples of various grains (winter wheat, spring wheat, summer barley, summer rye, and durum wheat) were obtained from the Federal Office and Research Centre for Agriculture, Vienna, Austria (Hoesch, 1999). The pots for growing the cereals (after Kick-Brauckmann) were filled with 4 kg soil and 4 kg quartz sand (1:1 w/w). The soil of two areas in Lower Austria was taken: soil from Zwettl (Soil A) and from Hirschstetten (soil B). Soil A was a CAMBISOL of pH 5.9 from silicate rock, containing 16% of clay size fraction. Soil B was a calcic CHERNOZEM of pH 7.5, containing 15% lime and 18% of clay size fraction. An artificial basal fertiliser (14% P_2O_5, 38% K_2O and 5% MgO) was added to each pot. Additionally, the soil was amended using a mineral fertiliser containing 20% N, 8% P_2O_5, and 8% K_2O, which had been added in two steps of 3 g each. This fertiliser was either supplemented using sodium selenate or not. The final concentration of selenium was 30 mg Se kg^{-1}. Forty grains per pot were

seeded. The pots were kept at ambient temperatures and supplied with as much water as needed to ensure optimal growth (Hoesch, 1999).

2.3.1. Aqueous extraction of selenium compounds from agricultural samples

Approximately 0.4 g of the powdered grain was weighed to 0.1 mg into 50-ml polyethylene tubes. Milli-Q water (10 ml) was added. The tubes were shaken for 14 h. The mixtures were centrifuged at 3000 rpm. The water extracts were filtered through 0.22-μm Millex-GS cellulose ester filters (Millipore, Bedford, USA) and chromatographed on the HPLC–ICP–MS system.

2.3.2. Enzymatic hydrolysis

Approximately 0.4 g of the cereal samples was weighed to 0.1 mg into 50 ml polyethylene tubes together with 20 mg protease (pronase, Sigma, P-5147) and either 10 ml Milli-Q water or 10 ml 0.1 M citrate/phosphate buffer. The tubes were shaken at 37°C in a water bath for 4 h. After centrifugation and filtering the extracts were chromatographed on the HPLC–ICP–MS system.

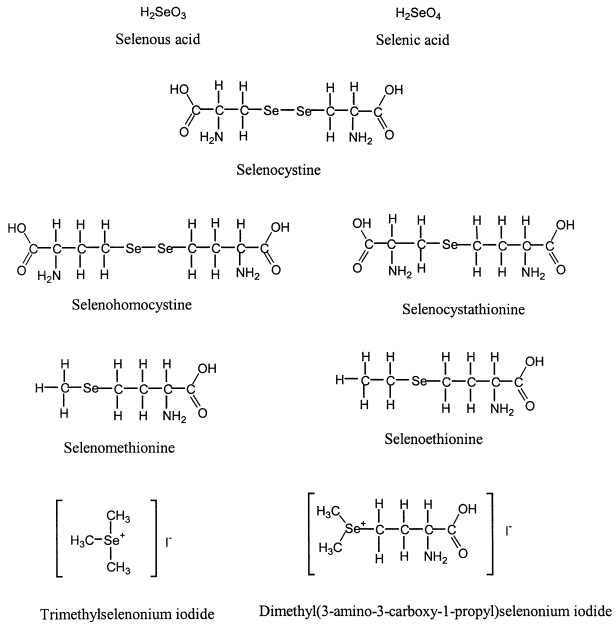

Fig. 1. Formulas for organic selenium compounds.

3. Results and discussion

Fertilisation of agricultural products with selenium supplemented products is currently not allowed in Austria. Experimental data concerning the uptake rate for plants as well as possible repercussions on agricultural soils are also not yet available.

Therefore, various laboratory experiments of growing cereals under controlled conditions to evaluate the extent of selenium uptake by plants was undertaken by the Federal Office and Research Centre for Agriculture, Vienna, Austria.

3.1. Separation technique

For the identification and quantification of selenium compounds in natural samples the development of a reliable separation technique is needed. An anion exchange HPLC coupled on-line to an ICP–MS was chosen for this work. Ion exchange columns allow the use of largely aqueous mobile phases. Higher amounts of organic solvent could destabilize or extinguish the argon plasma of the ICP–MS (Pedersen & Larsen, 1997). To prevent salt build-up on the sampler and skimmer cones the chromatographic mobile phase should contain preferably salts with organic anions, such as acetates, formiates, or citrates.

In literature most often carbonate, phosphate (Guerin, Astruc, Astruc, Batel, & Barsier, 1997), salicylate (Pedersen & Larsen, 1997), or citrate (Ge et al., 1996) were used as mobile phases for the anion chromatographic separation of selenium compounds. In this work a citrate buffer was used as mobile phase. Citric acid is a strong tricarboxylic acid, all three pK-values are in the acidic pH range ($pK_1 = 3.14$, $pK_2 = 4.77$, $pK_3 = 6.39$; Martell & Smith, 1977). At pH 8 the acid is fully deprotonated. A polymer-based PRP-X100 anion-exchange column (Hamilton) was used for the investigation of the retention behavior of selenium compounds.

Selenous acid (selenite), selenic acid (selenate), selenocystine (Secys), selenomethionine (Semet), selenohomocystine (Sehcys), selenoethionine (Seet), trimethylselenonium iodide (TmSe), dimethyl(3-amino-3-carboxy-1-propyl) selenonium iodide (DmpSe), and selenocystathionine (Secysta) were all present in solution as cations, anions or zwitterions (Fig. 1). The Hamilton PRP-X100 strong anion-exchange column has strongly basic quaternary ammonium groups as exchange sites, which are bound to the polymeric stationary phase. Negatively charged compounds, such as selenous acid and selenic acid, interact with the quaternary ammonium sites, and lipophilic selenium compounds with the hydrophobic backbone of the stationary phase. As selenoamino acids are amphoteric compounds, they can be present in solution as cations, anions, or zwitterions. Therefore, the pH of the mobile phase determines the charge and subsequently the retention behavior of these compounds. Fig. 2 shows the species distribution diagrams for selenic acid, selenous acid, selenomethionine, selenocystine, and citric acid.

The dependence of retention times on the pH of the mobile phase (5 mM citric acid) in the pH range 3.0–8.0 was also investigated using the Hamilton PRP-X100 polymer-based anion-exchange column to evaluate the optimal pH for the separation of organic (Fig. 3) and

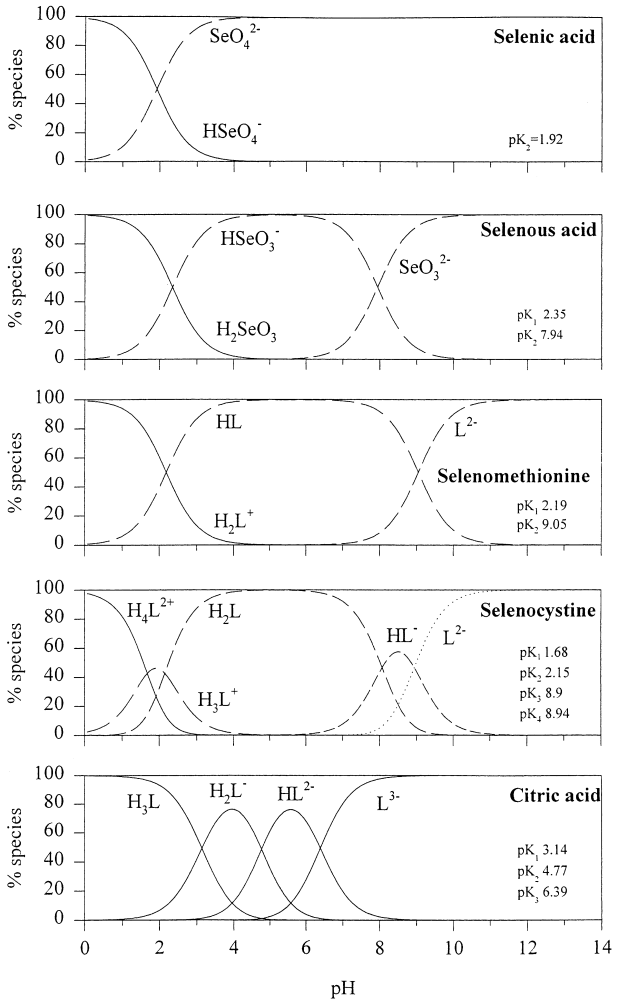

Fig. 2. Species distribution diagrams for selenic acid, selenous acid, selenomethionine, selenocystine, and citric acid in the pH range from 0 to 14. pK values taken from Martell and Smith, (1977). Rivail da Silva et al., (1997). Smith and Martell (1976).

inorganic selenium compounds (Fig. 4). According to literature (Abegaz, 1997) a buffer concentration of 5 mM citrate is well suited for the separation of selenium compounds in a reasonably short time.

DmpSe and TmSe carry positive charges at the pH-range studied. These compounds are not retained in the column and elute early. The selenoamino acids Secys, Semet, Seet, Sehcys, and Secysta are positively charged at relatively low pH-values, but will be zwitterionic (ammonium group, carboxylate group) at intermediate pH, and become anionic at higher pH. Between pH 3 and 8 all amino acids will be mainly zwitterionic. Thus, the retention times of TmSe, DmpSe, and the selenoamino acids do not show a strong dependence on the pH of the mobile phase. Retention of these compounds is mainly attributed to hydrophobic interaction with the polymeric backbone of the column.

DmpSe, TmSe, Secys, and Secysta cannot be separated easily at pH-values ranging between 5 and 8

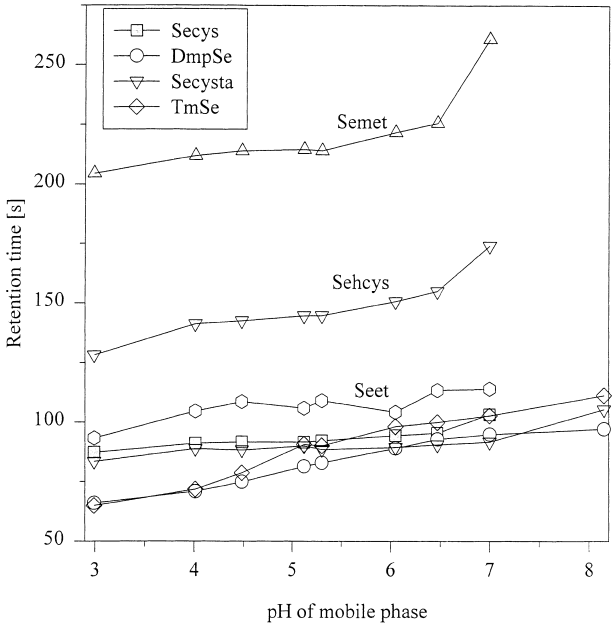

Fig. 3. Depencence of the retention times of selenocystine (Secys), selenohomocystine (Sehcys), selenocystathionine (Secysta), selenomethionine (Semet), selenoethionine (Seet), trimethylselenonium iodide (TmSe), and dimethyl(3-amino-3-carboxy-1-propyl)selenonium iodide (DmpSe) on the pH of the mobile phase (Hamilton PRP-X100 column; 20 ng ml^{-1} each; 100 μL injected; 5 mM citric acid as mobile phase; flow rate of 1.5 ml min^{-1}; ICP–MS as selenium-specific detector, m/z 82).

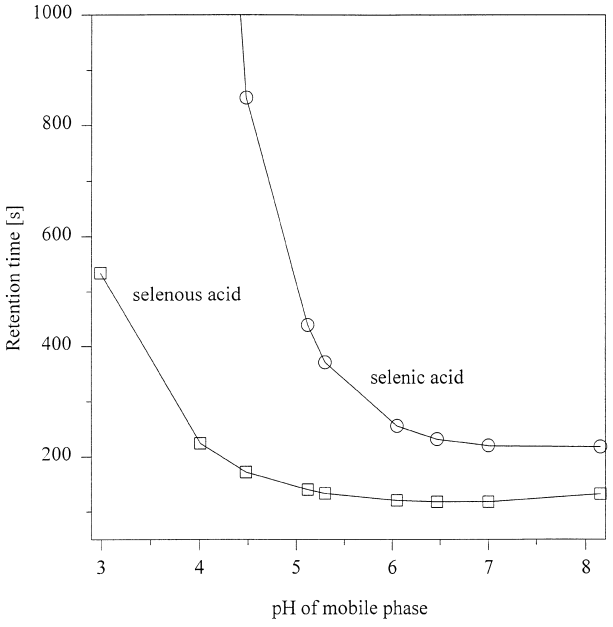

Fig. 4. Dependence of the retention times of selenic acid and selenous acid on the pH of the mobile phase, experimental parameters see Fig. 3.

(Fig. 3). At more acidic pH DmpSe and TmSe are eluted at the front (66 s at pH 3) and can thus be separated from Secys and Secysta (85 s at pH 3). The retention behavior of Secys and Secysta is quite similar, because the compounds differ only by one additional

CH_2-group. The homologous selenoamino acid selenohomocystine contains two additional CH_2-groups compared to selenocystine; with a retention time ranging from 128 (pH 3) to 174 s (pH 7); it is well separable from the other two compounds. Selenomethionine and selenoethionine are both dialkyl selenides, where one alkyl residue carries the amino and the carboxylate group. Both compounds are stronger retained in the column than Secys, which may be explained by interaction of the hydrophobic part of the molecules (alkyl chain) with the polymeric backbone. The retention time of Semet ranges from 205 s at pH 3 to 261 s at pH 7, whereas Seet elutes 100 s after injection. Obviously, the methyl group of Semet is more accessible for the hydrophobic part of the column than the ethyl group of Seet. As the signal intensity of the selenoamino acids Semet, Seet, Secys, and Sehcys decreased with increasing pH, the retention time of these compounds could not be investigated anymore at pH 8.

The retention behavior of the inorganic selenium compounds selenite and selenate (Fig. 4) is governed by the pH-controlled protonation of the Se anions and the citrate anions of the mobile phase (Fig. 2). These anions compete for the ammonium groups of the stationary phase. In the pH range studied selenic acid is fully deprotonated. Thus, the dissociation behaviour of citric acid determines the retention of selenate. At pH 3.0 50% of the fully protonated citric acid and 50% of the primary citrate anion are in equilibrium. The dinegative selenate ion could not be eluted anymore. With a pH of 4.0 the primary citrate anion is the predominant species, selenate elutes as broad peak 33 min after injection. Competition between selenate and citrate becomes important in the pH range between 4.0 and 8.0. At pH values higher than 8.0 no changes in the retention time of selenate (3.7 min at pH 8.0) can be expected anymore, because all of the citric acid is fully deprotonated.

Hydrogen selenite is the predominant species between pH 3.0 and 7.0. The retention time decreases from pH 3.0 to 6.0. At higher pH values the elution behavior does not change anymore, because the main buffer species are secondary and tertiary citrate ions.

This work was focussed to optimize the chromatographic separation of selenite, selenate, Semet and Secys, as the other selenium compounds under investigation are quite rare in nature.

Best separation of these compounds was found at pH values ranging between 4.5 and 5.0. The chromatographic signal of selenomethionine, however, is quite small and sometimes not visible even at concentrations up to 50 μg Semet l^{-1}.

The addition of small amounts of organic solvents to the mobile phase may enhance the signal intensities for selenium considerably (Goessler et al., 1997). When using a 5 mM citrate buffer pH 5.0, the four selenium compounds may be separated within 8 min (Fig. 5a); the

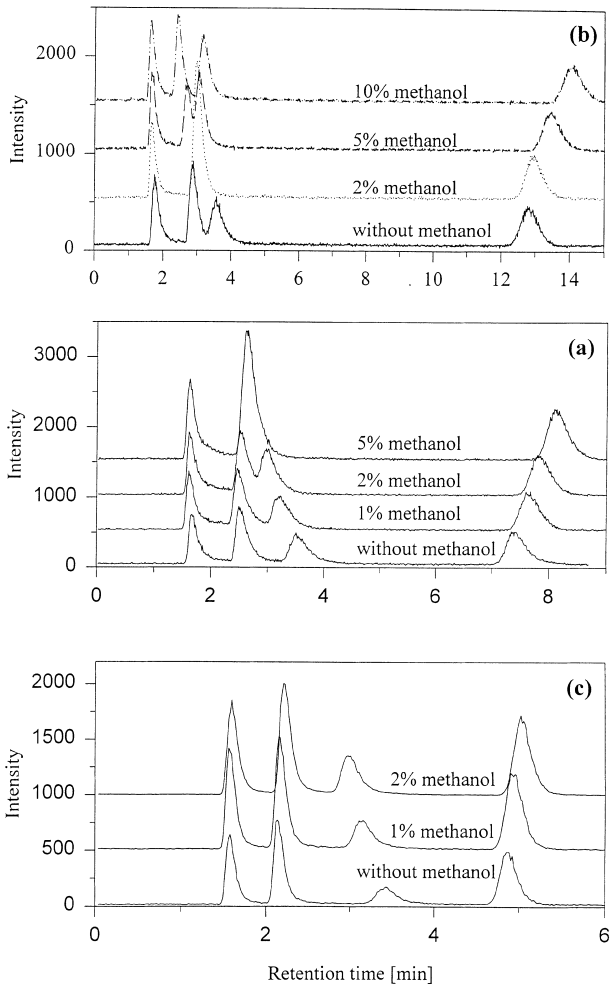

Fig. 5. Chromatograms obtained for selenocystine, selenite, selenomethionine, and selenate with mobile phases containing different amounts of methanol. Mobile phase: (a) 5 mM citric acid pH 5, (b) 2.5 mM citric acid pH 5.0, (c) 10 mM citric acid pH 5.0. (Hamilton PRP-X100 column, concentration of selenium compounds: 10 ng ml^{-1} each, 50 μl injected; flow rate of 1.5 ml min^{-1}; ICP–MS as selenium specific detector, m/z 82).

retention time of selenomethionine being 3.5 min. Upon addition of methanol, the elution of Semet shifts to a shorter retention time. The interaction of the alkyl chain of Semet with the hydrophobic backbone of the column is reduced when organic solvents are added to the mobile phase. Additionally, higher retention times of selenite and selenate are observed when the methanol concentration increases, due to a dilution of the buffer salt. Thus, with the applied buffer concentration and pH, selenite and Semet are not baseline separated anymore when 2% methanol is added. When the buffer contains 5% methanol both selenite and Semet elute with a retention time of 2.6 min.

With lower buffer concentrations selenite is shifted to longer retention times. When taking 2.5 mM citrate pH 5, selenite and Semet overlap completely at a methanol concentration of 2% (Fig. 5b). At a concentration of

10% methanol they are baseline separated again, Semet elutes at 2.4 min (compared to 4.1 min without methanol), selenite at 3.2 min. However, low buffer concentrations cause longer retention times of anionic compounds in the column; the chromatogram has to run for 15 min to elute all four compounds. Therefore, a higher buffer concentration was investigated as eluent, as the retention time of selenite decreases to a larger extent with increasing buffer concentration than the retention time of Semet. With a buffer concentration of 10 mM citrate pH 5, the four selenium compounds are eluted within 6 min (Fig. 5c). The retention time of selenite is shifted to 2.2 min compared to 3.4 min with 2.5 mM citrate, whereas for the elution of Semet it was 3.4 min (4.1 min at 2.5 mM citrate). The addition of 2% methanol to the mobile phase produces a shift of Semet to 3.0 min; thus, the chromatographic signals of selenite and Semet are well baseline separated.

The ideal case of a separation is baseline separation of the compounds with reasonably short retention times to obtain sharp signals and keep the analysis time short. All further measurements on the Hamilton PRP-X100 anion-exchange column a mobile phase containing 10 mM citric acid (pH 5) and 2% methanol was used.

3.2. HPLC of cereal extracts

The total concentration of selenium in various cereals from the growing experiment were determined by Hydride-Generation-ICP–MS (Stadlober, Goessler, & Sager, submitted). The results are listed in Table 1. The samples, which were grown under the same conditions but without the amendment with selenate (Hoesch, 1999), were very low in selenium. Amendment of the soil with Se-supplemented fertilisers resulted in an enhancement of the concentration of selenium by approximately one order of magnitude.

Water-soluble selenium compounds (selenite or selenate) are taken up from animal and human organisms easily. For investigation of the percentage recovery rates of water-soluble Se in the cereal samples, aqueous extracts were chromatographed using HPLC–ICP–MS for the identification and quantification of the compounds.

Chromatograms of aqueous extracts of the cereal samples, which were grown without supplemented selenate, did not show any evaluable signals. The total concentrations range between 10 and 25 μg Se kg^{-1}. Water extracts of cereals, which had been amended with sodium selenate supplemented fertilisers upon growing were chromatographed as well. Three to four very small signals at the retention times for selenocystine, selenite, selenomethionine and selenate were observed, the dilution factor in the extracts being ca. 25. Fig. 6 (a–d) shows a few sample chromatograms of aqueous extracts of cereal samples. Quantification of these signals, however, is difficult, because the concentrations are well below 1 μg

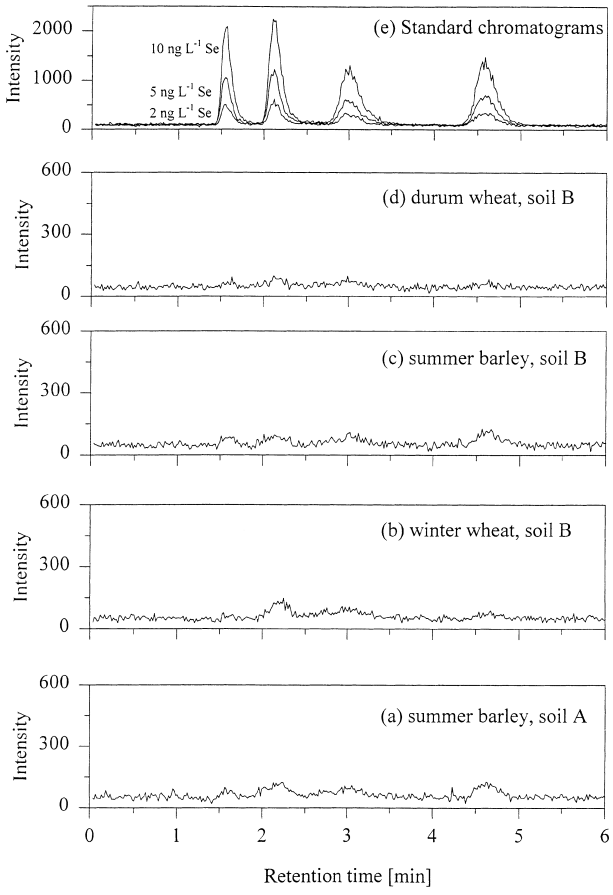

Fig. 6. Chromatograms of aqueous extracts of cereal samples, which were grown with supplemented selenate, (a) summer barely, soil A, (b) winter wheat, soil B, (c) summer barely, soil B, (d) durum, soil B, (e) standard chromatograms obtained for selenocystine, selenite, selenomethionine, and selenate, concentration of selenium compounds: 2, 5, 10 ng ml^{-1} each, experimental parameters see Fig. 5c.

Se l^{-1}. In Fig. 6(e) standard chromatograms are shown for comparison of the signal intensities. Only 3–9% of the total selenium concentration of cereal samples could be identified chromatographically in aqueous extracts.

To evaluate, whether the residual selenium is incorporated into proteins as seleno amino acids, an enzymatic extraction procedure was made. For enzymatic hydrolysis a non-specific protease (pronase) was chosen as enzyme. The pronase is able to break the peptidic bonds of any protein present in biological materials.

As extraction solvents either a buffer solution (0.1 M citrate/phosphate pH 7.3) (Gilon, Potin-Gautier, & Astruc, 1996; Olivas, Donard, Gilon, & Potin-Gautier, 1996) or pure water (Bird, Uden, Tyson, Block, & Denoyer, 1997) were tested. Both solvents released the same amount of seleno amino acids. Therefore, water was used as extraction solvent for enzymatic hydrolysis. Fig. 7 shows some sample chromatograms of enzymatic extracts of the cereals. The enzymatic hydrolysates of the cereal samples, which were grown without selenate supplemented fertilisation, exhibited two small signals

Table 1
Determination of selenium compounds in enzymatic hydrolysates of cereal samples[a]

No.	Cereals	Soil	Se-amendment[b]	Total Se (ng g^{-1} ± S.D.)[c]	Secys (ng g^{-1})	Semet (ng g^{-1} ± S.D.)[c]	Selenate (ng g^{-1})	% Recovery as Semet	% Recovery of total enzymatic extract
3	Winter wheat	Soil A	+	204 ± 19 (3)	~15	148 ± 6 (3)		73	80
5	Winter wheat	Soil A	–	11.9 ± 0.8 (3)		~11			
8	Spring wheat	Soil A	+	168 ± 8 (3)	~22	124 ± 14 (3)		74	87
10	Spring wheat	Soil A	–	6.0 ± 1.1 (3)		~6			
13	Summer barley	Soil A	+	206 ± 13 (3)	~20	127 ± 5 (3)	~16	62	79
15	Summer barley	Soil A	–	10.1 ± 2.0 (3)		~12			
18	Summer rye	Soil A	+	181 ± 14 (3)	~22	150 ± 5 (3)		83	95
20	Summer rye	Soil A	–	12.7 ± 0.6 (3)		~12			
28	Winter wheat	Soil B	+	238 ± 9 (3)	~16	162 ± 25 (3)	~12	69	80
30	Winter wheat	Soil B	–	23.1 ± 2.7 (3)		~11			
33	Spring wheat	Soil B	+	183 ± 14 (3)	~16	155 ± 8 (3)		85	93
35	Spring wheat	Soil B	–	12.2 ± 0.7 (3)		~11			
38	Summer barley	Soil B	+	188 ± 8 (3)	~16	148 ± 4 (3)		79	87
40	Summer barley	Soil B	–	6.5 ± 1.1 (3)		~5			
48	Durum wheat	Soil B	+	218 ± 30 (3)	~18	187 ± 4 (3)		86	94
50	Durum wheat	Soil B	–	24.1 ± 3.2 (3)		~15			

[a] Comparison with total selenium concentration determined with HG–ICP–MS (Stadlober et al., 1999). Experimental parameters for HPLC–ICP–MS: 10 mM citric acid pH 5, 2% methanol as mobile phase, 50 µl injected, flow rate of 1.5 ml min^{-1}, ICP–MS as selenium specific detector, m/z 82.
[b] +, Addition of 30 mg Se(VI) kg^{-1} to the multinutrient fertiliser; –, multinutrient fertiliser without selenate.
[c] Number of replicates in parentheses.

[Fig. 7(a)]. The chromatographic peak at a retention time of 3.0 min, which is more pronounced than the second one, can be identified as selenomethionine. The smaller signal with a retention time of 1.6 min could possibly be assigned to the existence of traces of selenocystine in the samples.

The cereal samples, which were amended with selenate supplemented fertilisers, took up selenate from soil, so that their selenium concentration increased by approximately one order of magnitude. Some sample chromatograms of enzymatic extracts of these cereals are shown in Fig. 7(b). The chromatographic signal corresponding to selenocystine increased only to a small extent, and sometimes a small signal of selenate appeared, similar to that observed in the chromatograms of the aqueous extracts. The signal corresponding to selenomethionine, however, increased to a large extent. The concentrations found with HPLC-ICP–MS are listed in Table 1. Obviously, almost all of the selenate taken up by the plant is converted quantitatively to selenomethionine. The recovery rates as Semet obtained with enzymatic hydrolysis range from 70 to 85%. The total extraction yields range between 80 and 95%. Enzymatic hydrolysis with non-specific proteases yielded excellent recovery rates of plant selenium as selenomethionine.

The results obtained in this work show that plants take up selenium as selenate to a large extent, when they are grown in pot experiments. The bioavailability of selenium for the human body is highly dependent on the selenium compound; the total amount of the element is not a measure for the degree of absorption (Fairweather-Tait & Hurrel, 1996). Selenium compounds

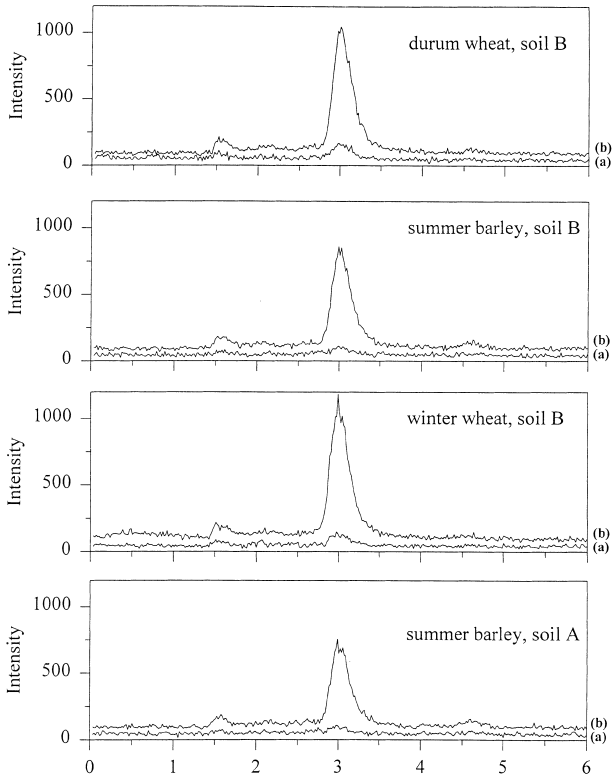

Fig. 7. Chromatograms of enzymatic hydrolysates of cereal samples, (a) cereals grown without supplemented selenate, (b) cereals grown with supplemented selenate, other experimental parameters see Fig. 5c.

that are insoluble or have low digestibility pass through the digestive tract and are excreted in the faeces. Compared to inorganic forms selenomethionine is absorbed very rapidly through the small intestine. Therefore, the

high conversion rate to selenomethionine in the cereal products suggests a good bioavailability for humans and animals.

With regard to the decision of amending nitrogen fertilisers with selenate, field experiments need to be carried out to investigate the selenium uptake rate under conditions, where the mobility of selenate and wash-out rates have to be taken into account. Additionally, the agricultural soils and the ground water in the vicinity of the rural areas should be analysed with respect to a possible contamination with selenium.

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