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Articles

Identification of Selenium-Containing Glutathione S-Conjugates in a Yeast Extract by Two-Dimensional Liquid Chromatography with Inductively Coupled Plasma MS and Nanoelectrospray MS/MS Detection

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An approach for the identification of unknown selenium-containing biomolecules was developed, enabling the identification of selenodiglutathione (GS-Se-SG) and the mixed selenotrisulfide of glutathione and cysteinylglycine (GS-Se-SCG) in aqueous yeast extracts. The method consists of two-dimensional liquid chromatography, inductively coupled plasma mass spectrometry (ICPMS) and nanoelectrospray tandem mass spectrometry. Analytes were separated by size-exclusion chromatography followed by preconcentration and separation on a porous graphitic carbon HPLC column. The HPLC effluent was monitored for selenium by ICPMS, and two selenium-containing fractions were isolated and analyzed by nanoelectrospray MS. The nanoelectrospray technique has a low sample consumption of ∼80 nL/min, enabling a preconcentration of the sample to a few microliters. Mass spectra of the two fractions showed the characteristic Se isotopic pattern centered at *m*/*z* 693.1 and 564.0 for the $[M + H]^+$ ^{80}Se ions. MS/MS spectra of adjacent parent ions confirmed the presence of Se. The two selenium species were identified as GS-Se-SG and GS-Se-SCG by collision induced dissociation (CID). The accurately measured masses of the most abundant 691 and 693 u parent ions are in good agreement (differences = 3 ppm) with the theoretical masses. To our knowledge, this is the first identification of GS-Se-SG and GS-Se-SCG in biological matrixes by MS/MS.

Since recent studies have shown that selenium supplementation in the diet can reduce the risk of several forms of cancer in humans and animals,[1−4] there is an increasing interest in the biochemistry of this essential trace element. Absorption, tissue distribution, bioavailability, and cancer preventive properties depend on the chemical species in which Se occurs in food and supplementary products,[3−5] raising a demand for analytical methods capable of identifying and determining selenium species.[6]

Although the knowledge of selenium metabolism is increasing, it is still not completely understood.[7,8] According to a reaction first described by Painter,[9] selenite can react in vitro with thiols (RSH), yielding selenotrisulfides (RS-Se-SR). Glutathione (GSH) is an abundant low-molecular-weight thiol in cells and, hence, a prime candidate for the thiol in this reaction, leading to the suggestion that selenodiglutathione (GS-Se-SG) is a key intermediate in the selenium metabolic pathway.[10−12] Gyurasics et al.[13] injected [^{75}Se] selenite into rats and identified GS-Se-SG in the bile by HPLC equipped with a radioactivity detector. But since the mixed selenotrisulfides of glutathione and cysteine or cysteinylglycine (GS-Se-SC or GS-Se-SCG) have the same retention time as GS-Se-SG, the authors could not exclude that their detected signals might be attributed to these compounds, as well. To our knowledge, this is the only paper describing the possible identification of GS-Se-SG in vivo. Hasegawa et al.[14] used HPLC with fluorometric detection to identify GS-Se-SC as an initial metabolite in mouse intestinal cytosol after oral administration of selenocystine. GS-

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Se-SG has been shown to be a strong inhibitor of erythroleukaema and neoplastic cell growth and of protein synthesis.[15−18]

Owing to the low volatility of most selenium compounds and the high sensitivity of ICPMS, selenium speciation analysis is often carried out by HPLC/ICPMS.[4,19−28] The main disadvantage of using an element-specific detector is that molecular information of the analyte is lost in the inductively coupled plasma. Therefore, the identification of species relies on a comparison of retention times and depends on the availability of commercially available matching standards. However, the analysis of real samples often reveals the presence of several selenium species, for which no matching standard is available for identification.[20−28] A promising approach to identify such selenium compounds is the use of mass spectrometry and tandem mass spectrometry. Casiot et al.[29] identified a selenium species with a molecular mass of 432 u in a HPLC fraction of an aqueous selenized yeast extract as Se-adenosylselenohomocysteine using electrospray tandem mass spectrometry (ESI-MS/MS). Kotrebai et al.[30] identified selenomethionine and Se-adenosylselenohomocysteine in an enzymatic yeast extract and γ-glutamyl-Se-methylselenocysteine in an aqueous garlic extract by HPLC/ESI-MS comparing retention times and the molecular ion masses (197, 432, and 312 u) with standards. Another selenium compound in the garlic extract, having a molecular weight of 326 u, was attributed to γ-glutamylselenomethionine. However, lacking a matching standard, no confirmation of the identity was possible. Se-methylselenocysteine was identified in an enzymatic garlic extract using the same technique.[31] McSheehy et al. confirmed the presence of γ-glutamyl-Se-methylselenocysteine in a selenium-containing HPLC fraction of an aqueous garlic extract after size-exclusion chromatography by ESI-MS/MS.[32] Using the same approach, McSheehy et al. also found three new selenium-containing species with molecular masses of 562, 584, and 603 u in aqueous selenized yeast extracts.[33] The authors demonstrated by collision-induced dissociation (CID) that these compounds contain a Se−S bridge between glutathione and three not identified selenocompounds. Another selenium species with a molecular mass of 372 u was shown to contain glutamine, but could not be identified completely, either. Montes-Bayón et al.[34] determined the molecular mass of a selenium-containing fraction of an enzymatic *Brassica juncea* extract as 344 u by HPLC/MS and suggested a Se−S-bridged seleno amino acid with a structure similar to cystine.

The main disadvantage of electrospray mass spectrometric detection is the lower sensitivity, as compared to ICPMS. To improve the detection power of selenium compound identification in real samples, the present work investigated a method using nanoelectrospray MS. Advantages of nanoelectrospray MS are the low sample consumption as well as a higher tolerance toward the presence of elevated levels of salts or buffers.[35] Therefore, the volume of HPLC fractions can be reduced to a few microliters, resulting in a considerable sample preconcentration. To reduce interfering compounds in the sample matrix, a two-dimensional chromatographic approach was chosen, separating a selenium-containing fraction obtained by size-exclusion on a second chromatographic column, consisting of porous graphitic carbon (PGC). This material has recently been used as a relatively new stationary phase for the separation of amino acids and hydrophilic peptides[36,37] and seleno amino acids[38] without a derivatization step. It exhibits not only strong reversed-phase behavior but can also retain polar compounds, possibly because of polarization of graphite π electrons. The objective of the present work was to separate and identify new selenium species in a yeast extract. In a first step, HPLC/ICPMS was applied to collect selenium-containing fractions, followed by nanoelectrospray MS/MS for structural identification of the selenium species. A hybrid quadrupole time-of-flight mass spectrometer was employed for this purpose, facilitating the identification of compounds by collision-induced dissociation over a broad mass range without significantly affecting detection limits negatively. In addition, the high mass resolution of the time-of-flight mass analyzer enables a comparison of theoretical masses with accurately measured parent and daughter fragment ion masses.

EXPERIMENTAL SECTION

Instrumentation. An Alliance 2690 separations module (Waters; Milford, MA) equipped with a vacuum solvent degasser was used as the HPLC system. Separations were carried out on a Hypercarb porous graphitic carbon (PGC) stationary phase (Thermo Quest Hypersil, Cheshire, U.K.). Selenium was monitored on-line in the effluent with a Micromass Platform ICP mass spectrometer (Micromass; Manchester, U.K.), which enables the reduction of spectral interferences by the addition of helium/

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hydrogen mixtures in a collision/reaction cell. Samples were introduced with a self-aspirating concentric Micromist nebulizer (Glass Expansion, Victoria, Australia). Mass spectra of the selenium-containing fractions were acquired on a Micromass Q-Tof-2 hybrid quadrupole time-of-flight tandem mass spectrometer (Micromass; Manchester, U.K.) equipped with a Z-spray nanoelectrospray ion source. Samples of a few microliters in size were injected into nanoflow probe tips (type F, Micromass), consisting of small glass tubes with a capillary end and a conducting coating, which were inserted into the ion source.

Chemicals. Formic acid (90%, AnalaR grade) was provided by BDH (Toronto, Canada), and heptafluorobutyric acid (99%) was purchased from Aldrich (Milwaukee, WI). Methanol (optima grade) was provided by Fisher Scientific (Fair Lawn, NJ), and Sephadex G-50 (20−80 μm), by Sigma (St. Louis, MO). Aqueous solutions were prepared with deionized water (18 MΩ cm). Selenium supplementary tablets (labeled as Selenium from Selenium Yeast, containing 200 μg Se/tablet and vitamin E) were purchased in a local drugstore in Ontario, Canada. The acidic solutions were handled with the usual safety care. Skin and eye contact were avoided as well as inhalation of fumes.

Extraction Procedure. A-24 g portion of a selenium-enriched yeast supplementary product (40 tablets) were extracted with 30 mL water in a hot water bath at 95 °C for 1 h. The extract was centrifuged and decanted. To remove solid particles, these two steps were repeated two times, and the solution was filtered (0.45-μm membrane filter).

Fractionation and Preconcentration by Size-Exclusion Chromatography. In a first chromatographic step, 5 mL of the filtered extract were introduced onto a column filled with Sephadex G-50. Chromatographic conditions are summarized in Table 1. Fractions of 4 mL size were collected during the 30-min chromatographic run. Selenium was determined in each fraction by ICPMS by self-aspirating nebulization at a flow rate of ∼30 μL/min. ICPMS conditions are listed in Table 1. The elution profile showing the ^{78}Se signal in each fraction can be seen in Figure 1. Fractions 17, 18, and 19, which had the highest selenium content, were combined, and the volume was reduced to 2 mL under a stream of nitrogen.

Chromatography of Selenocompounds on a Porous Graphitic Carbon Stationary Phase. After filtration (0.45-μm membrane filter), an aliquot of 200 μL from the preconcentrated size-exclusion eluate was injected onto a Hypersil Hypercarb column consisting of porous graphitic carbon. Fractions of this second chromatographic step were collected while intensities of different Se isotopes were observed simultaneously by ICPMS. This was achieved by T-splitting the HPLC effluent. Experimental HPLC and ICPMS conditions are summarized in Table 1. The recorded HPLC/ICPMS chromatogram is shown in Figure 2.

Nanoelectrospray Mass Spectrometry. Two fractions eluting from the porous graphitic carbon column at retention times between 29.0 and 30.7 min (fraction A) and between 24.8 and 26.4 min (fraction B) were analyzed by nanoelectrospray MS and MS/MS in order to obtain structural information of the selenium species. The solvent of the isolated fractions was evaporated under a stream of nitrogen. After dissolution in 15 μL of a solution containing 0.2 M formic acid in 10% methanol (v/v), the samples were transferred into nanoflow probe tips, which were inserted into the electrospray source of the Q-Tof-2 mass spectrometer. To obtain the highest signal intensity, the position of the probe tip was adjusted with micrometer screws. Spectra were recorded over an m/z range of 50−1550 u. MS/MS spectra were acquired in the CID mode for ions potentially containing selenium, as suggested by the observed characteristic Se isotope patterns. MS and MS/MS conditions are summarized in Table 1.

Table 1. Experimental Conditions

first chromatographic step	
stationary phase (size-exclusion)	500 × 20 mm; packed with Sephadex G-50; 20−80 μm; packing height, 300 mm.
mobile phase	300 mM formic acid
injected volume	5 mL
flow rate	3.8 mL/min
second chromatographic step, Waters Alliance 2690	
stationary phase	Hypersil Hypercarb 20 × 4 mm, 5 μm
mobile phase gradient	0−12 min, 300 mM formic acid; 12−32 min, linear ramp to 100% methanol
injected volume	0.2 mL
flow rate	0.2 mL/min
ICPMS conditions, Micromass Platform	
forward power	1600 W
plasma shielding	activated
spray chamber	Scott-type, glass, cooled to −8 °C
nebulizer	Micromist nebulizer (concentric)
nebulizer gas	Ar, 0.85 L/min
intermediate gas	Ar, 0.75 L/min
cool gas	Ar, 14 L/min
collision cell gas	He, 4 mL/min and H_2, 6 mL/min
isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se
dwell time	200 ms
ESI-MS and MS/MS conditions, Micromass Q-Tof 2	
ionization mode	electrospray, positive mode
source	Z-spray source, equipped with nanoflow interface
source temperature	70 °C
cone gas	N_2, 100 L/h
cone voltage	35 V
capillary voltage	800 V
collision gas for MS/MS	Ar, 0.3 Pa

RESULTS AND DISCUSSION

Size-Exclusion Chromatography. To reduce the matrix prior to the ESI-MS investigation, a two-dimensional chromatography approach was necessary, consisting of a size-exclusion chromatographic step and a second chromatographic separation on a porous graphitic carbon column. The first preparative scale size-exclusion chromatography serves as sample cleanup, reduces the matrix introduced onto the porous graphitic carbon column, improves the separation on this column, and decreases the noise level in electrospray mass spectrometry.

The size-exclusion elution profile of ^{78}Se shown in Figure 1a is consistent with the profile of the ^{76}Se, ^{77}Se, ^{80}Se, and ^{82}Se isotopes and their relative natural abundances, indicating that these signals are not caused by spectral interferences. Figure 1b exemplarily shows the good matching of the relative intensities of the selenium isotopes measured in fractions 8, 17, 18, and 19 with the natural abundances. As can be from Figure 1a, the extracted selenium species elute in two main fractions. The first

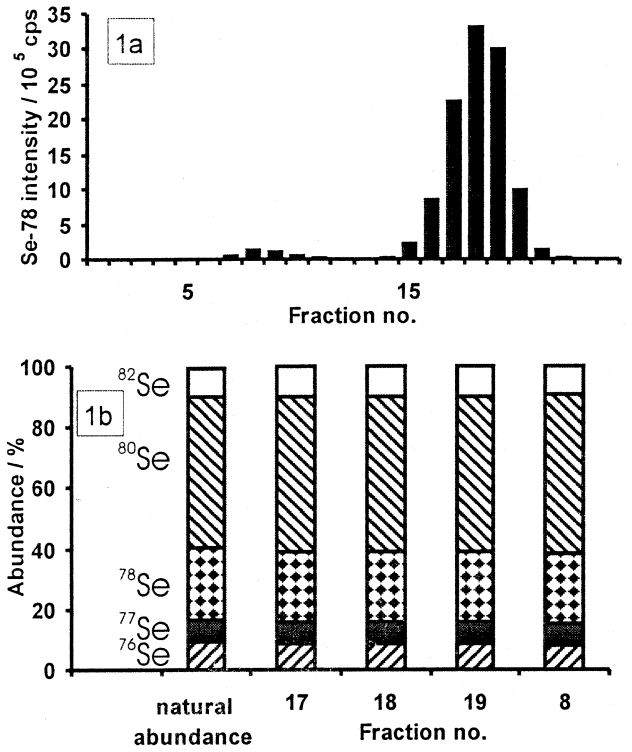

Figure 1. Elution pattern of selenium-containing compounds in yeast extract using size-exclusion chromatography monitored by ICPMS (1a). Fractions 17, 18, and 19 were combined for the second chromatographic step. The relative intensities of different selenium isotopes in fractions 8, 17, 18, and 19 are compared with the natural abundances in 1b.

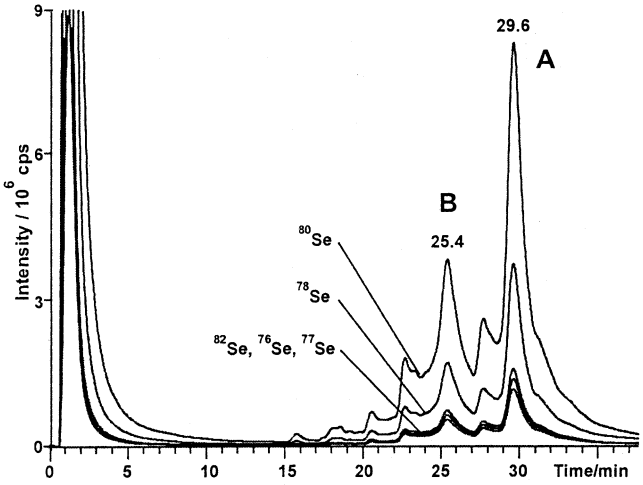

Figure 2. HPLC/ICPMS chromatogram of the combined size-exclusion chromatography fractions (17−19) using a Hypercarb column. Fractions A (29.0−30.7 min) and B (24.8−26.4 min) were collected for further analysis by nanoelectrospray MS.

high-molecular-weight fraction elutes close to the exclusion volume of the column while a more abundant low-molecular-weight fraction elutes near the total column volume. To separate selenium species in the low-molecular-weight fraction (combined fractions 17, 18, and 19), a second chromatographic step was used.

Separation of Selenium-Containing Species on a Porous Graphitic Carbon HPLC Column. Lindemann and Hintelmann[38] have demonstrated that porous graphitic carbon as a HPLC stationary phase enables the separation of selenomethionine, selenoethionine, selenocystine, selenocystamine, and Se-methylselenocysteine using a 100 × 2.1 mm column. Employing ICPMS for detection, none of these commercially available selenium species could be detected in the combined fractions from size-exclusion chromatography. Therefore, experiments were carried out using a shorter column (20 × 4 mm) to accomplish the elution of selenium species, which are retained on the column more strongly. With chromatographic conditions listed in Table 1, the chromatogram shown in Figure 2 was obtained. Formic acid was used as a mobile phase, because it can easily be removed by evaporation during the following preconcentration step. The chromatograms obtained for the ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se isotopes have the same shape, indicating that the signals are not caused by spectral interferences. Although selenite and selenate elute in the void volume, several selenium-containing peaks with retention times between 15 and 35 min can be observed. To identify the main peaks, two fractions, A and B, with the highest intensity at 29.6 and 25.4 min were collected and analyzed by nanoelectrospray MS.

Identification of Selenium Species by Nanoelectrospray MS and MS/MS. Since electrospray mass spectrometry is less sensitive than ICPMS, fractions A and B eluting from the porous graphitic carbon column had to be preconcentrated. This was achieved by evaporation of the solvent under a stream of nitrogen followed by dissolution in 15 μL of 0.2 M formic acid in 10% methanol, obtaining a preconcentration factor of approximately 20. Formic acid was used to protonate molecules to increase the intensity of $[M + H]^+$ ions in the positive ionization mode. Methanol reduces the surface tension and, therefore, enhances the formation of small droplets during the electrospray process. Nanoelectrospray,which is capable of handling these small amounts of sample, was used as the sample introduction system for the mass spectrometry. When a capillary voltage of 800 V was used, the low flow rate of 80 nL/min provided sufficient time (approximately 3 h/sample) to record MS/MS spectra of several parent ions.

Identification of Selenodiglutathione in Fraction A. The nanoflow electrospray mass spectrum of the preconcentrated fraction A is shown in Figure 3. This spectrum was analyzed for the presence of the characteristic selenium isotopic pattern, which was eventually detected, centered at m/z 693.1 for the $[M + H]^+$ ^{80}Se ion. As already noticed by McSheehy et al.,[33] the ESI-MS signals are difficult to distinguish from the noise. To our knowledge, no selenium-containing compound with a molecular mass of 693.1 u has been identified previously by mass spectrometry. To obtain structural information of this selenium species, MS/MS spectra of the protonated molecular ions containing different Se isotopes (m/z 689.1, 690.1, 691.1, 693.1, and 695.1) were acquired in the collision-induced dissociation mode. As can be seen in Figure 4, the masses of the selenium-containing fragment ions from adjacent parent ions (printed in bold) show the characteristic mass shift depending on the corresponding selenium isotope in the parent ion, whereas the daughter ions that do not contain selenium (underlined in Figure 4) have the same masses in all five MS/MS spectra. This was considered to be proof for the presence of Se in these molecular ions. The interpretation of the spectra reveals the presence of several amino acids, indicating a peptide structure.

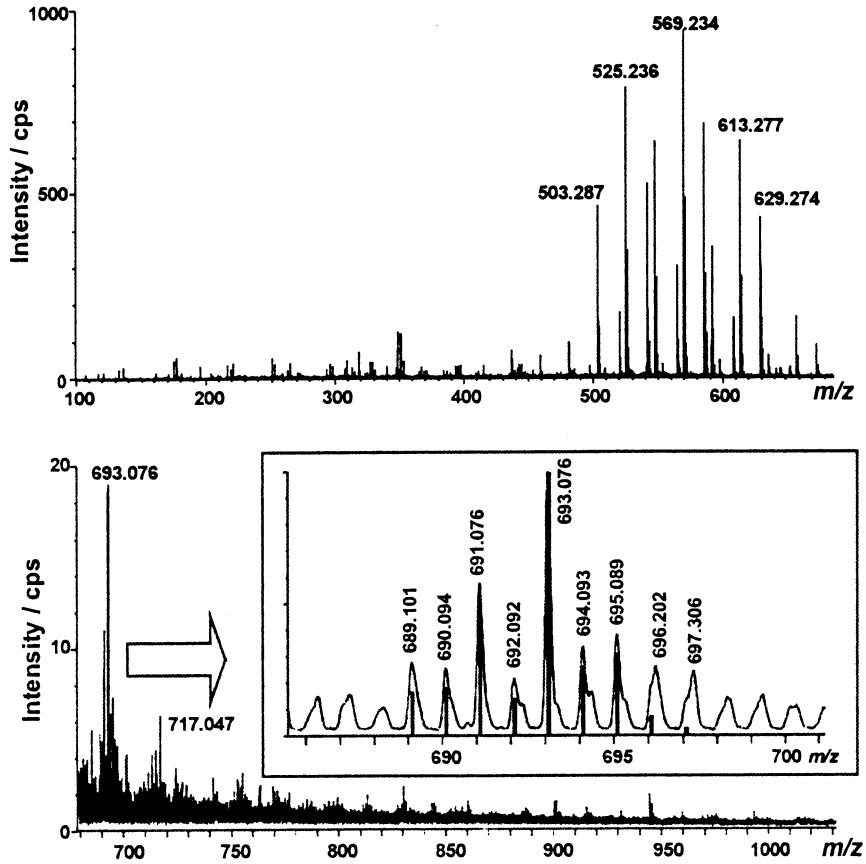

Figure 3. Nanoelectrospray MS spectrum of fraction A. The inset shows the section of the spectrum where the molecular ions showing the characteristic Se isotope pattern were detected, including the modeled isotope distribution of selenodiglutathione (bars).

The following discussion refers to the MS/MS spectrum of the protonated 693.1 u (^{80}Se) molecular ion, as shown in Figure 5. The most intense peaks correspond to a loss of two γ-glutamic acid residues (Glu + Glu, −258 u, m/z 435) and to the protonated cysteinylglycine dipeptide (H-Cys-Gly-OH) at m/z 179. The daughter ion at m/z 618 is caused by the loss of C-terminal glycine (Gly + H_2O, −75 u), and the daughter ion at m/z 564 is caused by the loss of a N-terminal γ-glutamic acid residue (Glu, −129 u). Other significant daughter ions include the loss of glycine and γ-glutamic acid (Gly + H_2O + Glu, −204 u, m/z 489), the loss of glutathione (H-Glu-Cys-Gly-OH, GSH, −307 u, m/z 386), the loss of glutathione and γ-glutamic acid (GSH + Glu, −436 u, m/z 257), and the loss of glutathione and C-terminal glycine (GSH + Gly + H_2O, −382 u, m/z 311). These daughter ions, the protonated glutathione fragment (GSH + H^+, m/z 308), and the γ-glutamyl-cysteinyl cation (H-Glu-Cys$^+$, m/z 233) lead to the hypothesis that selenodiglutathione is the selenium-containing species in fraction A. This was confirmed by comparing the accurate molecular ion masses obtained in MS mode (Figure 3) with the isotopic distribution modeled with the Micromass Masslynx 3.5 software for different Se, C, N, O, and S isotopes, considering their natural distribution. The TOF mass spectrometer measures molecular masses with an accuracy of ±5 ppm for molecules <1000 Da as long as they are not interfered by matrix compounds and count rates are high enough to obtain appropriate counting statistics. The accuracy is based on the stability of the reflectron time-of-flight mass analyzer and the high-resolution power ($m/\Delta m$ = 10.000 full width at half-maximum) of the instrument. The differences between measured and calculated masses were <3 ppm for the most abundant ions of 691 and 693 u (Table 2). Deviations for the ions at 689, 690, 692, 694, and 695 u were slightly higher, because these signals exhibited some interference by matrix components. These accurate results confirm the identification of selenodiglutathione as the selenium species in fraction A. This identification is further supported by the fact that the daughter ion masses measured in MS/MS mode show a good agreement with the masses calculated (Table 3). The differences (up to 130 ppm) are higher than those measured in MS mode, since the instrument was calibrated in MS mode to obtain the most accurate masses for the molecular ions. Additionally, the lower signal intensities of the fragment ions affected counting statistics negatively.

Table 2. Comparison of Measured and Theoretical Masses of Protonated Selenodiglutathione

meas mass [u]	theor mass [u][39]	$\Delta m/m$ [ppm]	molecular ion (unprotonated)
689.101	689.079	32	$C_{20}H_{32}N_6O_{12}S_2{}^{76}Se$
690.094	690.080	21	$C_{20}H_{32}N_6O_{12}S_2{}^{77}Se$
691.076	691.077	−2	$C_{20}H_{32}N_6O_{12}S_2{}^{78}Se$
692.092	692.081	17	$^{12}C_{19}{}^{13}CH_{32}N_6O_{12}S_2{}^{78}Se$
693.076	693.076	0	$C_{20}H_{32}N_6O_{12}S_2{}^{80}Se$
694.093	694.080	19	$^{12}C_{19}{}^{13}CH_{32}N_6O_{12}S_2{}^{80}Se$
695.089	695.077	18	$C_{20}H_{32}N_6O_{12}S_2{}^{82}Se$

Identification of the Mixed Selenotrisulfide of Glutathione and Cysteinylglycine in Fraction B. To identify the selenocom-

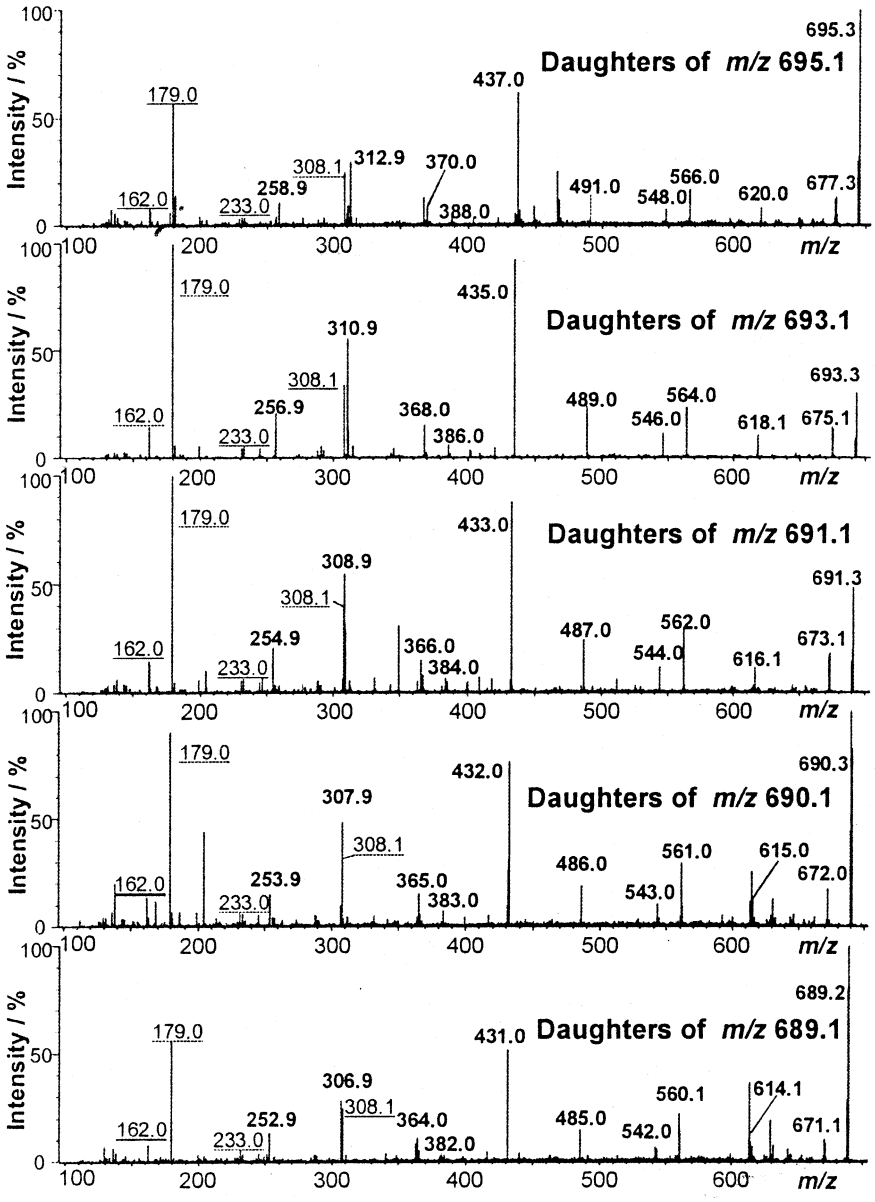

Figure 4. Collision-induced dissociation mass spectra (MS/MS) of the molecular ions in fraction A (selenodiglutathione) containing different Se isotopes. Se-containing daughter ions are annotated in bold, masses of daughter ions without Se are underlined. Collision energy: 30 eV.

Table 3. Comparison of Measured and Theoretical (Protonated) Daughter Ion Masses of Seleneodiglutathione (*m/z* 693.1, ^{80}Se)

meas mass [u]	theor mass [u][39]	$\Delta m/m$ [ppm]	molecular ion (unprotonated)
162.040	162.023	108	$C_5H_7NO_3S$
179.070	179.049	117	$C_5H_{10}N_2O_3S$ (Cys-Gly)
233.090	233.060	130	$C_8H_{12}N_2O_4S$ (Glu-Gly)
256.980	256.950	117	$C_5H_8N_2O_3SSe$
308.120	308.092	92	$C_{10}H_{17}N_3O_6S$ (GSH)
310.990	310.960	95	$C_8H_{10}N_3O_4SSe$
368.030	367.982	131	$C_{10}H_{13}N_3O_5SSe$
386.030	385.993	97	$C_{10}H_{15}N_3O_6SSe$
435.030	434.983	107	$C_{10}H_{18}N_4O_8S_2Se$
489.040	489.002	78	$C_{13}H_{20}N_4O_7S_2Se$
546.070	546.023	86	$C_{15}H_{23}N_5O_8S_2Se$
564.060	564.034	47	$C_{15}H_{25}N_5O_9S_2Se$
618.100	618.044	90	$C_{18}H_{27}N_5O_{10}S_2Se$
675.110	675.066	66	$C_{20}H_{30}N_6O_{11}S_2Se$

pound in fraction B, a nanoelectrospray mass spectrum of this preconcentrated fraction was acquired (Figure 6). The characteristic selenium isotopic pattern was found centered at *m/z* 564.0, although it is interfered by another larger signal at *m/z* 564.3. To obtain structural information of this selenium species, MS/MS spectra of the molecular ions *m/z* 560.0, 561.0, 562.0, 564.0, and 566.0, corresponding to the adjacent Se isotopes, were recorded in the collision-induced dissociation mode (Figure 7). The selenium-containing daughter ions from adjacent parent ions (masses printed in bold) show the characteristic mass shift corresponding to the selenium isotope in the parent ion, but daughter ions that do not contain selenium (masses underlined in Figure 7) have the same masses in all five MS/MS spectra. This was considered to be a proof for the presence of selenium in this compound. Similarly to the MS/MS spectra of the selenium compound in fraction A, these spectra show the presence of several amino acids.

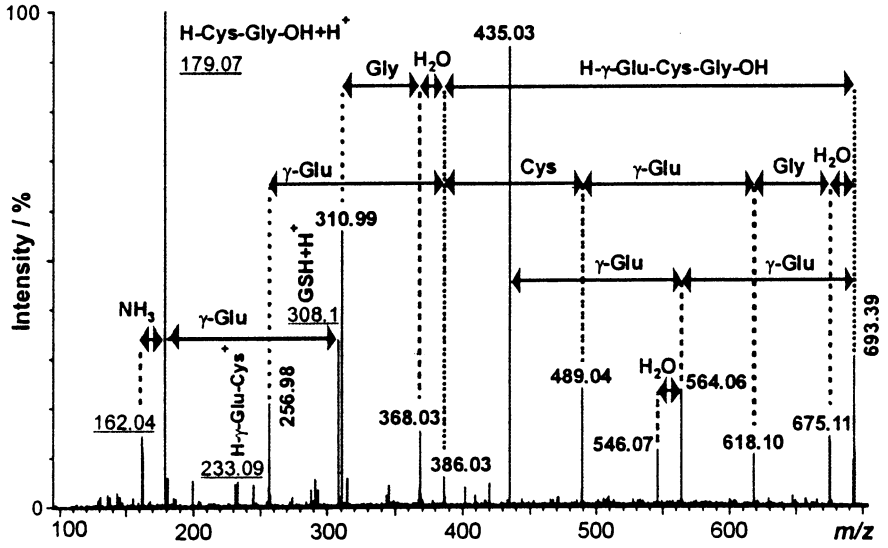

Figure 5. Identification of the selenocompound in fraction A by collision-induced dissociation of the 693.1 molecular ion as selenodiglutathione. Collision energy: 30 eV.

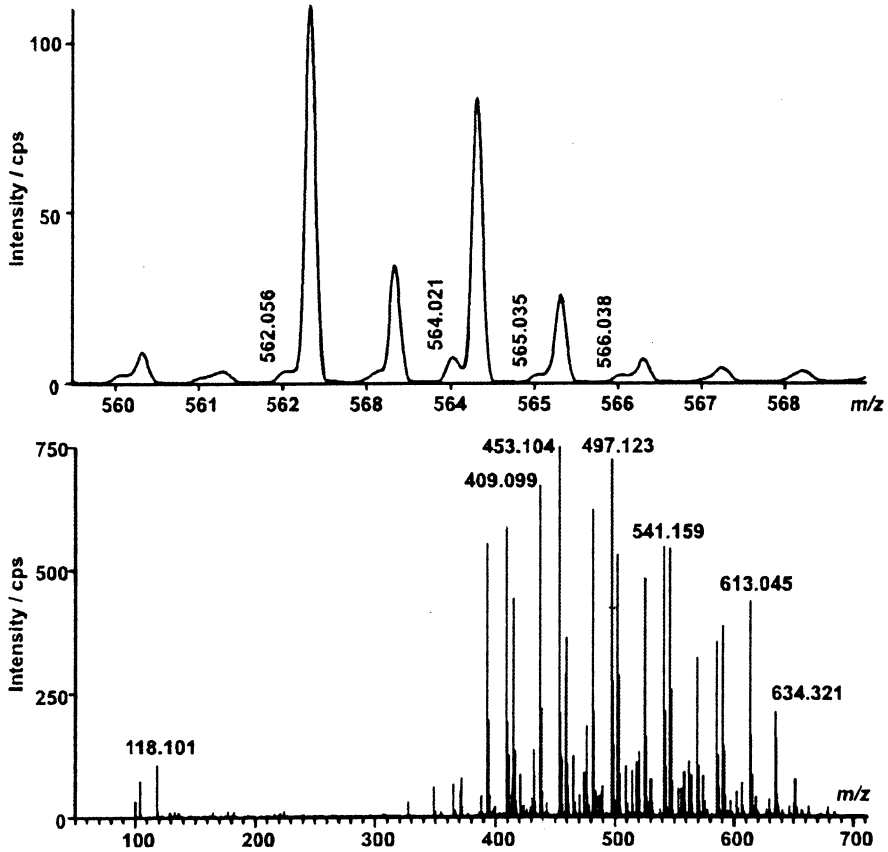

Figure 6. Nanoelectrospray mass spectrum of fraction B. The section of the spectrum where the molecular ions showing the characteristic Se isotope pattern were detected is shown on the top, including the modeled isotope distribution of the mixed selenotrisulfide of glutathione and cysteinylglycine (bars).

The most intense peaks in the MS/MS spectrum of the 564.0 parent ion (Figure 8) correspond to the protonated cysteinylglycine dipeptide (H-Cys-Gly-OH) at m/z 179 and to the loss of a N-terminal γ-glutamic acid residue (Glu, −129 u, m/z 435). Other daughter ions were caused by the loss of C-terminal glycine (Gly + H_2O, −75 u, m/z 489), partly followed by a loss of cysteinylglycine (H-Cys-Gly-OH, −178 u, m/z 311) and by protonated glutathione (GSH + H^+, m/z 308) and by the γ-glutamylcysteinyl cation (H-Glu-Cys^+, m/z 233). This fragmentation pattern leads to the hypothesis that the parent ion is the mixed selenotrisulfide of glutathione and cysteinylglycine (GS-Se-SCG). This suggestion was further confirmed by the fact that the accurate masses obtained in MS mode (Figure 6) are in good agreement with the isotopic distribution modeled with the Micromass Masslynx software (Table 4). The mass differences of ±38 ppm are somewhat higher than those for selenodiglutathione, owing to stronger signal

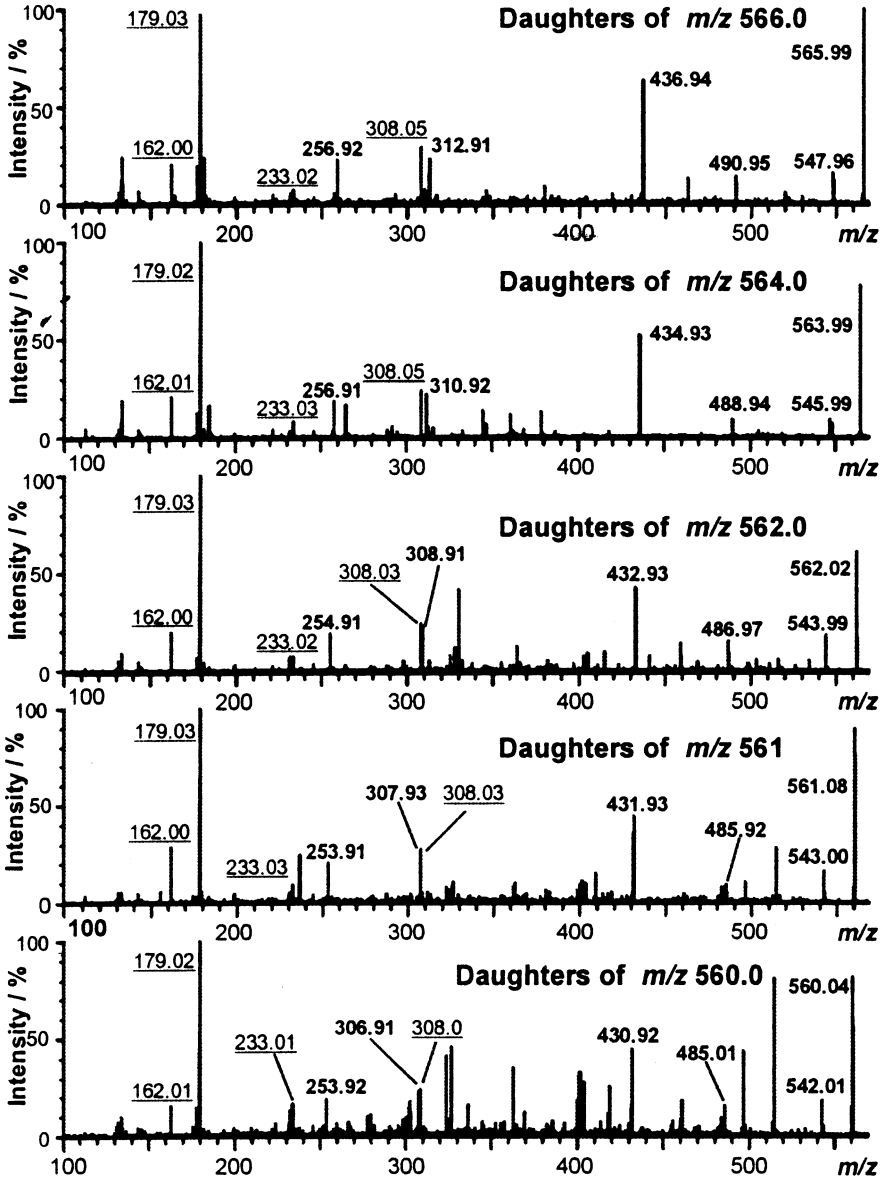

Figure 7. Collision-induced dissociation mass spectra (MS/MS) of the molecular ions in fraction B containing different Se isotopes. Se-containing daughter ions are annotated in bold; masses of daughter ions without Se are underlined. Collision energy: 25 eV.

Table 4. Comparison of Measured and Theoretical Masses of Protonated GS-Se-SCG

meas mass [u]	theor mass [u][39]	$\Delta m/m$ [ppm]	molecular ion (unprotonated)
562.056	562.035	38	$C_{15}H_{25}N_5O_9S_2{}^{78}Se$
564.021	564.034	−23	$C_{15}H_{25}N_5O_9S_2{}^{80}Se$
565.035	565.037	−4	$^{12}C_{14}{}^{13}CH_{25}N_5O_9S_2{}^{80}Se$
566.038	566.034	7	$C_{15}H_{25}N_5O_9S_2{}^{82}Se$

Table 5. Comparison of Measured and Theoretical (Protonated) Daughter Ion Masses of GS-Se-SCG (*m/z* 564.0, ^{80}Se)

meas mass [u]	theor mass [u][39]	$\Delta m/m$ [ppm]	molecular ion (unprotonated)
162.010	162.023	−77	$C_5H_7NO_3S$
179.020	179.049	−162	$C_5H_{10}N_2O_3S$ (Cys-Gly)
233.030	233.060	−127	$C_8H_{12}N_2O_4S$ (Glu-Gly)
256.910	256.950	−155	$C_5H_8N_2O_3SSe$
308.050	308.092	−135	$C_{10}H_{17}N_3O_6S$ (GSH)
310.920	310.960	−130	$C_8H_{10}N_3O_4SSe$
434.930	434.991	−141	$C_{10}H_{18}N_4O_8S_2Se$
488.940	489.002	−126	$C_{13}H_{20}N_4O_7S_2Se$
545.990	546.023	−61	$C_{15}H_{23}N_5O_8S_2Se$

interference by matrix components, which are not completely separated. Additional support for the identification of GS-Se-SCG are the calculated masses of the fragmentation products that are in good agreement with the masses measured in MS/MS mode (Table 5). These results confirm the identification of GS-Se-SCG as the selenium species in fraction B.

CONCLUSIONS

The use of HPLC/ICPMS in combination with MS/MS is an attractive arrangement to identify selenium species, for which no matching standards are available. Selenium-containing compounds are detected by HPLC/ICPMS, and the molecular structure of selenium species in isolated and concentrated fractions are determined by MS and MS/MS. Nanoelectrospray MS is a

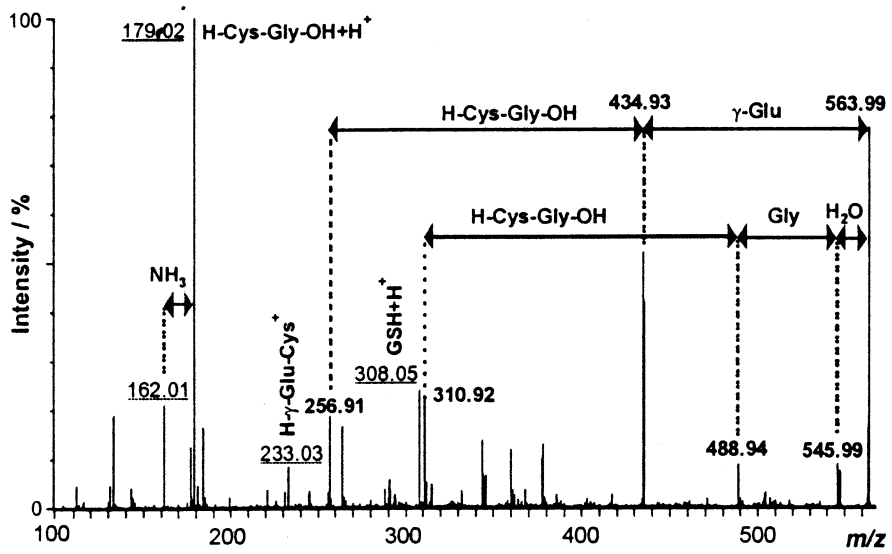

Figure 8. Identification of the selenocompound in fraction B by collision-induced dissociation of the 564.0 u molecular ion as GS-Se-SCG. Collision energy: 25 eV.

promising technique for the acquisition of mass spectra, when only small sample volumes are available. This allows an effective preconcentration of the analyte, resulting in a substantial increase in the sensitivity of the method. A two-dimensional chromatographic separation was necessary to reduce matrix and noise interferences during MS detection.

The use of a hybrid quadrupole time-of-flight mass spectrometer enables data acquisition over a broad mass range without significant loss of sensitivity and allows the acquisition of MS/MS spectra by collision-induced dissociation. The high mass resolution and accurate determination of parent and daughter ion masses allow a comparison with theoretical masses, which provides further confirmation when identifying new compounds. With the combination of these techniques, we were able to ascertain GS-Se-SG and GS-Se-SCG in an aqueous yeast extract. These findings support the hypothesis that selenodiglutathione is a key intermediate in the selenium metabolic pathway.

Abbreviations. GS-Se-SG, selenodiglutathione; GSH, glutathione; GS-Se-SCG, mixed selenotrisulfide of GSH and cysteinylglycine; GS-Se-SC, mixed selenotrisulfide of GSH and cysteine; γ-Glu, γ-glutamic acid residue, $NHCH(COOH)CH_2CH_2CO$; Cys, cysteinyl residue, $NHCH(CH_2SH)CO$; Gly, glycine residue, $NHCH_2CO$.

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