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Selenium Speciation by High-performance Liquid Chromatography with On-line Detection by Atomic Absorption Spectrometry

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Several approaches to the determination of selenomethionine, selenocystine, selenite and selenate by high-performance liquid chromatography with on-line detection by atomic absorption spectrometry are described. The *N*-2,4-dinitrophenyl derivatives of selenomethionine, selenoethionine, selenocystine and phenylmercury(II) cystineselenoate were recovered from aqueous solution, separated on a Nucleosil 5-NO_2 reversed-phase HPLC column with a methanolic mobile phase containing acetic acid and triethylamine, and detected with a quartz thermochemical hydride-generating interface–atomic absorption spectrometry (AA) system. The restriction of having to perform chromatography with an organic mobile phase (to support the combusion process) was overcome with a new interface design capable of operation with either organic or aqueous HPLC mobile phases. Using aqueous acetic acid (0.015% v/v) containing 0.1% (w/v) ammonium acetate delivered at 0.5 $cm^3 min^{-1}$, selenate, selenite, selenomethionine, selenocystine and selenoethionine were separated virtually to baseline on a cyanopropyl-bonded phase HPLC column. Other selenium compounds which were investigated included methane seleninic and methane selenonic acids as well as the crude oxidation product mixtures resulting from the treatment of selenomethionine and selenocystine with hydrogen peroxide. A procedure for extracting selenate, selenite, selenomethionine, selenocystine and selenoethionine from spiked water or ground feed supplement into liquefied phenol resulted in acceptable recoveries for the latter four analytes but was unacceptably low for selenate.

Keywords: selenium speciation; high-performance liquid chromatography–atomic absorption spectrometry; seleno oxyanions; selenoamino acids

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INTRODUCTION

Selenium is an essential ultratrace element for mammals, as well as for avians and several species of bacteria, yet exposure to and utilization by organisms of the different compounds containing this element are characterized by a surprisingly narrow rate for optimal biological activity.[1–3] Ingestion/exposure to higher levels of selenium compounds has resulted in well-characterized incidences of toxicity. Since the first reports[4–6] in 1957 of the essentiality of selenium, continued progress has been made in identifying the natural selenium compounds in biological media and in elucidating their function. Selenium has been identified as a constituent of numerous proteins and enzymes yet, collectively, the identified compounds typically account for only a small fraction of the total selenium present in many samples. Since food is the primary environmental medium through which man and animals are exposed to selenium, analytical methods which will provide reliable estimates of the component selenium-containing compounds which collectively make up the total concentration of this element in the food/feed are an essential prerequisite to predicting the availability of this micronutrient/toxicant to ingesting organisms.

Analytical techniques for the determination of total levels of selenium[7] have been developed to the point where they are considered to provide reliable estimates, and progress consists of fine-tuning procedures for specific matrices. There has also been continued progress in analytical methods for the determination of specific selenium compounds.[8]

One popular approach to the determination of specific selenium analytes has been to couple a chromatographic separation with on-line detection by atomic spectrometry. The use of various forms of HPLC for identification and quantitation of selenium compounds has been reviewed by

several authors.[9–11] We had previously reported the use of a thermochemical hydride-generating (THG) interface for the determination by atomic absorption spectrometry (AA) of selenonium compounds in HPLC column eluate.[12,13] The principal shortcoming of the THG device was the requirement for an organic-rich mobile phase (≥60% methanol) to support the pyrolysis process which, in turn, severely limited the separatory modes which could be used for the chromatographic stage of this hyphenated technique. This limitation has been circumvented with a new interface design which provides sensitive responses to arsenic or selenium[14] or a variety of heavy metals[15] (cadmium, zinc, lead, copper or mercury) contained in either an organic or an aqueous mobile phase. The objective of the current study was to develop rapid screening procedure(s) for readily extractable selenium compounds in natural matrices.

MATERIALS AND METHODS

Instruments

The chromatographic system used in these studies consisted of a Beckman Model 100A pump connected in series with an autosampler (LKB Model 2157), and an HPLC column. Column effluent was directed,via an all-silica T-tube interface, to an atomic absorption spectrometer (Philips PU9100 set to 196.0 nm) equipped with a high-energy selenium hollow-cathode lamp (Super Lamp, Photron Pty, Victoria, Australia) and a deuterium background correction system. The T-tube interface served to nebulize and pyrolyse the column eluate and to conduct/direct the pyrolysis product(s) through the optical beam of the spectrometer. Chromatograms were developed by reversed-phase chromatography on either a silica-based *p*-nitrophenyl stationary phase (5 μm Nucleosil 5-NO_2, 15 cm × 0.46 cm, CSC Ltd, Montréal, Qué., Canada) column or a 5 μm silica-based cyanopropyl (15 cm × 0.46 cm, LC-CN, Supelco Inc., Bellefonte, PA, USA) column or by ion-exchange chromatography on a strong anion-exchange phase (IC PAK HR, 7.5 cm × 0.46 cm, Millipore Waters Chromatography, Montréal, Qué., Canada, or PL-SAX, 8 μm, 15 cm × 0.46 cm, Polymer Laboratories, Amherst, MA, USA). The continuous signal from the AA provided a selenium-selective chromatogram which was recorded with the recording integrator (Hewlett–Packard, Model 3390A).

Two different all-silica interface designs were used during separate parts of these studies. The thermochemical hydride-generating (THG) interface (prototype 1) was as described[12] previously. In operation, liquid column eluate contained in a silica capillary transfer line (20 cm × 0.050 mm i.d.) was nebulized by thermospray effect into an oxygen-rich atmosphere and pyrolysed in a diffused flame maintained within a pyrolysis chamber. Hydrogen added to the downstream portion of this chamber enhanced the formation of hydrogen selenide,[12] which was swept by the expanding gases through a second cool microflame maintained just upstream from the unheated optical tube of the interface. Optimized gas flows to this interface were 650 and 1700 $cm^3 min^{-1}$ of oxygen and hydrogen to the pyrolysis chamber and 170 $cm^3 min^{-1}$ oxygen to the analytical flame.

The second interface[14] (prototype 2) represented a modification and simplification of the THG design. Eluate from the HPLC column was nebulized by thermospray effect[12,14] into a combustion chamber containing a diffused flame maintained by separate flows of oxygen and hydrogen to the base of the chamber. External radiative heating was provided by a heating coil which surrounded the combustion chamber. Combustion products were entrained directly into the unheated optical tube, which was positioned within the optical beam of the spectrometer. A maximum response to selenium compounds, delivered directly to the interface at 0.7 $cm^3 min^{-1}$ in an aqueous mobile phase, was obtained with 60 and 1950 $cm^3 min^{-1}$ of oxygen and hydrogen, respectively. In addition to a somewhat enhanced response to selenium analytes, this design was compatible with either organic or aqueous mobile phases.

Procedures

Preparation of N-DNP selenoamino acids

To 20 μg (as Se) of selenomethionine, selenocystine or phenylmercury(II) (*ϕ*-Hg) cysteineselenoate contained in 5 cm^3 phosphate buffer (pH 9.0; ionic strength, 0.2 mol dm^{-3}) was added 1 cm^3 of freshly prepared 10% (w/v) methanolic 2,4-dinitrofluorobenzene (DNFB). The reaction was maintained under nitrogen in the dark for 2 h, then extracted three times with 5 cm^3 benzene to remove excess DNFB. The reaction mixture

was acidified to pH 2 with 1 mol dm^{-3} HCl and further extracted three times with 5 cm^3 diethyl ether. The ether extracts were combined, dried over anhydrous sodium sulphate and filtered, and the filtrate was evaporated, at 40 °C, to dryness under a gentle stream of nitrogen. The residue wass redissolved in 3 cm^3 methanol and stored in the dark at 4 °C to await analysis by HPLC–THG–AA.

Phenol extraction

Ground plant material (0.5 g), in a 25-cm^3 centrifuge tube, was vortexed for 1 min with 7 cm^3 distilled deionised (DD) water which had been heated to 80 °C then centrifuged at 2000 rpm. The supernatant fraction was removed and replaced with 7 cm^{-3} fresh hot solvent and the extraction repeated twice. The three supernatant fractions were combined, transferred quantitatively to a separatory funnel, mixed with 1.5 cm^3 glacial acetic acid (17.4 mol dm^{-3}), then diluted to approximately 25 cm^3 with water. Alternately, an aqueous sample (20–25 cm^3) was acidified with 1.5 cm glacial acetic acid. The acidified aqueous solution was extracted three times with liquefied phenol (1×10 cm^3 and 2×5 cm^3). The phenol washes were combined, diluted with 70 cm^3 diethyl ether, then back-extracted three times with 5 cm^3 DD water. The combined aqueous extracts were washed three times with 5 cm^3 diethyl ether. The aqueous phase was evaporated to dryness under reduced pressure at 37 °C and the residue was resolubilized in 2 cm^3 DD water, filtered through a 0.45 μm filter and analysed by HPLC–AA.

Reactions of selenomethionine and selenocystine with hydrogen peroxide

Aqueous selenomethionine or selenocystine (5 cm^3, 20 μg cm^{-3}) was mixed with 0.1 cm^3 30% hydrogen peroxide and reacted overnight. The resulting solutions were evaporated virtually to dryness and the residue, dissolved in 5 cm^3 water, was analysed by HPLC–AA. Mobile phase [0.1% (w/v) ammonium carbonate (which had been adjusted to pH 8.0 with aqueous ammonia)] was delivered, at 0.6 cm^3 min^{-1}, to the PL-SAX column (15 cm × 0.46 cm i.d.).

Synthesis of standards

***Warning*: Since many of the reagents and intermediates used in the synthetic procedures described below are reactive and noxious, these preparations should be carried out in an efficient fume hood.**

Methaneseleninic acid

Following the method of Bird and Challenger,[16] sodium formaldehydesulphoxylate (2.5 g, 21.1 mmol), sodium hydroxide (2.5 g, 62.5 mmol) and powdered selenium (3.2 g, 40.5 mmol) suspended in 25 cm^3 water were stirred for 30 min (by which time the selenium had dissolved completely), then amended with the dropwise addition of 2.1 cm^3 (22.4 mmol) dimethyl sulphate. The reaction mixture was refluxed gently at 40–50 °C for 4–5 h, then amended with 10 cm^3 water, which caused a red oil (dimethyl selenide) to separate from the reaction mixture. After removal of the aqueous phase, 6.5 cm^3 of hydrogen peroxide (30% v/v) was added to the residual oil and the mixture was refluxed for a further 30 min. The crude product mixture was concentrated to a small volume and set aside to crystallize as white needles. Repeated crystallization from heptane/methanol furnished an analytical sample, m.p. 131–134 °C (lit. m.p. 131–133 °C).[17,18]

Potassium methaneselenoate

Following the method of Bird and Challenger,[16] methaneseleninic acid (1 g, 7.9 mmol) and potassium hydroxide (0.15 g, 2.7 mmol) in 10 cm^3 DD water was treated with potassium permanganate (0.84 g, 5.3 mmol, in 10 cm^{-3} DD water), added in small portions during 10 min. The product mixture was cooled in ice–water and filtered, and the filtrate was evaporated to dryness. The residue was crystallized from ethanol to afford white crystals. This material has been reported[16] to decompose vigorously on heating.

Triphenylphosphine selenide

Following a method for the preparation of triphenylphosphine sulphide,[19] a suspension of triphenylphosphine (1.66 g, 6.3 mmol) and powdered selenium (0.5 g, 6.3 mmol) in 20 cm^3 diethyl ether was stirred overnight. Toluene (30 cm^3) was added to the crude mixture to dissolve crystals of the product selenide which had separated. Filtration removed metallic selenium and the filtrate was evaporated (37 °C, partial pressure) nearly to dryness. The residue, dissolved in 40 cm^3 of 3 : 1 (v/v) ethanol/toluene, was refrigerated to crystallize the product. Recrystallization from ethanol furnished an analytical standard (m.p. 185–186 °C, lit. m.p. 184–186 °C).[16,20]

Phenylmercury(II) cysteineselenoate

Following a method[21] for the preparation of methylmercury(II) cysteineselenoate, excess

$NaBH_4$ (50 mg) slurried in water was slowly added to a solution of selenocystine (50 mg, 0.15 mmol) in 15 cm^3 water containing 1 mol dm^{-3} sodium hydroxide. During 20 min stirring under nitrogen, the mixture gradually became colourless. Sufficient 1 mol dm^{-3} HCl was added to destroy excess tetrahydroborate and to reduce the pH to 4. Phenylmercuric acetate (100 mg, 0.3 mmol) and the reaction mixture were stirred together for a further 3 h. Filtration to remove metallic selenium, evaporation of the solvent almost to dryness and resolubilization of the residue in 20 cm^3 ethanol resulted in white crystals when cooled. The product was recovered by filtration and washed sparingly with ethanol. Concentration of the mother liquor and ethanol washes furnished a second crop of crystals. The product which migrated as a single spot on silica gel TLC plates [$R_f = 0.5$ in butanol/acetic acid/water (4:1:1, by vol.); $R_f = 0.3$ aqueous hydrogen peroxide followed by 1% (w/v) diphenylcarbazide in 95% ethanol followed by exposure of the treated TLC plant to an ammonia atmosphere. The presence of both selenium and mercury in the product was corroborated by HPLC–AA using either a selenium or a mercury hollow-cathode lamp.

Reagents and samples

Sodium selenate, sodium selenite, selenomethionine, selenocystine and selenoethionine, purchased from Aldrich Chemical Co., Milwaukee, WI, were used without further purification. Selenium pellets, 99.995%, were purchased from Fluka Chemical Co., Milwaukee, WI. All other reagents were ACS Reagent grade or better and solvents were 'distilled in glass' or 'HPLC' grade. Plant samples (mixed feedstuff consisting mainly of wheat) which had been ground to pass a 50-mesh screen were kindly supplied by E. Chavez, Department of Animal Science, Macdonald Campus of McGill.

RESULTS AND DISCUSSION

Determination of selenoamino acids by *N*-2,4-dinitrophenylation

Initial efforts were directed to the development of a method for the determination of traces of selenomethionine [$CH_3SeCH_2CH_2CH(NH_3^+)COO^-$], selenocystine [$^-O_2CCH(NH_3^+)CH_2Se]_2$ and selenocysteine [$^-O_2CCH(NH_3^+)CH_2SeH$]. Dinitrofluorobenzene (DNFB) has been used routinely to produce somewhat less polar *N*-2,4-dinitrophenyl (DNP) derivatives of amino acids. However, commonly used procedures have two disadvantages; long reaction times and less than quantitative yields. It has been reported[22] that variations in the rates of reaction of DNFB with glycine, proline or *N*-phenylglycine were accounted for entirely by the effect of pH on the degree of ionization of the amino acid. There was no evidence for specific base catalysis in these substitution reactions. Figure 1 presents the variation in the yield of *N*-2,4-DNP-selenomethionine with pH of the phosphate buffer medium, ionic strength 0.1 mol dm^{-3}. The procedure followed to recover the DNP product in these trials was approach I of Table 1.

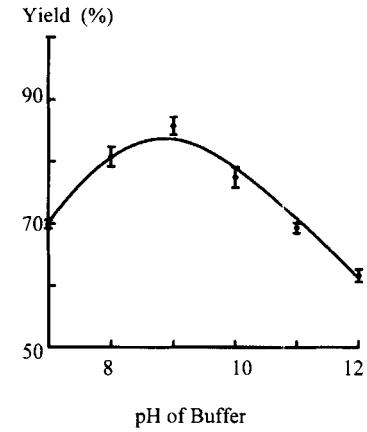

Figure 1 Variation in the yield of *N*-2,4-DNP selenomethionine with the pH of the phosphate buffered reaction medium.

Recovery of product *N*-2,4-DNP-amino acids from the reaction medium typically involves a preliminary extraction to remove excess DNFB

Table 1 The influence of the identities of the extracting solvent(s) on the two-stage recovery procedure for *N*-2,4-DNP selenomethionine

Approach	First extraction[a]	Second extraction[b]	Yield[c] (%)
I	Diethyl ether	Benzene	87.0 ± 1.5
II	Diethyl ether	Toluene	85.3 ± 2.7
III	Benzene	Benzene	86.7 ± 0.25
IV	Benzene	Diethyl ether	99.8 ± 1.3

[a] Three successive 5 cm^3 washes of the crude reaction mixture. [b] Followed by three successive 5 cm^3 washes after pH adjustment to 2. [c] Mean ± 1 RSD based on three replicate trials.

from the crude reaction mixture, then pH adjustment, followed by a second extraction to recover the derivatized analyte(s). The identity of the organic solvent used to effect each of these extractions had an appreciable influence on the recovery of product, as indicated in Table 1. The benzene–ether combination provided a virtually quantitative recovery of *N*-2,4-DNP-selenomethionine and of *N*,*N*-di-DNP-selenocystine (97.9 ± 1.3%). The recovery (99.3 ± 1.1%) of *N*-2,4 DNP-selenomethionine (0.25 μmol) was unaffected by the presence of 20-fold excess of other amino acids (0.5 μmol of each of ten other amino acids which included methionine, cystine, cysteine, α-alanine, glutamic acid, histadine, lysine, phenylalanine, proline and tyrosine. However the recovery (86.4 ± 1.3%) was decreased somewhat by a 100-fold excess of these same amino acids. No response to the DNP-amino acid mixture was obtained by HPLC–THG–AA, and co-injection of the DNP-amino acid mixture with 2,4-DNP-selenomethionine or di-DNP-selenocystine had no effect on the response to either of these selenium analytes.

In contrast to the formation of *N*-DNP-selenomethionine and *N*,*N*-di-DNP-selenocystine, selenocysteine reacts with DNFB to form *Se*-DNP-selenocysteine, which is freely soluble in water. In mildly basic media this derivative is converted via an intramolecular rearrangement (Smiles rearrangement[23]) to the *N*-substituted derivative. This transformation is somewhat difficult to control, principally because of competing intermolecular transfers to other nucleophiles. In consequence, the *Se*-DNP derivative must be purified to a high degree prior to effecting the rearrangement. In addition, blocking the liberated selenol provides a derivative which is similar in chromatographic behaviour to the derivatives of selenomethionine and selenocystine. Previous investigators have used aliphatic reagents such as iodoacetic acid to block free selenol groups in selenoproteins. An alternative approach would be to block the free selenol with phenylmercuric (φHg) acetate to form φHg cysteineselenoate prior to derivatization with DNFB. This approach was attractive in that final product would be appreciably less polar than the starting material. Mercury binding to selenols is stronger than to thiols[16, 24] and the introduction of a second element which could be monitored separately by detection system might be of benefit in the identification of selenol and/or thiol residues. Relative to the methylmercury isologue, the φHg derivative was anticipated to be (a) less polar than, and (b) less likely to be a constituent of, most samples. Selenocysteine was smoothly converted to φHg cysteineselenoate by reaction with phenylmercuric acetate in aqueous medium at pH 4 and then reacted with DNFB in the presence of selenomethionine and selenocysteine. The mixture of product DNPs was separated to baseline on the Nucleosil 5-NO_2 column with a methanolic mobile phase containing 0.6% (w/v) tetrabutylammonium nitrate and 0.9% (v/v) triethylamine. Alternatively the mixture was separated (Fig. 2) with a methanolic mobile phase containing acetic acid and triethylamine (0.05 and 0.8 μl cm^{-3}, respectively).

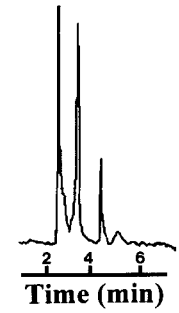

Figure 2 HPLC–THG–AA chromatogram of *N*-2,4-DNP derivatives of φ-Hg cysteineselenoate [retention time (r_T), 2.14 min], selenomethionine (r_T, 3.49 min) and selenocystine (r_T, 5.11 min) separated with a methanolic mobile phase containing acetic acid and triethylamine (0.05 and 0.8 μl cm^{-3}, respectively) delivered to the Nucleosil 5-NO_2 column at 0.5 cm^3 min^{-1}.

Although the 2,4-DNP derivatization approach did provide a method for the determination of selenoamino acids at trace levels and potentially a route to the determination of selenol residues, it did not permit the determination of selenium oxyanions which were also of interest. These latter analytes did not react with the derivatizing reagent and were not soluble in the diethyl ether used to recover the derivatives from the crude product mixture. Moreover, the quantitation was somewhat complicated by the fact that the THG interface–detector system provided different responses to the introduction of equimolar quantities of different selenium compounds. This route was abandoned in favour of a new silica T-tube interface[14, 15] design (prototype 2) which was compatible with either aqueous or methanolic mobile phases and which, potentially, could obviate the requirement for analyte derivatization prior to chromatography. A simple aqueous extract might

be sufficiently pure to be analysed directly with the HPLC–AA system. This new design combined the pyrolysis and the atomization processes into a single stage (the diffused flame within the pyrolysis chamber of the new design served not only to volatilize/pyrolyse the analyte(s) but apparently effected the atomization as well).

The response of the THG (prototype 1) interface–AA system to the introduction of selenate (SeO_4^{2-}) in a flow injection mode was only 18% of the response to an equimolar quantity of selenomethionine.[12] Presumably, this result reflected an inefficient generation of selenium compound(s) which could be atomized when passing through the second microflame. In the absence of the second flame, no signal was observed for any of the selenium analytes. By contrast, under prototype 2 operating conditions, which provided a maximal response to selenomethionine, the new interface provided equivalent responses to equimolar quantities of analytes containing selenium in different formal oxidation states (Table 2). The response to different selenium analytes was unaffected by the magnitude of the currents supplied to the heating coils surrounding the thermospray tube and the pyrolysis chamber, provided that sufficient heat was suplied to generate a stable combustion process and a thermospray

Table 2 Relative interface–AA responses and chromatographic limits of detection for representative selenium compounds

Selenium analyte	Relative response[a] (%)	Chromatographic LOD[b] (ng)
Selenomethionine	100.0 ± 0.7	1.0
Selenocystine	103.1 ± 0.3	1.1
Selenoethionine	97.3 ± 0.5	1.4
Selenate	98.0 ± 0.5	1.3
Selenite	100.8 ± 0.3	1.3
Trimethylselenonium iodide	99.5 ± 0.4	
Triphenylphosphine selenide	98.1 ± 0.6	

[a] Detector response was determined in a flow injection mode using distilled water as the mobile phase.

[b] Chromatographic LOD = estimated limit of detection for chromatography on the cyanopropyl-bonded phase column eluted with 0.5 $cm^3 min^{-1}$ mobile phase consisting of 0.01% (v/v) aqueous acetic acid and 0.05% (w/v) ammonium acetate. $LOD = 3[s_B^2 + s_i^2 + (i/S)^2 s_S^2]^{1/2}/S$ where S, i, s_S and s_i are the slope and intercept and their respective standard deviations of the best-fit calibration plot obtained by linear regression, and s_B represents the standard deviation of the detector blank signal.[21]

effect. The same quartz device has been used virtually daily for up to nine months without appreciable loss (≤30%) of response. Moreover, more than 95% of the initial detector response to selenium analytes was restored by simply washing the inner surfaces with 60% hydrofluoric acid.

Chromatographic separations of selenium compounds

The aims of this phase of the research was to develop a method to determine representative selenium-(II), -(IV) and -(VI) compounds using automated chromatography with on-line detection by AA. This method might then serve as a rapid screening technique for extractable selenium residues in biological samples. For these studies, selenoethionine [$C_2H_5SeCH_2CH_2CH(NH_3^+)CO_2^-$] was to be included among the analytes. It was anticipated that this analyte might serve as an internal standard since, to our knowledge, it has not been identified as a natural product. In addition, it was postulated that free selenocysteine could be ethylated (by adding an appropriate alkylating reagent to the sample) prior to extraction of the analytes. The other target analytes were selenomethionine, selenocystine, selenite, selenate, methaneseleninate ($CH_3SeO_2^-$) and methaneselenonate ($CH_3SeO_3^-$).

Selenocystine, selenomethionine and selenoethionine were separated to baseline on a Nucleosil 5-SA column with 0.5 $cm^3 min^{-1}$ of either 0.1% (v/v) aqueous acetic acid containing 0.05% triethylamine or 0.12% aqueous ammonium acetate. Although the chromatography was highly repeatable and the relative responses to different selenium analytes in the same chromatographic run were constant, the magnitude of analyte responses was dependent on the mobile phase (up to 30% difference in response for the same quantities of analytes injected into different mobile phases). Similar results were observed if these same analytes were separated on a Nucleosil C_{18} column using 0.6 $cm^3 min^{-1}$ of either 0.05% (v/v) aqueous acetic acid containing 0.2% (v/v) tetramethylammonium hydroxide or 0.06% (v/v) aqueous ammonium acetate. However, selenate was not separated from selenite with any of these chromatographic conditions, principally because of a lack of retention.

Methaneseleninate was readily separated from methaneselenonate with a strong anion-exchange material (PL-SAX column) and 0.02% (w/v) aqueous ammonium carbonate. Selenite could

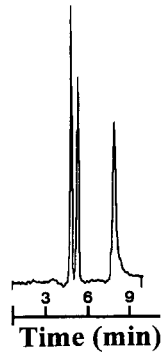

Figure 3 HPLC–AA chromatogram of methaneseleninate [retention time (r_t), 4.54 min], methaneselenonate (r_t, 5.12 min) and selenite (r_t, 8.31 min) separated with 0.1% (w/v) aqueous ammonium carbonate delivered to the PL-SAX column at 0.6 cm^3 min^{-1}.

also be eluted from the column by increasing the ammonium carbonate concentration of the mobile phase to 0.1% (w/v) (Fig. 3) but selenate was totally retained under these conditions. Selenite was separated from selenate on this column with the same mobile phase if the pH of the mobile phase was adjusted to 9.0 with aqueous ammonia, but methaneseleninate and methaneselenonate co-chromatographed under these conditions. Although it is presumed that a baseline separation of the four oxyanions could have been achieved with a solvent programme, this was not verified experimentally.

The combination of anionic analytes (selenium oxyanions) with amino acids, which conventionally are resolved on cation-exchange materials, was separated on a cyanopropyl-bonded stationary phase. This column proved to have a greater resolving power for the three selenoamino acids (Fig. 4) than either the Nucleosil 5-SA or the C_{18}

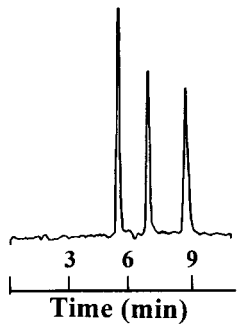

Figure 4 HPLC–AA chromatogram of 10 ng each (as Se) of selenocystine [retention time (r_t), 5.20 min], selenomethionine (r_t, 6.68 min) and selenoethioine (r_t, 8.45 min) eluted with 0.04% (v/v) acetic acid delivered, at 0.5 cm^3 min^{-1}, to the cyanopropyl column.

Figure 5 HPLC–AA chromatogram of 20 ng each (as Se) of selenate [retention time (r_t), 2.67 min], selenite (r_t, 3.07 min), selenocystine (r_t, 3.94 min), selenomethionine (r_t, 4.36 min) and selenoethionine (r_t, 4.81 min). Analytes were eluted from the cyanopropyl column with 0.6 cm^3 min^{-1} aqueous mobile phase containing 0.015% (v/v) acetic acid and 0.1% (w/v) ammonium acetate.

column. Moreover, selenate, selenite, selenocystine, selenomethionine and selenoethionine (in order of elution, Fig. 5) were resolved virtually to baseline with an aqueous mobile phase containing 0.015% (v/v) acetic acid and 0.1% (w/v) ammonium acetate. With these chromatographic conditions, the limits of detection (LODs) for the analytes as estimated with a first-order error propagation model[25] have been added to Table 2. The linear calibration models of peak area with quantity of analyte injected were highly correlated ($0.9971 > r > 0.9986$) in the range studied (5–50 ng as Se). With a 0.05% (v/v) acetic acid mobile phase, methaneselenonic acid, selenite, methaneseleninic acid, selenocystine, selenomethionine and selenoethionine were separated on the cyanopropyl column (Fig. 6).

Determination of selenium analytes

Several methods for extracting free selenoamino acids have been described, including the use of trichloroacetic acid,[26] hot 80% ethanol,[27] hot acidic ethanol[28] (0.1 mol dm^{-3} HCl/EtOH, 2:8, v/v) and hot water.[29] However, stability trials[30] of selenate, selenite and selenomethionine during storage in water suggested that these analytes might be partially decomposed during extraction(s) with hot solvents. A standard mixture of the five analytes was spiked into DD water or into tap-water or into 80% (v/v) ethanol/water. The resulting solutions (0.2 μg cm^{-3} per analyte as Se) were heated to 80 °C for 0.5 h, then evaporated to dryness at room temperature, the residues were resolubilized in DD water and the resulting solu-

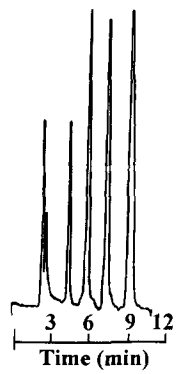

Figure 6 HPLC–AA chromatogram of methanoselenonic acid [retention time (r_T), 2.42 min], hydrogen selenite (r_T, 2.67 min), methaneseleninic acid (r_T, 4.52 min), selenocystine (r_T, 6.00 min), selenomethionine (r_T, 7.49 min) and selenoethionine (r_T, 9.28 min). Analytes were eluted, at 0.5 $cm^3\ min^{-1}$, from the cyanopropyl column with 0.05% (v/v) aqueous acetic acid.

tion was analysed by HPLC–AA. The results of the analyses are recorded in Table 3. Whereas the recovery of selenite was apparently unaffected by any of these treatments, the recoveries of seleno-amino acids were somewhat reduced, presumably because of their sparing solubilities in diethyl ether (Table 3, Procedure B vs A) and the recovery of selenocystine was appreciably reduced by Procedures C or D. If tap-water was spiked with the mixed standards (0.2 $\mu g\ cm^{-3}$ as Se of each analyte; data not included in Table 3), evaporation of the solvent at room temperature and resolubilization of the residue in hot water resulted in virtually quantitative recovery of each analyte. If DD water replaced the spiking solution, no selenium response was observed in the HPLC–AA chromatogram. The immediate chromatography of extracts which resulted from the extraction of mixed feed samples (primarily ground wheat) with either hot (80 °C) water or hot 80% (v/v) ethanol/water proved to be unsatisfactory. The plant extracts appreciably changed both the chromatographic behaviour of co-injected standards and the detector response to these standards, which necessitated the development of a pre-chromatographic clean-up procedure.

A method involving the sequential partitioning of selenonium[31] or arsonium[32] compounds or arsenic oxyanions[14] into liquid phenol and their subsequent repartitioning back into water following dilution of the phenol base with diethyl ether was investigated for the selenium analytes of the current study. It was demonstrated that, for recoveries from tap-water, reducing the pH of the sample medium to 3.0 with acetic acid improved analyte recoveries relative to pH adjustment to 3.0 with either formic or hydrochloric acids. By contrast, if the pH was increased to 10.0, only selenomethionine and seenoethionine were partially recovered. Whereas selenite, selenomethionine, selenocystine and selenoethionine were recovered efficiently (>85%) from tap-water which had been acidified to pH 3.0 with acetic acid, selenate remained in the aqueous phase (Table 4). Application of this optimized recovery procedure to ground wheat which had been spiked at 4 $\mu g\ g^{-1}$ (as Se) with each of the five selenium analytes resulted in a moderate decrease in the recoveries of the other analytes but an increase in selenate recovery (Table 4). Although appreciably less than quantitative, the recoveries of

Table 3 Apparent stabilities of selenium standards to simulated extraction procedures with solvents at 80 °C

	Recovery ± 1 RSD[a] (%)			
Procedure[b]	A	B	C	D
Selenate	95.1 ± 1.9	97.9 ± 0.7	70.8 ± 0.7	103.3 ± 0.8
Selenite	94.9 ± 2.3	95.1 ± 2.6	98.5 ± 2.1	98.6 ± 0.8
Selenocystine	89.0 ± 2.0	80.4 ± 1.3	69.1 ± 0.7	65.2 ± 1.0
Selenomethionine	94.9 ± 2.2	80.8 ± 1.5	90.7 ± 1.6	90.0 ± 0.6
Selenoethionine	93.2 ± 2.1	77.3 ± 3.4	91.5 ± 1.3	90.8 ± 0.3

[a] mean recovery ± 1 RSD based on three replicate trials.
[b] A: analytes in DD water were heated at 80 °C for 30 min. B: analytes in DD water were heated at 80 °C for 30 min, then extracted three times with 5 cm^3 diethyl ether. C: analytes in tap-water were heated at 80 °C for 30 min. D: analytes in 80% (v/v) ethanol/water were heated at 80 °C for 30 min.

Table 4 Recoveries using the phenol extraction procedure of selenium analytes from tap-water (20 cm^3) or ground wheat (1 g) which had been spiked with 4 μg (as Se) of each of the five selenium standards

Selenium analyte	Recovery (%)	
	Tap-water	Ground wheat
Selenate	—	35.3
Selenite	89.5	72.9
Selenocystine	88.0	57.5
Selenomethionine	96.7	60.0
Selenoethionine	95.3	73.2

standards and their chromatographic behaviour in the presence of the plant extract (Fig. 7) were considered sufficient to permit the phenol partitioning procedure to form the basis of a preliminary screening technique to detect readily extractable selenium residues in ground plant samples. The relatively high level of spiking to the ground wheat sample was necessitated by the total selenium content, which had been determined to be 2 μg g^{-1}. When 0.5 g subsamples of the composite ground wheat were subjected to the identical extraction procedure, no residues of any of the analytes were detected, suggesting that all the selenium in this composite sample was protein-bound.

Other selenium analytes which might have been isolated with the phenol extraction procedure included selenoamino acid oxidation products selenocysteic acid and the selenoxide and/or selenone of selenomethionine. Treatment of selenomethionine with hydrogen peroxide at room temperature resulted in one major selenium product [retention time (r_T), 3.52 min] and a small quantity (less than 5%) of a second selenium product [r_T, 6.07 min] which co-chromatographed with selenite [r_T, 6.05 min] when the crude product mixture was eluted from the PL-SAX column with 0.6 cm^3 min^{-1} of 0.1% (w/v) ammonium carbonate which had been adjusted to pH 8.0 with aqueous ammonia. Apparently, neither methaneseleninate nor methaneselenonate was present in this mixture. Similar treatment of selenocystine resulted in approximately equal quantities of two major selenium products [r_T, 3.52 min, 6.01 min (possibly selenite)] and traces (≈1%) of a third selenium product (r_T, 10.18 min). The determination of elemental selenium which might be accomplished by converting this analyte to triphenylphosphine selenide was not investigated in detail.

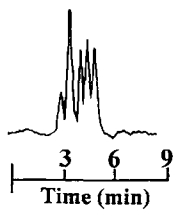

Figure 7 HPLC–AA chromatogram of the phenol extract from a ground dried wheat sample which had been spiked [at 4 μg each (as Se) g^{-1}] with selenate, selenite selenocystine, selenomethionine and selenoethionine (in order of elution), then acidified to pH 3 prior to extraction. The extract was eluted with aqueous acetic acid (0.015%, v/v) containing 0.1% (w/v) ammonium acetate and delivered at 0.5 cm^3 min^{-1} to the cyanopropyl column.

Collectively, these studies demonstrate that on-line monitoring by AA provides a sensitive, inexpensive and versatile means of detecting and quantifying selenium compounds in HPLC column eluate. However the challenge remains to devise procedures which liberate the selenium compounds efficiently from biological samples. Enzymic digestion followed by HPLC determination of the selenium-containing proteolytic fragments would seem to be a promising approach in this regard.

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