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WORKING METHODS PAPER

Speciation of selenoamino acids by on-line HPLC ETAA spectrometry

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An analytical method was developed to determine selenoamino acids in the presence of other compounds. Separation has been achieved by High Performance Liquid Chromatography (HPLC) using electrothermal atomic absorption (ETAA) spectrometry as a very sensitive and element-specific detector. On-line HPLC ETAAS speciation of selenocystine and selenomethionine has been studied, using a laboratory made interface. Analytical characterization of the method has been realized with standard solutions. Using a 100 µl sample loop, the detection limits were calculated as 8 µg l^{-1} for selenomethionine and 10 µg l^{-1} for selenocystine with repeatability and reproducibility of 4% and 7% respectively. The method has been applied to the determination of selenoamino acids in an extract of white clover (CRM402) certified for total selenium.

Keywords: Selenoamino acids, HPLC ETAAS, speciation, environment, determination

INTRODUCTION

The role of selenium as both a toxic agent and a micronutrient was recognized as early as 1957. It is widely accepted by most investigators that it is essential for vegetation and cattle growth as well as for human health.[1] As for other elements, its toxicity and bioavailability depend on its physicochemical forms, i.e. its speciation. In order to evaluate the exact impact of selenium in a definite environment it is necessary to identify and determine its different species. Selenoamino acids are essential in the selenium biogeochemical cycle.[2-4] Previous studies[5,6] have shown that selenite and selenate ions in soil are taken up by vegetation, selenites being converted into amino acids.

Amino acids are introduced in animals either by ingestion of vegetation or by supplementary food intake, or they are synthesized from other selenium species.

Primary selenoamino acids seem to be the main forms viz., selenomethionine, selenocysteine and selenocystine.[2,4,7-9] Some authors noted also the presence of compounds such as selenocystathionine, selenohomocystine and, especially, methylated primary acid derivatives.[3,7,8,10] The main identified role of selenium is as a constituent of glutathione peroxidase enzyme, whose active site is formed from selenocysteine.[11,12] This enzyme inhibits the oxidative role of peroxides and hydroperoxides, thereby protecting immunocompetent cells and slowing down ageing processes.[2,9] Numerous questions remain to be solved about the specific role of other selenium active-site enzymes.

The development of analytical methods to study the speciation of selenium appears to be a necessary step to understanding the biogeochemical cycle, mobility, transfer and uptake of this element as well as its toxicity.

Two separation methods have been suggested in the literature, involving either liquid or gas chromatography (see Ref. 13 for a review). Liquid chromatographic separation of selenoamino acids in the literature is based on either ion exchange or reversed-phase HPLC after derivatization. However most of the publications in this area deal with qualitative identification of selenium species or analysis of standard solutions, or they follow the evolution of radioactive organic selenium introduced in living organisms. It is only very recently that methods of determination with very low detection limits (ng) of selenoamino acids by derivatization, reversed-phase HPLC, thermochemical hydride generation and AAS were proposed.[14,15] The objective of this work has been to propose a simpler procedure using only commercially available equipment. We have therefore chosen to use a chromatographic procedure that does not necessitate a derivatization

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step, coupled on-line to a graphite furnace atomic absorption detector. The Limit of Detection (LOD) in the nanogram range could be expected *a priori* due to the high sensitivity of this detector.

We have developed a direct HPLC-ETAAS determination based on the idea of F. E. Brinckman,[16,17] with a very low-volume laboratory made interface that will periodically be sampled for injection into the furnace, the period being defined by the thermal cycle of the furnace assembly; the ETAAS measurements are then a discrete series of quasi-instantaneous data on the composition of the LC effluent. This hyphenated technique has already been used for speciation of butyltin compounds.[18–20] In this paper we present a new application of this method to the determination of selenoamino acids in the presence of selenite and selenate ions.

MATERIALS AND METHODS

Reagents

DL-Selenocystine and DL-selenomethionine (SeCys, SeMet) were purchased from Sigma. These products were used without further purification (90% purity for SeCys). Stock solutions (1000 mg l^{-1} as Se) in deionized water (Millipore 18 Ω) were stored at 4 °C in the dark. 3% hydrochloric acid (HCl) is necessary to dissolve selenocystine. Working standards (200 μg l^{-1} as Se) were obtained daily by dilution in deionized water and stored in the dark. Solutions of tetraethylammonium bromide (TEABr) (Kodak) in deionized water (Millipore) and acetonitrile (Prolabo 99/1, v/v) were prepared daily; pH was adjusted by addition of nitric acid (Merck, Suprapur).

Nickel nitrate used for matrix modification in ETAAS determination was purchased from Prolabo (Normapur). A mixed solvent, chloroform (Merck), methanol (Prolabo Normapur) and deionized water (1/2/0.8 by vol.), was used for extraction of amino acids from white clover. This clover (reference material CRM 402) is available from BCR (Community Bureau of Reference, Commission of European Communities) as a bottled dry powder.

Equipment

A Varian 5020 liquid chromatograph with a Hamilton PRP1 5 μm column (styrene divinylbenzene 150 × 4.1 mm) was coupled through a 300 μl home-made interface[21] to a Varian ETAAS assembly (AA 30, GTA 96). The matrix modifier, pushed by a Gilson Minipuls 2 peristaltic pump, was mixed in a T-tube with the chromatographic effluent before introduction into the interface. A slow air flow was pushed by the same pump to bubble in the interface to ensure total mixing.

Table 1 ETAAS temperature cycle

Step	Temperature (°C)	Time (s)	Gas flow (1 min^{-1})	Gas type	Read command
1	95	10	3	Normal	No
2	115	5	3	Normal	No
3	250	5	3	Normal	No
4	400	10	3	Normal	No
5	400	15	3	Normal	No
6	650	10	0	Normal	No
7	2400	1.2	0	Normal	Yes
8	2400	2	0	Normal	Yes
9	2600	1	3	Normal	No

Optimization of the ETAAS detection

ET AAS temperature program

Injection

The absorbance signal remains constant for injection temperatures between 40 and 55 °C and decreases significantly at higher values (−36% at 90 °C).

Ashing step

The ashing temperature must not be too high. The absorbance signal decreases regularly (65%) from 300 °C to 900 °C.

Atomization step

A maximum absorbance signal is obtained for 2400 °C with 22% fluctuation between 2200 and 2600 °C.

Temperature program

The GTA 96 automatic injection device automatically sampled 20 μl from the interface with a delay fixed by the temperature cycle of the graphite furnace and the software. The temperature cycle, reduced to a minimum (59.2 s), is presented in Table 1, the injection temperature being 50 °C.

The overall time period between two measurements, including cooling down and injection delays, is 78.2 s. Using a pyrolytic carbon platform in the pyrolytic carbon furnace leads to a +40% improvement of sensitivity.[22]

In these conditions the platform and the furnace may be used for approximately 1200 and 600 atomizations respectively.

Matrix modifier

A nickel nitrate solution is efficient as matrix modifier[22,23] in improving the sensitivity of the method, probably through the formation of nickel selenide in the furnace (Table 2). A 0.1% concentration of $Ni(NO_3)_2$ in the furnace was retained as a most convenient compromise between improved sensitivity and reduced solution salinity.

As the flow rate of the chromatographic mobile phase is typically 0.4 ml min^{-1} in this work, a 0.8% (w/v) nickel nitrate solution was added at a flow rate of 0.06 ml min^{-1} to minimize dilution.

Hollow cathode lamp intensity

Maximum sensitivity is obtained with an intensity of 7 mA.

Optimization of chromatographic conditions

Previous workers[7,24] have used ion-exchange chromatography for separation of selenoamino acids. We retained partition chromatography based on formation of ion-pairs following Juang and Houk[25] who separated the sulphur amino-acids, cysteine and methionine.

The conditions for practical analysis were as follows: the column was equilibrated with a 99:1 (v/v) water/acetonitrile solution containing 10^{-2} mol l^{-1} tetraethylammonium bromide at pH 4 and 0.4 ml min^{-1} flow rate. Volume injected was 100 µl.

Figure 1 presents an example of a chromatogram obtained for the analysis of a 500 µg l^{-1} (Se) selenocystine and selenomethionine solution. The overall LC ETAAS peak, as obtained on a y–t recorder, is a series of parallel bars, the height of which reflects the individual absorbance measurements. These bars are separated by a time delay which is the time necessary for the accomplishment of the whole thermal cycle of the spectrometer. The quantitative use of this chromatogram is preferably made by the determination of peak area obtained by summation of the individual bars rather than peak height which is quite sensitive to several parameters.[21]

Retention times are determined at the maximum peak height. We used a 0.4 ml min^{-1} flow rate as the best compromise between analysis time, peak broadening and the limitation of resolution by the ETAAS detector.

Table 2 Variations of ETAAS sensitivity with concentration of $Ni(NO_3)_2$ matrix modifier 20 µl injection of a 20 µg l^{-1} solution of selenomethionine

$Ni(NO_3)_2$ concentration (%)	Sensitivity (absorption m units ng^{-1})
0	21
0.05	67
0.1	137
0.15	164
0.2	176

Effect of pH

A pH titration by sodium hydroxide indicated the following acidity constants of the selenoamino acids: selenocystine ($pK_{a_1}=2.4$; $pK_{a_2}=8.9$) and selenomethionine ($pK_{a_1}=2.6$; $pK_{a_2}=8.9$). In the range $4<pH<8$ studied, the amino acids are in the form of zwitterions. This is well inside the PRP1 columns recommended stability domain (pH 1 to 13).

Retention times are independent of pH but sensitivity is best at pH 4, the value retained for further work.

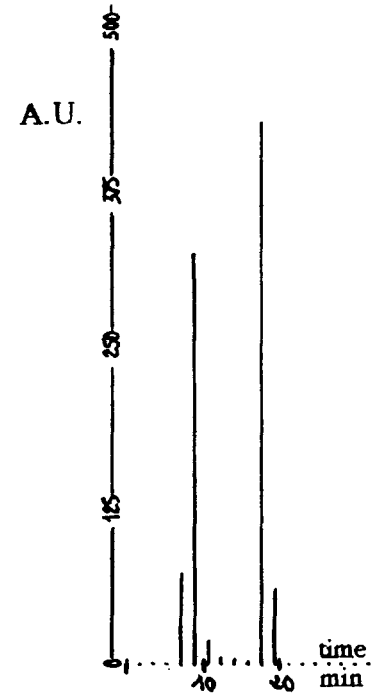

Figure 1 HPLC ETAAS chromatogram of a standard mixture of selenocystine and selenomethionine (500 µg l^{-1} selenium each).

Effect of ionic strength (μ)

Retention times observed in the presence of various concentrations (10^{-4}–10^{-2} mol l^{-1}) of tetraethylammonium bromide are surprisingly constant and similar to those evaluated in the absence of any ion-pairing reagent. Moreover retention times decrease when tetrabutylammonium bromide is added instead of tetraethylammonium bromide. These results led us to think the retention mechanism of selenoamino acids as being a reverse-phase rather than an ion-pairing separation process. This is in contradiction with the assertions of Juang and Houk.[25] The retention of SeMet is higher than that of SeCys as the alkyl chain of the former is more accessible and as the latter molecule contains polar and ionized functions at its end.

We have already noted[18] when studying the liquid chromatographic speciation of organotin compounds that at trace level (μg l^{-1}) the chromatographic separation mechanisms often differ from those predicted at higher concentrations (mg l^{-1}).

Inorganic species

Inorganic forms such as selenite and selenate may also be present in the different compartments of the environment and must then be separated from selenoaminoacids. Selenite and selenate are retained on PRP1 columns in the presence of the counter ion but are not well separated from one another. Their retention times are intermediate between those of SeCys and SeMet and their common peak slightly interferes on both. Further improvement of the chromatographic separation will be necessary to allow a perfect simultaneous determination of the four species. In the present state of the chromatographic procedure preliminary removal of inorganic Se species is necessary to obtain precise SeCys and SeMet determinations. An example of a chromatogram is presented in Fig. 2 obtained with a PRP1 column 400 mm long, and a 6.25×10^{-4} mol l^{-1} solution of TEABr at pH 4 in water/acetonitrile (99:1) solution as mobile phase.

Analysis of standard solutions

Calibration graphs established from HPLC ETAAS peak areas for the analysis of standard solutions containing selenocystine and selenomethionine are perfectly linear in the range of concentrations studied (0–700 μg l^{-1}), but sensitivities are quite different (Table 3).

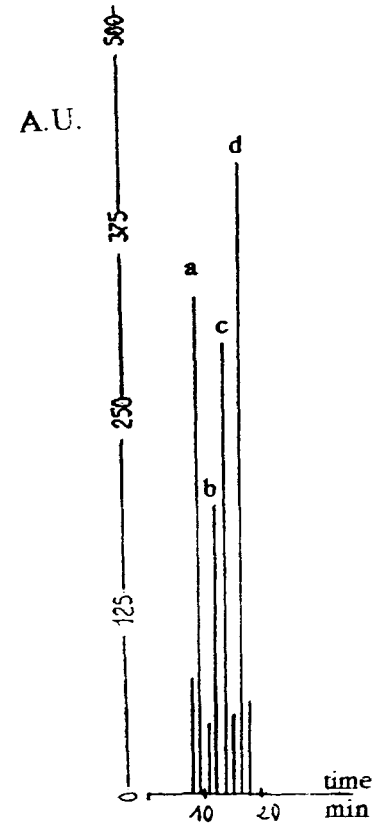

Figure 2 HPLC ETAAS chromatogram of a standard mixture of (a) selenocystine, (b) SE(IV), (c) Se(VI) and (d) selenomethionine (500 μg l^{-1} selenium each).

Repeatability of the chromatographic procedure was examined by six replicate injections of a 500 μg l^{-1} solution of selenomethionine. The relative standard deviation RSD was 4%. Reproducibility was evaluated to be 7% by the analysis of six independent selenomethionine solutions (500 μg l^{-1}).

Table 3 Calibration parameters of the HPLC ETAAS analysis of individual solutions of various selenium species (0–700 μg l^{-1})

Compound	m^a	r^b
Selenocystine	0.85	0.998
Selenomethionine	1.04	0.980
Se(IV)	0.84	0.975
Se(VI)	0.73	0.991

[a] Slope of the calibration curve in milliabsorption units μg^{-1} (Se) l: (HPLC ETAAS sensitivity) with a 100 μl sample injection loop. [b] Correlation coefficient.

Concentration detection limits (C_L) have been estimated by the formula $C_L = 3\,S_B\,m^{-1}$, where S_B is the standard deviation of the blank determined from 20 blank measurements (20 µl) sampled in the graphite furnace. C_L values are respectively 10, 8, 10, 12 µg l^{-1} (as Se) for selenocystine, selenomethionine, selenium (IV) and selenium (VI) respectively.

RESULTS

Application to environmental samples

This analytical procedure has been applied to the analysis of an extract of a white-clover sample (CRM 402) certified by the BCR for its total selenium content (6.69 ± 0.25 mg kg^{-1})[26] and the subject of an intercalibration exercise organized between ten French laboratories by MRT.[27]

The first and critical step is to extract selenoamino acids from the sample without changing their chemical forms. We used a process similar to that proposed by Martin *et al.*[8] for the extraction of these compounds from seeds of *Astragalus*. A portion of CRM 402 white clover was dried during 24 h, then ground. About 0.4 g of this powdered sample was placed in a 50 ml Pyrex flask with a ground glass stopper together with a mixture of deionized water, chloroform and methanol (2:3:5, by vol.) and shaken during 5 h. The suspension was centrifuged (6000 rpm, 10 min), evaporated to dryness then dissolved in 10 ml of the chromatographic mobile phase. The same procedure has been applied to standard solutions of SeCys and SeMet. HPLC ETAAS analysis did not show evidence of degradation of the selenoamino acids and allowed us to achieve yields of $90 \pm 1\%$.

A direct determination of total selenium in the clover extract by ETAAS, using standard additions, led to a determination of a concentration of 5.0 ± 0.4 mg kg^{-1}, i.e. 75% of the total selenium concentration expected from total dissolution of the clover material. Furthermore the absence of inorganic selenium forms in this extract has been proved by two different techniques: an electrochemical redox speciation method for selenite and selenate compound[27–29] and a quartz-furnace atomic absorption method following hydride generation,[30] sensitive only to selenium(IV).

The chromatogram obtained by HPLC ETAAS (Fig. 3) displays only one peak at the retention time of selenocystine. Standard additions of selenocystine increase this signal, and the determination of selenocystine led to 4.7 ± 0.3 mg kg^{-1} (Se).

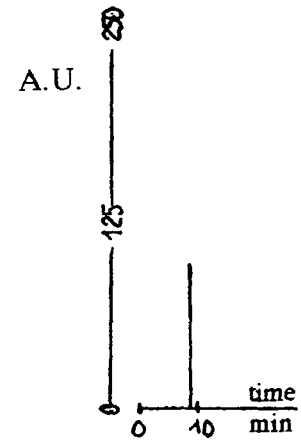

Figure 3 HPLC ETAAS chromatogram of a CRM402 white clover extract (conditions in text).

DISCUSSION

Many reports on selenium availability in food have been published; however, the chemical forms have not been strictly identified. Selenomethionine has been shown as predominant in wheat grain,[31,32] soybean[33] and selenium yeast.[34] Up to 50% selenium(VI) has been found in beet or cabbage leaves and garlic.[35]

In this context, our finding of the absence of selenomethionine or inorganic selenium species in white clover deserves some comment. First of all, approx. 25% of total selenium escaped extraction by the soft extraction procedure used (water–chloroform–methanol). Also selenocystine and selenocysteine are easily oxidized during sample pretreatment procedures[33] or hydrolysed[36] and, taking special precautions, Broderick *et al.*[37] identified selenocysteine in proteins. Martin and Gerlach[8] showed evidence of selenocystathionine as a major species in seeds of *Astragalus pectinatus* and *Astragalus osterhouti* but not in Astragalus racemosus, together with some selenocystine and methylselenocysteine, they did not find any selenomethionine.

There seems thus to be a great variability of Se speciation in vegetation. Our preliminary finding that selenocystine, or at least some organoselenium compound with a similar retention time,

represents ≥75% of total Se in white clover must be examined in this context.

Further investigations will be necessary to confirm the identity of this compound. Further work is also needed to improve selenium extraction yield from the white clover sample and to evaluate whether the residual fraction that was not extracted in this work, has the same composition or contains different selenium species.

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