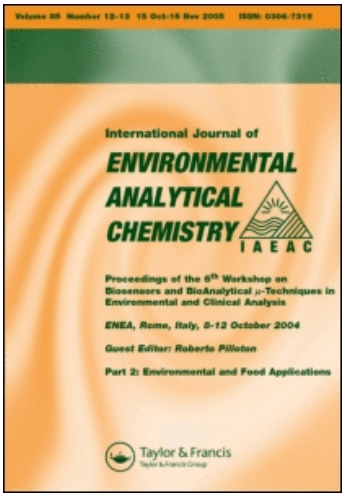

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COMPARISON OF SELENIUM EXTRACTION PROCEDURES FOR ITS SPECIATION IN BIOLOGICAL MATERIALS

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Extraction procedures of selenium compounds applied to solid biological samples (CRM 186: pig kidney, CRM 402: white clover and a selenium enriched yeast) are discussed. Enzymic hydrolysis led to high recoveries (57% to 92%, depending on the sample). A hyphenated High Performance Liquid Chromatography-Electrothermal Atomic Absorption Spectrometry technique is used to study the speciation of selenium in the enzymic extracts. Organic compounds: selenomethionine and selenocystine are found as major components in these samples, 39%, 56% and 77% for clover, kidney and yeast, respectively.

Keywords: Selenium; extraction; speciation; HPLC-ETAAS

INTRODUCTION

Selenium is an essential trace element for most living organisms. It has oxidation states of -II, O, IV, and VI and can occur as Se(-II) (selenide), Se (elemental selenium), SeO_3^{2-} (selenite) and SeO_4^{2-} (selenate). Present in rocks (volcanic rocks, shales, limestone)[1] it is transferred to soils through physicochemical degradation processes. Soluble species identified in soils are selenite, selenate and organic selenide. Selenate and organic selenium are more available for microorganisms and plants uptake[2]. Selenium is incorporated in their tissue as organic species and volatilized[2]. Selenium in soils and parent rocks is also leached and found in groundwaters and surface waters where the element is mainly present as

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selenite and selenate anions. Selenium occurring in plants enters the human food chain through crops, flours and animal feedstuff. When present in yeast or livestock it enters directly into human nutrition. In all these biological materials selenium is mainly found linked to proteins[3].

Selenium is considered as essential and toxic within a narrow range of concentrations. The recommended dietary allowance is 55-70 $\mu g.day^{-1}$ for an adult whereas the toxic level is estimated to 800 $\mu g.day^{-1}$[4]. Species also vary greatly in their bioavaibility and toxicity to humans[5], thus the speciation studies of selenium in biological materials is now highlighted. Selenium is present at significant levels in all environmental compartments. Recent studies have investigated food items, the mean values ranges from 1.1 $\mu g\ Se.kg^{-1}$ in fruits to 295.0 $\mu g\ Se.kg^{-1}$ (on a wet weight basis) in fish[6]. Concerning plants the concentration is closely correlated to the soil content and the nature of the plant. Non accumulators contain low amounts in the order of 1 mg $Se.kg^{-1}$ (on a dry weight basis) some accumulators may tolerate levels of 4000 mg $Se.kg^{-1}$[7].

Minor trace element speciation in solid samples undergoes an extraction step which should not affect the species distribution in the matrix. Some extracting methods are based on a matrix fractionation, as it is commonly carried out on soils[8]. Solvents are employed to partially solubilize the sample. Such extraction procedure involves the use of ether and water[9], a methanol and chlorform mixture[10], hot ethanol[11] or acidic ethanol[12]. Selenocompounds are extracted from the solid matrix using usual aminoacid hydrolysis with concentrated sodium hydroxide solutions[13] or hydrochloric acid[14]. A recent work has driven to the identification of selenocysteine in dolphin liver using acid hydrolysis[15]. Enzymic digestion is also achieved to fractionate the protein containing materials. Proteolytic cleavage of selenoamino acids has been carried out on soybean proteins using successively pepsin, pancreatine and pronase[16]. Meanwhile this method was applied to other organometallics speciation[17-20]. We have applied some simple procedures to the extraction and speciation of selenium from spiked clover, a selenium enriched yeast and two reference materials: CRM 186 (pig kidney) and CRM 402 (white clover). These two later samples are certified for their total selenium content.

Selenium speciation using high performance liquid chromatography (HPLC) separation followed by a highly specific detection has been focused in the past decade and extensively reviewed[21-23]. Atomic absorption spectrometry (AAS) under all its different forms is employed for the sensitive determination of both organic and inorganic selenium species. Flame AAS is reported for the on line detection of selenite and selenate with detection limits in the range of 10 ng[24]. Thermochemical hydride generation AAS coupled to a reverse phase separation mechanism gives a significant sensitivity improvement[25]. The introduction of

an on-line microwave reduction between the end of the column and the hydride generator-AAS detector led to a similar sensitivity in the ng range for Se(VI) and Se(IV) determination[26]. Kölb and coworkers describe the use of an electrothermal atomic absorption detector coupled on line to an ion-exchange chromatography, detection limits are in the ng range[27]. We reported a similar system and compared both ion pairing and ion exchange mechanisms for the determination of selenite, selenate, selenomethionine and selenocystine[28]. The ion exchange separation coupled to ETAAS led to an efficient separation with low detection limits.

Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) is also employed as a specific chromatographic detector but sensitivity is still high, in the 0.1 μg range[29].

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) is actually one of the most studied detectors. Liquid chromatographic separations of selenoamino acids and inorganic species are reported with low detection limits in the pg range[30].

Capillary electrophoresis is now applied to the separation of selenocompounds. Various detectors are employed: UV[31], conductimetry[32], and more recently ICP-MS[33].

The present work deals with the comparison of extraction reagents in view of the selenium speciation in the extracts. Two enzymic hydrolysis, an acid treatment and an organic solvent extraction are studied. The proteolysis is reported to give high recoveries in the three materials: white clover (CRM 402), pig kidney (CRM 186) and enriched yeast. The speciation is carried out on the three samples using HPLC-ETAAS.

EXPERIMENTAL

Reagents

Seleno -D,L-cystine (SeCyst) and seleno -D,L- methionine (SeMet) were purchased from Sigma. Sodium selenite is a Merck suprapur reagent and sodium selenate (98%) were obtained from Aldrich. The 1000 $mg.l^{-1}$ stock solutions were prepared monthly in deionised water (Milliro-MilliQ system from Millipore). Selenocystine was dissolved in 3% hydrochloric acid (Merck suprapur reagent). Nitric acid (65%) and hydrogen peroxide (30%) were pro analysis reagent from Merck.

Chromatographic Analysis

A Varian 5020 liquid chromatograph equipped with a 100 µl loop was used with a Hamilton PRP-X100 column (250X 4.1 mm i.d., 10 µm particles) whose stationary phase consists in a poly(styrene-divinylbenzene) with trimethylammonium sites. The ion exchange mobile phase was a gradient elution of nickel acetate (Merck, pro analysis reagent): 100% for 19 min, to nickel sulfate 100% in one minute (Normapur-prolabo). Precolumn of the corresponding stationary phase was used in the analysis of natural matrices. Acetate and sulfate were found efficient as chromatographic mobile phase, whereas nickel was chosen to be used as matrix modifier.

ETAAS

The chromatograph was coupled to an ETAAS assembly through a home made interface (Unicam 939QZ-FS 90) equipped with Zeeman background correction. The selenium hollow cathode lamp is a photron super lamp (λ = 196 nm, Instrument power supply I = 11 mA, Boost current: I = 15 mA). The furnace program is divided in: a drying step at 90°C (5s), an ashing step at 800°C (10s) and atomization at 2200°C (3s). Nickel solutions were used as matrix modifiers.

Spiking procedure

A portion of wet freshly cut off clover is weighed and partially lyophilized prior to being powdered. The volume of the spiking solution is calculated to rehydrate the material. It is added dropwise during 1 hour while the material is cooled in a crushed ice bath.

Extraction procedures

Water Extraction A 250 mg portion of the material is placed together with 5 ml of deionized water (CRM 402, 8 ml) the mixture is magnetically stirred overnight at 37°C. It is centrifuged (20 min. at 6000 rpm), filtered (0.45 µm), and mineralized with a H_2O_2/HNO_3 mixture before direct analysis using ETAAS.

Acidic Extraction A 250 mg portion of the material is placed together with 5 ml of HCI 0.01 mol.l^{-1} CRM 402, 8 ml) the mixture is magnetically stirred overnight at 37°C. It is centrifuged (20 min. at 6000 rpm), filtered (0.45 µm), and mineralized with a H_2O_2/HNO_3 mixture before direct analysis using ETAAS.

Solvent Extraction A 250 mg portion of the material is placed together with 20 ml of a mixture consisting in methanol (10 ml) chloroform (6 ml) and water (4 ml). The pH is adjusted to 2 with hydrochloric acid and the suspension is shaken 5 hours and filtered (0.45 μm) before direct analysis with ETAAS.

Enzymic hydrolysis

Pepsin A 250 mg portion of the material is placed together with 5 ml of HCl 0.01 $mol.l^{-1}$ and pepsin 25 mg the suspension is magnetically stirred overnight at 37°C. It is centrifuged (20 min at 6000 rpm), filtered (0.45 μm), and mineralized with a H_2O_2/HNO_3 mixture before direct analysis using ETAAS.

Pronase/Lipase A 250 mg portion of the material is placed together with 5 ml of nickel acetate, pronase 25 mg and lipase 10 mg, the suspension is magnetically stirred overnight at 37°C. It is centrifuged (20 min. at 6000 rpm), filtered (0.45 μm), and mineralized with a H_2O_2/HNO_3 mixture before direct analysis using ETAAS.

RESULTS AND DISCUSSION

In our study, reference materials have been selected for their high selenium content and also for their relation with the human food chain. White clover (CRM 402, with a certified selenium content of 6.7 ± 0.3 $mg.kg^{-1}$) is a grass type sample located at the beginning of the food chain. Pig kidney (CRM 186, certified selenium value: 10.3 ± 0.5 $mg.kg^{-1}$) was chosen because it is an accumulating organ of livestock. Finally, selenium enriched yeast (indicative value of 1050 $mg.kg^{-1}$) commonly enters the composition of several medications[4].

Analytical Procedure

The analytical procedure we used is presented in Figure 1 and was already used in previous works[20,28]. The solid sample is mineralized to determine its total concentration of selenium. All calculations are carried out on a dry weight basis. Results obtained for reference materials are in good agreement with the reference values (CRM 186 found: 10.4 ± 0.25 $mg.kg^{-1}$, certified value: 10.3 ± 0.5 $mg.kg^{-1}$; CRM 402 found: 6.72 ± 0.2 $mg.kg^{-1}$, certified value 6.7 ± 0.25 $mg.kg^{-1}$) which validates the mineralization and analysis procedures.

Mild extracting procedures were used on the material in order to solubilize selenocompounds without modifying their nature and partition. Extracted sele-

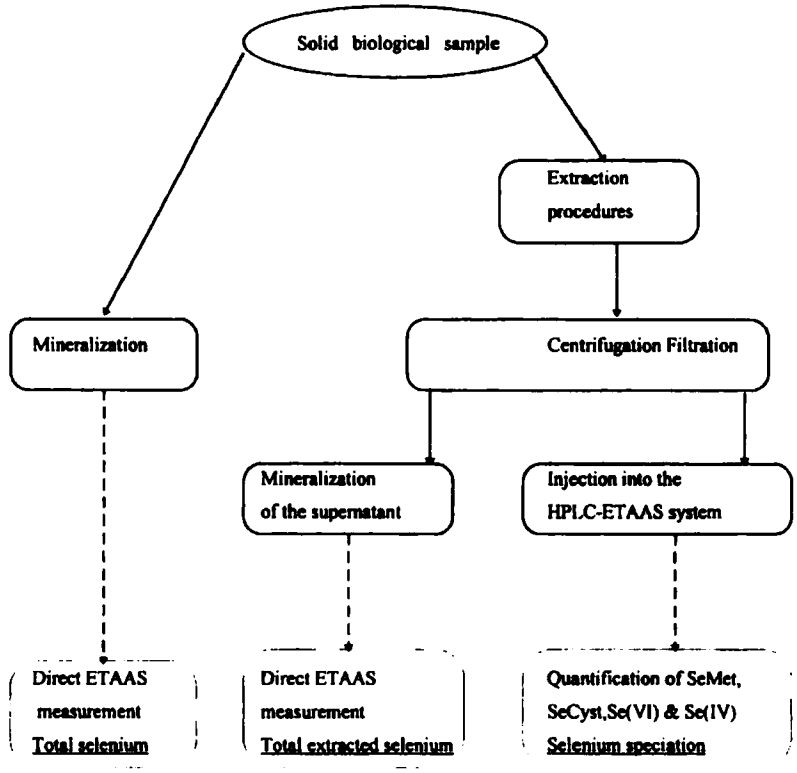

FIGURE 1 Application to the study of biological samples

nium is measured in the extracts supernatant. Mineralization of these solutions reduces the matrix effects that could inhibit ETAAS measurements. Recovery is calculated with respect to the total selenium concentration.

Speciation in the extracts is carried out with direct chromatographic injection of the solutions. The separation is achieved using an ion-exchange chromatography. The ETAAS spectrometer ensures a specific selenium detection. The chromatographic parameters were previously optimized to achieve an efficient separation of selenocystine, selenomethionine, selenite and selenate[28]. An ion exchange mechanism was determined to ensure high resolution with good detection limits when a nickel containing mobile phase is employed. The chromatogram is a series of discontinuous measurements, an example is given in Figure 2. Each peak, corresponding to a compound is defined by a series of values, the sum of the values represents the peak response[34]. For species quantification, the standard addition method is used. Analytical criteria of the technique are summa-

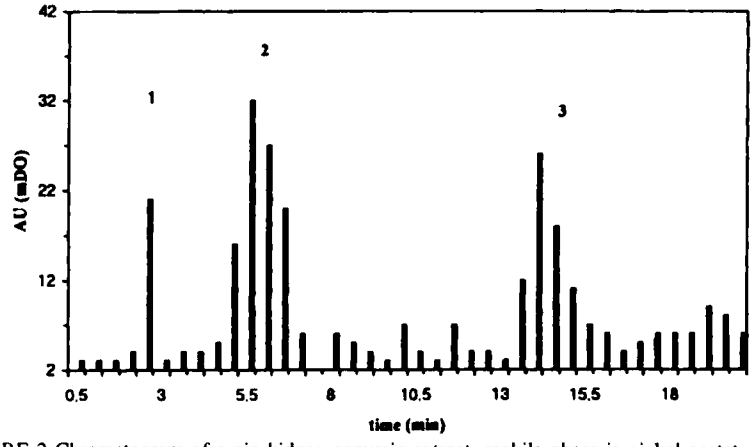

FIGURE 2 Chromatogram of a pig kidney enzymic extract, mobile phase is nickel acetate 2 $g.l^{-1}$ pH = 7, flow rate = 0.8 $ml.min^{-1}$, peak 1 corresponds to an unretained selenated compound, peak 2 is selenocystine (SeCyst) and peak 3 is selenomethionine (SeMet)

rized in Table I. Detection limits were calculated following IUPAC recommendation (DL = 3σd/m, where DL is the detection limit, σd the standard deviation evaluated on the basis of 20 blank measurements and m is the slope of the calibration curve). Reproducibilities were calculated on the basis of measurements of six consecutive injections of a 200 $\mu g\ l^{-1}$ standard solution[28].

The speciation recoveries are expressed as a function of total selenium concentration in the sample, the identified selenium fraction is expressed with respect to both extracted and total selenium concentrations of the sample.

TABLE I Analytical criteria of the HPLC-ETAAS

	SeCyst	*SeMet*	*Se(IV)*	*Se(VI)*
Relative detection limit (μg.l-1)	8	15	17	12
Absolute detection limit (ng)	0.8	1.5	1.7	1.2
Retention time (min)	6	15	20	29.5
Reproductibility (RSD %)	5	4	4	2

Extraction Procedures

Several extraction procedures, already reported in the literature, were carried out on these materials. These were investigated because they implied no derivatization and the minimum sample handling in order to avoid any loss or degradation of the compounds. The selected extracting agents were solvents[10], and two

enzymes, pepsine[16] and a pronase/lipase mixture[18-20]. The extraction capacity of the different media in which enzymes are reacting was evaluated (water, nickel acetate and hydrochloric acid). Table II summarises the results obtained for the three different matrices.

TABLE II Total selenium measurements in the different extracts

Matrix	*Water*	*Solvents*	*HCl*	$Ni(CH_3COO)_2$	*Pepsin*	*Pronase/ Lipase*
CRM 402 *white clover*						
level (mg kg^{-1})	1.32 ± 0.05	2.6 ± 0.3	1.6 ± 0.1	2.98 ± 0.02	4 ± 0.3	3.84 ± 0.07
recovery (%)	19.8 ± 0.8	39 ± 4	23 ± 2	29.6 ± 0.3	60 ± 5	57.4 ± 0.9
CRM 186 *pig kidney*						
level (mg kg^{-1})	1.2 ± 0.1	ns	5.5 ± 0.3	1.0 ± 0.4	8.7 ± 0.6	8.2 ± 0.8
recovery (%)	11.2 ± 0.8	ns	53.0 ± 3.0	9.5 ± 4	85 ± 5	80 ± 10
Enriched yeast						
level (mg kg^{-1})	200 ± 8	117 ± 8	84 ± 2	613 ± 13	854 ± 50	955 ± 7
recovery (%)	20 ± 0.8	11 ± 0.7	8 ± 0.2	59 ± 1.3	82 ± 5	92 ± 0.7

ns: not significant, recovery is 4 ± 5%

The recoveries appear as highly matrix dependant. With regard to extracting solutions, less than 20% of selenium is water soluble in every case. Solvents are not extracting kidneys and poor recoveries are obtained for other materials. Thus, high yields are reported in the literature when free amino acids present in supplement mixtures are extracted in organic solvents[10-12]. It seems that in a complex matrix where selenium should be covalently bounded solvents are not efficient enough. Nickel acetate provides a low extracting solution for enzymic hydrolysis, with recoveries of 9.5% and 29% for reference materials. Proteolysis carried out in this media with pronase/lipase is resulting in high recoveries for both CRM 186 and yeast, whereas only 57% of the selenium was removed from CRM 402. High protein content of the first two samples could explain this result. The nearly total solubilization of the tissues is in good agreement with this hypothesis, on the contrary clover was weakly digested by any enzymic procedure. Pepsin appears as a good extractant (60% to 85%) probably enhanced by the solubilization capacity of its reaction medium: hydrochloric acid at pH = 2. When only the HCl solution is used, 23% and 53% of extraction on CRM402 and CRM 186 respectively are obtained. Nevertheless, injections on the chromatograph gave no single amino acid peaks so that, despite the high yields obtained for pepsin extraction, an uncomplete hydrolysis was suspected. A pronase/lipase mixture gave best results in every case.

Speciation Study

Speciation studies were carried out on the pronase lipase extracts. Firstly, standard solutions of the selenocompounds were treated with pronase/lipase mixture and measurements gave a mean 90% conservation of the species. Time of reaction was also investigated and an overnight reaction time was chosen as a compromise. Table III summarizes the results obtained for the three matrices. Organic selenium is found as major constituant of the three samples: CRM 186, CRM 402 and enriched yeast. Se(IV) and Se(VI) represent less than 10% in every case.

Selenomethionine is identified as the major constituant of yeast. This result is in good agreement with other authors[35]. Selenocystine represents 33% of the total amount whereas only 3% occurs as Se(IV). A 13% extracted fraction gave no chromatographic response and thus could not be identified. Selenocystine and selenomethionine were identified in kidney tissue. Selenomethionine is the major way of selenium storage in living organisms[3] whereas selenocysteine, present in glutathione, was reported as major selenoamino acid in mouse kidney[37-38]. Nevertheless, a comparative study of selenium form after selenite or selenomethionine absorption has given different results. Selenium was incorporated as selenocysteine in the first case whereas selenomethionine was found in the second[39]. Unidentified selenium (24% in CRM 186) might represent unhydrolysed proteins that could still contain some of the studied seleno compounds or others that could not be detected in our chromatographic conditions.

TABLE III Speciation in the pronase/lipase extracts

Matrix	*SeCyst*	*SeMet*	*Se(VI)*	*Se(IV)*	*∑Identified/ Extracted*	*∑Identified/ Total*
CRM 402	2.0 ± 0.4	0,6 ± 0,04	0.201* ± 0.001	$<l_d$	2.8 ± 0.4	2.8 ± 0.4
	30 ± 4	9,2 ± 0,7	3.0 ± 0,1	$<l_d$	73 ± 11	42 ± 6
CRM 186	1.5 ± 0.2	4.2 ± 0.6	0.49* ± 0.04	$<l_d$	6.2 ± 0.8	6.2 ± 0.8
	15 ± 2	41 ± 6	4.8 ± 0.4	$<l_d$	75 ± 9	61 ± 8
Yeast	343 ± 20	457 ± 21	$<l_d$	29.5 ± 2.5	831 ± 30	831 ± 30
	33 ± 2	44 ± 2	$<l_d$	3 ± 0.3	87 ± 3	80 ± 3

*values obtained wilh Differential Pulse Cathodic Stripping Voltammetry measurements using conditions from ref 29.

Selenocystine was identified in withe clover with low recovery (31%) together with Se(VI) (3%). Unreproducibilities in the speciation of this extract has led to further investigations. A clover sample collected in the area was spiked and

extracted to study what reaction could occur in the sample after rehydration. Clover was spiked with standard solutions of selenocystine, selenomethionine, Se(VI) and Se(IV). Recoveries after enzymic extraction using pronase/lipase are presented in Table IV. Extraction of the selenocystine is not quantitative (72%) and Se(IV) seems to be more available than Se(IV). Degradation of selenom-ethionine occured readily in the presence of clover, resulting in a cationic or neutral unknown species eluted into the dead volume of the chromatographic conditions. This compounds appeared also when selenomethionine is placed in contact with clover without enzymes. Selenomethionine is an intermediary metabolite implied in the volatilization of selenium by non-accumulator plants species. A hypothesis that could be stated is that, even after a partial lyophilisation, the plant enzymes responsible for selenium assimilation and particularly selenomethionine metabolisation are not inhibited and could react on this selenated species[2].

TABLE IV Speciation in spiked clover.

	SeCyst	*SeMet*	*Se(IV)*	*Se(VI)*	*∑Identified*
normal ($\mu g\ g^{-1}$)	7.0 ± 0.3	4.4 ± 1.3	7.1 ± 0.5	10.1 ± 0.3	29 ± 2
case (%)	70 ± 3	44 ± 13	71 ± 5	101 ± 3	71.5 ± 6

sample was spiked wilh 10 $\mu g\ g^{-1}$ as each species.

CONCLUSION

The selective determination of selenocompounds in environmental matrices is achieved with sample dependant recoveries (57% for clover, 80% for pig kidney and 92% for yeast). Enzymic extraction seems to be of a real interest for the analysis of highly proteinic samples whereas it is less effective on plants. The identification of selenomethionine and selenocystine as major components of proteinic samples is in agreement with literature[35-36].

Nevertheless, unidentified extracted selenocompounds still represent an important part of samples: 13% for yeast, 25% for kidney and over 40% for clover. The use of this speciation method together with complementary analytical methods such as analysis of volatile and cationic species should give more information on natural materials.

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