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SPECIES-SELECTIVE DETERMINATION OF SELENIUM COMPOUNDS IN BIOLOGICAL MATERIALS

(Technical Report)

Prepared for publication by
R. LOBINSKI[1,2], J. S. EDMONDS[3], K. T. SUZUKI[4], and P. C. UDEN[5]

[1]Warsaw University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, ul. Noakowskiego 3, 00-664 Warszawa, Poland; [2]CNRS UMR 5034, Hélioparc, 2, av. Pr. Angot, 64 000 Pau, France; [3]Department of Chemistry, De Montfort University, The Gateway, Leicester LE1 9BH, UK; [4]Faculty of Pharmaceutical Sciences, Chiba University, Inage, Chiba 263-8522, Japan; [5]Department of Chemistry, University of Massachusetts, Amherst, MA 01003-4510, USA

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Species-selective determination of selenium compounds in biological materials (Technical Report)

Abstract: There is substantial evidence of the complexity of selenium speciation in living organisms and of the importance of the selective determination of the particular species of this element in order to understand its metabolism and biological significance in clinical chemistry, biology, toxicology, and nutrition. The state-of-the-art of analytical techniques available for this purpose is critically evaluated with particular emphasis on the element-selective detection and identification of the detected selenium compounds. Whereas there are a number of techniques available that are able to detect various selenium species in living organisms selectively, few techniques exist that are able to identify and to characterize the species detected.

INTRODUCTION

Selenium has been shown to be essential for life and to be toxic at levels little above those required for health. Indeed, dietary levels of the desired amount of Se are in a very narrow range: consumption of food containing less than 0.1 mg kg^{-1} of this element will result in its deficiency, whereas dietary levels above 1 mg kg^{-1} will lead to toxic manifestations [1]. The essentiality of selenium results from its presence as a necessary component to form the active center, selenol group (–SeH), of glutathione peroxidase, thioredoxin reductase and of other selenoenzymes [2,3]. Powerful cancer chemopreventive effects are seen for inorganic selenium salts, selenoamino acids, and various synthetic organoselenium compounds. Monomethylated forms of selenium such as methylselenol (CH_3SeH) are a critical class of chemopreventive selenium metabolites [4]. Selenium has also attracted attention because of its apparent ability, usually when administered as inorganic salts, to ameliorate the toxic effects of heavy metals such as mercury and cadmium [5,6].

In the living body, in contrast to metal–protein complexes, selenium is not bound by coordination but forms covalent carbon–selenium (C–Se) bonds. Selenium species in the living body can be grouped as shown in Table 1. They can be divided into two categories: enzyme products and gene products. The former are present as products of enzymatic reactions such as reduction, methylation, and of reactions leading to selenoamino acid synthesis. Selenium is incorporated into the gene products, according to the UGA codon which encodes the selenocysteinyl residue. Selenoproteins will therefore

Table 1 Selenium species in living organisms

Selenium in proteins	
Selenoproteins	selenocysteinyl residues
Se-containing proteins	selenomethionyl residues
Non-protein selenium species	
Inorganic selenium	selenite (SeO_3^{2-}), selenate (SeO_4^{2-})
Methylated selenium	monomethylselenol, dimethylselenide, trimethylselenonium ions
Selenoamino acids	selenocystine, selenomethionine, *Se*-methylselenocysteine, selenoglutathione

contain selenium in the form of selenocysteinyl residues; proteins that contain this element in the form of selenomethionyl residues are not formally classified as selenoproteins.

The myriad of selenium species present in biological systems represents a challenge to the analytical chemist. Analytical techniques for the determination of selenium species were reviewed [7–10]; the coverage of selenoprotein analysis in animals was considered by Behne et al. [11,12]. Critical analysis of these reviews leads, however, to the conclusion that the majority of work (except that on selenoproteins) was driven by the intent of analysts to develop new instrumental techniques to be applied to the commercially available standards rather than to address a particular biochemical problem. Consequently, very limited information on the distribution of Se species in biological systems is available because of the obvious lack of standards.

The objective of this report is to present the current need for species-selective information concerning Se in biomaterials, to identify the key analytical challenges, and to evaluate the extent to which analytical instrumentation and methodology available can tackle them. Particular attention is given to the identification and characterization of selenium species in biological materials.

SPECIES OF INTEREST

The major fields of interest include (*i*) determination of methylselenium species in urine, (*ii*) species-selective determination of selenoaminoacids in microorganisms and plants, and (*iii*) characterization of selenoproteins in mammals. Chemical formula of the principal species of interest are given below.

Selenite	SeO_3^{2-}
Selenate	SeO_4^{2-}
Trimethylselenonium ion	Me_3Se^+
Dimethylselenide	Me_2Se
Selenocysteine	$H_3N^+–CH(COO^-)–CH_2–SeH$
Selenocystine	$H_3N^+–CH(COO^-)–CH_2–Se–Se–CH_2–CH(COO^-)–NH_3^+$
Selenomethionine	$H_3N^+–CH(COO^-)–CH_2–CH_2–Se–Me$
Se–Methylselenocysteine	$H_3N^+–CH(COO^-)–CH_2–Se–Me$
γ-Glutamyl-*Se*-methylselenocysteine	$H_3N^+–CH(COO^-)–CH_2–CH_2–CO–NH–CH(COO^-)–CH_2–Se–Me$
Selenocystathionine	$H_3N^+–CH(COO^-)–CH_2–CH_2–Se–CH_2–CH(COO^-)–NH_3^+$
Selenohomocysteine	$H_3N^+–CH(COO^-)–CH_2–CH_2–SeH$
Selenocystamine	$H_2N–CH_2–CH_2–Se–Se–CH_2–CH_2–NH_2$
Se–Adenosylselenohomocysteine	$NH_2CH(COOH)CH_2CH_2SeCH_2C_4H_5O_3C_5N_4NH_2$

The reduction of Se at the intrinsic ionic potential followed by its methylation to methylselenol, dimethylselenide, and the trimethylselenonium ion is the primary pathway of the metabolism of selenite by animals and man. Some of these and other unidentified compounds of this type can be excreted in urine [13,14]. Trimethylselenonium accounts for a few percent of Se in urine in normal subjects but it becomes predominant if the nutritional intake of selenium increases. Monomethylselenium was found in untreated rats but selenium-treated rats contained trimethylselenonium. Three peaks were observed in reversed-phase HPLC chromatograms of the basal human urine but their identification by retention time matching was unconvincing [15]. At least five species have been found in urine but only two organoselenium compounds trimethylselenonium and monomethylselenol, have been identified so far [13].

In addition to the common selenate and selenite species, a number of selenoamino acids have been identified in microorganisms and plants [16]. Selenoamino acid analogues are present in general in nonreactive forms containing C–Se–Se–C and C–Se–C linkages, but not in the reactive C–SeH form.

In the most widely investigated material, Se-enriched yeast, more than 20 selenium compounds including selenocysteine, selenomethionine, *Se*-methylselenocysteine, *Se*-adenosylselenohomocysteine, and inorganic forms appear to be present [17–21]. Five selenium species and several unknown peaks were observed in selenium-enriched garlic, onion, and broccoli [22, 23]. Several seleno-analogues of sulfur-containing amino acids and their derivatives have been identified in terrestrial plants, especially in so-called "selenium accumulator plants" [24–26]. Among the six organoselenium compounds detected in seagull eggs the major compounds were selenocysteine and selenocystamine [27]. Another area of interest in species-selective determination of amino acids is their production by enzymolysis of selenoproteins [28–31]; this step is also a central one in sample preparation of selenium materials for HPLC determination.

In mammals, speciation of Se usually involves the determination of the different Se-containing proteins [32–39]. The metabolic fate of selenium in the human body is schematically illustrated in Fig. 1 [6]. Inorganic selenium is reduced stepwise to the assumed key intermediate hydrogen selenide, and it or a closely related species is either incorporated into selenoproteins after being transformed to selenophosphate and selenocysteinyl tRNA according to the UGA codon encoding selenocysteinyl residue, or is excreted into urine after being transformed into methylated metabolites of selenide. As a result, selenium is present mostly in the forms of covalent C–Se bonds in mammals. The most important may be selenoprotein P, a major protein which is sometimes used as a biochemical marker of selenium status [33], selenoenzymes such as several glutathione peroxidases (enzymes that catalyze the reduction of peroxides and thus protect the cells from oxidative damage), type 1-iodothyronine de-iodinase (which catalyzes the de-iodination of thyroxine to triiodothyronine) [32,33,37] and thioredoxin reductase which may trigger cell signaling in response to oxidative stress [2].

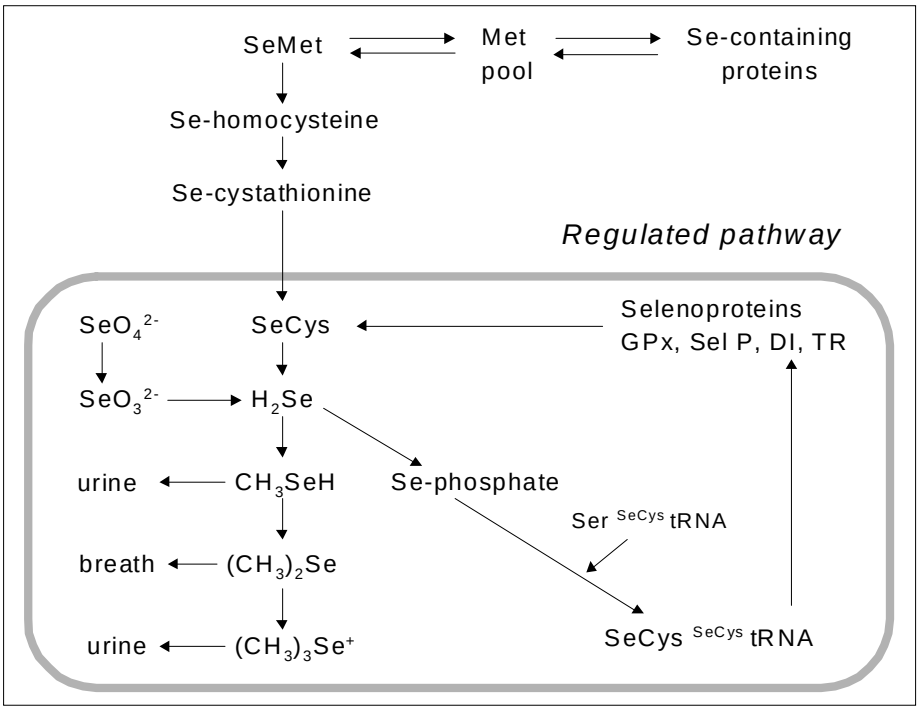

Fig. 1 The metabolic fate of selenium in the human body.

A number of other selenoproteins were identified by SDS-PAGE followed by autoradiography [40] after the injection of ^{75}Se-tracer into selenium-depleted animals: 28 different selenium-containing proteins or protein sub-units with molecular masses between 8000–116 000 Da (unified atomic mass units) were found [12,37]. Differences between different tissues were observed indicating specific functions of some of the selenium-containing proteins in certain tissues. Differences were also obtained between the subcellular fractions indicating that some selenoproteins are involved in different intracellular processes [12,41]. In human breast milk four selenium species with the apparent molecular weights of 15, 60, 1500 and >2000 kDa were detected [42].

ANALYTICAL TECHNIQUES

In order to be viable, an instrumental method must be specific to a particular selenium species or the species must be separated in time or space prior to arriving at a selenium-specific detector. The first group of techniques includes, in particular, radioimmunological assays (RIA) for selenoproteins which offer very low detection limits but require the proteins to be isolated in amounts sufficient for antibody production [33]. The second approach, using so-called hyphenated techniques, is based on the coupling of an electrophoretic or chromatographic separation technique with an atomic spectrometric or other Se-specific technique. This coupling can be realized off-line, e.g., SDS-PAGE with instrumental neutron activation analysis (INAA), or on-line, e.g., HPLC-ICP-MS. The principle of coupled techniques has been widely discussed elsewhere [43–46]. Selenium species (Se(IV), Se(VI), selenomethionine, and selenocysteine) were often used as examples in experimental developments. Below, the limitations of these techniques to unravel the metabolism of selenium in living organisms are evaluated.

Polyacrylamide gel electrophoresis with selenium-specific detection

Flatbed electrophoresis offers a much better resolution than HPLC for the separation of selenoproteins. The fact that Se is bound via a covalent bond allows the use of SDS-PAGE without the risk of Se losses [11,12,37,40,41,47–49]. SDS-PAGE may assure the complete dissociation of multimeric proteins into their subunits, and thereby it may give rise to several labeled bands originating from the same compound. Tissues may contain proteins with similar migration velocities, and complementary separation mechanisms are necessary. For example, the isolation of the protein fraction by anion-exchange chromatography was recommended prior to PAGE [50].

Autoradiography is the principal technique used for the detection of selenoproteins in gels strips [11,12,37,40,41,47,49]. It requires that the organisms studied are radiolabeled by injection of the tracer into selenium-depleted animals; the ^{75}Se nuclide ($t_{1/2}$ = 120.4 d and emitting γ-rays in the range 10 and 280 keV) is well suited for such experiments. The tissue homogenate of the labeled animals is leached with an aqueous buffer, and the selenoproteins in the supernatant are separated by SDS-PAGE prior to autoradiography [11,12,37,40,41,47,49]. The advantage is the very low limit of detection (in the sub-picogram range) which may be controlled by the uptake and retention of the labeled selenium. The disadvantage is the use of a radioactive isotope. Also, it may be difficult to be sure that after an initiation phase the tracer activity reflects the distribution of the native selenium. Reliance on radiotracers is not compatible with the analysis of field-collected samples which is critical to the understanding of selenium biogeochemistry and ecotoxicology. Most studies concern selenoproteins in rats [11,12,37,40, 41, 47,49] and soybean radiolabeled with ^{75}Se [50,51].

Alternatively, selenoproteins separated by PAGE can be Western blotted onto nitrocellulose membranes in which Se is determined by any appropriate method [52]. Some instrumental techniques such as NAA [40, 41, 51, 52] and XRF [54] allow the direct determination of selenium in polyacrylamide gel strips obtained after electrophoresis of selenoproteins. The absolute detection limits of these techniques were 0.1 ng and 2–30 ng, respectively.

HPLC WITH SELENIUM-SPECIFIC DETECTION

Applications of HPLC with selenium-specific detection for the analysis of biological samples are summarized in Tables 2 and 3. Separation mechanisms used have included size-exclusion, ion-exchange (anion and cation) and ion-interaction reversed-phase. Size-exclusion HPLC (often in combination with affinity chromatography) was mainly used for the determination of selenoproteins and studies of interactions of selenium with proteins in the animal and human body. Ion-exchange and reversed-phase HPLC have been mainly applied to the separation of Se(IV), Se(VI), selenomethionine, and selenocysteine (for an exhaustive list of references see [17,18,55]). In recent studies a large number of synthetic organoselenium compounds were used [24–26].

Inductively coupled plasma mass spectrometry (ICP MS) is the primary detection technique in HPLC of selenium species despite the fact that this element is often quoted as an exception to the excellent ionization yield and sensitivity of ICP-MS. Detection limits obtained are at the low ng ml^{-1} level. A ten-fold improvement of detection limits can be achieved by the use of an ultrasonic nebulizer MS [56], post-column hydride generation [57], or the use of a HR ICP mass spectrometer [15].

Significant isotopic overlaps on the most abundant isotopes (^{75}Se, ^{80}Se) require the use of the relatively non-abundant ^{82}Se but isobaric interferences are much less pronounced when ICP MS is employed as a chromatographic detector than when it is used for the determination of total selenium. The coupling of HPLC with ICP MS is relatively straightforward. However, in reversed-phase HPLC, when the concentration of organic modifier in the mobile phase exceeds 5%, the use of a cooled spray chamber, auxiliary oxygen gas, and platinum sampler and skimmer cones is recommended. A valuable feature of ICP MS is the possibility of discrimination between the particular selenium isotopes. It allows therefore the differentiation of endogenous selenium and external tracers without the need for radioactive isotopes [71–73].

Neither ICP AES nor AAS coupled directly to HPLC can offer detection limits sufficient to match the concentrations of Se species in biological samples. The detection limits can be improved by post column conversion of selenium into H_2Se which is then transported into the quartz furnace for AAS [57], or into the ICP for AES detection [57,74,75]. Detection limits in the low ng range can be achieved. An alternative is the use of GF AAS off-line [67]. Se-containing proteins are more difficult to digest on-line [57].

Table 2 Analytical methods for species-selective analysis of seleno compounds in samples of plant origin by HPLC with element selective detection

Sample	Column	Mobile phase	Detection	Ref
nutritional supplements	CE: Supelcosil LC-SCX (250 × 4.6 mm)	20 mM pyridine (pH 4.7) + 5 mM citric acid (pH 5.95) (1.5 ml min^{-1})	ICP MS	[58]
nutritional supplements	RP: Nucleosil (200 × 4 mm × 5 µm)	0.05% triethylamine (0.65 ml min^{-1})	HG AAS	[59]
corn and rice	Dionex DC6A	not given	radioactivity	[31]
Se-enriched garlic, onion, and broccoli	Hamilton PRPX-100 (150 × 4.6 mm)	5 mM ammonium citrate (pH 4.8) in 2% MeOH	ICP MS	[22]

Table 2 *Continued*

Sample	Column	Mobile phase	Detection	Ref
Se-enriched yeast	SE: Superdex-200 HR (10 × 300 mm × 13 µm) AE: Hamilton PRPX-100 (250 × 4.1 mm × 10 µm) RP: Inertsil ODS-2 (150 × 4.6 mm × 5 µm)	SE: 30 mM Tris-HCl (pH 7) AE: gradient elution with ammonium phosphate buffer RP: 0.1% TFA in 2% MeOH	ICP MS	[22]
Se-enriched yeast	RP: Inertsil ODS-2 (150 × 4.6 mm × 5 µm)	0.1% TFA in 2% MeOH	ICP MS ES MS/MS	[21]
Se-enriched yeast and garlic	AE: Hamilton PRPX-100 (250 × 4.1 mm × 10 µm) RP: Brownlee C18 (22 × 2.1 mm) IRP: Zorbax SB-C8 (150 × 4.6 mm)	AE: 5 mM ammonium citrate buffer (pH 4.8) containing 2% MeOH (1 ml min^{-1}) RP: 0.034% TFA in 60% MeOH IRP: 0.1% TFA in 2% MeOH	ICP MS	[17]
Se-enriched yeast, garlic	IRP: Symmetry Shield RP8 (150 × 3.9 mm × 5 µm)	0.1% heptafluorobutanoic acid in % MeOH	ICP MS	[26]
Se-enriched yeast, garlic	IRP Symmetry Shield RP8 (150 × 3.9 mm × 5 µm)	1% MeOH with 0.1 % TFA (1 ml min^{-1})	ICP MS ES MS	[23,24, 25]
Se-enriched yeast	IRP: Zorbax SB-C8 (150 × 4.6 mm)	0.1% TFA in 2% MeOH (1 ml min^{-1})	ICP MS	[18]
Se-enriched yeast enzymatic hydrolyzate	IRP: Hamilton PRP-1 (250 × 4.1 mm)	sodium heptanesulfonate in 10% CH_3CN (pH 2.4)	GF AAS	[28]
White clover BCR 402	AE: Polyspher IC AN-2 (120 × 4.6 mm × 10 µm)	6 mM salicylate in 3% MeOH (1 ml min^{-1})	FAAS, ICP MS	[60]
White clover CRM 402	AE: PAX-100 or Ion-Pac AG10 (50 × 2 mm)	$(NH_4)_2CO_3$ (pH 10) in 2% MeOH (80 µl min^{-1})	DIN - ICP AES, GFAAS	[61,62]
White clover CRM 402	IRP: Hamilton PRP-1 (150 × 4.1 mm)	10 mM tetrabutylammonium bromide in % CH_3CN (pH 4) (0.4 ml min^{-1})	GF AAS	[63]
Se-exposed cyanobacterium	CE: Nucleosil 100-5SA (250 × 4.6 mm)	A: 25 mM $NH_4H_2PO_4$ (pH 3.0); B: 25 mM $(NH_4)_2HPO_4$ (pH 7.0). Gradient elution: 0–20 min 0–67%B	ICP MS	[64]

Table 3 Analytical methods for species-selective analysis of seleno compounds in samples of animal and human origin by HPLC with element-selective detection.

Sample	Column	Mobile phase	Detection	Ref
seagull eggs	RP: 5 µm C18 Nucleosil	30 mM $HCOONH_4$ in 5% MeOH (pH 3) (1.2 ml min^{-1})	HR ICP MS	[27]
enzymatic extract of cooked cod	AE: polysphere IC AN-2 column (120 × 4.6 mm × 10 µm)	5 mM salicylate - Tris (pH 8.5) (0.75 ml min^{-1})	ICP MS	[30]
rat plasma	SE: Asahipak GS520 or GS320	50 mM Tris-HCl buffer (pH 7.4) (1.0 ml min^{-1})	ICP MS	[5,14]
human plasma	SE: Asahipak GS520 HQ	10 mM sodium phosphate buffer (pH 7.0) (0.6 ml min^{-1})	ICP MS	[38]
human and mouse plasma	tandem HPLC: heparin affinity (AFPak) and SE (Asahipak GS520 HQ)	sodium heparin in 20 mM phosphate buffer (pH 7.5) containing 0.01% EDTA	ICP MS	[39]
human liver	Sephadex 200 (10 × 300 mm × 13 µm)	not given	INAA	[65]
serum, breast milk	CZE and CIF of SEC-purified fraction		ICP MS	[66]
human erythrocyte	SE: G3000 SWXL (300 × 7.8 mm × 10 µm)	10 mM Tris buffer (pH 7) containing 0.1 M NH_4NO_3 (0.25 ml min^{-1})	On-line GF AAS	[67]
serum, urine	IRP: Hamilton PRP-1 (150 × 4.1 mm × 5 µm)	0.1 mM sodium pentanesulfonate in 2 % MeOH (pH 4.5) (1 ml min^{-1})	ICP MS	[68]
rat urine	SE: Asahipak GS520 or GS320	50 mM Tris-HCl buffer (pH 7.4) (1.0 ml min^{-1})	ICP MS	[69,70]
urine	IRP: Hamilton PRP-1 (150 × 4.6 mm × 5 µm)	5 mM tetrabutylammonium phosphate in 3% MeOH (pH 7.6) (1.7 ml min^{-1})	ICP MS	[56]

Detection of selenoproteins by HPLC-ICP MS

Size-exclusion (SE) HPLC with on-line detection by ICP MS appears to be the primary technique that allows the detection of the presence of metals bound to macromolecular ligands in an unknown sample [76,77]. Despite the fact that SE HPLC was the first to produce evidence of the presence of selenoprotein-P in human plasma, it is considered to lack the resolution to permit the separation of the major Se-proteins; in addition, the large dilution factor limits detection sensitivity. SE HPLC-ICP MS of a human serum sample yielded three signals, none of which, however, co-eluted with the glutathione peroxidase activity [38]. SE HPLC of human breast milk whey produced four Se signals corresponding to species

with apparent molecular weights of 15, 60, 1500 and >2000 kDa [42].

The principal speciation approach for selenoproteins in serum is based on affinity chromatography; column packing materials being available that allow the separation of serum Se-proteins [32,35,36]. Affinity chromatography using a heparin-Sepharose CL-6B column in series with a column of reactive blue 2-Sepharose CL-6B with off-line detection, was proposed for the separation of selenoproteins in serum [32,35]. Recently, a combination of affinity chromatography with SE HPLC and on-line ICP MS detection was reported to separate the three major Se-containing proteins (albumin, glutathione peroxidase, and selenoprotein P) found in human plasma [39]. It was also reported that the three proteins in rat serum could be separated in one step by SE HPLC-ICP MS [71].

HPLC-ICP MS with enriched stable isotopes was used to study speciation of both endogenous Se and the external ^{82}Se tracer. It allowed the simultaneous differentiation of the fate of injected and endogenous selenium, the injected element being incorporated efficiently into selenoprotein P and, to an extent, into extracellular glutathione peroxidase [13,70,71,73,78]. However, the full identification of selenium peaks was not possible; several of them were assigned on the basis of their molecular sizes in the selenium SE HPLC profiles [13,70,72,78].

Selenium incorporation into cyanobacterial metallothionein induced under heavy metal stress was studied using SEC HPLC - ICP MS; existence of two pathways was indicated [64]. SEC was used to assign selenium in human erythrocyte lysates to two (35 and 100 kDa) proteins [67].

Detection of small seleno compounds by HPLC-ICP MS

A plethora of methods claimed to be useful for speciation of Se(IV), Se(VI), selenomethionine, and selenocysteine exist, as can be seen from the review papers [7–10]. The reason for this choice of analytes seems to be the commercial availability of standards for these compounds since many of the methods developed have never been applied. Paradoxically, the opposite approach, i.e., one based on the screening of a sample for the presence of stable selenium species by HPLC with selenium-specific detection, leads not only to the conclusion that the actual number of species present exceeds 20 but also that in some instances none of the four above-mentioned standard compounds is apparently the dominant species [17–21].

The employed separation mechanisms have included ion-exchange (anion and cation) and ion-pairing reversed-phase chromatography, the degree of separation being strongly dependent on the pH of the mobile phase [17,30,58,61]. Anion-exchange [20] and cation-exchange [55] are complementary separation mechanisms, neither of which used alone are able to assure satisfactory resolution of all the seleno compounds in extracts of biological samples. In the cases when the interest is limited to the quantitation of inorganic selenium species or selenomethionine (enzymatic extracts) anion-exchange HPLC seems to be the technique of choice. The chromatographic mobile phase should possess a pH-buffer effect in the range of the p*K*a values of the anionic Se compounds and, in order to prevent salt buildup on the ICP-MS sampler and skimmer cones, the eluting molecular species should preferably be organic in nature [60].

Most organoselenium compounds show insufficient hydrophobicity to be retained and separated on C_8 and C_{18} reversed-phase stationary phases. HPLC with a salt-free aqueous mobile phase is a convenient method to separate the relatively hydrophobic adenosyl-substituted selenoamino acids from the matrix salts eluting with the dead volume [21]. Retention and separation of selenoamino acids can be increased by using an ion-pairing reagent: trifluoroacetic acid [17,20] or octanesulfonate [79]. In particular, perfluorinated carboxylic acids offer good resolution and should be given preference for the characterization of samples containing many seleno compounds [23–25]. Another possibility is the derivatization of the selenoamino acids to *N*-2,4-dinitrophenyl derivatives by reaction with 2,4-dinitrofluorobenzene [17].

Despite the fact that, in theory, SE-HPLC separation should be based on the molecular weights of the analytes, secondary adsorption and ion-exchange effects apparently play an important role, and separation of small compounds with similar molecular masses can be achieved in addition to the possibility of the detection of selenoproteins [20].

Capillary zone electrophoresis with ICP MS detection

Because of the large number of theoretical plates obtainable, capillary zone electrophoresis is a very promising technique for the definitive verification of the chromatographic purity of the target compound. The advantages of CZE, such as the possibility of analyzing for relatively labile species because of the absence of chromatographic packing, and high resolution, are outweighed by the need for ultrasensitive detection, such as high-resolution ICP MS because of the small sample amount injected. A disadvantage is the lack of a commercial interface.

The potential of CZE-ICP MS was exemplified by the identification and determination of selenoglutathione in human milk, and by the differentiation of methionine and selenomethionine and of cystamine and selenocystamine in milk [66,80–85]. The selenoamino acids were identified by comparison with standards [80,81]. In some other studies, however, migration times differed from those of aqueous standards [82].

Gas chromatography of selenoamino acids

Selenomethionine is the predominant selenium moiety in plants, whereas selenocysteine is formed by conversion of selenomethionine in mammalian tissues. There are some analytical approaches that are based on the degradation of the originally existing species to the above two amino acids followed by their determination. For this purpose, gas chromatography with element-selective or mass spectrometric detection was preferred to HPLC because of the higher sensitivity.

Selenoamino acids were derivatized with isopropyl chloroformate and bis(*p*-methoxyphenyl) selenoxide [86], with pyridine and ethyl chloroformate [87] or silylated with bis(trimethylsilyl)-acetamide [88]. Selenomethionine forms volatile methylselenocyanide with CNBr [89,90]. A derivatization method was based on esterification of the carboxylic group with propan-2-ol and subsequent acylation of the amino group with heptafluorobutyric anhydride [91]. Mass spectrometric or Se-specific detection is necessary because of the possible interference from the sulfur analogues.

The determination of selenomethionine in the proteinaceous fraction is carried out after acid digestion and silylation, the compound being identified on the basis of both the GC retention time and the mass fragmentation pattern. Quantification should be performed with the standard addition method to correct for the response from the protein digest matrix. The highest recovery of selenomethionine (84%) was obtained by carrying out the digestion with 4 M HCl at 50 °C for 50 h under nitrogen [91].

Identification of selenium species

HPLC-ICP MS and CZE-ICP MS offer sufficient sensitivity and selectivity for the detection of seleno compounds in biological samples but do not provide structural information for the identification of known, unknown, or unpredicted compounds. This drawback becomes more important as the wider availability of more efficient separation techniques and more sensitive detectors makes the number of unidentified species grow. The identification of the species in chromatographic techniques is based on their migration time through a chromatographic support. The identifying parameter is the retention (migration) time or the position after a certain time on a PAGE plate. The availability of the authentic standards of seleno compounds is therefore mandatory for retention-based identification.

However, except for simple compounds such as selenate, selenite, selenomethionine, selenocysteine, and selenoethionine, authentic standards of selenium compounds are rarely available. The major approaches to address this problem are based on the (i) preparation of synthetic standards and retention (migration) time matching, and (ii) isolation of the purified seleno compound from the sample for its further characterization, e.g., by electrospray (ES) [21,23–25,29,30,83]) or matrix-assisted laser desorption ionization (MALDI) [50].

The successful identification of a selenium species by matching its retention (migration) time with that of an authentic standard depends critically on two factors. The first is to predict what compound should be synthesized, and the second is the assurance that the resolution of the chromatographic technique used produces at a given retention time a signal corresponding to this compound only. Also, in the presence of a biological matrix the retention time may shift because of interactions of seleno compounds with sample components.

The enzymatic synthesis of selenomethionine metabolites (from the transmethylation and polyamine synthesis pathways): adenosylselenomethionine, adenosylselenohomocysteine, decarboxylated adenosylselenomethionine, and methylselenoadenosine was described; the simultaneous assay of these compounds together with the corresponding methionine metabolites was developed [79]. Fan et al. [29] synthesized the selenium metabolite compounds *Se*-methylselenomethionine, *Se*-methylselenocysteine, and dimethylselenonium propionate, and employed a combination of 2D multinuclear NMR, electrospray MS and GC-MS methods to identify Se-compounds in synthetic preparations [29]. Uden et al. synthesized a number of selenoamino acids and tried to match their retention times with signals produced by extracts of yeast supplements [18,23,24,25,26]. The success of these approaches so far has been moderate.

A more promising way to identify selenium compounds is the use of (tandem) mass spectrometry and/or NMR on selenium containing fractions. The most sensitive method is GC-MS, but this method is not suited for metabolites that cannot be made volatile and is handicapped when authentic standards are not available [29]. Nevertheless, a number of examples of successful quasi-direct determination of selenoamino acids in biological samples [29,91] and in selenoprotein hydrolysates [88] have been demonstrated. The technique is also valuable for the identification of alkylselenium compounds [92,93].

For the nonvolatilizable metabolites, the major shortcoming is the discrepancy between the sensitivity of MS techniques and the sensitivity required for the detection of Se in real samples. These techniques allow the characterisation of synthetic standards but, in order to be successfully applied to real-world samples, purification and preconcentration of Se-species is mandatory.

Electrospray MS that allows a precise (±1 Da) determination of the molecular mass of a species is an invaluable tool for the identification and a prerequisite for further characterization of compounds in speciation analysis. So far, ESI MS has been applied mostly to commercial standards [30,83] or synthetic preparations of *Se*-methylselenomethionine, *Se*-methylselenocysteine, and dimethylselenonium propionate (precursors of volatile alkylselenides) [29]. Recently, improved pneumatically-assisted ESI MS was used to identify *Se*-adenosyl-homocysteine in an extract of selenized yeast [21]. A number of other organoselenium compounds were identified in yeast, garlic, and plant extracts [23–25]. It should be emphasized that when authentic standards are not available the use of tandem MS is mandatory. Once a hypothesis on the structure is put forward on the basis of ESI MS/MS data, the fragmentation patterns may be confirmed by corresponding sulfur-containing compounds for most of which standards are commercially available.

Sample preparation

Plasma, milk whey, and urine samples can be injected on a HPLC column directly, usually after filtration and dilution. Since selenoamino acids are water-soluble, leaching with hot water has been judged sufficient

to recover selenium species which are not incorporated into larger molecules. The sample is homogenized with water, sonicated or heated and ultracentrifuged. The typical recovery of selenium extracted in this way is ca. 10% [17,18,20,28,58] but it can be total in the case of some selenized yeast samples. Free selenoamino acids were separated by ultrafiltration (breast milk) [66] or dialysis (algal extract) [29]. Selenocysteine and some other selenoamino acids are highly susceptible to oxidative degradation, because the selenol group has a significantly lower oxidation potential than its sulfur counterpart. The carboxymethyl derivative was synthesized (by addition of iodoacetic acid) to stabilize selenocysteine and thus to prevent its degradation [64].

The low yields from the aqueous leaching procedure for some species and samples promoted more aggressive leaching media to be used by some workers. A trade-off is always necessary between the recovery of Se from a solid matrix and the preservation of the original Se species. As shown by Casiot et al. [20] the addition of sodium dodecyl sulfonate (SDS) to the leaching mixture increases the yield of Se by releasing protein-bound selenoamino acids. The recovery of selenoamino acids can increase to above 95% by degrading the species originally present with a mixture of proteolytic enzymes [28]. Leaching with an aqueous buffer was also used to extract selenoproteins from hydroponically grown, intrinsically labeled soybeans [50].

Care is advised in the interpretation of literature data since the results depend on the way in which the sample was prepared. This applies in particular to the frequently used statement the majority of Se is present as selenomethionine, when describing the result of a procedure involving an enzymic digestion. Actually, much of the selenomethionine usually constitutes a part of larger stable selenoproteins or is bound to non-selenium proteins that had been destroyed during the sample preparation procedure.

CONCLUSIONS

The development of procedures for the direct analysis of biologically important selenoamino acids and selenoproteins in plant and animal tissues and human body fluids remains a great challenge. Whereas much has been done recently in terms of the detection of the plethora of selenium species in these matrices by PAGE-autoradiography and HPLC-ICP MS, little information is still available on the identity of the signals measured. The prerequisite is wider use of techniques giving direct access to the structural information such as NMR and electrospray (tandem) mass spectrometry. The insufficient sensitivity and vulnerability to matrix interferences of these techniques can be overcome by the classical approach of natural products chemistry which involves the concentration, purification, and isolation of the seleno species detected. More substantial work on synthesis of selenium species is necessary to make standards available to analysts. Only then can the barriers to a molecular-level understanding of Se clinical chemistry, ecotoxicology, and nutrition be eliminated.

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