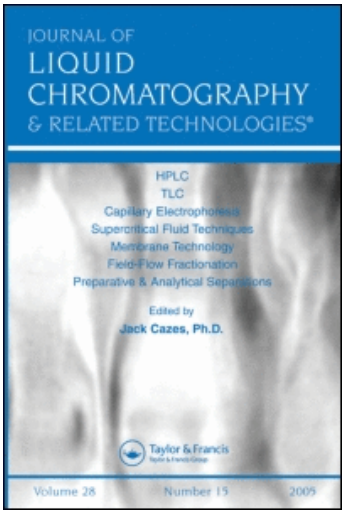

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SPECIATION OF SELENIUM IN BIOLOGICAL SAMPLES BY ION CHROMATOGRAPHY WITH INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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SPECIATION OF SELENIUM IN BIOLOGICAL SAMPLES BY ION CHROMATOGRAPHY WITH INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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□ *A comprehensive review is presented addressing recent trends in the speciation and determination of selenium in biological sample matrices, including important analytical aspects such as sample clean up, pre-concentration, method development, and removal of instrumental interferences. A brief outline of analytical principles, together with an overview of the recent developments and applications of selenium speciation determination is included. The newer methods for detecting selenium, including preparation schemes are also discussed together with speciation methodology based on ion chromatography hyphenated with inductively coupled plasma mass spectrometric techniques.*

Keywords sample pre-treatment, selenium, separation method, speciation, spectroscopy

INTRODUCTION

Selenium (Se) has been recognized as having many important roles in plant and animal physiology as well as being beneficial for human health. Selenium is a trace element well known as both an essential and toxic element to animals and humans.[1] The biological interest in Se covers a large variety of compounds, ranging from small volatile molecules, inorganic ions, and non-volatile amino acids to macromolecules such as

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selenoproteins.[1] There is strong evidence that diets supplemented with Se can reduce the incidence of certain types of cancer, such as prostate and breast cancer.[2] For example, through metabolic processes, Se enriched dietary supplements can be converted to anti-carcinogenic metabolites such as methylselenol (CH_3SeH).[3] As a consequence, there has been an increasing use of Se in food and food supplements. In turn, this has resulted in a need for analytical methods providing characterization of Se species. In most cases, Se species constitute very small portions of the total sample. Thus, sample preparation, analyte extraction, and preconcentration play key roles in practical method development. Since target species in complex matrices are found at trace to ultratrace (sub-ppb) levels, their determination requires development of robust analytical techniques with extraordinary specificity and sensitivity.[1]

The wide variety of hyphenated techniques used for speciation analysis, as well as the applications for which they have been employed have been previously reviewed.[4] In conjunction with HPLC, ICP-MS has been the most widely used instrument in recent years for element-specific detection[5] as it provides high sensitivity, allows high sample throughput and has multi-element detection capability with little interference. Hence, a number of excellent reviews for Se speciation analysis have been published.[6–10]

Uden[6] reviewed modern trends in the speciation of Se by hyphenated techniques, where these techniques were evaluated with particular emphasis on separation and identification of the Se-containing compounds. B'Hymer and Caruso[7] reviewed Se speciation using inductively coupled plasma-mass spectrometry (ICP-MS). This review also covered some of the background of Se in the environment and living systems, and, more importantly, some of the currently used separation methodologies, including both chromatography and electrophoresis. However, the emphasis was on applications of Se speciation techniques using ICP-MS detection. Polatajko et al.[8] presented state of the art systematic developments concerning different aspects of qualitative and quantitative Se speciation in biological samples. A review by Dumont et al.[9] provided a detailed picture of the current technological advances in Se speciation in food sources, in human tissues, and body fluids. More recently, Pedrero and Madrid[10] critically evaluated current trends in Se speciation, including hyphenated techniques, with a primary focus on biological matrices such as nutritional and food products. All of these speciation techniques were based on coupling chromatographic separation with inductively coupled plasma mass spectrometry (ICP-MS) and molecular mass spectrometry (ESI-MS, ESI-MS–MS, and MALDI-TOF) to allow identification, quantification, and structural characterization of Se species. Particular attention was paid to the development of Se-enriched food and nutritional products and how

the application of the techniques mentioned previously was mandatory to obtain reliable results on Se metabolites in these particular matrices.

This review differs substantially from some of these earlier reviews[6–10] in that the focus is broad and not restricted to just analytical techniques but also mechanisms and applications. It thus details the current state and progress in Se speciation in biological matrices by ion chromatography with inductively coupled plasma mass spectrometry up to 2010. First, the review examines in detail various sample pretreatment methods and techniques which were not emphasized in previous reviews. Second, ion chromatographic methods used for the separation of Se species are addressed and, subsequently, ICP-MS for the detection of Se is also discussed. The methods used to detect Se are summarized, together with a critical examination of the analytical methods currently available for Se speciation.

SELENIUM SPECIATION

Selenium exists naturally in different oxidation states and as various compounds (forms) in biological systems. Organic forms of Se in biological systems (animals, plants, and microorganisms) are variously reported as volatile methylated compounds, selenoamino acids, selenoproteins, or their derivatives. Selenium can biologically form covalent carbon (Se-C) and sulfur (Se-S) bonds and is found as enzyme or gene components. It can be incorporated into such substances by enzymatic actions including reduction and methylation, which lead to seleno-amino acid synthesis or Se gene products. For example, the UGA codon encodes for the selenocysteinyl residue. Identified Se species of major interest to date are summarized in Table 1.

SAMPLE PRE-TREATMENT

The successful preparation of the sample is crucial to successfully assessing the concentration and range of a Se species. An important requirement for reliable speciation is to retain the concentration and structure of the original chemical species in the sample. The direct analysis of Se species by either atomic spectroscopic or separation techniques is difficult because of the relatively low Se concentrations when compared to other metallic elements and the complex matrices of biological samples, which can interfere in the determination of low level Se species. The extraction of the species of interest with the highest recovery is the next challenge prior to analysis using chosen techniques. The successful extraction of Se compounds will depend on whether they are water soluble, protein bound, or polysaccharide bound.

TABLE 1 Principle Selenium Species in Environmental and Biological Systems

IUPAC Name	Abbrev.	Formula	Sample Type
Selenous acide(selenite)	Se(IV)	SeO_3^{2-}	Natural water, soil
Selenic acid(selenate)	Se(VI)	SeO_4^{2-}	Natural water, soil
Methylselenol		CH_3SeH	Urine
Dimethylselenide	DMSe	CH_3-Se-CH_3(volatile)	Breath, Se-enriched broccoli, garlic, onions
Dimethyldiselenide	DMDSe	CH_3-Se-Se-CH_3(volatile)	Breath, Se-enriched broccoli, garlic, onions
Dimethylseleniumsulfide		CH_3-Se-S-CH_3(volatile)	Breath, Se-enriched broccoli, garlic, onions
Diethylselenide	DESe	CH_3-CH_2-Se-CH_2-CH_3	Air, sediment
Trimethylselenonium ion	$TMSe^+$	$(CH_3)_3Se^+$	Urine
Selenocysteine	SeCys	HSe-CH_2CH(NH_2)-COOH	Seagull eggs, Se-enriched broccoli, garlic, onions
Selenocystine	$SeCys_2$	HOOC-CH(NH_2)CH_2-Se-Se-CH_2CH(NH_2)-COOH	Se-enriched yeast, broccoli, garlic, onions
Se-Methylselenocysteine	SeMC	CH_3Se-CH_2CH(NH_2)-COOH	Se-enriched yeast, broccoli, garlic, onions, chives
Selenomethionine	SeMet	CH_3Se-CH_2CH_2CH(NH_2)-COOH	Se-enriched yeast, broccoli, garlic, onions, seleniferous corn, wheat, soybeans, Brazil nuts, mushrooms, sesame seeds, chives
Se-Methylselenomethionine		$(CH_2)_2Se^+$-CH_2-CH_2-CH(NH_2)-COOH	Se-enriched yeast, broccoli, garlic, onions
Γ-Glutamyl-Se-methylselenocysteine		$H_2NCH_2CH_2$-CO-NHCH(COOH)CH_2-Se-CH_3	Se-enriched yeast, broccoli, garlic, onions
Selenohomocysteine		HSe-CH_2-CH_2-CH(NH_2)-COOH	Se-enriched yeast, broccoli, garlic, onions
Selenocystathionine		HOOC-CH(NH_2)-CH_2-CH_2-Se-CH_2-CH(NH_2)COOH	Se-enriched yeast, broccoli, garlic, onions
Selenocystamine	SeCystm	H_2N-CH_2-CH_2-Se-Se-CH_2-CH_2-NH_2	Human urine, seagull eggs, milk
Se-adenosylselenohomocysteine	AdoSeHcy	H_2N-CH(COOH)-CH_2-CH_2-Se-CH_2-$C_4H_5O_3C_5N_4$-NH_2	Se-enriched yeast
Selenourea	SeUr	$Se=C(NH_2)_2$	Urine
Selenosugars		Various sugar structures	Urine
Selenoproteins		Various proteins and enzymes	Milk, soybean, yeast, plasma, tissues

Liquid extraction is the most conventional approach to extract Se species[11] and a variety of different solutions have been used to extract species from selected matrices. Further discussion in this review will focus on the extraction of Se from biological samples. The main extraction methods for biological samples as listed in Table 2 include aqueous extraction, enzymatic hydrolysis, ultrasonic probe proteolytic hydrolysis, *in vitro* digestion, and sequential treatment.

Aqueous Extraction

Low recoveries (10–40%) from protein material have generally been observed when using simple methods such as hot water extraction. This has stimulated interest and promoted the development of higher efficiency methods for Se extraction. Some approaches have included mineral as well as organic acid attack. Methanesulfonic acid was used for selenomethionine extraction from yeast and Brazil nuts under reflux.[12] Since the SeMet found in these matrices is in a bound form, the acid liberates the bound analyte, thus giving a higher efficiency extraction than hot water. However, acid digestion was less favored for examining biological systems as some important organic forms of Se may not remain intact under such harsh conditions.

Enzymatic Hydrolysis

Enzymatic hydrolysis focuses on the extraction of Se protein bound compounds, either mechanically or via coordination complexes in biological matrices. A number of procedures have been developed. Driselase is a commercial enzyme preparation from *Basidiomycetes sp.* that is usually used to destroy cell-wall components.[2] Non enzymatic reagents like sodium dodecyl sulphate (SDS) are more suitable for membrane-protein containing Se[2]. Proteinase K or proteolytic enzymes (Protease XIV) have often been used for water-insoluble Se fractions in various complicated matrices, including human blood serum, cod muscle, wheat flour, etc.[13]

The efficiency of enzymatic hydrolysis was relatively high but variable (40%–90%). The efficiency of extraction depended on some key parameters such as the choice of enzymes, hydrolysis solution pH, temperature, and hydrolysis time. For Se based food supplements, the main extraction problems were caused by bulking agents and binders used in the manufacture of the food supplements.

Ultrasonic Probe Proteolytic Hydrolysis

Traditionally, enzymatic hydrolysis is time consuming and requires incubation.[14] Thus, ultrasonic probe proteolysis has been offered as an

TABLE 2 Extraction Efficiency of Se from Biological Samples

Sample	Extraction Method	Extraction Efficiency (% of Total Se)	Ref
Se-enriched shiitake mushroom	(1) Water in an ultrasonic bath for 30 min at 25°C, then incubated for 3 h at 37 or 95°C	68 (water)	[58]
	(2) 20 mg pronase Tris-HCl (pH 7.2) at 37°C overnight	77 (pronase)	
Natural & selenised *Agaricus* mushrooms	Water (37 or 85°C)	40–68	[59]
Enriched green onions (*Allium fistulosum*)	(1) Alkaline extraction: 0.1 M NaOH for 15 min	(1) 55	[25]
	(2) Enzymatic hydrolysis: 5 mg pronase E shaken at 25°C for 24 h	(2) 78	
Dill (*Anethum graveolens L.*) root, stem, leaf	(1) 0.1 M HCl were stirred for 24 at 25°C	31–35	[60]
Brazil nuts	(1) water microwave	(1) 9	[34]
	(2) 0.5 M HCl microwave	(2) 37	
	(3) 25 mg Proteinase K incubated at 37°C for 20 h	(3) 83	
Brazil nuts	4 M methanesulfonic acid at reflux for 8 h	75 (found as SeMet)	[12]
Six different brands of Se food supplements	Water with microwave for 2 h	15–95 (water)	[18]
	2 M HCl with microwave for 2 h	22–99 (HCl)	
Edible mushroom (*Agaricus bisporus*)	Step 1: water at 37°C for 3 h Step 2: 45 mg pepsin in Tris-HCl(pH 2.1) stirred at 37°C for 20 h Step 3: 45 mg trypsin in phosphate buffer (pH 7.6) stirred at 37°C for 20 h	75	[61]
Yeast supplements (*Saccharomyces cerevisiae*)	(1) *In vitro*, simulated gastric fluid (SGF) (2) Simulated Intestinal fluid (SIF)	55–80	[62]
Se-enriched fresh and dried radish	*In Vitro* gastrointestinal digestion	73–84 (gastric juice) 93–110 (intestinal juice)	[63]
Laboratory reference material from Brazil nuts	15 mg Pronase-E in phosphate buffer (pH 7.4) stirred at 37°C for 24 h	98	[64]
Brassica juncea (Indian mustard)	(1) 1 M HCl; (2) Tri-HCl, pH 8; (3) proteinase K; (4) protease XIV	(1) 54 (2) 46.5 (3) 73 (4) 70	[65]

Antarctic krill	20 mg Pronase-E in Tris-HCl (pH 7.5) incubated at 37°C for 24 h and with ultrasound-assisted for 15 min	87 (without ultrasound) 98 (with ultrasound-assisted)	[66]
Yeast certified reference material (CRM)	20 mg pronase and 10 mg lipase at 37°C for 24 h	30–50 (obtained for SeMet)	[19]
^{77}Se-enriched yeast	(1) 30 mg β-glucosidase in a water bath for 3 h, followed by 15 mg mixture of endo- and exopeptidases at 50°C for 24 h (2) 20 mg Protease XIV at 37°C for 24 h	(1) 95 (2) 90	[28]
Yeast-based supplements	(1) 1 mg protease XIV in Tris-HCl at pH 7 for 20 h at 37°C (2) 4% driselase in Tris-HCl for 4 h in shaking bath, then 1 mg protease XIV for 20 h incubated at 37°C (3) *in vitro* gastrointestinal model: 100 µL gastric juice in a shaking bath at 37°C for 4 h and 100 µL gastrointestinal juice incubated for further 4 h	(1) 87 (2) 92 (3) 87–103	[67]
Selenised yeast	Sequential leaching: Water 3 h, then the residue in 4% Driselase in Tris- HCl (pH 7.5) for 18 h, and the last residue in 4% SDS in Tris-HCl (pH 7.5) for 1 h	32 (water) 60 (Driselase) 8 (SDS)	[68]
Se-enriched plants (garlic and Indian mustard)	25 mM Ammonium acetate(pH 5.6) by ultrasonic probe 3 min	80	[69]
Chicken muscle, liver and kidney	Method 1: Pronase-E incubation at 37°C for 48 h in water and Tris-HCl at pH 7.5) Method 2: ProteaseXIV and Tris-HCl at pH 7.5, probe sonication extraction for 2 min	Method 1: 30–67 in water and 81–97 in buffer Method 2: 93–97	[70]
Dietary supplement	Sequential extraction: 10 mM Tris-HCl (pH 8) for water soluble fraction, 4% SDS (pH 7) for the residue 1, 5% driselase on the residue 2, last step for adding protease inhibitors in supernatant.	85	[45]

efficient alternative to reduce extraction time for catalyzing the breakdown of Se containing proteins (peptide bonds) into selenoamino acids. For example, the release of selenomethionine from selenized yeast using a ultrasonic probe was completed in only 30 seconds.[14]

In Vitro Digestion

In vitro gastrointestinal digestion was used to assess bioaccessibility of Se compounds from fish samples (swordfish, sardine, and tuna).[15] The digestion method was studied in gastric juice at 37°C on a water bath, with shaking for a few hours and then adding intestinal juice for a few more hours under the same conditions. Selenium solubility in the gastrointestinal supernatants was higher in swordfish and sardine (76 and 83%, respectively) than in tuna (50%). Simulated human gastric and intestinal digestion led to the identification of selenomethionine (SeMet) in the three digested fish samples.

Sequential Treatment

Sequential treatment with enzymes combined with conventional techniques may also improve extraction efficiency. This strategy was applied to Se-enriched yeast material[16] using Tris-HCl buffer for water-soluble fractions, followed by Tris-HCl buffer with SDS for solubilization of the protein fraction and, finally, concentrated HNO_3 for the dissolution of the resulting solid residue. After the three steps, total Se recovery reached 91.5%. In another example, mushrooms were treated by the sequential enzymatic processes with the highest efficiency (89%) being obtained by a 3-step procedure using lysing enzyme and pronase.[17]

A number of such procedures, including up to fourteen extraction methods, were compared by B'Hymer and Caruso[18] and Yang[19], although the true extraction efficiency obtained from these different methods is difficult to compare due to the absence of certified reference materials (CRM) for different Se species and sample matrices of varying complexity. The extraction methods and their efficiencies applied to biological samples are summarized in Table 2.

ION CHROMATOGRAPHIC SEPARATION METHODS AND APPLICATIONS

As mentioned previously, Se species in biological samples are at very low levels and the concentration of each species needs to be established instead of only the total Se concentration. After sample extraction, and before

instrumental detection of Se, separation/pre-concentration procedures of each species in the extraction solution become essential.

With hyphenated technique development, the on-line coupling of separation with an element-specific detector has become a highly effective technique for Se speciation.[6,20,21] Liquid chromatography (LC) is most commonly used for separating complex Se mixtures into individual components. GC and electrophoresis have also been applied to Se speciation, albeit to a lesser extent. Based on the nature of Se compounds (inorganic species, small metabolites, amino acids, or even polypeptides containing Se) and the detection demands of the speciation study, ion chromatographic techniques with inductively coupled plasma mass spectrometry (IC-ICP-MS) have become the most powerful separation and detection method for Se species due to its high selectivity and sensitivity.[2,7–9,22] The advantage of combined systems consisting of ion chromatography and ICP-MS selective detection of Se is obvious as they provide flexibility and broad applicability, at the disadvantage of increased complexity. Species equilibrium can be altered drastically during separation, and species transformation and destruction are possible.[4] The main forms of ion chromatographic separation for Se speciation are described as follows, including ion exchange, ion pairing reversed phase, and size-exclusion chromatography (SEC).

Ion-Exchange Chromatography

Ion exchange chromatography (IEC) has often been used for the separation of Se species owing to its high separation efficiency compared to SEC.[4] In this approach, the separation is based on the exchange equilibrium between charged solute ions and the oppositely charged surface of the stationary phase. Solute ions and ions of equivalent charge in the mobile phase compete for oppositely charged spaces on the stationary phase. The difference in interaction between analytes with the charged stationary phase brings about their separation. Selectivity in separation can be achieved by optimizing the ionic strength of the solute or the mobile phase, pH of mobile phase, temperature, and flow rate.[23]

Fritz[24] proposed a mechanism of ion-exchange chromatography resulting from enforced pairing effects (EP). Enforced pairing effects resulted when stronger ion pairing occurred with the stationary phase compared to that in the aqueous solution and was a combination of effects that included hydrophobic attraction, hydrogen bonding, lowered dielectric constant, and "water-structure induced ion pairing."

Both cation and anion-exchange HPLC have been employed in Se speciation. Isocratic and gradient elution were used to separate inorganic

and organic Se compounds.[25,26] Inorganic species (selenite and selenate) can be easily separated on IC, which could not be easily accomplished with reversed-phase or ion-pair reverse phase HPLC. For example, the inorganic selenium (Se) species, selenite, selenate, and selenocyanate, in sea and rain water were separated by anion exchange chromatography using a gradient of 0.1 M NaOH as mobile phase and determined by hydride generation-inductively-coupled plasma-dynamic reaction cell-mass spectrometry (HG-ICP-DRC-MS) with instrumental detection limits of 0.15, 0.27, and 0.19 ng Se L^{-1}, respectively.[27]

Cation exchange chromatography was used for analyzing ^{77}Se-enriched yeast in a human absorption study.[28] Using a range of pyridinium formate buffer from 0.75 to 8 mM with 3% methanol as mobile phase in a gradient elution, ten organic Se compounds were successfully separated (Figure 1). But, the method was not suitable for the separation of inorganic selenite and selenate.

The possibility of simultaneous separation of selenoamino acids and inorganic species has been investigated. Ion-chromatography with microwave assisted on-line species-conversion hydride generation atomic absorption spectrometry (IC-HG-ICP-MS) demonstrated the application of the technique for the quantification of selenite, selenate, SeMet, and SeCys in white clover using two columns for separation.[29] Detection limits were 1.0–1.6 ng g^{-1}.

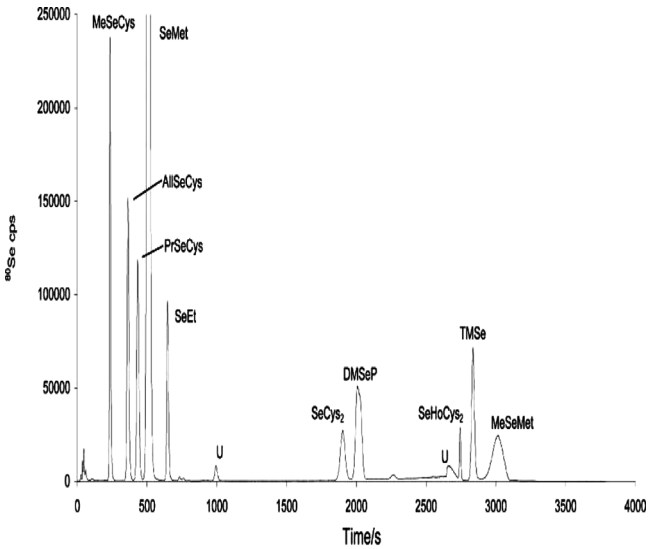

FIGURE 1 Cation exchange HPLC separation of mixture of 10 Se standard compounds[28] (AllSeCys: Se-allylselenocysteine, PrSeCys: Se-propylselenocysteine, other compounds see Table 1).

Juresa et al.[30] investigated three chromatographic systems (anion, cation, and reversed-phase chromatography) with hydride generation ICP-MS for examination of Se metabolites in human urine. Though hydride generation was employed to obtain better sensitivity and lower detection limits, it had a more complicated interface and has not been frequently used in conjunction with ICP-MS compared to AAS/AFS.[31]

Larsen et al. subsequently combined cation and anion exchange chromatography to separate a mixture of 12 selenium species comprising selenoamino acids, selenonium ions, and inorganic Se.[32] Cationic species were separated using a cation-exchange column and gradient elution with aqueous pyridinium formate at pH 3 as the mobile phase. The anionic species were separated by anion-exchange column and isocratic elution with an aqueous salicylate-Tris mobile phase at pH 8.5. Two additional species, selenomethionine-Se-oxide (oxidative degradation of SeMet) and dimethylselenoniumpropionate (DMSeP), were also detected in selenized yeast and an algal extract. Combination of anion and cation exchange chromatography was applied to identify the trimethylselenonium ion and SeMet in oyster samples.[33]

Compatibility of mobile phase of IC with ICP-MS is a serious consideration. Mobile phases that have been used include ammonium citrate, pyridinium formate, ammonium phosphate, and salicylate-Tris. For ICP-MS, excessive salt (sodium or potassium phosphate) or organic solvent (methanol) introduced into the plasma is undesirable because high concentrations of both salt and solvent can change the sensitivity over the experimental period when salt or carbon deposits build up on the sampler and skimmer cones. High levels of salt and solvent also alter the ionization characteristics of the Ar plasma and cause plasma instability, which can eventually extinguish the plasma. Therefore, lower concentration substance such as ammonium salts which can be converted to volatile components under high ICP torch temperatures are suggested for Se speciation when using ion-exchange HPLC with ICP-MS.[7]

The applications of IC-ICP-MS in analysis of Se are listed in Table 3. Despite the regular use of IC-ICP-MS for analysis of Se species, this technique has some limitations which need to be addressed. One major limitation is that organic modifiers at concentrations lower than 10% may also influence the selectivity of the separation by affecting the mechanism controlling the hydrophobic interaction of the solutes with the matrix.[7] Another is that the employed eluents often have high ionic strength. Consequently, the high salt load causes severe problems for interfacing to ICP-MS.

Ion-Pair Reversed Phase Chromatography (IP-RPC)

IP-RPC is a useful technique for the simultaneous separation of mixtures of anionic and cationic ions, as well as neutral molecules. It is a

TABLE 3 Applications of Ion-Exchange Chromatography for Se Speciation

Sample	Se Target Molecules	Mobile Phase	Detection	Ref
Human urine after supplemented with SeMet	Se(IV) and Se(VI)	Anion exchange Dionex Ionpac AG11-HC column in series with an AS11-HC column, 25 mM NaOH and 2% MeOH	ICP-MS	[71]
Yeast and wheat flour	$SeCys_2$, SeMC, SeMet, Se(IV) and Se(VI)	Hamilton PRP-X100 colum, 5 mM ammonium citrate in 2% MeOH by gradient and isocratic	Isotope dilution ICP-MS	[55]
^{77}Se-enriched yeast	SeMC, Se-allylselenocysteine, Se-propylselenocysteine, SeMet, SeEt, $SeCys_2$, dimethylselenoniumpropionic acid, methylselenomethionine, TMSe, Selenohomocystine	A silica-based strong cation exchange HPLC column, gradient elution by mobile phase 0.75–8 mM pyridinium formate with 3% MeOH	Dynamic reaction cell (DRC)-ICP-MS	[28]
Yeast and tablets	Methaneseleninic acid, Se(IV), Se(VI), SeMC, $SeCys_2$ and SeMet	Hamilton PRP-X100, 15–20 mM parahydroxybenzoic acid (PHBA) in 1% MeOH at pH8, gradient	ICP-MS	[72]
Wheat-based food	$SeCys_2$, SeMC, SeMet, Se(IV) and Se(VI)	Hamilton PRP-X100, (1) gradient of acetic acid and triethylamine, pH 4.7 (2) 5 mM ammonium hydrogen citrate (pH 5.9) containing 2% MeOH	ICP-MS	[52]
Enriched green onions (*Allium fistulosum*)	Se(IV), SeMC, SeMet, $SeCys_2$ and Se(VI)	(1) Silica-based strong cation exchange column, 2 mM pridinium formate, pH 2.8 as mobile phase, isocratic elution (2) Anion exchange column, Hamilton PRP-X100, 10–50 mM $NH_4H_2PO_4$ (pH 5.0) in 1% MeOH by gradient elution	ICP-MS	[25]

Se-enriched yeast material	Low molecular mass selenocompounds (≤10000)	Anion exchange column Dionex AS10, mobile phase A: 5 mm ammomium phosphate, pH 8.0; B: 50 mM ammonium acetate, pH 8.0 gradient elution	ICP-MS	[16]
Certified reference material (NIES CRM 18 human urine)	DMSe, DMDSe, three selenosugars, TMSe, $SeCys_2$, SeMet, SeCystm. Methaneseleninate, Se(IV) and Se(VI)	(1) Anion-exchange PRP-X100 column, 10 mM citric acid at pH 4.8 (2) Reversed-phase C18 column, 20 mM ammonium formate at pH3.0 containing 3% MEOH (3) Cation exchange PRP-X200 column, 10 mM pyridine at pH 5.0	Hydride generation (HG)-ICP-MS	[30]
Se-enriched supplements	$SeCys_2$, Se(IV), SeMet and Se(VI)	Hamilton PRP-X100, 5 mM ammonium hydrogen citrate (pH 5.9) in 2% MeOH	Ultrasonic and pneumatic nebulisation ICP-MS	[73]
Se-enriched yeast	SeMet	PRP-X100 column, gradient elution 10–100 mM phosphate buffer (pH 7) in 2% MeOH	Isotope dilution analysis-ICP-MS	[74]
Yeast	Seleno compounds	(1) PRP-X100, 20–200 mM actic acid, 10–100 mM triethylamine (pH 4.7) gradient elution (2) Cation-exchange Supelco SCX column, 20 mM pyridine formate (pH 3), isocratic	ICP-MS	[26]
Broccoli (*Brassica oleracea*)	Seleno compounds	PRP-X100 column, 10 mM citric acid, 2% MeOH, pH5	ICP-MS	[75]

variation on reversed phase chromatography. The separation mechanism depends on analyte ions in solution being paired or neutralized using low concentrations of appropriate ion-pairing reagents also in solution, for separation on a reversed phase column. Selectivity and retention of the analytes can be increased by counter-ions of the ion pairing reagents. The resolution of Se species depends on the concentration of the ion-pairing reagent, flow rate, ionic strength, and pH of the mobile phase, as well as the properties of the stationary phase.[4,6–10]

Anion-pairing (e.g., alkylsulfonates) and cation-pairing (e.g., tetraalkylammonium salts) reagents and long-chain carboxylate ions (e.g., perfluorinated carboxylic acids) have often been used as ion-pairing reagents.[22] A C-18 column has typically been used for selenoamino acid analysis, while C-8 has been described for selenoprotein or long peptide analysis.[7]

Hexanesulfonic acid has been used as an anion-pairing reagent for the analysis of Se in Brazil nut extracts on a C8 column.[34] SeMet was found to be the most abundant compound with some unidentified Se containing species. Organic species were separated by ion-pairing, but the technique proved less sensitive for selenite and selenate. Difficulties were experienced with poor separation or co-elution of inorganic Se and organic species. Marchante-Gayón et al.[35] developed tetrabutylammonium acetate (TBAA) as cation-pairing reagent to separate the anionic selenite and selenate from other seleno compounds ($SeCys_2$, SeMet, and SeEt) (Figure 2).

Trifluoracetic acid (TFA) and perfluoroaliphatic acids were used as pairing reagents for the separation of selenoamino acids, anionic, cationic, and neutral Se compounds in Se-enriched yeast, garlic, mushrooms, and

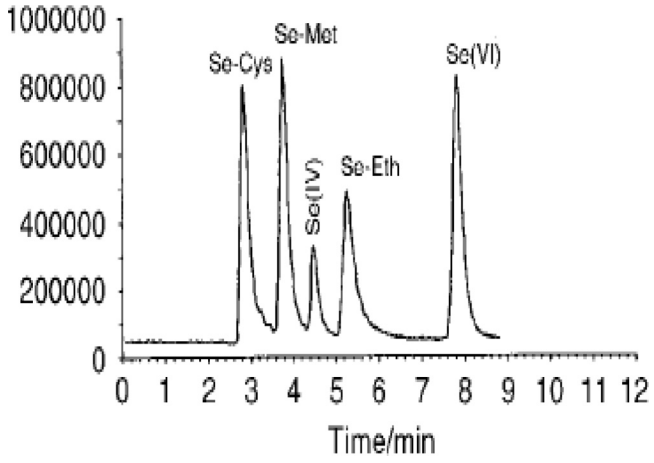

FIGURE 2 Ion-pairing reverse phase chromatography separating a mixture of 5 Se standard solutions by ICP-MS (5 ng/mL of each seleno.compound; ^{78}Se monitored).[35]

ramps.[36] Heptafluorobutanoic acid (HFBA) was used as a superior ion-pairing reagent to improve the resolution of many organoselenium species compared to either pentafluoropropionic acid (PFPA) or TFA.[37] For example, a mixture of standards of 20 Se compounds was separated on a C-8 column using isocratic 1% aqueous methanol and 0.1% HFBA as the mobile phase, obtaining good resolution of the individual species.[37] A mobile phase containing HFBA was also used in parallel studies with detection of ESI-MS to gain structural identification of Se species.[38] IP-RPC has similarly been used for Se speciation in animal tissues and serum.[39]

Mixed ion-pairing reagents (butanesulfonic acid and tetramethylammonium hydroxide) were also used to simultaneously separate inorganic and organic species. With good resolution of seven species (selenite, selenate, $TMSe^+$, $SeCys_2$, SeMet, selenoethionine, and selenocyatamine).[40] To obtain high efficiency, resolution, and speed of separation of selenocompounds, ultra-performance liquid chromatography was utilized.[41] Examples of using ion-pairing reverse-phase chromatography procedures are listed in Table 4.

The advantages of IP-RPC include flexibility and potential for variability of this separation mode. Efficient separation is possible for a wide range of Se species, including both inorganic and organic Se. However, limitations are faced with Se speciation when hyphenated techniques are used since organic solvents and ion-pairing reagents used in the mobile phase are not compatible with the ICP plasma. This is a consequence of the alteration of the ionization characteristics of the Ar plasma and production of polyatomic ions, as well as changes in structure of selenium species. Therefore, concerns about analyte stability during separation require more consideration in IP-RPC than in RPC alone.

As mentioned before, organic solvents used in RP or IP-RPC can cause serious interferences with the ICP plasma. Gradient elution can reduce sensitivity in ICP-MS. To overcome these problems, micro and nano-flow nebulizers have been introduced which have low solvent load.[35] Alternatively, post-column dilution or post-column addition of standards has been described. Oxygen addition has been recommended and Pt cones may be used instead of the common Ni cones.[42]

The main IP-RPC-ICP-MS methods and techniques currently being utilized for selenium speciation using ion-pairing reagents are summarized in Table 4.

Size-Exclusion Chromatography

Size exclusion chromatography (SEC) is often used for the separation of various selenoproteins from different sample matrices and provides

TABLE 4 Applications of Ion-Pairing Reverse-Phase Chromatography for Se Speciation

Sample	Se Target Molecules	Mobile Phase	Detection	Ref
Se-accumulating *Brassica juncea* (Indian mustard) and selenized yeast	Se(IV), $SeCys_2$, SeMet and SeEt	Four columns: XTerra RP-C18 (3.5 and 5 μm), Symmetry Shield RP-C18 and C8, 0.1% HFBA, 1% MeOH	ICP-MS	[76]
Dill (*Anethum graveolens L.*) root, stem, leaf	$SeCys_2$, MeSeMet, SeMC, TMSe, SeMet, Se(IV) and Se(VI)	Allima C8 column, mobile phase: 0.15% HFBA, 5% MeOH pH 2.1	ICP-MS	[60]
Nutritional supplements and Chinese tea	Se(IV), Se(VI), $SeCys_2$, SeMet and SeEt	Nucleosil 120 A, C18, 30 mM ammonium formate, pH 5.0, 5% MeOH, 10 mM TBAA, isocratic	Hexapole collision/reaction cell ICP-MS with meinhard, microconcentric and hydraulic high pressure nebulisers	[35]
Human Urine	TMSe, $SeCys_2$, Se(IV), SeUr, SeMet and SeEt	C8 column, 13 mM tetrabutylammonium hydroxide, 1.3% MeOH, pH 5.7–5.8	ICP-MS	[77]
Yeast-based supplements	SeMet, S-(methylseleno)cysteine and selenomethionine Se-oxide	RP-C8 column, 0.1% HFBA, 1% MeOH	ICP-MS	[78]
Biofortified products	SeMet, $SeCys_2$, Se(IV) and Se(VI)	C8, 0.1% HFBA, 10% MeOH	Species-unspecific isotope dilution ICP-MS	[79]
Human urine	Se(VI), SeUr, SeMet, SeEt and $TMSe^+$	RP C18, mixed ion-pairing mobile phase containing 2.5 mM sodium 1-butanesulfonate and 8 mM tetramethylammonium hydroxide	ICP-MS	[80]
Urine from before and during supplementation with SeMet	SeMet, MeSeMet,SeMC, TMSe and selenogammaaminobutyric acid (SeGaba)	Luna C8, mobile phase is (1) 10 mM HFBA, 20% MeOH (2) 3 mM nonafluoropentanoic acid (NFPA), 20% MeOH (3) 0.2 mM tridecafluoroheptanoic acid (TFHA), 20% MeOH	ICP-MS using a laboratory made direct injection nebuliser for allowing high MeOH concentration	[81]

Brazil nuts	SeMet, SeEt, $SeCys_2$ and unidentified Se species	Alltima C8, 5 mM citric acid and 5 mM hexanesulfonic acid, pH 3.5, 10% MeOH	ICP-MS	[34]
Se-enriched garlic (*Allium sativum*)	SeCystm, SeMet, SeMC, γ-glutamyl-SeMC and two unknown Se compounds	XTerra MS C18, (1) 0.01% tetraethylammonium chloride (TEACl), 2% MeOH, pH 4.5 (2) 2% MeOH	ICP-MS and ESI- MS-MS	[82]
Se-enriched chives (*Allium schoenoprasum*)	Se(IV), Se(VI), $SeCys_2$, SeMC and SeMet	(1) Sperdex Peptide HR 10/300, 30 mM Tris buffer, pH7.5 (2) Alltima C8, 0.1% heptafluorobutyric acid, 5% MeOH, pH 2.5 (3) Daicel Crownpak CR(+), 0.1 M perchloric acid, pH 1.0	ICP-MS	[83]
Se-enriched onion leaves	Se(IV), SeCys2, SeMC and SeMet	Alltima C8, 5 mM citric acid, 5 mMhexanesulfonic acid, pH 4.5, 5% MeOH	ICP-MS	[84]
Se-enriched yeast, ramp, garlic, onion, *Astragalus praleongus* and B*rassica juncea*	Se compounds, unidentified species	Symmetry Shield RP C8, (1) 0.1% HFBA, 1% MeOH (2) 0.1% TFA, 1% MeOH	ICP-MS and ESI-MS	[37]

preliminary information about the distribution of Se in a sample since the retention of Se species in SEC depends on molecular size.[4,6–10] Size exclusion chromatography is ideal for high molecular weight compounds (1000 and 10 K Da) such as polymers, peptides, and proteins. In addition, because of its high matrix tolerance, SEC can be used as sample purification step prior to other kinds of HPLC (such as IP-RPC and IC) and electrophoresis. Multidimensional approaches combining SEC are often employed in characterizing particular Se species by ESI-MS.[43]

The enzymatic digests of both water-soluble and insoluble Se protein fractions in biological samples (tuna, trout, krill, oyster, mussel, white clover, wheat flour, and selenized yeast) were analyzed by SEC-ICP-MS and UV spectrophotometry.[33,44] Two different SEC columns were used in combination with a cation-exchange column. A Biosep-SEC-2000 column for the effective separation of 300-1 kDa peptides and a Superdex Peptide HR 10/30 column for 0.1–7 kDa were employed for high molecular weight selenoproteins (150-50 kDa) present in the samples.[43] Another separation scheme combined size exclusion and anion-exchange chromatographies coupled with ICP-MS for Se speciation in dietary supplement.[45] Some unidentified high molecular weight Se compounds (22%) in the samples were observed from a sequential extraction steps and separated by SEC.[45]

Affinity chromatography combined with SEC-ICP-MS has been used to separate three major Se-containing proteins (albumin, glutathione peroxidase, and selenoprotein P) from human plasma.[46] Selenium incorporation into cyanobacterial metallothionein induced under heavy metal stress has been investigated by SEC-ICP-MS.[47] Selenite and selenate metabolism in rats were successfully studied using SEC, with exchange of endogenous and dietary selenium examined in brain, liver, and kidneys.[48]

Compared to other LC separation modes for Se speciation, SEC advantages include the determination of the molecular mass for any unknown Se species and it normally does not result in a change of species during separation owing to the gentle nature of SEC. Currently SEC has been used for the separation of labile and weak metal-complexed biopolymers.[4,6–10] However, the main limitations of SEC when used for the separation of selenium species is that separation efficiency of multi-selenium species is poor, leading to poor resolution between species.

INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS) FOR SE DETECTION

In general, inductively coupled plasma mass spectrometry (ICP-MS) is a powerful technique for the detection of Se species separated by ion

chromatography (IC). This very sensitive and selective method allows for the detection of Se species in a wide variety of samples due to its element specificity, wide linear range, and low detection limits. A number of authors have discussed the use of IC-ICP-MS for Se speciation.[4,6–10] However, the most abundant selenium isotopes, such as ^{80}Se (49.6%) and ^{78}Se (23.8%), suffer from Ar plasma interferences caused by $^{40}Ar^{40}Ar^{+}$ and $^{38}Ar^{40}Ar^{+}$. To address this issue, ICP with high-resolution MS can differentiate these interferences,[7] although it finds less commercial application than a conventional ICP with low resolution MS owing to its high cost. For this reason, ICP-MS equipped with a reaction/collision cell has been developed as a complimentary technique to ICP with high-resolution MS.[7] In this application various gases, including hydrogen (H_2), helium (He), ammonia (NH_3) and oxygen (O_2), can be used as reaction/collision gas to reduce spectral interferences.[7] In practice, only He and H_2 options are commonly available during commercial ICP-MS analysis.

The removal of polyatomic interferences may be achieved by means of collision/reaction cell technology without significant loss in sensitivity and increase in the signal to noise ratio for those isotopes impossible to be monitored by low resolution quadrupole mass analyzers. Increasing application of reaction cell techniques has resulted in relatively interference free responses and lower detection limits for trace elements.

A collision cell typically consists of six (hexapole) or eight (octopole) rods to which a RF only voltage is applied. While there are a few different designs from different manufacturers, all use the same basic principle. An octopole reaction system (ORS) is incorporated in Agilent devices 7500c, 7500cs, and GV instruments platform, a hexapole (HEX) reaction cell is favored by Thermo Elctron X-Series, while a dynamic reaction cell (DRC) is used in Perkin-Elmer Sciex instruments.

The collision/reaction cell is operated by pressurizing the cell interior with gases such as H_2 and He in reaction and collision mode, respectively. In reaction mode, the interferent of the analyte ions undergo chemical reactions with a gas.[49,50] This technique helps prevent interfering ions from spectrally overlapping with the target isotopes. Helium is an inert gas and is often used in the collision cell regardless of element. Hydrogen is the most commonly used reaction gas for element analysis in ORS, because it gives lower detection limits compared to He and dinitrogen oxide. The reaction of the argon dimer with hydrogen is assumed as interference association, where hydrogen associates with argon dimer by charge transfer, proton transfer, or atom transfer.

Charge transfer:

$$ArH^{+} + H_2 \rightarrow H_3^{+} + Ar \text{ (Removes interference with } {}^{39}K^{+})$$

Proton transfer:

$$Ar_2^+ + H_2 \rightarrow Ar_2H^+ + H \text{ (Removes interferences with } {}^{78}Se^+ \text{ and } {}^{80}Se^+)$$

Atom transfer:

$$ArO^+ + H_2 \rightarrow H_2O^+ + Ar \text{ (Removes interference with } {}^{56}Fe^+)$$

While in collision mode, helium gas can remove polyatomic interferences by one of two mechanisms: (1) collision induced dissociation; (2) kinetic energy discrimination. Dissociation of polyatomic ions into individual components happens when the energy associated with a collision is greater than the bond energy. In most cases, the second mechanism is followed. Kinetic energy discrimination is based on the fact that polyatomic ions undergo more energy reducing collisions with He gas than analyte ions owing to their larger cross-section. Stopping potential is then applied between the octopole and quadrupole to reject the lower energy interferent ions, while allowing the higher energy analyte ions to enter the quadrupole mass analyzer.[49–51]

Spectral interferences are a serious problem when detecting Se by conventional ICP-MS. These arise from isobaric and polyatomic, as well as nonspectral, interferences coming mostly from sample matrix (such as carbon and chloride in the case of Tris-HCl eluent). Se has six isotopes: ^{80}Se(49.61%), ^{78}Se(23.77%), ^{76}Se(9.37%), ^{82}Se(8.73%), ^{77}Se(7.63%), and ^{74}Se(0.89%). In argon plasma, several dimer interferences of $^{40}Ar^{36}Ar^+$, $^{40}Ar^{38}Ar^+$ and $^{40}Ar^{40}Ar^+$ have identical mass as the abundant isotopes ^{76}Se, ^{78}Se, and ^{80}Se, respectively. Also, polyatomic spectral interferences $^{40}Ar^{37}Cl^+$ and $^{81}Br^1H^+$ affect ^{77}Se and ^{82}Se when samples contain traces of chloride or bromide. In most cases, $^{40}Ar^{40}Ar^+$ is unavoidable and is reflected in higher background counts leading to higher detection limits, so other less abundant isotopes such as ^{82}Se(8.73%) or ^{77}Se(7.63%) are often monitored and detection limits are compromised, making Se determination more difficult.[31]

Recently, ICP-MS using ORS or dynamic reaction cell (DRC) through chemical resolution for spectra interferences has improved mass resolution and sensitivity for Se speciation. The gases used in collision/reaction cells for Se speciation are hydrogen, helium, dinitrogen oxide, methane,[52] and deuterium.[53] For example, Reyes et al.[54] performed Se determination in biological reference materials and serum samples using H_2 reaction gas in ORS. Huerta et al.[55] determined Se species in yeast and wheat flour samples using the same system. Marchante-Gayón et al.[56] used H_2 and He in a hexapole collision cell in urine samples before and after supplementation with different commercial tablets. In this study, He was

especially useful to improve the ion transmission while H_2 was useful to reduce polyatomic argon ions and increased sensitivity by orders of magnitude. Darrouzès and co-workers[57] reported Se detection limit was twice improved in monitoring ^{80}Se when using a collision/reaction cell. Using the DRC system, Larsen et al.[32] determined selenoamino acids in yeast and algal extracts at trace level after enzymatic digestion. Se speciation in human plasma, urine and feces was also performed by DRC-ICP-MS.

CONCLUSIONS

The determination of Se speciation is of great importance in understanding the biological and physiological functions of Se, as well as its potential health benefits. Hence, Se speciation analysis by IC-ICP-MS will continue to rapidly grow in importance in the near future because of its maximum sensitivity, low detection limits, and identification of element specific species. Despite recent advances in speciation analysis, direct determination of trace Se in complexes matrix is still very difficult and, therefore, separation/pre-concentration techniques are frequently required prior to any analytical determination. In addition, various ion chromatographic separation modes can be coupled with ICP-MS, but factors such as separation mechanisms, mobile phase, pH, and the steps involving sample preparation must be carefully considered to prevent the inter-conversion of species and to ensure accurate characterization of sample. Furthermore, the interference from matrix and argon gas can be improved by use of dynamic reaction/collision cell technology, and this area will probably continue to develop and grow more rapidly in the near future.

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