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REVIEW

Heidi Goenaga Infante · Ruth Hearn · Tim Catterick

Current mass spectrometry strategies for selenium speciation in dietary sources of high-selenium

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Abstract This document reviews the most relevant mass spectrometry approaches to selenium (Se) speciation in high-Se food supplements in terms of qualitative and quantitative Se speciation and Se-containing species identification, with special reference to high-Se yeast, garlic, onions and Brazil nuts. Important topics such as complexity of Se speciation in these materials and the importance of combining Se-specific detection and molecule-specific determination of the particular species of this element in parallel with chromatography, to understand their nutritional role and cancer preventive properties are critically discussed throughout. The versatility and potential of mass spectrometric detection in this field are clearly demonstrated. Although great advances have been achieved, further developments are required, especially if "speciated"certified reference materials (CRMs) are to be produced for validation of measurements of target Se-containing species in Se-food supplements.

Keywords Selenium speciation analysis · Se-enriched food supplements · Mass spectrometry strategies · High Se-yeast · Cancer preventive properties

Introduction

Selenium (Se) is a crucial nutrient for higher organisms including humans. It is a component of a number of important Se-containing proteins and enzymes required for such functions as antioxidant defence, reduction of inflammation, thyroid hormone production, DNA synthesis, fertility and reproduction [1, 2]. Se requirements can be satisfied by 0.1 mg kg^{-1} of the diet [3]. On the basis of this estimate, daily Se intakes for humans that vary according to the country or region, age and sex have been recommended and recently compiled by Rayman [4]. However, the dose at which selenium has to be taken is very critical, as Se can be toxic at levels little above those required for health [5–8].

Recent studies have shown that Se supplementation in the diet can reduce the risk of several forms of cancer (in humans and animals) [9–22] and other human diseases and may prevent the development of AIDS in HIV-positive individuals [2]. The interest in Se as a food supplement and the marketing of it to the public has therefore increased in recent years.

Se supplements commercially available include the inorganic forms, sodium selenite, sodium hydrogen selenite and sodium selenate and the organic forms, selenomethionine (SeMet), selenium-methyl-Se-cysteine (SeMC) and Se-enriched yeast [23]. SeMet is the principal form of Se in most foods and is well absorbed and stored within the human body [24]. The Se ingested as free SeMet (major component of Se-yeast) or selenised yeast has been shown to be more bioavailable than selenite in lactating rats [25]. In these studies, selenium bioavailability of the Se compounds was estimated not only on the basis of determination of Se absorption but also on the measurements of tissue Se concentration and glutathione peroxidase activities. Fractional absorption and retention of SeMet and Se-yeast in humans [26–30] were found to be higher than those of inorganic Se; inorganic selenium is generally excreted more rapidly and is more toxic than SeMet. The SeMC, a naturally occurring selenoamino acid synthesised by garlic, broccoli and onions, has been shown to be more efficacious than the most extensively studied organoselenium compounds (e.g. SeMet) in mammary cancer chemoprevention in rats [14, 15]. More recently, it has become available to the public as a dietary supplement of Se due to the great efficacy as a chemopreventive agent that this Se species has shown in experimental models of breast cancer and its promising potential for use in human populations [16].

H. G. Infante (✉) · R. Hearn · T. Catterick
LGC Limited, Queens Road, Teddington,
Middlesex, TW11 OLY, UK
E-mail: Heidi.Goenaga-Infante@lgc.co.uk
Fax: +44-20-89432767

The anti-carcinogenic effects of Se have been associated with certain Se-metabolites such as methylselenol (CH_3SeH) [31–35]. However, the relationship and mechanism by which Se supplementation may reduce certain cancer risks is not fully understood. Absorption, tissue distribution, bioavailability, and cancer preventive properties depend on the chemical species in which Se occurs in food and supplements.

A typical metabolic fate of SeMet and other organic Se compounds from the human diet (e.g. selenocysteine (SeCys) and SeMC), as shown in Fig. 1, emphasises the complexity of Se speciation [4]. SeMet from Se-enriched food supplements (e.g. Se-yeast) and food proteins can be incorporated non-specifically into proteins such as albumin and haemoglobin by replacing methionine (Met). Alternatively, after being released from the protein pool, it can be transformed via SeCys to hydrogen selenide (H_2Se) [36]. The metabolism of SeMC species, mostly studied in animals, indicates that SeMC is not incorporated into any proteins in place of Met. It can be thus easily used for the synthesis of Se-containing enzymes as well as converted to metabolites such as methylselenol, which is thought to be potently anti-carcinogenic as stated above. Gamma-glutamyl-SeMC, recently identified as the major Se-compound in natural garlic and selenised garlic and onion, serves primarily as a carrier of SeMC [36]. Oxidised inorganic forms of selenium undergo reductive metabolism yielding H_2Se, the starting point for the production of selenoproteins [4]. Excess Se is detoxified by successive methylation of H_2Se, yielding methylselenol (CH_3SeH), dimethyl selenide ($(CH_3)_2Se$) and trimethyl selenonium ion ($(CH_3)_3Se^+$), the latter two of which are excreted in breath and urine, respectively. Identification of $(CH_3)_3Se^+$ as the main Se product in urine is based on experiments with rats [35] after administration of large doses of Se. However, new studies involving the identification of Se compounds in urine by MS techniques have shown that the main Se metabolite in rats urine as well as in human urine, after Se supplementation in sub-toxic doses, is Se-methylseleno-*N*-acetylgalactosamine [37–39]. More recently, two minor metabolites were identified as Se-methylseleno-*N*-acetylglucosamine and Se-methylselenogalactosamine in human urine from individuals who had been supplied with Se as Se-yeast [40]. The latter appeared to be one of the main metabolites in basal human urine from individuals who were not supplied with Se.

The speciation analysis of inorganic and organoselenium compounds in food supplements is desirable, to define biological roles and cancer prevention efficacy and to assure batch-to-batch reproducibility in these products. This requires the development of ''state of the art'' mass spectrometric techniques capable of identifying and determining selenium species in Se-enriched food matrices and in samples with natural Se concentration levels (control samples) [41]. The measurement of the low concentrations of the element, associated with minor species, in the complex biological matrices and prevention of species transformation during the analysis are considered to be the main challenges.

Selenised yeast has probably been the most widely investigated food supplement containing Se [4, 9, 41–78]. It is the only form of Se to date to have shown

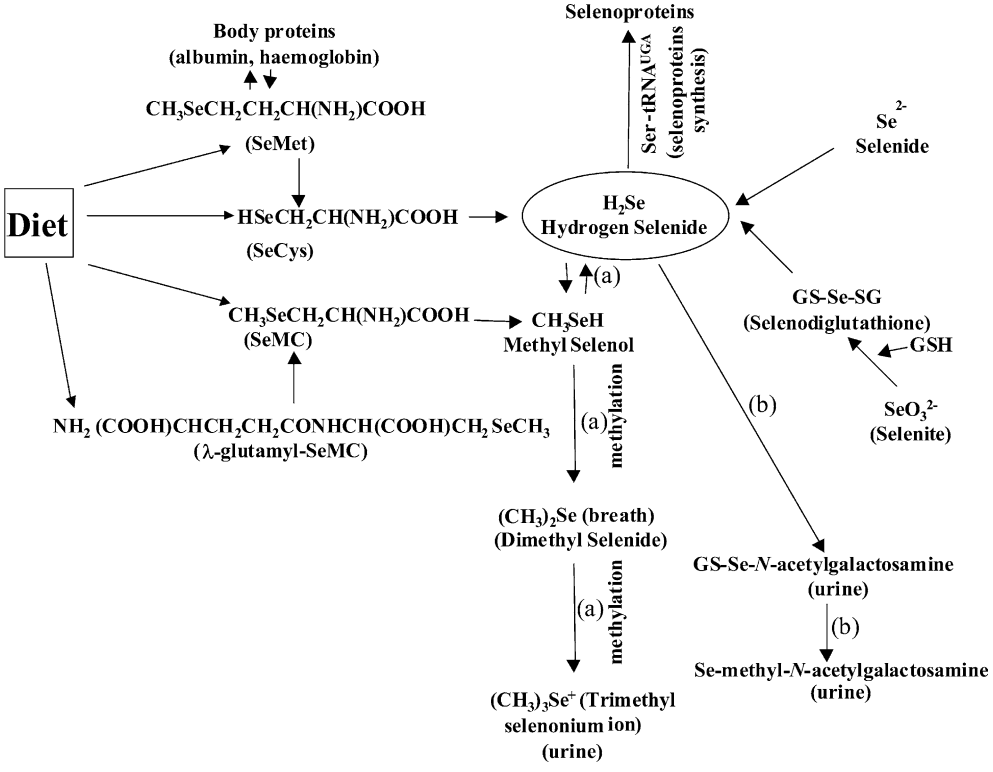

Fig. 1 Pathways of Se metabolism (adapted from Rayman [4] and supplemented with the results recently reported in Refs. [37, 39, 40]. **a** Toxic and **b** sub-toxic dose

efficacy as an intervention agent in human cancer prevention studies [9, 76, 77]. To a lesser extent, different species of *Allium* plants (Se-enriched garlic and Se-enriched onion) have also been investigated as dietary supplements because of their perceived medical value, especially in the context of cancer and anticardiovascular diseases [79–92]. Other dietary Se sources are Brazil nuts since these foodstuffs can contain very high levels (as high as 35 μg g^{-1}) of naturally occurring Se [93–98]. The efficacy of selenium, delivered naturally in this food form, in cancer chemoprevention was demonstrated in experiments with rats [97, 98]. Much effort has been devoted to characterisation of these materials in terms of species quantitation and identification and isotopic composition determination (the last only applies to isotopically enriched Se-yeast). Modern MS approaches to Se speciation in the above-mentioned dietary supplements are reviewed in this paper. Moreover, different extraction protocols are compared in terms of Se extraction yield and preservation of the identity of Se-compounds. Applications of chromatography with Se-specific and molecule-specific detection for the analysis of dietary sources of Se are summarised in Table 1.

Modern trends in the speciation analysis of Se in high-Se yeast by hyphenated techniques

Cereals and forage crops convert Se mainly into SeMet and incorporate it into proteins in competition with Met. In a similar manner, *Saccharomyces cerevisiae* (baker's yeast) may assimilate during its growth up to 3000 μg g^{-1} Se as selenite, which is converted into a safer and highly bioactive species with improved nutritional properties, the major product being SeMet [44].

Se-yeast is attractive as a supplementary source of Se owing to its low cost, its ability to act as a precursor for selenoprotein synthesis and its high content of SeMet, a form of Se found in most foods. However, the variability of Se-yeast with respect to Se content and speciation and the lack of knowledge of the identity of the Se species that occur in this food supplement remain a problem. There is therefore an essential need to characterise and quantify the Se-species present in selenised yeast.

The literature on the Se speciation analysis of selenised yeast is abundant (see Table 1) and has been, to some extent, reviewed [4, 41, 42]. The analytical methods used have been based on the coupling of liquid chromatography (usually size-exclusion followed by ion-exchange and ion-pair reversed-phase HPLC) [48–51], gas chromatography [52, 53], capillary electrophoresis [54], and 2D gel electrophoresis (for Se-containing proteins) [55] with Se-specific detection, usually by inductively coupled plasma mass spectrometry (ICP-MS). Electrospray ionisation (ESI) MS [56–58] and matrix-induced laser desorption ionisation (MALDI) MS [58, 73] have been used for the confirmation and/or identification of Se-compounds detected. The GC-MS has been used for molecular mass determination of Se-amino acids after their extraction from yeast and derivatisation [52, 68].

Despite the increasing number of publications about the speciation of Se in high Se-yeast, the preservation of the identity of the Se-compounds during their extraction from the solid matrix prior to their analysis using chromatography and mass spectrometry techniques remains a challenge. Water has been used for extraction of the intact low and high molecular weight compounds from the solid sample prior to their analysis using MS techniques [48–50]. Unfortunately, the extraction efficiency with this method was low (10–20% of the total Se in yeast was found in the water-soluble fraction). Alternatively, extraction of cell-wall bound compounds has been achieved by using cell-wall degrading enzymes (e.g. Driselase) [49]. To characterise membrane-protein containing Se, leaching with sodium dodecylsulphate (SDS) was utilised [49]. To release the Se-compounds from proteins, which are present in any of the yeast fractions, enzymatic [47, 49, 51, 59, 60, 93] or, to a lesser extent, acid digestions [3, 42, 89] were carried out. Table 2 summarises the extraction efficiency of Se from Se-yeast as a result of various extraction methods. A critical comparison of extraction methods for quantification of SeMet and Met in yeast has recently been reported by Yang et al. [70]. The enzymes used in most digestion protocols are Proteinase K and Protease XIV [49, 94]. Digestion with enzymes has led to 55–85% [45, 47, 51, 52, 62, 69] recovery of the total Se content in the dry yeast (mostly found as SeMet). The extraction efficiency of the enzymatic methods of hydrolysis critically depends on the type of enzyme and the extraction conditions (e.g. incubation time and sample to enzymes ratio), as demonstrated by Yang et al. [70]. Furthermore, the SeMet content/total Se value (used for calculation of the extraction efficiency) might be dependent on the source of yeast analysed and can be affected by the degree of oxidation of SeMet during the entire analytical procedure. Therefore large variations in extraction efficiencies for SeMet with different enzymatic protocols have been reported for yeast-based materials [45, 47, 51, 52, 62, 69, 70]. A more efficient liberation of SeMet from yeast was observed by hydrolysis with methanesulphonic acid compared to the most commonly used enzymatic approaches [72, 93]. A different technique, using cyanogen bromide to liberate SeMet from yeast was reported by Wolf et al. [71]. However, the incomplete degradation of proteins with cyanogen bromide could possibly cause the low extraction efficiency observed with this method, as shown by results from a recent study [72].

In terms of species identification, consistent results have been reported for some Se compounds in yeast using MS techniques. With size-exclusion LC-ICP-MS, supported by peptide mapping approaches and atmospheric pressure ionisation MS, SeMet was found as the major Se species in yeast. In contrast to previous findings, a large part of it was found to be physically associated with a number of macromolecules, especially cell wall constituents rather than bound into Se-containing

Table 1 Analytical strategies for species-selective analysis of target Se-compounds in high-Se dietary sources by chromatography with MS techniques

Dietary source of Se	Target Se-compounds	Sample preparation	Separation conditions	Detection	Reference
High-Se yeast	Se-containing proteins (SIP18 and HSP12)	Extraction in water followed by tryptic digestion of water-soluble protein SEC fractions	> Column: Supelco C_4 (250 × 4.6 mm × 5 μm). Buffer A: 0.1% TFA in H_2O. Buffer B: 0.1% TFA in MeOH. Elution gradient: 5%B (5 min), 10% B (5–10 min), 45% B (10–55 min)	ICP-MS, MALDI-TOF MS, direct infusion-electrospray Q-TOF MS	50
High-Se yeast	Non-peptide species including Se-adenosyl-Se-methionine	Extraction in water followed by fractionation of the water extract using semi-preparative size exclusion chromatography and fraction lyophilisation.	> Column: Pharmacia Hiload 26/60 Superdex 30 Prep; mobile phase: 10 mM ammonium acetate at pH 9.5 (2 ml min^{-1}) (water extract fractionation) > Column: Pharmacia Superdex Peptide 10/30; mobile phase: 10 mM ammonium acetate at pH 9.5 (0.7 ml min^{-1}) (fraction purification)	MALDI MS using post-source decay and orthogonal Q-TOF detection	73
High-Se yeast	Glutathione *S*- conjugates with Se-compounds with molecular masses of 562, 584 and 603 u, SeMet and Se-adenosylhomocysteine	Extraction in water	> Column: Pharmacia Sephadex G-75 (700 × 16 mm) (fraction collection). Mobile phase: 1% HAc at pH 2.97 (0.7 ml min^{-1}) > Column: waters Spherisorb (250 × 4.6 mm × 5 μm) (fraction purification) Mobile phase: 0.3% HAc at pH 3 (0.9 ml min^{-1})	ICP-MS, direct infusion ESI-MS/MS	48
High-Se yeast and garlic	SeMet (in yeast), λ-glutamyl-Se-methyl-Se-cysteine and λ-glutamyl-Se-methyl-Se-methionine (in garlic)	Protein hydrolysis using enzymes	> Column: symmetry shield RP_8 (150 × 3.9 mm × 5 μm) Mobile phase: 1% MeOH with 0.1% TFA (1 ml min^{-1}) > Column: agilent R_X–C_8 column (150 × 2.1 mm id × 5 μm) and Mobile phase: (98 + 2) water-methanol mixture containing 0.1% (v/v) TFA (0.15 ml min^{-1})	ICP-MS, ESI-MS ICP-MS, on-line ESI-MS/MS	56 78
High-Se yeast	SeMet and its oxidation product, Selenomethyl-Se-cysteine	Protein hydrolysis using enzymes	> Column: Chrompack Ionosphere-C (100 × 3 mm) (cation-exchange); mobile phase: buffer A: 0.75 mM pyridinium formate (pH 3) Buffer B: 8.0 mM pyridinium formate (pH 3.2) Elution gradient: 100% A (7 min), 100% B (30 min) > Column: ION-120 polymeric (125 × 4.6 mm) (anion-exchange) Mobile phase: 4 mM sodium salicylate-Tris at pH 8.5 in (1 ml min^{-1})	ICP-dynamic reaction cell (DRC)-MS	61
High-Se yeast	Seleno-methyl-Se-cysteine	Protein hydrolysis using enzymes followed by fraction collection and lyophilisation	> Column: Synergi Polar-RP column (250 × 2.0 mm i.d. × 4 μm) Mobile phase: (98 + 2) water–methanol containing 0.1% (v/v) TFA (0.15 ml min^{-1})	ICP-MS, on-line ESI-MS/MS	78

High-Se yeast	Selenite, Se-Lanthione, Se-Cystine, Se-Cystathionine, Se-methyl-Se-cysteine, λ-glutamyl-Se-methyl-Se-cysteine	Protein hydrolysis using enzymes and hot water extraction	> Column: symmetry shield RP_8 (150 × 3.9 mm × 5 μm) Mobile phase: 0.1% heptafluorobutanoic acid (HFBA) or 0.1% TFA in (1 + 99) MeOH-water	ICP-MS	62
	Se-methionine, Se-Adenosylselenohomocysteine			ICP-MS, ESI-MS	62
High-Se garlic	Selenate, Se-Cystathionine			ICP-MS	62
	Se-methyl-Se-Cysteine, Se-methionine, λ-glutamyl-Se-methyl-Se-cysteine-Se-cystine			ICP-MS, ESI-MS	62
High-Se onions	Selenate, Se-cystine, Se-methyl-Se-Cysteine, Se-methionine			ICP-MS	62
	λ-glutamyl-Se-methyl-Se-cysteine			ICP-MS, ESI-MS	62
High-Se yeast	S-(selenomethyl)-cysteine, Se-methionine-Se-oxide	Protein hydrolysis using enzymes. Derivatisation of the Se-compounds in the enzymatic extract for GC-AED and GC-MS	> HPLC column: symmetry shield RP_8 bonded hybrid stationary phase (150 × 3.9 mm × 5 μm) or Xterra RP-C_{18}(150 × 4.6 mm × 5 μm) Mobile phase: (99 + 1) water-MeOH with 0.1% TFA or HFBA > GC column: HP 1 (25 m × 0.32 mm × 0.17 μm) Conditions: isothermal separation at 160°C, He as a carrier gas (2 ml min^{-1})	ICP-MS, AED, MS	52
High-Se yeast	Se-diglutathione and the mixed Se-trisulphide of glutathione and cysteinylglycine	Extraction in water followed by fraction collection using size-exclusion chromatography and fraction lyophilisation.	> Column: SEC (500 × 20 mm) packed with Sephadex G-50 Mobile phase: 300 mM formic acid (3.8 ml min^{-1}) (extract fractionation) > Column: Hypersil Hypercarb (20 × 4 mm × 5 μm) (fraction purification) Mobile phase: buffer A: 300 mM formic acid, buffer B: MeOH Elution gradient: 100% A (012 min), linear ramp to 100% B (12–32 min) at 0.2 ml min^{-1}	ICP-MS, direct infusion nanoESI-MS	63
High-Se yeast	SeMet, selenite, selenate	Protein hydrolysis using enzymes	> Column: Hamilton PRP-X100 (250 × 4.1 mm × 10 μm) Mobile phase: 5 mM ammonium citrate in 2% MeOH (0.9 ml min^{-1})	ICP-octopole reaction system (ORS)-MS	60
High-Se yeast and Brazil nuts	SeMet	Protein hydrolysis with methanesulphonic acid	> Column: Superdex Peptide HR 10/30 Mobile phase: 10 mM formic acid/NH_3 at pH 3.5 (0.7 ml min^{-1}) > Column: C_8 Alltima (150 × 4.6 mm × 5 μm) Mobile phase: (5 mM citric acid/NaOH, 5 mM hexanesulphonic acid pH 4.5)-MeOH (95 + 5) at (0.8 ml min^{-1})	ICP-MS	93
High-Se yeast	Met and SeMet	Protein hydrolysis with methanesulphonic acid followed by Se-compounds derivatisation	GC column: Isomass DB-5MS (30 m × 0.25 mm × 0.25 μm) Conditions: 120–260°C at 20°C/min (hold 2 min), He as a carrier gas (1.5 ml min^{-1})	IDMS	68

Table 1 (Contd)

Dietary source of Se	Target Se-compounds	Sample preparation	Separation conditions	Detection	Reference
High-Se garlic	λ-glutamyl-Se-methyl-Se-cysteine	Extraction in water followed by fractionation of the extract by preparative SEC	> Column: Pharmacia Sephadex G-75 (700 × 16 mm) (preparative SEC for extract fractionation) Mobile phase: 1% HAc at pH 2.97 (0.7 ml min^{-1}) > Column: Supelco Spherisorb (250 × 4.6 mm × 5 μm) (fraction purification) Mobile phase: 0.3% HAc at pH 3 (0.9 ml min^{-1})	ICP-MS Direct infusion ESI-MS/MS	82
High-Se onions	Se-cystine, seleno-methyl-Se-cysteine, SeMetλ-glutamyl-Se-methyl-Se-cysteine	Protein hydrolysis using enzymes	> Column: Pharmacia Superdex Peptide HR 10/30 (SEC separations) Mobile phase: 10 mM CAPS buffer at pH 10 (0.6 ml min^{-1}) > Column: Alltech Alltima C_8(150 × 4.6 mm × 5 μm) Mobile phase: 0.1% heptafluorobutyric acid, 5% MeOH at pH 2.5 (0.9 ml min^{-1})	ICP-MS ESI-ion trap (IT)-MS	79 79
Brazil nuts	SeMet	Protein isolation by dissolution in NaOH and precipitation with acetone followedby protein hydrolysis using enzymes	> Column: Alltech Alltima C_8(150 × 4.6 mm × 5 μm) Mobile phase: 5 mM citric acid NaOH, 5 mM hexanesulphonic acid pH 4.5)-MeOH (95+ 5) (0.9 ml min^{-1})	ICP-MS	96

proteins [49]. The combination of orthogonal size exclusion-reversed-phase chromatography with ICP-MS and MALDI TOF MS or ESI-MS/MS allowed for the identification of a family of Se-containing proteins; a salt stress-induced protein SIP-18 (*Mr* 8874) and a heat-shock protein HSP12 (*Mr* 11693) and of a low molecular weight compound Se-adenosyl-homoselenocysteine in the water-soluble extract [49, 50]. More recently, the potential of MALDI tandem (Q/TOF) MS for the speciation of Se in selenised yeast aqueous extracts was investigated and compared with that of ESI tandem MS [73]. The MALDI was found to offer a competitive performance to ESI tandem MS for the identification of non-peptide Se species in yeast extracts, owing to its ability to handle minute volumes of samples with complex matrices. An unknown Se compound with a molecular mass of 388 u was detected and the product ion spectrum from the precursor ion (*m*/*z* 388) was obtained. However, the MS data alone did not provide enough evidence of structural confirmation [73].

Using size-exclusion chromatography and ESI-MS/MS, McSheehy et al. [48] found three new Se-containing species with molecular masses of 562, 584 and 603 u in aqueous selenised yeast extracts. The authors demonstrated by collision-induced dissociation (CID) that these compounds contain a Se-S bridge between glutathione and three unidentified Se-compounds. Another Se species with a molecular mass of 372 u was shown to contain glutamine, but also could not be completely identified.

Identification of SeMet as the main compound in enzymatic yeast extracts by combination of chromatography with ICP-MS and on-line ESI-MS has been accomplished by Uden and co-workers [56]. With no fragmentation of the molecular ion possible, the identification was only based on retention time matching with authentic standards and the molecular weight of the species. Structural characterisation of SeMet in yeast enzymatic digests was recently accomplished by on-line ion-pair reversed-phase HPLC with ICP-MS and ESI-MS/MS detection [78]. The CID was used to obtain the product ion spectrum of the *m*/*z* 198 precursor ion for the SeMet peak. The MS results were consistent with the presence of SeMet in the yeast digests. Other attempts to identify SeMet and its oxidation product (SeOMet) in the unpurified enzymatic yeast hydrolysates by on-line cation-exchange HPLC and ESI-MS were not successful, possibly due to the ionisation suppression by the sample matrix in the ES ion source [61].

The presence of some Se species such as SeMC, selenocystine ($Se(Cys)_2$), selenite, γ-glutamyl-Se-methyl-SeCys (γ-glutamyl-SeMC), Se-cystathionine and Se-lanthione in Se-yeast extracts has been detected by chromatography and ICP-MS on the basis of comparison of retention times with a matching standard [52, 61, 62]. However, verification of these species by molecule-specific mass spectrometry was not possible because of the low concentrations and matrix, which affected ionisation. Note that although assignments based on retention

Table 2 Extraction efficiency of Se from selenised yeast as a result of various sample preparation methods

Extraction method	Extraction efficiency (% of total Se in yeast)	References
Leaching with hot water	10–20	[44, 48–50, 58, 63, 65, 78]
Extraction with Driselase (for cell-wall components digestion)	27–60 (mostly found as SeMet)	[44, 49]
Digestion with enzymes (for enzyme type and conditions used, see Ref. [70])	55–85 (mostly found as SeMet)	[45, 47, 49, 51, 52, 59, 60, 62, 69, 70, 78, 93, 94]
Hydrolysis with methanesulphonic acid	66 (mostly found as SeMet)	[70, 72, 93]
Hydrolysis with hydrochloric acid	73 (mostly found as SeMet)	[7, 71]
Protein degradation with cyanogen bromide	43 (mostly found as SeMet)	[70–72]

times suggest the presence of $SeCys_2$, it is unlikely that this Se species occurs in yeast because of the absence of the UGA codon, which initiates the biosynthesis of the SeCys monomer, in the yeast genome [51]. In such a case, the use of complementary chromatographic methods combined with ICP-MS to achieve a selective separation/detection [51] and/or of molecular information has proven to be essential for verification of retention based identification. For SeMC, verification of its presence in yeast digests on the basis of retention time, molecular mass determination for the $[M+H]^{+80}$ Se ions (m/z 184) and detection of its product ions by ESI-MS/MS was recently reported by Goenaga-Infante et al. [78]. To do this it was necessary to develop an efficient sample cleanup method prior to analysis by on-line ion-pair reversed-phase HPLC with ESI tandem MS. However, the sensitivity of the RP-IP HPLC-ESI-MS/MS method was found still poor, probably due to the lack of compatibility of TFA (ion-pairing reagent) with ESI, which is known to affect the on-line mass spectral confirmation of Se-compounds by molecule-specific techniques with ESI source. The presence of SeMC is of interest, as this species is believed to be metabolised in animals and humans to methyl selenol (CH_3SeH), which appears to be a key anti-tumorigenic metabolite as stated above.

The use of ion-pair reversed-phase HPLC with ICP-MS and gas chromatography (after derivatisation of selenoamino acids) with atomic emission detection (AED) was useful for the detection of both SeMet-Se-oxide and Se-S bonded S-(selenomethyl)-cysteine (previously unidentified species) in archived nutritional yeast [52]. Confirmation of the presence of Se-S bonded S-(selenomethyl)-cysteine in the yeast sample was achieved using GC-MS.

Lindemann and Hintelmann [63] used nanoelectrospray MS to improve the detection power of Se compound identification in yeast extracts. The use of two-dimensional liquid chromatography combined with ICP-MS and off-line nanoelectrospray tandem MS enabled identification of Secontaining Glutathione S-Conjugates (selenodiglutathione and the mixed selenotrisulphide of glutathione and cysteinylglycine) in aqueous yeast extracts.

Two-dimensional gel electrophoresis for protein separation followed by laser ablation-ICP-dynamic reaction cell-MS for Se detection and protein characterisation by ESI-MS was reported and enabled the characterisation in terms of molecular mass, of up to 10 Se-containing proteins [55]. The strategy showed a promising potential for application to the possible establishment of a 2D reference map for Se-containing proteins in yeast. However, despite the high level of Se incorporation (0.2%), this [5] and other recent MS approaches [50, 66] have yielded limited results with respect to the elucidation of the incorporation of SeMet (presumably non-specifically incorporated into protein in the place of Met) in the yeast proteome. This has been attributed to the low abundance of the SeMet residues; a third of the Met residues, which have a relatively low abundance (about 1–2% of all amino acid residues) in the yeast proteome, are present as SeMet in Se-yeast [68]. This low abundance makes the identification of proteins, in which SeMet is incorporated, challenging. McSheehy et al. [75] have recently provided definitive evidence for the non-specific incorporation of SeMet in the yeast proteome using a two-dimensional liquid chromatography-MS based proteomic approach. By comparing the product ion spectrum of the precursor molecular ion for a Met-containing peptide (as previously identified in a LC fraction of a protein tryptic digest) with that of the same precursor molecular ion plus 48 mass units (the difference between the most abundant isotopes of S and Se), the presence of SeMet in the peptide could be confirmed if 'matching' spectra were observed. Moreover, the detection of the characteristic Se isotopic pattern helped in the identification of Se-containing peptides. The results suggested that the yeast support a high degree of SeMet incorporation (by replacement of about 30% of all Met with SeMet). However, the application of this approach to identification of proteins in yeast is restricted to higher abundance proteins with a relatively high Met content in sequence.

In terms of quantitative speciation of Se and determination of Se isotopic composition (of isotopically enriched Se-yeast), the attractive features of collision cell ICP-MS instruments (high sensitivity, selectivity and absence of spectral interferences that affect the ICP-MS detection of the most abundant Se isotopes) have been exploited in combination with chromatography for the analysis of yeast extracts [51, 60, 69]. Although most quantification approaches are based on calculations of

percentage Se distributions, as expressed as the total Se area response of eluted peaks [51, 52], species-unspecific isotope dilution (ID) has recently been used in combination with collision cell ICP-MS for accurate quantitative determination of different Se-containing species in yeast enzymatic digests [60]. Species-specific isotope dilution analysis in combination with HPLC-ICP-MS [69], GC-ICP-MS and GC-MS [68, 71, 72] has recently been proposed for the accurate determination of SeMet in Se-yeast. With the latter approach the simultaneous determination of SeMet and Met was achieved using ^{13}C-enriched SeMet (available from Sigma, St.Louis MO, USA) and Met spikes [68]. This has found promise in the study of the incorporation rate of Se into the yeast proteome [68, 75].

Despite the increasing use of isotope dilution techniques for validation of methodologies for Se speciation analysis, the accurate determination of Se-compounds identified in Se-yeast, other than SeMet, still remains a challenge. This is due to the lack of commercially available isotopically enriched Se-containing species (to perform IDMS) and of "speciated" yeast certified reference materials for validation of measurements of target Se-containing species. Furthermore, information available on the identity of the molecules incorporating or binding Se in selenised yeast is still scarce because of the complexity of the system, the low concentration of the Se-compounds and the lack of analytical approaches allowing their identification in the absence of any suitable standards. Although the major component of Se-yeast has been identified as SeMet, the speciation studies have indicated that this food supplement is a source of other organoselenium compounds, some of which may be more potently anti-carcinogenic than SeMet. Therefore, further studies should be pursued to verify the presence of these species in the complex yeast sample.

Mass spectrometry techniques for Se speciation in vegetables from *Allium* family

In the case of vegetables from *Allium* family, the research interests have been mostly focused on Se-enriched garlic and onions.

Se-methyl-Se-cysteine (the main Se form in Se-enriched garlic) and Se-enriched garlic were shown to be more-effective anti-carcinogens than Se-yeast, in animal studies [81]. Attempts to identify the Se-containing compounds with cancer chemopreventive activity in Se-enriched garlic have led to a number of reports on Se speciation studies in these dietary supplements.

M. Kotrebai et al. [62] identified SeMC as the main compound in Se-garlic enzymatic extracts using ion-pair reversed-phase HPLC combined with ESI-MS comparing retention times and the molecular ion masses with standards. Using the same technique, γ-glutamyl-SeMC was identified in an aqueous garlic extract by the same authors [56]. Another Se compound in the garlic extract, having a molecular weight of 326 u, was attributed to γ-glutamyl-SeMet. However, lacking a matching standard, no confirmation of the identity was possible. Again, with no fragmentation of the molecular ion possible, identification is often ambiguous. The lack of compatibility of the ion-pairing reagents used in these studies with ESI could possibly hamper the on-line mass spectral confirmation of Se-compounds by ESI-MS/MS. Moreover, the complexity of the matrix and the low analyte concentration added to the difficulty of identification. The same authors found that the total Se content affects the Se distribution in samples of the same type with different Se levels. The speciation results obtained for analysis of Se-enriched garlic showed the presence of γ-glutamyl-SeMC as the main species, the same as was found in samples containing low concentration levels of Se. Moreover, the contribution of SeMC increased with the increasing total Se content [62, 82].

McSheehy et al. [82] confirmed the presence of γ-glutamyl-SeMC in a Se-containing HPLC fraction of an aqueous garlic extract after size-exclusion chromatography by ESI-MS/MS.

The presence of SeMC (as the major compound), SeMet, inorganic Se and $Se(Cys)_2$ was reported by Bird et al. [83] using three chromatographic approaches (ion-exchange, reversed-phase and ion-pair reversed-phase HPLC) combined with ICP-MS and matching standards. However, full identification of these species detected by ICP-MS was not possible in the absence of a molecule-specific detector such as ESI-MS or ESI-MS/MS.

Selenium incorporation into onions has apparently improved the potential of these vegetables from the *Allium* family as a dietary source of Se compounds.SeMet, SeCys and SeMC have been identified as present in onions [79, 84]. Caruso and co-workers [79] have recently provided some evidence for the presence of $Se(Cys)_2$, SeMC, SeMet, and inorganic Se in enzymatic extracts from combined bulbs and leaves of Se-enriched green onions (*Allium fistulosum*) using ion-pair reversed-phase HPLC and size-exclusion chromatography coupled with ICP-MS and matching the chromatographic retention times with commercially available standards. ESI-ion trap (IT)-MS confirmed the presence of γ-glutamyl-SeMC in green onions as has been reported in other onion types and Se-enriched garlic. In an attempt to investigate the incorporation of Se in a particular part of the plant, the leaves of Se-enriched onions (*Allium cepa* L.) were analysed for Se speciation by Wrobel et al. [84]. Size-exclusion HPLC-ICP-MS analysis of the plant extract obtained using sodium hydroxide showed the incorporation of Se mostly in high molecular weight fractions (apparent molecular weight higher than 10 kDa). The combination of ion-pair reversed phase HPLC with ICP-MS allowed for detection of the presence of SeMC, as the principal organoselenium species, in the enzymatic extracts of the leaves. The existence of SeMC might be relevant to the anticarcinogenic potential of Se-enriched *Allium* plants for the reasons mentioned above.

Characterisation of Se species in Brazil nuts by chromatography and MS techniques

Brazil nut is one of the very few consumable products with naturally occurring exceptional high levels of Se. The high bioactivity of Se from this food source for cancer prevention and selenoenzyme maintenance has been demonstrated in studies with rats [97]. In order to elucidate which species could be responsible for the disease preventive effects of Se the characterisation of Se-containing species in this dietary source of Se has been undertaken [93–98]. The low molecular weight fraction, extracted with perchloric acid from the nut matrix, was found to contain 3–15% of the total Se in different types of nuts. Proteins were dissolved with sodium hydroxide, precipitated with acetone and re-dissolved in phosphate buffer at pH 7.5 prior to Se speciation analysis using size exclusion HPLC with on-line ICP-MS. To release Se-compounds from proteins enzymatic digestion with proteinase K [96] or protein hydrolysis with methanesulphonic acid [93] were carried out. The use of ion-pair reversed-phase HPLC-ICP-MS for analysis of the enzymatic digests has led to 19–25% recovery of the total Se content in nuts (mostly found as SeMet). Better cleavage of SeMet (75% of total Se) was observed using hydrolysis with methanesulphonic acid compared to the enzymatic hydrolysis. It therefore seems probable that Brazil nuts could be a valuable dietary source of SeMet. Further studies should be pursued for identification of unknown Se species in nuts. The recent preparation of a candidate reference material from Brazil nuts (*Bertholletia excelsa*), which has been certified for SeMet and total Se and passed the relevant homogeneity and stability tests reccommended by BCR, may be of relevance to quality control purposes of Se speciation in this food-matrix of high-Se [94].

Outlook and future trends

Further Se speciation studies in high-Se food will be required to establish fully the links between health benefits and particular Se species. The production of new isotopically enriched Se-compounds should be pursued because it may help in the characterisation of candidate ''speciated'' reference materials via isotope dilution techniques and in the validation of speciation methodologies. The need to develop chromatographic separation methods that can be sequentially coupled on-line to both elemental and molecular mass spectrometry drive continued development in the field of Se speciation analysis by chromatography and MS techniques. The choice of chromatographic conditions that are fairly compatible in terms of mobile phase flow rate (μl min^{-1}) and composition with ESI to overcome the lack of sensitivity of ESI MS will require advanced interfaces to be used between the LC system and ICP-MS. The identification of target species at low concentration levels in such complex matrices will require the development of efficient sample cleanup and preconcentration methods. The attractive features of nano-ESI MS, in terms of low sample consumption as well as a relatively high tolerance toward the presence of elevated levels of salts or buffers, may help to improve the detection power of Se compounds identification in a few microlitres of partially purified and preconcentrated HPLC fractions. Identification of unknown Se-compounds for which authentic standards are not available demands the combined use of a range of analytical techniques to elucidate their structural characteristics. Chemical and physicochemical data, in addition to the MS data, are often required to propose a reliable structure of the detected Se species. The wider use of complementary techniques (e.g. NMR) that give access to structural information is important. Confirmation of Se speciation revealed by means of chromatography and element-specific detection may involve the accurate and precise molecular mass measurement of the unknown isolated species (the better the accuracy the less the ambiguity) and of the product ions obtained from the precursor ion after CID (tandem MS). When the result of accurate mass measurement is used for empirical formula confirmation, the mass accuracy required will depend on the m/z to be measured; with increasing m/z the number of possible formulae dramatically increases making identification more and more difficult [99, 100].

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