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Speciation, Quantification and Stability of Selenomethionine, S-(methylseleno)cysteine and selenomethionine Se-oxide in yeast-based nutritional supplements

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The speciation and stability of organoselenium compounds present in the selenized yeast evaluated in National Cancer Institute human intervention trials were examined along with those of related supplements. Total selenium was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES), ICP-mass spectrometry (ICP-MS) and/or electrothermal atomic absorption spectrometry (ET-AAS) after microwave digestion. Enzymatic extraction enabled selenium speciation profiles to be obtained by ion-pair reversed phase high performance liquid chromatography (HPLC) with ICP-MS detection. Extracts were also subjected to chloroformate derivatization followed by Se and S specific GC analysis with atomic emission detection (GC-AED). Currently available and archived supplements show major differences in speciation for selenomethionine, selenomethionine *Se*-oxide and *S*-(methylseleno)cysteine with respect to time and conditions of storage. The formation of the latter compound is reported under different synthetic and elevated temperature conditions.

Introduction

The biochemistry, toxicology and nutritional importance of selenium have been reviewed regularly.^{1–5} Selenium (Se) is an essential micronutrient for humans and animals being found in many foods, with grain products having higher concentrations. Concentration varies with geographical origin of the agricultural products and on the selenium content of the soil. Human selenium deficiency has been noted in some global regions as a result of low soil selenium levels and dietary sources, a Se plasma level at or below 85 $\mu\text{g L}^{-1}$ being indicative.^{6,7} Selenium dietary deficiency has been linked to heart diseases,⁷ arthritis,⁸ cancer,⁹ and AIDS.¹⁰ Dietary intake of Se in the United States is typically 80 to 120 $\mu\text{g day}^{-1}$ and the recommended dietary allowance (RDA) is 55 $\mu\text{g day}^{-1}$; this exceeds Se intake in many world regions.⁶ For those seeking to increase their selenium intake, selenium-enriched foods have been recommended.^{11,12} Clark *et al.* reported that dietary supplementation by 200 μg of selenium per day, administered in high selenium yeast, significantly reduced lung, prostate, and colorectal cancer in the Nutrition Prevention of Cancer (NPC) trial, but all Se species in these supplements were not characterized.¹³ Further studies are on-going in major clinical trials, the European Prevention of Cancer by Intervention with Selenium (PRECISE)⁷ trial and the Selenium and Vitamin E Cancer Prevention Trial (SELECT).^{14,15} In the PRECISE trial, selenized yeast is the intervention agent. In the SELECT trial, the effect of selenomethionine (SeMet) is

to be evaluated, this agent being chosen in part by reason of the presence or formation in yeast of other organoselenium species in addition to SeMet. The marketing of selenium supplements in recent years has increased but regulation with respect to quality, storage conditions, stability and content of selenium supplements is problematic. Further selenium speciation for selenized yeast and selenium supplements is needed to quantify active components and potential anticarcinogenic components.^{16–23}

Prior to the determination of selenium species in yeast by HPLC with ICP-MS detection, an extraction technique is needed to liberate them from the yeast matrix. Water has been used to extract soluble low molecular-mass selenium species from yeasts, but with poor efficiency.²⁴ Higher extraction efficiencies have been achieved with proteolytic enzymes,²⁵ hydrochloric acid²⁶ and methanesulfonic acid.²⁷ The more widely employed proteolytic enzymes for species liberation, most closely resemble the physiological conditions in the human intestine, whereas harsher chemical hydrolysis may cause degradation or alteration of selenium species of physiological importance. Enzymatic hydrolysis usually requires long extraction periods but a rapid extraction procedure using focused ultrasound in conjunction with enzymatic hydrolysis, enzymatic probe sonication (EPS), for total selenium and selenomethionine determination in selenium-enriched yeast has been reported.²⁸ A multi-laboratory round-robin evaluation of a proposed selenized yeast reference material (SELM-1) (NRC, Canada) has recently emphasized the challenges inherent in the acquisition of accurate and precise data for selenomethionine and methionine content.²⁹ The need can be argued, however, for data for other selenium species, such as selenomethionine selenoxide and *S*-(methylseleno)cysteine,

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prior to any formalization of a standard reference selenized yeast, a goal which has been in view for a number of years. Without such a more comprehensive investigation and analysis, a fully speciated reference selenized yeast for method validation remains an unaccomplished goal. Integrated chromatographic strategies of size-exclusion, anion exchange and reversed-phase ion pair (RP-IP) in combination with ultrasonic nebulization, ICP-MS and electrospray MS-MS were used to optimize speciation of selenized yeast and *Se*-(methyl) selenocysteine based supplements.³⁰ Simultaneous speciation of Se-containing glutathione species in selenized yeast has also been recently reported in a similar study.³¹

In the present study, selenium nutritional supplements, including those administered in human intervention trials, and commercially available selenized yeast products were examined. The goals of the study were to characterize and quantify the selenium species present in the enzymatic hydrolysates of these materials and compare speciation profiles with regard to sample age, storage and subjected temperatures with particular emphasis on the formation of *S*-(methylseleno) cysteine, the recently identified Se-S aminoacid metabolite of selenized yeast.^{32,33}

Materials and methods

Instrumentation

Elan 5000 and subsequently Elan DRC-e inductively plasma mass spectrometers (Perkin-Elmer Sciex, Ontario, Canada) were used as HPLC detectors. Operating conditions for the spectrometers are shown in Table 1. The HPLC system has been described previously.³² A 5 μ m Symmetry Shield™ RP-C₈

Table 1 Operating conditions of the ICP-MS and ICP-OES instruments

ICP-MS	Perkin Elmer Elan DRC-e
RF power:	1100 W
Plasma Ar flow rate:	15 L min ⁻¹
Nebuliser Ar flow rate:	0.98 L min ⁻¹
Auxiliary gas flow rate:	1.20 L min ⁻¹
Cell gas (CH ₄) flow rate:	0.6 ml min ⁻¹
Isotopes monitored:	⁷⁸ Se, ⁸⁰ Se, ⁸² Se
Dwell time	80 ms
Nebulizer	Cross flow
Spray chamber	Scott
ICP-MS	Perkin Elmer Elan 5000
RF power:	1100 W
Plasma Ar flow rate:	15 L min ⁻¹
Nebuliser Ar flow rate:	0.88 L min ⁻¹
Auxiliary gas flow rate:	0.80 L min ⁻¹
Isotopes monitored:	⁸² Se, ⁷² Ge
Dwell time	1000 ms
Nebulizer	Cross flow
Spray chamber	Scott
ICP-OES	Perkin Elmer Optima 4300 DV
RF power:	1300 W
Plasma Ar flow rate:	15 L min ⁻¹
Nebuliser Ar flow rate:	0.80 L min ⁻¹
Auxiliary gas flow rate:	0.20 L min ⁻¹
Plasma view:	Axial
Wavelength monitored:	Se 196 nm
Nebulizer	Cross flow
Spray chamber	Cyclonic

column (3.9 mm $\times$ 15 cm) (Waters Corporation, Milford, MA, USA) were used. The injected sample volume was 20 μ L, and the flow rate of the mobile phase was 1 mL min⁻¹. The mobile phase composition was 0.1% heptafluorobutanoic acid (HFBA), 1% methanol and 99% water (v/v). A PerkinElmer Optima 4300 DV ICP-optical emission spectrometer was used for total selenium determination, operating conditions being shown in Table 1.

A PerkinElmer 4100ZL atomic absorption spectrometer equipped with longitudinal Zeeman-background correction and a transversely heated graphite furnace with a transversely heated graphite atomizer (THGA) tube, equipped with an integral L'vov platform (PerkinElmer part number BO504053) (ET-AAS) was used. A PerkinElmer AS-70 autosampler injected the standards and samples in to the graphite furnace. The selenium electrodeless discharge lamp (EDL) was powered by a PerkinElmer system 2 EDL and operated at 260 mA. A slit width of 2 nm and a wavelength of 196 nm were selected for the experiments. The inert carrier gas was argon (Meriam-Graves, high purity), the flow rate being 250 mL min⁻¹ during all stages of the furnace program, except during the atomization step when the gas flow was stopped. For total selenium determination the ET-AAS furnace heating programs were run under optimized conditions recommended by PerkinElmer.

A Hewlett Packard (Agilent) HP 5921A atomic emission detector interfaced to a HP 5890 II gas chromatograph (GC-AED) was used with hydrogen plasma reagent gas. Eluted selenium species were monitored at the 196 nm emission line. Sulfur and carbon-containing species may be simultaneously monitored at the adjacent emission lines 181 nm (S) and 193 nm (C), respectively. A 30 m SE-30 capillary column of 0.32 mm internal diameter and 0.25 μ m film thickness was employed with helium carrier flow rate 1 mL min⁻¹ and injection split ratio 30:1. The GC oven was held at 60 °C for the first 10 min and then heated to 300 °C at a rate of 5 °C min⁻¹ and held there for 2 min. The solvent vent on the detector was set at 7 min to avoid the introduction of excess solvent into the plasma.

Chemicals

All reagents used were of analytical reagent grade. Barnstead E-pure 18 M Ω water (Boston, MA, USA), methanol (HPLC grade) and HFBA (Aldrich, Milwaukee, WI, USA) were used throughout the experiments. Palladium (10 000 mg L⁻¹) and magnesium nitrate (10 000 mg L⁻¹) modifier solutions for ET-AAS analysis were from PerkinElmer. For microwave digestions, nitric acid and 30% (v/v) hydrogen peroxide (Fisher Scientific) were used. Sodium selenite [Na₂SeO₃], sodium selenate [Na₂SeO₄], DL-selenomethionine [CH₃SeCH₂CH₂CH(NH₂)COOH] and Protease XIV were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Stock standard solutions (1000 mg L⁻¹ Se) were prepared in ultra pure water (18 M Ω Barnstead E-pure) and solutions were stored between 0 and 4 °C and in the dark. *S*-(methylseleno)-cysteine [CH₃SeSCH₂CH(NH₂)COOH] was synthesized and characterized as reported previously.^{32,33} Selenomethionine *Se*-oxide (hydrate) [CH₃Se(OH)₂CH₂CH₂CH(NH₂)COOH]

was prepared by adding excess oxidizing agent, 50 μL of 30% (v/v) hydrogen peroxide to a 500 μL aliquot of a 0.1 M HCl solution of selenomethionine (1 mg L^{-1} Se) and left overnight in the dark. This and other oxidized selenium species have been characterized and authenticated in various selenized yeast and selenomethionine supplements.^{34–36}

Selenized yeast samples

A selenium yeast slurry sample (a liquid sample of live Se-yeast) and Selenolife yeast tablets (Lot #0094; 200 μg Se per tablet) were provided by LeSaffre Yeast Corporation-Red Star Nutritional Yeast (Milwaukee, WI, USA). Selenium-enriched yeast powder with a total selenium content of 1250 $\mu\text{g g}^{-1}$, dry matter ($\pm 10\%$), a minimum protein content of 50% (w/w), and a phosphate content in the range of 2.5–3.4% (w/w) (SelenoPrecise™ also designated SelenoExcell™) was obtained from Cypress Inc. (Fresno, CA, USA). The selenium supplements (in Table 2) were obtained from local retail stores.

Procedure

Microwave digestion. For the closed vessel microwave assisted acid digestion of the yeast samples a MDS 2100 microwave oven (CEM, NC, USA) with PTFE vessels was used. To determine the total selenium content, 0.2 g yeast samples were digested with 2 mL of HNO_3 and 1 mL of H_2O_2 . The microwave conditions used to digest the samples are reported elsewhere.³² After this treatment, the yeast digests were transferred and made up to volume with de-ionized water in 10 mL volumetric flasks.

Enzymatic digestion for HPLC and GC. Enzymatic extraction was as reported earlier and is briefly summarized.³⁴ Representative tablets were crushed using mortar and pestle, after manually removing titanium dioxide coatings if present. Approximately 0.2 g of the powdered sample was added to 0.02 g of the enzyme (Protease XIV) in a 15 mL centrifuge tube. After the addition of 5 mL of distilled deionized water to the centrifuge tube, the sample was shaken for 24 h at room temperature using LABQUAKE® Shakers and Rotators (Barnstead/Thermolyne Corporation, Dubuque, IA, USA). For the extraction of the liquid sample of live yeast 5 mL of the slurry mixture and 0.02 g of the enzyme were mixed and shaken for 24 h. After the extraction the sample was centrifuged (Fisher Scientific, Fair Lawn, NJ, USA) at 3000 g for 20 min. Then the supernatant was filtered through a 0.45 μm polypropylene filter (Whatmann®). The filtered supernatant was once again filtered through a 10 000 Dalton molecular weight cutoff Ultrafree-4-centrifugal filter and tube (Millipore Corporation, Bedford, MA, USA) by centrifugation for 50 min. The clear filtrate (900 μL) was mixed with 100 μL of concentrated HFBA before HPLC-ICP-MS analysis.

Derivatization of standard selenoamino acids for GC-AED.

Selenoamino acid standards were derivatized with ethylchloroformate (ECF) using procedures reported by a number of workers.^{37–41} Stock solutions of the amino acids were prepared by dissolving 10 mg in 1 mL of 0.1 M HCl to make 10 000 $\mu\text{g mL}^{-1}$ standard solutions. Then 1000 $\mu\text{g mL}^{-1}$ derivatized standard solutions were prepared as follows: 100 μL of the

Table 2 Commercial selenium supplements, selenium species determined by HPLC-ICP-MS

Commercial brand name	Ingredients ^a	Total Se ^d	Selenium species (%) ^{b,c}			
			1	2	3	4
Vitamin World—naturally inspired	Yeast free form of selenium as L-selenomethionine. Also contains dicalcium phosphate, cellulose (plant origin), vegetable stearic acid, vegetable magnesium stearate and croscarmellose	200	—	—	—	98 $\pm$ 1.2
Bio-Active Selenium/ Solaray Dietary Supplement	Natural source of selenium from Indian Mustard (<i>Brassica juncea</i>) stem and leaf. Also contains cellulose, gelatin, silica and magnesium stearate	200	1 $\pm$ 0.1	—	—	97 $\pm$ 1.0
Sundown	Vitamin C, bioflavonoids, selenium yeast chelate, brewer's yeast, citrus bioflavonoids, whey, ascorbic acid, orange peel extract and magnesium stearate	20	5 $\pm$ 0.2	—	81 $\pm$ 1.1	—
Selenomax (Nutrition 21)	High selenium yeast, rich source of natural and organic L-selenomethionine	200	22 $\pm$ 0.3	18 $\pm$ 0.4	53 $\pm$ 0.8	2 $\pm$ 0.1
Vitamin E 400 IU with selenium	Vitamin E (as <i>dl</i> -alpha tocopheryl acetate), selenium from selenium yeast. Also contains gelatin, USP water, hydrogenated vegetable oil, glycerin, soybean oil, yellow bees wax and carob extract	50	34 $\pm$ 0.6	7 $\pm$ 0.2	23 $\pm$ 0.3	35 $\pm$ 0.4
Schiff's Antioxidant Plus	Vitamins, minerals, herbs and botanical extracts among other ingredients. Selenium as L-selenomethionine	50	1 $\pm$ 0.1	2 $\pm$ 0.10	88 $\pm$ 1.2	13 $\pm$ 0.2

^a Selenium content per tablet specified on label (μg). ^b Selenium species identified by HPLC-ICP-MS in the present study after enzymatic hydrolysis: **1**: selenomethionine *Se*-oxide hydrate, **2**: *S*-(methylseleno)cysteine, **3**: selenomethionine, **4**: selenate. ^c The % selenium distributions (mean $\pm$ standard error; $n = 3$) are expressed in terms of total selenium content eluting from the column based on Se-specific peak areas.

10 000 $\mu\text{g mL}^{-1}$ selenoamino acid stock solution was treated with 1 mL of water–ethanol–pyridine mixture (60 : 32 : 8 by volume), 50 μL of ECF was added and the mixture shaken until gas evolution ceased. Then 1.0 mL of chloroform containing 1% ECF was added, and the derivatives were extracted into the organic phase by shaking for 30 min, which was evaporated to dryness. The residue was redissolved in 1.0 mL of chloroform and 1 μL was injected.

Yeast samples were derivatized with ethylchloroformate and analyzed by GC-AED. The procedure for the amino acids was scaled up for their determination in the yeast sample. To a 2 mL portion of the extract was added 5 mL of a water–ethanol–pyridine mixture (60 : 32 : 8 by volume) followed by 1 mL of ethyl-chloroformate, the sample was shaken and any evolved gas vented. Ethylated derivatives were extracted into 3 mL chloroform containing 1% ECF by shaking for 30 min. The chloroform layer was separated, solvent was removed with a flow of nitrogen and the residue was redissolved in 100 μL chloroform or hexane. One μL was injected.

Thermal treatment of selenized yeast. Selenized yeast powder (Selenoprecise™) was heated in a closed vial at 100 °C and 200 mg aliquots were analyzed after 1, 3 and 7 days. A commercial bakers yeast, spiked with selenomethionine, was also heated at 100 °C for 24 h, as was a selenomethionine-cysteine sample. Samples were subjected to enzymatic digestion, and selenium speciation profiles were obtained by HPLC-ICP-MS and GC-AED.

Results and discussion

Treatment of selenized yeast with proteolytic enzymes, followed by extraction, releases more than 90% of total selenium present as selenite, selenate and selenamino acids or their derivatives. Perfluoro acid ion pair reversed phase HPLC-ICP-MS affords good separation of inorganic selenium species from selenoamino acids.^{32–34} Except for cases wherein formulated supplements (yeast or yeast-free) incorporate inorganic selenium, the major organoselenium species identified in such studies was selenomethionine (SeMet).^{1,27,29,42} Other selenoamino acids, such as selenocystine, *Se*-lanthionine, *Se*-cystathionine, *Se*-methylselenocysteine, were observed in selenized yeast at levels <1% of SeMet, but at that time a number of minor selenium species remained unidentified.⁴² Qualitative identification of these relied in part upon retention time matching with available or synthesized standards, but in some cases direct confirmatory HPLC mass spectral data was obtained. The identity of two of these compounds in selenized yeast, selenomethionine *Se*-oxide hydrate^{20,32,33,36} and the *Se*-S bonded amino acid *S*-(methylseleno)cysteine has now been confirmed.^{32,33} The presence of the latter was also confirmed by ethylation derivatization and capillary GC with selenium specific atomic emission detection (GC-AED)³² and by ⁷⁷Se NMR.³³

It has become clear that these two compounds comprise the primary organoselenium compounds released upon proteolysis other than SeMet, and that they increase in relative proportions to it as selenized yeast ages. This was initially indicated by study of archived samples of yeast and supple-

ment tablets employed in the ‘Clark’ trials¹³ and stored subsequently for a number of years at ambient temperature.³²

In order to gain a perspective as to the presence of the three primary organoselenium species present in commercial selenized yeasts and supplements, four different brands of selenium nutritional supplements along with two non-yeast supplements were obtained locally, and showed substantially different speciation content (Table 2). The reported distribution numbers are percentage concentrations, relative to the total selenium response to the eluting selenium compounds, including selenate. With the exception of the “Sundown” material, summed percent data for designated eluted selenium species were in the 96–99% range of total eluted selenium signal over the elution time measured. The exception for the “Sundown” material suggests the presence there of *ca.* 15% of undefined eluted selenium species.

The data indicate that supplement labeling should be approached with caution. The supplement “Vitamin World” indicating selenomethionine content contained inorganic selenium (selenate) and “Bio-Active Selenium/Solaray Dietary Supplement” indicating a natural source of bio-available selenium also contained selenate as the major selenium species. The proportion of selenomethionine (taken together with Selenomethionine *Se*-oxide) was higher than 70% in the supplements with brand names “Sundown”, “Selenomax” and “Schiff’s Antioxidant Plus”. The low level of selenomethionine *Se*-oxide and absence of *S*-(methylseleno)cysteine in the “Sundown” supplement that also contained the reducing agent ascorbic acid (vitamin C) is noteworthy. In the supplements that also contained vitamin E (E Vitamin 400 IU with selenium), SeMet (23%), selenate (35%), *S*-(methylseleno)cysteine (7%) and selenomethionine *Se*-(34%) were all present.

In view of the studies described later concerning the changing ratios of the three organoselenium species with time and temperature, the wide variation among the proportions in these supplements is not unexpected. The variations among the selenium species present in selenized yeast products stored at room temperature for several years, and also of recently manufactured yeast products, suggested that speciation changes have occurred during storage or during yeast supplement manufacture.³² In order to test this hypothesis and to determine whether any previously unidentified organoselenium species were produced when selenized yeast was heated at an elevated temperature (100 °C), samples were heated before characterization by HPLC-ICP-MS and GC-AED. No attempt was made in this study to conform to any pharmaceutically accepted procedures for ‘accelerated stability studies’ such as would clearly be required for a full characterization of selenized yeast from a pharmaceutical standpoint.

Speciation experiments were first carried out to determine if *S*-(methylseleno)cysteine and selenomethionine *Se*-oxide were present in a typical selenized yeast slurry (yeast cream) that is used at the beginning of the yeast tablet manufacturing process. This slurry sample (a liquid sample of live *Se*-yeast) and the tablets manufactured from it (Selenolife yeast tablets) were subjected to standard enzymatic extraction, selenium species being determined by HPLC-ICP-MS and GC-AED.

Fig. 1 GC-AED chromatograms of the derivatized enzymatic extracts of (A) selenium yeast cream slurry and (B) Selenolife yeast tablet prepared from the yeast slurry.

A major advantage of GC-AED is the ability to obtain simultaneously a number of element specific chromatograms for different elements. We earlier reported comparative C, S and Se GC-AED chromatograms for ethylchloroformate-derivatized Clark yeast and established the retention characteristics for *S*-(methylseleno)cysteine by referencing both sulfur and selenium chromatograms.³² Fig. 1 shows a comparison of selenium specific gas chromatograms for derivatives of the yeast slurry and Selenolife yeast tablets prepared from it. A small peak corresponding to *S*-(methylseleno)cysteine appears in the chromatogram of the yeast tablet but is absent in that of the yeast cream. Selenomethionine *Se*-oxide is not observed in the chromatogram since it is derivatized to the same product as selenomethionine in the ethylation process.

By comparison, the HPLC-ICP-MS chromatograms for the selenium yeast cream slurry sample and the derived Selenolife yeast tablet are shown in Fig. 2. In the yeast cream chromatogram, a small peak corresponding to selenomethionine *Se*-oxide (1) is observed but *S*-(methylseleno)cysteine is absent. In the derived tablet, *S*-(methylseleno)cysteine (2) appears and it is noteworthy that selenomethionine *Se*-oxide hydrate is present at a higher level than in the yeast slurry. The two chromatograms correspond to different overall species levels due to the different analyte quantities sampled.

The chromatographic results for the thermal treatment of SelenoPrecise™ at 100 °C are shown in Fig. 3 (HPLC-ICP-MS) and Fig. 4 (GC-AED). Both HPLC and GC chromatographic patterns for selenomethionine, *S*-(methylseleno)cysteine and

selenomethionine *Se*-oxide (observed only in the HPLC chromatogram as discussed earlier) retain the same overall characteristics as observed previously and no other identifiable

Fig. 2 HPLC-ICP-MS chromatograms (Elan 5000) of the enzymatic extracts of (A) selenium yeast cream slurry and (B) Selenolife yeast tablet prepared from the yeast slurry. (1) selenomethionine *Se*-oxide, (2) *S*-(methylseleno)cysteine, (3) selenomethionine.

Fig. 3 HPLC-ICP-MS chromatograms (Elan DRC-e) of the enzymatic extracts of SelenoPrecise™ yeast stored at (A) 4 °C, and heated (B) 100 °C for 1 day; (C) 100 °C for 3 days and (D) 100 °C for 1 week. (1) selenomethionine *Se*-oxide, (2) *S*-(methylseleno)cysteine, (3) selenomethionine.

species are formed at appreciable levels. In Fig. 3, it is noted that a small peak (not identified) is present on the tail of peak 1 (selenomethionine *Se*-oxide), which introduces uncertainty into its quantification. The successive chromatograms (A, 4 °C storage), (B–D, 1, 3 and 7 days at 100 °C) show the overall trend that total selenomethionine content diminished over the period while the *S*-(methylseleno)cysteine percentage peak area (% of the total selenium eluted from column) increased during the heating period. The relative amount of selenomethionine *Se*-oxide present also increased. The total peak area corresponding to the three chromatographable species decreased to approximately one half of the initial summed area after one week heating but at that time the proportions of *S*-(methylseleno)cysteine in particular and also of selenomethionine *Se*-oxide were enhanced relative to residual selenomethionine. Table 3 shows percent distributions of selenomethionine, *S*-(methylseleno)cysteine and selenomethionine *Se*-oxide based upon total eluted selenium signal.

Fig. 4 shows the corresponding GC-AED chromatogram and the quantitative data are given in Table 4. The ‘selenomethionine’ peaks (3) correspond to the sum of selenomethionine and selenomethionine *Se*-oxide which form the same derivative species; the responses relative to *S*-(methylseleno)cysteine are thus larger than shown in the HPLC-ICP-MS analysis. The GC-AED and HPLC-ICP-MS data show the same trend with sample heating, but numerical differences from

Fig. 4 GC-AED temperature programmed chromatograms of the enzymatic extracts of SelenoPrecise™ yeast stored at (A) 4 °C, and heated (B) 100 °C for 1 day; (C) 100 °C for 3 days and (D) 100 °C for 1 week. (2) Selenomethionine (including selenomethionine *Se*-oxide) (3) *S*-(methylseleno)cysteine.

the former, notably in the ‘unidentified’ category, presumably arise from derivatization processes. These observations parallel the speciation observed earlier for archived selenized yeast from the Clark trials,^{13,32} lending some validity to the application of briefer more elevated temperatures in stability studies.

Selenized yeast samples are subjected to elevated temperatures during manufacture. To remove excess free selenium, yeast is washed 3 to 4 times at 90–95 °C, with each wash cycle lasting about 30 min. Next a process called drum drying takes place at 150–175 °C for 5 s to remove moisture from the yeast. The present stability study indicates loss of selenomethionine and the formation of *S*-(methylseleno)cysteine at such temperatures.

In order to ascertain any changes in the sample’s selenium content during the heating period, total selenium was measured before and after heating by ICP-MS and ICP-OES. The values obtained are shown in Table 5 and indicate that all initial selenium was still present after heating. There was no suggestion of the formation of volatile selenium compounds such as dimethyldiselenide during the heat treatment, so it may be concluded that selenium is converted at least in part to larger species not amenable to direct selenoamino acid analysis.

Formation of *S*-(methylseleno)cysteine in selenized yeast products

The overall elemental composition of bakers yeast indicates that sulfur (S) is the seventh most abundant element after

Table 3 Peak area percent of selenium compounds in enzymatic hydrosylates of SelenoPrecise™ yeast powder by HPLC-ICP-MS^a

Selenium species	4 °C storage	100 °C for 1 day	100 °C for 3 days	100 °C for 7 days
Selenomethionine <i>Se</i> -oxide	16.6 ± 1.0	27.6 ± 1.1	36.7 ± 0.6	39.2 ± 0.1
<i>S</i> -(methylseleno)cysteine	2.6 ± 0.1	11.0 ± 0.1	17.6 ± 0.2	23.4 ± 0.2
Selenomethionine	77.3 ± 1.2	58.7 ± 1.3	42.1 ± 0.5	34.2 ± 0.3
SUM	96.5	97.3	96.4	96.8
Unidentified	3.5	2.7	3.6	3.2

^a The % selenium distributions (mean ± standard error; *n* = 3) are expressed in terms of total selenium content eluting from the column.

Table 4 Peak height percent of selenium compounds in enzymatic hydrosylates of SelenoPrecise™ yeast powder by GC-AED^a

Selenium species	4 °C storage	100 °C for 1 day	100 °C for 3 days	100 °C for 7 days
<i>S</i> -(methylseleno)cysteine	8.3 ± 0.7	12.1 ± 0.5	13.6 ± 0.2	19.7 ± 0.2
Selenomethionine	88.5 ± 1.2	77.1 ± 0.4	73.5 ± 0.4	59.2 ± 0.6
SUM	96.8	89.2	87.1	78.9
Unidentified	3.2	10.8	12.9	21.1

^a The % selenium distributions (mean ± standard error; *n* = 3) are expressed in terms of total selenium content eluting from the column.

Table 5 Total selenium content (μg g⁻¹) of SelenoPrecise™ yeast powder before and after heating at 100 °C for 7 days

	ICP-MS (Elan DRC-e)	ICP-OES (Optima 4300-DV)
Before heating	1335 ± 6	1370 ± 24
Heated for 7 days 100 °C	1349 ± 56	1395 ± 65

^a Concentrations expressed as mean ± standard error, *n* = 15.

carbon (C), oxygen (O), nitrogen (N), hydrogen (H), potassium (K) and phosphorus (P). Sulfur constitutes 0.36% of yeast dry matter.⁴³ In the course of the growth cycle of *Saccharomyces cerevisiae* yeast, selenite that is potentially toxic and poorly bioavailable, is converted to bioactive species such as selenomethionine which have better nutritional properties. The formation of *S*-(methylseleno)cysteine during the yeast growth cycle is not apparent since the slurry selenium yeast did not indicate its presence.

However, the formation of *S*-(methylseleno)cysteine might be explained by degradation of selenomethionine to form dimethyldiselenide within the yeast structure, that could in turn react with cysteine and/or cystine (present in yeast) to form the Se–S bonded species. Such a reaction taking place in the solid phase would parallel that involved in the synthesis of

S-(methylseleno)cysteine,³³ although the yeast heating experiment did not indicate that any free dimethyldiselenide was formed. Experiments were performed with pure standards, selenomethionine and L-cysteine to test this hypothesis. Solid standards (selenomethionine 10 mg and L-cysteine 100 mg) were mixed with a few drops of deionized water and heated for 24 hours at 100 °C. For HPLC-ICP-MS analysis, the solid was dissolved in 10 mL of deionized water, and the chromatogram (Fig. 5) shows the formation of *S*-(methylseleno)cysteine and selenomethionine *Se*-oxide hydrate. The large tailing peak for selenomethionine is due to column overloading. A similar experiment was performed by spiking commercial bakers yeast

Fig. 5 HPLC-ICP-MS chromatograms (Elan 5000) of Selenomethionine and L-cysteine mixture after 24 h at 100 °C, upper: full scale, lower: zoom. (1) selenomethionine *Se*-oxide, (2) *S*-(methylseleno)cysteine, (3) selenomethionine. **Fig. 6** HPLC-ICP-MS chromatograms (Elan 5000) of a commercial bakers yeast, (A) spiked with selenomethionine and left at room temperature for 24 h; (B) zoom in of (A); and (C) spiked with selenomethionine and heated at 100 °C for 24 h. (1) Selenomethionine *Se*-oxide, (2) *S*-(methylseleno)cysteine, (3) selenomethionine.

with selenomethionine. The chromatogram for the spiked yeast heated at 100 °C for 24 h (Fig. 6) showing a peak for *S*-(methylseleno)cysteine. These preliminary experiments suggest that *S*-(methylseleno)cysteine forms slowly from selenomethionine through an intermediary such as dimethyl diselenide, which may react with L-cysteine and/or cystine in yeast.

Conclusions

Regulations governing the QA/QC protocols followed during nutritional supplement preparation within the United States and elsewhere are less rigid than those for drug substances, as is product labeling for the consumer. A case in point is that of 'accelerated stability' studies including thermal stability generally performed for pharmaceutical 'active' species. It may be pointed out that ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) stipulates protocols for stability studies including, for example, comparisons of testing between storage at 25 °C and 30 °C. The guidelines for 'accelerated', *i.e.* elevated temperature, studies are also specific and it is emphasized that our experiments in no way follow such protocols. However, our observations showing close similarities of speciation and quantitation between extended (many years) storage at ambient temperatures and brief heating at elevated temperatures are at least indicative of a consistent mechanism of speciation behavior.

Although progress on the designation of a 'standard reference selenized yeast (for speciation validation)' has been a goal for some time, it is not yet in place for independent validation. This remains an arduous task, since not only the major selenium species, but also minor (and potentially) variable minor components, must also be considered.

Of the different brands of selenium nutritional supplements analyzed, only two brands had high concentrations of selenomethionine and two brands appeared to contain mostly inorganic selenium (selenate), suggesting that label claims of "bioavailable selenium" or the presence of mainly selenomethionine may often be inaccurate. Selenomethionine selenoxide hydrate appears to form as soon as selenized yeast is dried and tableted. The wide range of the selenomethionine to selenoxide ratios observed in this and other studies certainly depends on storage time and conditions, notably exposure to air and elevated temperatures. *S*-(methylseleno)cysteine also appears to form soon after selenized yeast is made and appears at increasing ratios to selenomethionine in stored samples and in samples subjected to elevated temperatures for briefer periods. Analytical methods may not distinguish between selenomethionine and the selenoxide hydrate and indeed quantitation may usefully combine content for both species as they are probably utilized in the same manner physiologically. Any studies on the clinical, nutritional or toxicological properties of *S*-(methylseleno)cysteine remain to be initiated.

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