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Application of Isotope Dilution Analysis for the Evaluation of Extraction Conditions in the Determination of Total Selenium and Selenomethionine in Yeast-Based Nutritional Supplements

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Isotope dilution analysis (IDA) has been used to quantify total selenium, total solubilized selenium, and the selenomethionine (SeMet) amount in yeast and yeast-based nutritional supplements after acid microwave digestion and different enzymatic extraction procedures. For this purpose, both a ^{77}Se-enriched SeMet spike, previously synthesized and characterized in our laboratory, and a ^{77}Se-(VI) spike were used. In the analysis of the nutritional supplements, the SeMet spike was added to the sample and extracted under different conditions, and the ^{78}Se/^{77}Se and ^{80}Se/^{77}Se isotope ratios were measured as peak area ratios after high-performance liquid chromatography (HPLC) separation and inductively coupled plasma mass spectrometry (ICP-MS) detection. The formation of SeH^+ and mass discrimination were corrected using a natural SeMet standard injected every three samples. Similarly, total solubilized selenium was measured in the extracts after enzymatic hydrolysis using the ^{77}Se-enriched SeMet as a spike by direct nebulization without a chromatographic separation. To establish a mass balance, total selenium was also determined by IDA-ICP-MS on the yeast tablets after microwave digestion using ^{77}Se(VI) as a spike. Results showed that all enzymatic procedures tested were able to solubilize total selenium quantitatively from the solid. However, the recovery for the species SeMet, the major selenium compound detected, was seriously affected by the enzymatic procedure employed and also by the matrix composition of the supplement evaluated. For the yeast sample, SeMet recovery increased from 68 to 76% by the combined use of driselase and protease. For the nutritional supplements, the two most effective procedures appeared to be protease and driselase/protease, with a SeMet recovery ranging from 49 to 63%, depending upon the supplement evaluated. In the case of *in vitro* gastrointestinal enzymolysis, the results obtained showed 26−37% SeMet recovery, while the rest of selenium was solubilized as other unknown compounds (probably Se-containing peptides).

KEYWORDS: Nutritional supplements; total selenium; selenomethionine; enzymatic extraction procedures; isotope dilution analysis

INTRODUCTION

The nutritional functions of selenium are well-recognized (*1*, 2), and it has been reported that, in consuming a natural diet, daily selenium intake generally tends to be lower than the recommended value by the Food and Agricultural Organization/World Health Organization (55 μg/day for both men and women) (*3*). On the other hand, after Clark et al. (*4*) demonstrated the potential of selenium-enriched yeast for cancer prevention, different selenium supplements have became widely used. However, the mechanisms by which Se elicits chemopreventive properties have not been elucidated yet. Furthermore, it is not known which form(s) of Se exhibit activity. It has been hypothesized that selenomethionine (SeMet) may be a key link in understanding the proposed cancer preventive properties of yeast. *In vitro* and *in vivo* studies have shown that SeMet exhibits anticancer activity in models of cancer of the colon and prostate (*5*). Several selenized yeast products are found on the market but with different organic and inorganic selenium content. Therefore, there is a need for more reliable methods to determine different selenium species in those samples, thus increasing our ability to understand their biochemical pathways and nutritional effects.

The coupling of high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is a powerful technique for selenium speciation in biological samples using different chromatographic systems (*6*). This combination offers the advantage of easy coupling, high

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sensitivity, specificity, and the possibility of using isotope dilution analysis (IDA) for quantitative selenium speciation. However, the application of isotope dilution for speciation analysis is still limited by the commercial availability of isotopically enriched species (*7*). In a previous paper, ^{77}Se-enriched SeMet was obtained by yeast growth on a ^{77}Se-rich culture medium (*8*) and was used there for the determination of SeMet in selenized yeast by HPLC-ICP-MS. More recently, species-specific isotope dilution was applied to the determination of methionine and SeMet in selenized yeast by LC using MS and ICP-MS detection (*9*) or GC-MS (*10*). A further application was reported by Ruiz Encinar et al. on the determination of SeMet and selenocysteine in human blood serum using a ^{77}Se-labeled SeMet spike in capillary HPLC-ICP-MS (*11*). Polatajko et al. described for the first time the use of a ^{77}Se-labeled selenopetide (Asp-Tyr-^{77}SeMet-Gly-Ala-Ala-Lys) for the quantification of 12-kDa heat-shock protein in selenized yeast by capillary HPLC-ICP-MS (*12*).

One problem for selenium speciation analysis in biological materials is the sample preparation step, where the target is the quantitative extraction of the compounds maintaining the information on the original species (*13*−15). For this purpose, many different extraction procedures have been evaluated in selenized yeast. The most frequently applied method is the enzymatic hydrolysis based on the use of proteolytic enzymes resulting in a breakdown of peptide bonds in proteins, thus releasing free amino acids. The application of protease XIV (*16*−18), proteinase K (*13*, *19*), pepsin (*20*), or a mixture of proteolytic enzymes (*21*) in Se-speciation analysis has been reported in different papers. The main selenium species found in yeast enzymatic digest was SeMet (*22*). Recently, a method was developed allowing for the separation, detection, and identification of Se species extracted from *Saccharomyces cerevisiae* yeast supplements during simulated gastric or intestinal digestion processes (*23*). The major selenium species presented in those nutritional supplements was again identified as SeMet.

Although consistent results have been reported in the detection/identification of the main selenium species in yeast extracts, their quantification is still a challenge. Recently, improved recovery of Se species from biological samples was reported, taking advantage of the simultaneous and sequential use of proteolytic and nonproteolytic enzymes (*14*, *15*, *24*). Then, the application of cell-wall-degrading enzymes was shown to increase selenium extraction. In this case, driselase of fungi origin (*Basidiomycetes* sp.) has been applied to the speciation analysis of selenium compounds in selenium-enriched yeast (*16*). Again, the main compound extracted from cell walls was also identified as SeMet.

The objective of this paper was the development and application of isotope dilution ICP-MS methodologies able to determine both total extracted selenium and SeMet with a single ^{77}Se-enriched SeMet spike. These methodologies were applied to the evaluation of three different extraction procedures with hydrolytic enzymes for the accurate determination of total selenium and SeMet extracted from selenized yeast and yeast-based nutritional supplements. The total Se concentration in the solid was also determined by IDA-ICP-MS after microwave digestion for mass balance purposes.

MATERIALS AND METHODS

Instrumentation. The ICP-MS instrument used was an Agilent 7500c ICP-MS equipped with an octapole ion guide operated in rf-only mode (Agilent Technologies, Tokyo, Japan). The plasma and auxiliary argon flow rates were 15 and 1.12 L/min, respectively. The RF power was 1500 W. The torch shield was employed to minimize the plasma potential. The torch position and ion lens voltage settings were optimized daily for optimum sensitivity with a 10 ng/g Li, Co, Y, Tl, and Ce mixture in 1% (w/w) HNO_3 solution. The octapole cell was pressurized with 4 mL/min hydrogen to avoid the spectral interferences of argon (*25*). For total selenium determination, the acquisition mode selected was an isotope ratio using a 4 s integration time (per point) and 3 points per peak with five replicates. Masses 76, 77, 78, 80, 82, and 83 were monitored.

For HPLC separations, a Hamilton PRP-X100 (Reno, NV) column was used. The column was connected to a Shimadzu LC-10 AD VP HPLC pump (Kyoto, Japan) equipped with a Rheodyne injector (model 7125, Cotati, CA) having a 100 μL sample loop. The solution eluting from the column was introduced on-line by a short length of Tygon tubing to the ICP-MS through a Meinhard glass nebulizer attached to a Scott-type double-pass spray chamber (cooled to 2°C). The conditions for separation and detection of selenium compounds in the enzymatic hydrolysates by HPLC-ICP-MS were already published (*8*).

A Milestone (Socisole, Italy) model 1200 microwave digester with medium-pressure PTFE vessels were used for the acid digestion of yeast-based nutritional supplements. The following microwave digestion program was used: 250 W for 2 min and 450 W for 5 min using nitric acid and hydrogen peroxide.

Extraction of the selenium compounds from nutritional supplements using enzymatic hydrolysis was carried out in a digital control immersion thermostat model Digiterm 100 (J. P. Selecta, Barcelona, Spain) at 37 °C. A Biofuge stratos centrifuge (Heraeus, Hanau, Germany) was also used. A Memmert (Schwabach, Germany) oven model UM/SM 100 was used to determine the moisture content of the yeast sample.

Samples and spikes were weighed on a 0.01 mg balance (Mettler model AE 163, Cambridge Scientific Products, Cambridge, MA).

Reagents and Materials. All reagents used were of analytical grade. Ultrapure water was obtained from a Milli-Q System (Millipore Co., Bedford, MA). Standard solutions of 1000 mg/L of Se as SeO_2 stabilized in 2−3% (v/v) nitric acid Suprapur was purchased from Merck (Darmstadt, Germany). Enriched ^{77}Se was supplied from Cambridge Isotope Laboratories (Andover, MA) as elemental powder, and it was dissolved in the minimum volume of subboiled nitric acid and diluted to volume with ultrapure water. The concentration of this solution was established by reverse IDA (*25*).

Hydrogen peroxide and nitric acid (from Merck) were used for sample digestions. The nitric acid was additionally purified by subboiling distillation.

The ^{77}Se-enriched SeMet biosynthesis was described in a previous paper (*8*). The concentration of total selenium and SeMet obtained was calculated by reverse IDA on the yeast hydrolysate. A stock solution (100 mg/L Se) of seleno-DL-methionine (Sigma−Aldrich, >99% purity) was obtained by dissolving the appropriate amount of this compound in 0.1 M HCl to prevent oxidation. This solution was kept frozen at −20 °C.

The candidate yeast reference material (lyophilised yeast sample, Pharma Nord, Denmark) was supplied by the Danish Veterinary and Food Administration (Soeborg, Denmark). The concentration of total selenium and SeMet in this material has been determined by several laboratories in an intercomparison exercise (*21*), and those values were used as a reference. Three brands of yeast-based selenium food supplements were used in this study, and they were purchased from a local drugstore. The selenium-enriched supplements analyzed were Selenium Arkovital (Arkopharma, France), Selenium Verdalia (Naturland, France), and Selenium-ACE (Wassen, U.K.).

Protease XIV (from *Streptomyces griseus*), driselase (from *Basidiomycetes* sp.), bile salts and porcine enzymes (pepsin, α-amylase, and pancreatin), sodium bicarbonate, phenylmethylsulfonyl fluoride (PMSF), hydrochloric acid 37% (v/v), sodium chloride, and tris(hydroxymethyl)-aminomethane (Tris) were used for the enzymatic hydrolysis procedures. They were supplied from Sigma−Aldrich (Steinheim, Germany).

For the chromatographic separations, ammonium phosphate from Sigma−Aldrich was used. HPLC-grade methanol and 25% ammonia solution (v/v) were purchased from Merck (Darmstadt, Germany).

HPLC buffers were freshly prepared and degassed with helium prior to use. Samples were filtered through polyvinylidenedifluoride syringe filters (PVDF; 0.45 μm; Teknokroma, Barcelona, Spain).

Procedures. *Sample Handling for Commercial Nutritional Supplements*. A composite blend was made by crushing 10 randomly selected tablets of one bottle and mixing them thoroughly by mortar and pestle. Three samples of each brand were accurately weighted (ca. 0.1 g) and used for the total digestion assay. The other three samples for each brand were accurately weighed (ca. 10 mg) on a 0.01 mg balance and used for the enzymatic extraction procedure.

Extraction Procedures. Three extraction procedures were evaluated. Extraction recovery was calculated versus the total concentration of Se as measured by IDA-ICP-MS in the original product. Each experiment was performed 3 times with three HPLC injections each. The samples (ca. 10 mg) were mixed with the appropriate amount of the spike and placed in 7 mL glass vials. After vortex-mixing for 10 min, to ensure complete equilibration of the mixture, the following reagents were added to the mixture:

Enzymatic Hydrolysis with Protease XIV. The samples were incubated for 20 h at 37 °C in a shaking water bath with 1 mg of protease XIV in 100 mM Tris-HCl buffer (pH 7).

Enzymatic Hydrolysis with Driselase/Protease XIV. The samples were incubated in a shaking bath a 37 °C with 150 μL of 4% (w/v) driselase in 30 mM Tris-HCl buffer in the presence of 1 mM PMSF for 4 h. Then, 1 mg of protease XIV was added. The samples were incubated for a further 20 h at 37 °C.

In Vitro Gastrointestinal Enzymolysis. An *in vitro* gut model was used as described by Crews et al. (*26*). Enzymolysis was carried out in two steps corresponding to the conditions in the stomach and in the intestine. For that, the samples were incubated in a shaking bath at 37 °C for 4 h with 100 μL of gastric juice [1% (w/v) pepsin in 0.15 M NaCl acidified with HCl to pH 2.0]. After the pH was adjusted to 7 with 2 M $NaHCO_3$, 100 μL of gastrointestinal juice [3% (w/v) pancreatin, 1.5% (w/v) amylase, and 1% (w/v) bile salts in 0.15 M NaCl] was added. The samples were incubated for a further 4 h at 37 °C.

After extraction, the samples were centrifuged at 4000*g* for 40 min at 4 °C and the supernatant was filtered through a 0.45 μm PVDF syringe filter and frozen at −20 °C to avoid species transformation of the extracted substances. The samples were diluted with ultrapure water before injection in the HPLC system.

The moisture content of the yeast sample (reference material) was determined by drying three separate subsamples (0.5 g) until a constant mass was acquired at 100 °C.

Total Se Determination by IDA-ICP-MS. For the determination of total selenium in the yeast and nutritional supplements, microwave digestion was employed using the heating program indicated before. Approximately 0.1 g of samples was digested by using 3 mL of concentrated HNO_3 and 0.5 mL of 30% H_2O_2. The end solutions were diluted with ultrapure water to 20 mL. Because of the high concentration of selenium in the yeast and nutritional supplements, the samples were spiked with the appropriate amount of ^{77}Se(VI)-enriched isotope after digestion to minimize the amount of ^{77}Se needed. The total selenium concentration was determined by IDA-ICP-MS using the ^{78}Se/^{77}Se and ^{80}Se/^{77}Se isotope ratios (*25*). The correction for SeH^+ formation was carried out using mathematical equations by monitoring also the signal at masses 76, 82, and 83. All isotope ratio measurements were corrected using a dead time of 47 ns. A natural standard of selenium was also measured between the samples to calculate the mass bias correction factor using the exponential model of mass bias (*27*). The concentration of selenium was calculated using the isotope dilution equation previously described (*28*).

To evaluate the extraction efficiency and establish the mass balance, the total selenium concentration in the enzymatic extracts was also determined by IDA-ICP-MS. In this case, the enzymatic extracts, already spiked with the ^{77}Se-enriched SeMet standard, were filtered and diluted 20-fold with ultrapure water before direct nebulization in the ICP-MS using the instrumental conditions and acquisition parameters used for total selenium determination in the solid samples.

SeMet Determination by IDA-HPLC-ICP-MS. The determination of SeMet in yeast and nutritional supplement extracts was performed by IDA. Each experiment was repeated 3 times with three HPLC injections per experiment. Previous HPLC-ICP-MS conditions were used (*8*). Anion-exchange HPLC was employed for the separation of SeMet from other Se-containing compounds. HPLC separation parameters were optimized to minimize peak integration errors. An integration time of 250 ms per isotope for SeH^+ and mass bias correction was chosen, and the monitored masses were 76, 77, 78, 80, 82, and 83. For IDA, an integration time of 300 ms per isotope was chosen and the monitored masses were 76, 77, 78, and 80.

Table 1. Comparison of Calibration Results for Se(IV), Se(VI), and SeMet ($n = 5$)

calibration data ($n = 5$)	Se(IV)	Se(VI)	SeMet
slope (counts/ng of Se g^{-1})	107	109	110
SD slope (95%)	7	2	2
intercept (counts)	–574	–43	318
SD intercept (95%)	68	10	47
regression coefficient (r^2)	0.9992	1.0000	1.0000

The intensity chromatograms were first corrected for detector dead time (*25*). Then, the raw data of the transient isotope signals for SeMet were processed using the chromatographic software provided with the ICP-MS instrument (Hewlett−Packard, Avondale, PA). Quantitations were performed in a peak area mode. After adequate mathematical corrections were applied to correct for the SeH^+ interference (*25*), the ^{78}Se/^{77}Se or ^{80}Se/^{77}Se isotope ratios were then calculated and corrected for mass bias using an exponential model (*27*). Finally, the SeMet content in the sample was calculated by applying the isotope dilution equation (*28*).

RESULTS AND DISCUSSION

Comparison of ICP-MS Sensitivity for Different Selenium Species. The isotope dilution procedure to be applied in this paper is based on the assumption that different selenium species provide the same sensitivity in the ICP-MS; therefore, isotope equilibration prior to the ion source is not required. To check this assumption, calibration graphs were obtained for standard solutions of the natural abundance of Se(IV), Se(VI), and SeMet. The results obtained are shown in **Table 1**. As can be observed, no significant differences in the slope at the 95% confidence level were detected for the three species tested. This means that no species-dependent sensitivity factors need to be applied under the nebulizer and plasma conditions used in this paper. The first conclusion that can be drawn from these results is that a ^{77}Se spike containing Se(IV), Se(VI), SeMet, or mixtures of these species will, *a priori*, provide the same analytical isotope dilution results. This fact allows for the development of alternative isotope dilution procedures in which total selenium is measured by direct nebulization of the samples and SeMet is measured after a chromatographic separation of the same sample used for total selenium. For this purpose, the ^{77}Se-enriched SeMet spike needs to be characterized both for the SeMet content and for total selenium by reverse IDA.

Characterization of the ^{77}Se Spike for Total Selenium and SeMet. Different aliquots of the ^{77}Se-enriched yeast obtained previously (*8*) were kept frozen at −70 °C in Eppendorf tubes. For this study, one of these tubes containing ca. 50 mg of the yeast was hydrolyzed with protease XIV and the resulting extract (ca. 5 mL) was centrifuged, filtered, and stored at −18 °C. In this case, SeMet was not further purified by HPLC to avoid dilution of the spike. It is clear that this spike will contain a certain amount of free SeMet but also other Se-containing compounds. For this reason, we decided to characterize the ^{77}Se spike both for total selenium (by direct nebulization) and for SeMet content (after a chromatographic separation).

Table 2. Isotope Composition of the ^{77}Se-Enriched SeMet Solution Characterized for Total Se and SeMet

isotope[a]	natural isotopic abundance (%)	isotope abundance (%) total Se	isotope abundance (%) SeMet
76	9.37 ± 0.29	1.03 ± 0.07	0.9 ± 0.1
77	7.63 ± 0.16	91.1 ± 0.7	92.7 ± 0.4
78	23.77 ± 0.28	3.9 ± 0.3	3.5 ± 0.2
80	49.61 ± 0.41	3.4 ± 0.2	2.5 ± 0.2
82	8.73 ± 0.22	0.54 ± 0.04	0.39 ± 0.04

[a] The abundance of ^{74}Se was less than 0.01%.

The total Se concentration in the ^{77}Se-enriched SeMet spike was calculated using reverse IDA by spiking with natural abundance SeMet of a known concentration and purity and direct nebulization of the blend into the ICP-MS. Three independent isotope dilution experiments were carried out. The average total Se concentration of the spike solution turned out to be 5.00 ± 0.04 μg/g. For the determination of the SeMet content, the same blends were injected in the HPLC-ICP-MS system and the content of SeMet was calculated on the basis of peak area ratios. A 3.0 ± 0.2 μg/g of SeMet (as Se) in solution was obtained (ca. 60% of total selenium). As can be observed, approximately 2 μg/g of selenium is present in other unknown chemical forms in the spike solution.

For this double isotope dilution quantitation approach to work for real samples, we have to assume that the response of selenium in the plasma is independent of its chemical form as also described previously (*29*, *30*). The linear regression calibration was accomplished in the concentration range of 100−400 ng/g for Se(IV), Se(VI), and SeMet. From the results shown in **Table 1**, we can conclude that, at least, for Se(IV), Se(VI), and SeMet, this assumption seems to be correct. However, the ^{77}Se spike prepared here may contain other selenium compounds, e.g., small Se-containing peptides, for which no standards are available. This means that the assumption cannot be proven conclusively for all possible Se-containing compounds.

The isotopic composition of selenium in this spike solution was also determined both for total selenium and for SeMet and is given in **Table 2**. The minor differences observed between total selenium and SeMet could be due to a small contamination with natural abundance inorganic selenium, which will not affect the SeMet isotopic composition.

Total Se Concentration in the Nutritional Supplements by Microwave Digestion. According to the supplier specifications, selenium-enriched yeast is the source of selenium present in the evaluated supplements. The total selenium concentration in the yeast-based nutritional supplements was determined using IDA-ICP-MS after microwave digestion with HNO_3 and H_2O_2 using inorganic ^{77}Se(VI) as a spike. The determination of the total Se content was validated by the analysis of Test SEAS 6 yeast Candidate Reference Material, and the results are given in **Table 3**. Good agreement for total Se content in the yeast reference material between our results and the interlaboratory mean value was observed.

The total Se concentrations in the nutritional supplements is also listed in **Table 3**. The results obtained for both ^{78}Se/^{77}Se and ^{80}Se/^{77}Se isotope ratios, respectively, after adequate correction of SeH^+ interference were in good agreement (between 0.2 and 2.5% difference). Total selenium levels were between 101 and 117% of the label value, on the basis of a 10 tablet composite assay.

Table 3. Determination of Total Selenium in Commercial Yeast-Based Supplements by IDA-ICP-MS

sample	total Se, μg/g (indicative value)	total Se, μg/g ($n = 3$)[a] ^{78}Se/^{77}Se
SEAS 6 yeast sample	1374 ± 101[a,b]	1288 ± 31
Arkovital	100	117 ± 7
Verdalia	100	108 ± 2
Selenium-ACE	200	202 ± 1

[a] Uncertainties given as standard deviation. [b] Interlaboratory value from the intercomparison exercise (see ref *21*).

Table 4. Total Se Extraction Efficiency in SEAS 6 and Commercial Yeast-Based Supplements (Referred to Total Se in the Solid Sample)

	total Se extraction efficiency (%), $n = 3$[a]		
sample	procedure A (protease)	procedure B (driselase/protease)	procedure C (*in vitro* enzymolysis)
yeast (SEAS 6)	87 ± 6	92 ± 4	
Arkovital		105 ± 10	97 ± 6
Verdalia		108 ± 10	103 ± 2
Selenium-ACE		59 ± 2	87 ± 3

[a] Uncertainty given as 95% confidence interval, taking into account also the uncertainty of total selenium (last column in **Table 3**).

Total Se Concentration in the Enzymatic Extracts. The total amount of Se extracted by the different enzymatic digestion procedures was determined by IDA-ICP-MS using the ^{77}Se-enriched SeMet as a spike by direct nebulization. Three extraction procedures were compared in terms of their extraction efficiency of total selenium from the solid: (a) protease extraction, (b) driselase/protease extraction, and (c) *in vitro* gastrointestinal enzymolysis, and the results are given in **Table 4**.

For the extraction of selenium from the yeast sample (SEAS 6, candidate reference material), the first two procedures were compared. In this case, driselase was additionally used to release the possible selenium compounds trapped in the cell walls, either mechanically or as coordination complexes. Driselase is a commercial enzyme preparation from *Basidiomycetes* sp. that is usually used to destroy the cell-wall components. This new method (procedure B) showed that the extraction efficiency for total Se increased to 92 ± 4%, which was a bit higher than the results obtained for the enzymatic hydrolysis with only protease XIV (87 ± 6%). However, these differences cannot be considered statistically significant.

In the case of the three studied nutritional supplements, only the procedures with driselase/protease (procedure B) and *in vitro* gastrointestinal enzymolysis (procedure C) were compared. The general trends are similar for both driselase/protease and *in vitro* enzymolysis for the brands Verdalia and Arkovital: the extraction efficiencies reached approximately 100% of total selenium. For Selenium-ACE, the recoveries were lower by both procedures probably because of the more complex matrix of the sample. However, for the *in vitro* enzymolysis procedure, the recovery of selenium observed for this last supplement was still a remarkable 87 ± 3%. This *in vitro* gastrointestinal enzymolysis model was previously evaluated by Dummont et al. (*23*) for the extraction of selenium in the same nutritional supplement. They obtained a total selenium extraction efficiency of 55%, which is much lower than the results obtained here using IDA. However, these differences could be due to the different mode in which the gastrointestinal digestion was applied. In our case, the intestinal digestion was applied after the gastric digestion,

while Dummont et al. (*23*) applied either gastric or intestinal digestion but never in combination.

We can conclude that the *in vitro* enzymolysis procedure, which broadly simulates gastric digestion in the stomach plus in the small intestine, provides quantitative or nearly quantitative solubilization of selenium for the three nutritional supplements evaluated. Although the results obtained can only approximate the processes occurring in living organisms, this model is simple, easy to control, and provides useful information for decisions concerning dietary indications. Thus, in the next part of this paper, we will evaluate how much of this extracted selenium is in the form of SeMet (speciation).

Evaluation of Driselase/Protease for the Extraction of SeMet from the Yeast Candidate Reference Material SEAS 6. IDA of SeMet was applied to a yeast sample obtained from an intercomparison exercise (SEAS 6). The sample was analyzed by following procedure B, extraction with driselase/protease XIV, as described in the Materials and Methods. The obtained results were also compared with previous data for that sample obtained in our group using only protease XIV (*8*). Three independent spiking experiments were carried out to analyze the candidate reference material, and each sample solution was injected 3 times in the HPLC-ICP-MS system. At the beginning and between each triplicate sample determination, a natural SeMet standard was injected and measured to correct for mass bias and SeH^+ formation as described previously (*8*).

Typical peak area precision for the SeMet peaks ranged between 1 and 5% relative standard deviation (RSD) for three injections of the same sample. Also, the chromatograms obtained were identical to those reported previously for the same yeast sample using protease extraction (*8*). Several authors have indicated that selenomethionine selenoxide (SeOMet) could be observed in the chromatograms in yeast enzymatic hydrolysates digested with protease XIV (*16*, *17*, *18*) as a peak close to the retention time of SeMet. However, we did not observe this oxidation product of SeMet.

After the driselase/protease extraction, the concentration of SeMet obtained was 978 ± 9 μg/g as Se (76 ± 2% of total selenium, uncertainty given as a standard deviation of $n = 9$ measurements), while the results obtained previously using procedure A were 869 ± 48 μg/g as Se (68 ± 2% of total selenium) (*8*). As can be observed, the levels of SeMet found by procedure B were significantly higher than those obtained using procedure A and also higher than the intercomparison value (874 ± 105 μg/g), where the uncertainty is also given as a standard deviation of all individual measurements. These results prove that the release of SeMet from yeast by the combined action of the driselase/protease enzymes is more efficient than the use of only protease. Similarly, Yang et al. (*10*) obtained SeMet recoveries approaching 66% of total selenium when increased amounts of protease XIV were used for digestion. In their comparison of extraction methods (*10*), these high SeMet recoveries were also obtained using 4 M methanesulfonic acid (*9*, *10*) but all other extraction procedures tested provided lower recoveries. The use of driselase in combination with protease was not evaluated in their paper (*10*).

However, it should be pointed our that the real concentration of SeMet in solid yeast could be even higher than that reported here because we cannot be completely sure that the extraction procedures tested liberate SeMet quantitatively from all of its possible compounds and those that could still contain bound SeMet, perhaps in the form of small peptides. Therefore, the quantitative speciation of selenium in yeast is still a challenge.

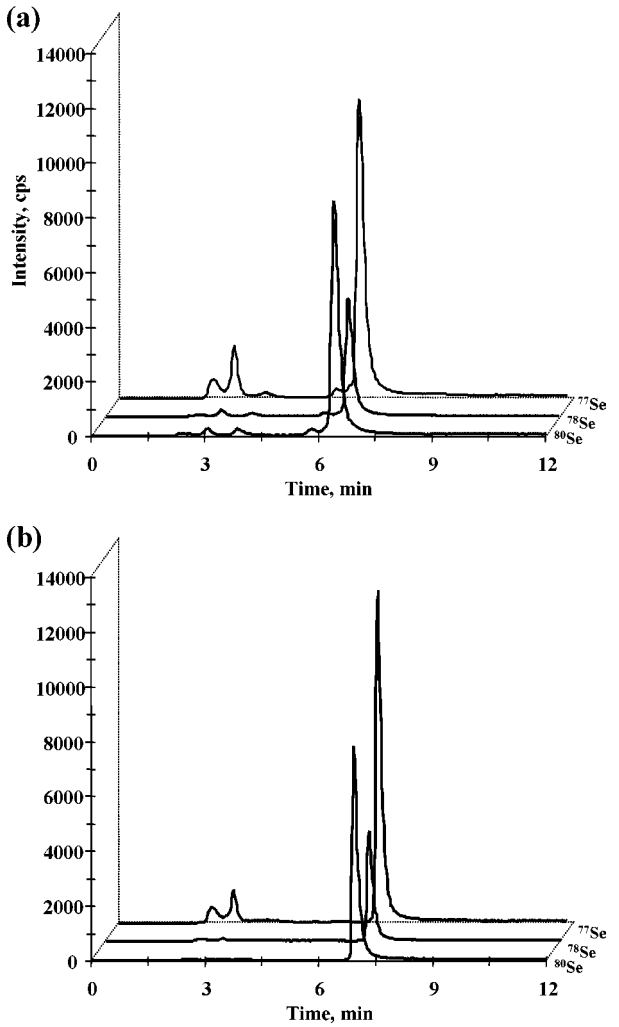

Figure 1. Chromatograms obtained for the Selenium-ACE extract spiked with ^{77}Se-enriched SeMet solution after enzymatic hydrolysis with (a) driselase/protease and (b) *in vitro* gastrointestinal enzymolysis. Chromatograms acquired at different masses were shifted for clarity.

Evaluation of Protease, Driselase/Protease, and *in Vitro* Enzymolysis for the Extraction of SeMet from the Studied Yeast-Based Nutritional Supplements. Finally, the proposed isotope dilution methodology was applied to the determination of SeMet extracted from three yeast-based nutritional supplements. HPLC-ICP-MS measurements showed that, for all samples and under all extraction procedures evaluated, the primary compound extracted had a retention time equal to that of the SeMet standard solution. As an example, the obtained chromatograms for Selenium-ACE, after (a) driselase/protease hydrolysis and (b) *in vitro* enzymolysis, are shown in **Figure 1**, where the altered isotope abundances for SeMet at ca. 7 min retention time can be seen. The early elution peaks observed in **Figure 1** are unknown impurities in the ^{77}SeMet spike. Please also note that for the diselase/protease hydrolisis (**Figure 1a**) a small peak appears just before the main SeMet peak. This peak has been adscribed to SeOMet by other authors (*18*). Additionally, SeMet has also been identified by Dumont et al. (*23*) as the major Se compound extracted from Selenium-ACE by *in vitro* gastric or intestinal digestion. In addition, those authors identified two other compounds, SeOMet and $Se(Cys)_2$ (selenocystine), as degradation products. We did not detect those compounds in the gastrointestinal digestions (**Figure 1b**).

The SeMet recoveries obtained for the three supplements and extraction procedures by IDA-HPLC-ICP-MS are included in

Table 5. SeMet Recovery in Commercial Yeast-Based Supplements (Referred to Total Se in the Solid Sample)

	recovery (% of total selenium in the solid), $n = 3$[a]		
sample	procedure A (protease)	procedure B (driselase/protease)	procedure C (*in vitro* enzymolysis)
yeast (SEAS 6)	68 ± 2	76 ± 2	
Arkovital	54 ± 4	60 ± 4	37 ± 3
Verdalia	58 ± 9	63 ± 6	26 ± 2
Selenium-ACE	51 ± 1	49 ± 4	26 ± 1

[a] Uncertainty given as 95% confidence interval, taking into account also the uncertainty of total selenium (last column in **Table 3**).

Table 5. The data for the SEAS 6 yeast are also included for a comparison. As can be observed, for the nutritional supplements, lower recoveries were obtained than for the yeast SEAS 6 sample when comparing procedures A and B. On the other hand, although one would expect a more complete SeMet extraction and hydrolysis with driselase/protease than with only protease, no significant differences between both procedures for the nutritional supplements were apparent. The results obtained for Selenium-ACE using protease agree with those obtained by Zheng et al. (*31*) (using ion-pair reversed-phase chromatography), which showed a SeMet recovery of 55% of total selenium. It has to be noted that the observed reduced extraction efficiency using procedures A and B, as compared with results for the yeast candidate reference material, indicates that the supplement additives may interfere on the enzymatic activity. If we compare data shown in **Tables 4** and **5** for procedure B (using driselase/protease), we can conclude that for Arkovital and Verdalia samples, where selenium was extracted quantitatively, there is ca. 40% of selenium in a soluble form different from SeMet (probably as small peptides), while for Selenium-ACE, most of the solubilized selenium seems to be in the form of SeMet (49% of SeMet of 59% solubilized).

Data obtained using *in vitro* enzymolysis (procedure C) showed the lowest SeMet recovery, despite its high solubilization ability for total selenium (see **Table 4**). As can be observed, only between 26 and 37% of selenium was measured as SeMet in such extracts for the three supplements evaluated. In other words, most of the soluble selenium should be present as chemical forms other than free SeMet after gastrointestinal digestion.

It is clear that the elucidation of the chemical nature of these other soluble forms of selenium, which may be absorbed by the organism and could possess biochemical properties different from those of SeMet, by resorting to MALDI-TOF and ESI-Q-TOF techniques is now an important next step in these investigations.

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