

# Determination of Selenomethionine in Nutritional Supplements using HPLC coupled to the XSeries<sup>II</sup> ICP-MS with CCT

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## Introduction

Selenium is recognized as an essential trace element that provides antioxidant properties, preventing a number of ailments associated with physiological and environmental oxidative stress. Selenium stores in the human body supply the expression and function of selenoenzymes, which specifically incorporate the amino acid, selenocysteine. Selenoenzymes include glutathione peroxidases, iodothyronine 5'-deiodinases and thioredoxin reductases. However, enzymatic activity is compromised in the average human due to the low level of selenium found in the diet.

The major sources of selenium in the average diet are cereals, meat and fish. Low selenium content in soil however, can lead to the production of foods, low in selenium. Although geographically it is only certain areas in Asia, Russia and Africa, which experience severe deficiencies, the general population is also at risk as a large portion will not receive the recommended daily levels from their regular diet. Low selenium levels are linked to deficiency syndromes such as Keshan and Kaschin-Beck disease as well as diminished immune function due to susceptibility from free radical attack. Supplementation, therefore, is becoming more commonplace as a solution to overcome deficiencies. The use of selenium supplementation in livestock feeds has been common practice for a number of decades and combats debilitating deficiency diseases such as nutritional muscular dystrophy. Additionally, Se supplementation has been associated with chemoprevention for certain cancers, anti-aging and improved immunity against viruses.

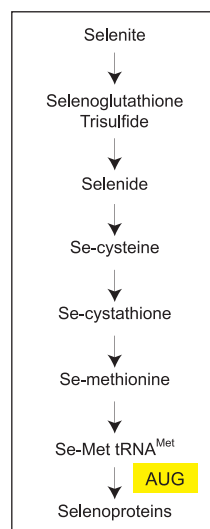
The recommended daily intake of Se provided by the Food and Nutrition Board of the National Academy's Institute of Medicine is 55 µg/day. However, the supranutritional intake (greater than the amount required for selenoenzyme expression and which is reported to reduce cancer risk) is closer to 300 µg/day. In addition to intake, the form in which the Se is supplemented has an effect on the nutritional significance of selenium. L-selenomethionine (SeMet) is the major species naturally occurring in most foods and is generally thought of as the most bioavailable and bioactive form of Se. Yeast has been exploited as the most economical way of generating bioactive Se, as it produces L-selenomethionine as well as other, minor Se-containing compounds when grown in selenized media. Figure 1 (adapted from several sources) outlines the uptake and metabolism of inorganic selenium by yeast due to the invasion or mimicry of the sulfite biotransformation pathway.

Selenized-yeast is often employed in nutritional supplements as a bioactive form of Se. However, the quality of some food supplements is of concern as some may not contain yeast which has metabolized inorganic selenium to the bioactive form, but instead contains yeast spiked with inorganic selenium. Some labeling, such as 'organic selenium' or 'selenium yeast' are ambiguous with respect to the forms of selenium contained in the supplement. As it is the bioactive form found in selenized-yeast that is reported to bestow the anti cancer properties, it is important to establish quality control procedures to determine the speciation of Se and the Se content of supplements.

This note presents a procedure for the analysis of Se-containing nutritional supplements and the determination of SeMet therein by HPLC-ICP-MS. Se is a multi-isotopic element, where the major isotope at  $m/z$  80 is interfered

by the argon dimer ( $^{80}\text{Ar}_2$ ). This note demonstrates the use of the XSeries<sup>II</sup> ICP-MS Collision Cell Technology to remove this interference and permit acquisition of chromatographic data with the most abundant Se isotope. Although a certified reference material (CRM) is not yet commercially available, a selenized-yeast material currently undergoing a certification procedure was used for method validation.

Figure 1. Schematic presenting the metabolism of inorganic selenium by yeast. The last steps present the ultimate formation of SeMet and its non-specific incorporation into the proteome.



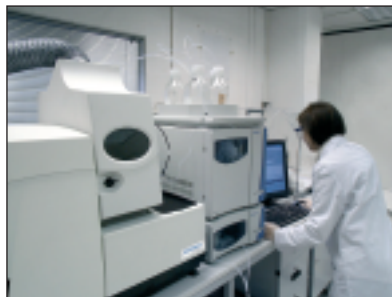
## Instrument configuration

A Finnigan<sup>TM</sup> Surveyor<sup>TM</sup> HPLC pump with autosampler was coupled to the XSeries<sup>II</sup> ICP-MS with the aid of a Thermo HPLC-ICP-MS Coupling Pack (P/N 4600485) and Finnigan Surveyor LC Wiring Harness (P/N 4600487). The HPLC-ICP-MS coupling pack includes all the required components to establish electrical and analytical connections between the HPLC pump and ICP-MS instrumentation. The PlasmaLab software and

## Key Words

- HPLC
- ICP-MS
- Nutritional Supplements
- Selenium
- Speciation

External Trigger Card (P/N 4600261) enable automated HPLC accessory control using bi-directional communications and intelligent peak integration facilities. The instrument was configured with a Peltier cooled quartz impact bead spray chamber, Meinhard concentric nebulizer and a Quartz torch with a standard 1.5 mm i.d. injector torch and a PlasmaScreen. The instrument was operated in collision cell mode using 8% H<sub>2</sub> in He collision gas under non-iced conditions.



### Analytical Conditions for HPLC-ICP-MS

The Finnigan Surveyor HPLC pump with autosampler was programmed from the XSeries<sup>II</sup> ICP-MS PC using Atlas software to enable separation of the selenium species. The XSeries<sup>II</sup> ICP-MS was performance tested, tuned and optimized as required for HPLC-ICP-MS analysis using the automated PlasmaLab Performance Test and Autotune facilities. The selenium-containing species were separated using an anion-exchange HPLC column and eluted with an ammonium acetate mobile phase at pH 5. HPLC parameters and analytical conditions for HPLC-ICP-MS are shown in Table 1.

Column	Polymeric Anion Exchange (250 x 4.6 mm, 10µm)
Injection volume	20 µL
Flow rate	1.5 mL min <sup>-1</sup>
Buffer A	20mM ammonium acetate:acetic acid, pH 4.7
Buffer B	200mM ammonium acetate:acetic acid, pH 4.7
Gradient	
0 min	100% Buffer A
20 min	100% Buffer B
Forward Power	1350 W
Nebulizer Gas Flow	0.8 L min <sup>-1</sup>
Auxilliary Gas Flow	0.8 L min <sup>-1</sup>
Cool Gas Flow	13 L min <sup>-1</sup>
Data Acquisition Mode	PlasmaLab Transient Time Resolved Analysis (TRA)
Isotopes and dwell times, ms	<sup>78</sup> Se, <sup>80</sup> Se, <sup>82</sup> Se (100 ms)
Channels per AMU	1
Timeslice duration	306 ms
Transient acquisition time	1800 s
Spray chamber	Quartz impact bead
Nebulizer	Glass concentric
Cones	Xt
Collision Cell gas	8% H <sub>2</sub> in He at 7.25 mL min <sup>-1</sup>
Cell Conditions	Non-iced

Table 1. HPLC-ICP-MS conditions

### Preparation of Selenium-Containing Standards

Stock solutions of 1000 µg Se g<sup>-1</sup> of each selenium standard (selenite, selenate, selenomethionine and selenocystine) were prepared by dissolving the appropriate quantity of the commercially available salt in milli-Q water (18.2 MΩ). The stock solutions were diluted to produce daily working standards of 1 µg g<sup>-1</sup>. The stock solutions were kept at 4°C in the dark.

### Preparation of Selenized Yeast and Nutritional Supplements

#### Acid Hydrolysis

As SeMet is incorporated into the proteome, it is important to hydrolyze completely the protein to liberate the amino acids for quantification. This can be achieved with enzymatic digestion or by acid hydrolysis. The latter is often a cheaper and less time consuming protocol and a method developed by Wrobel *et al.*<sup>(1)</sup> was selected for this study.

A reagent blank, a sub-sample (250 mg) of selenized-yeast and a nutritional supplement capsule/tablet were digested by acid hydrolysis, each day, for 3 days. Thus, digestions of three independent replicates of selenized-yeast, three independent replicates of reagent blanks and three different nutritional supplements (A, B and C) were performed. The digestion was performed with 4 M methanesulfonic acid (16 mL) under reflux for 8 h. The digests were left to cool and were transferred to 50 mL volumetric flasks where they were diluted. After dilution, the samples were centrifuged for 20 m at 2000 rpm. The supernatant was decanted and appropriate dilutions of each sample were performed prior to analysis. The reagent blanks were used to determine the method limit of detection.

#### Aqueous Extraction

Selenium incorporated in the proteome will not be extracted in aqueous media. Indeed, it has been found that aqueous soluble selenium species account for only 10-25% of the total selenium present in selenized-yeast. Thus it is of interest to perform an aqueous reaction to determine if selenized-yeast was used as a source for Se in the supplements.

A reagent blank, a sub-sample (250 mg) of selenized-yeast and three different nutritional supplement capsules/tablets (A, B and C) were extracted with 10 ml of milli-Q water (18.2 MΩ), for 2 hours in an ultrasonic bath at approximately 50 °C. The extracts were centrifuged at 2000 rpm for 20 m and the supernatant transferred to a clean vial for storage prior to analysis.

### Results and Discussion

The chromatographic data is displayed automatically in the XSeries<sup>II</sup> PlasmaLab software package following analysis and an example of the chromatographic separation of selenium-containing standards is shown in Figure 2(a.). The HPLC protocol allowed the baseline separation of selenocystine (SeCy), selenomethionine (SeMet), selenite (Se<sup>IV</sup>) and selenate (Se<sup>VI</sup>) with a sample run time of 30 m (including re-equilibration time of the analytical column).

The acid hydrolysis of selenized-yeast (Figure 2(b.)) yielded one major species, identified as SeMet from the correlation in retention time (3 m 51 s) with the analytical standard. Several peaks can be observed eluting prior to SeMet near to the void volume of the column, one of which correlates to the retention time of SeCy (1 m 32 s). However, the peak is not baseline resolved and this could be due to the coelution of other selenium-containing species. For example, a SeMet standard solution which is not freshly prepared will yield a species with the same retention time of SeCy. It is highly probable that this is due to an oxidation of the SeMet. Therefore, it is not possible to fully quantify the peak with the same retention time as SeCy. A minor species eluting later in the chromatogram is identified as selenate due to the retention time match of 845 s to the analytical standard. However, this selenate is also found in the method blank as well as the other samples at an equal concentration and is most probably due to contamination from the analytical reagents used in sample preparation.

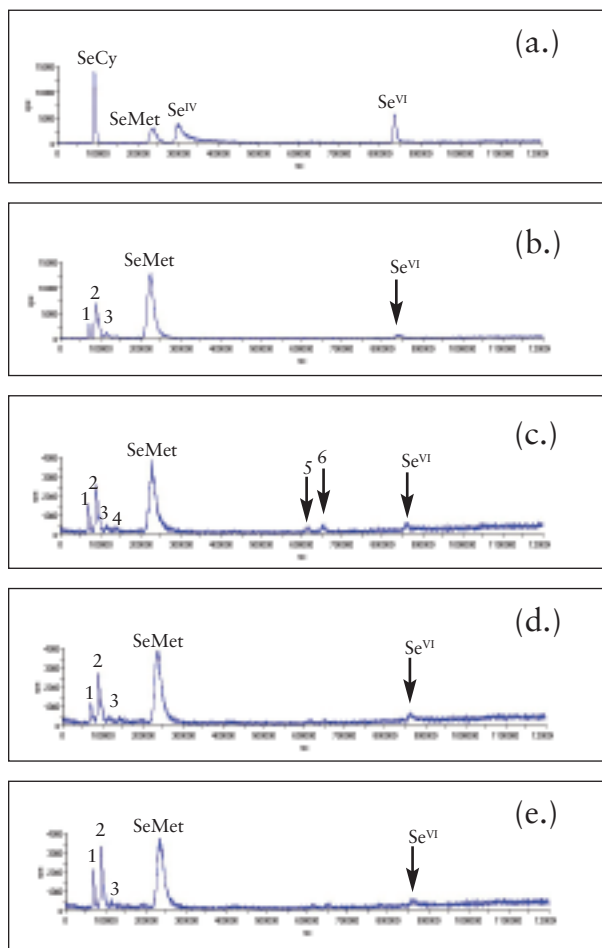


Figure 2. Chromatograms of (a.) commercially available selenium-containing standards, and acid hydrolyzed digests of (b.) selenized-yeast, (c.) nutritional supplement A, (d.) nutritional supplement B and (e.) nutritional supplement C

Similar elution profiles were obtained from acid hydrolysis of the nutritional supplements A, B and C (presented in Figure 2 (c.), (d.) and (e.) respectively). SeMet is the major species after acid hydrolysis of the nutritional supplements.

SeMet in the hydrolyzed selenized-yeast was quantified by standard addition. The standard addition calibration curve for one replicate extraction is presented in Figure 3 (a). The slope (or sensitivity) was then used to calculate the concentration of SeMet in the remaining replicate extractions and the results are presented in Table 2. Similarly, SeMet in the nutritional supplements A, B and C was also determined by standard addition (calibration curve presented in Fig 3 (b.)) and the data is presented in Table 2 as micrograms of selenium per tablet.

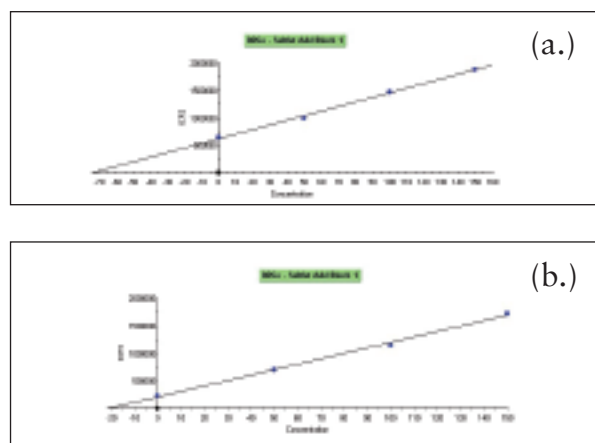


Figure 3. Standard addition calibration curves for SeMet in (a.) selenized-yeast and (b.) nutritional supplement C

For selenium-containing species present in the digests whose standards were not available (numbered 1-6 in Figure 2), quantification was performed by using the semi-quantitative feature in the PlasmaLab software. Calibration curves (presented in Figure 4.) were generated for the four standards (SeCy, SeMet, Se<sup>IV</sup> and Se<sup>VI</sup>) using a blank and mixed calibration standards at five concentration levels. The mean sensitivity of these fully quantitative calibration curves was then used to enable Compound Independent Calibration for uncharacterized species and data for the selenized-yeast and the nutritional supplements are presented in Table 2. The data presented are based on three replicate extractions of the selenized-yeast and one replicate extraction for each of the nutritional supplements A, B and C.

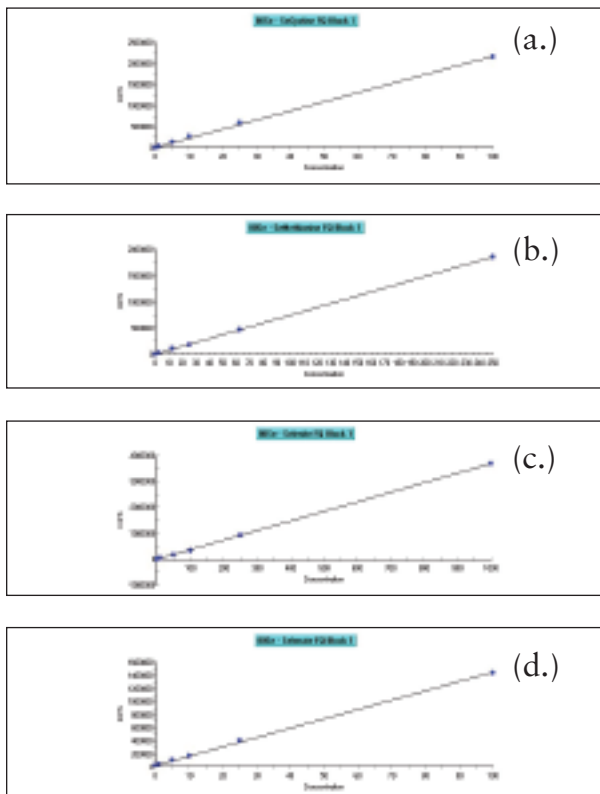


Figure 4. Calibration curves for (a.) Selenocystine, (b.) Selenomethionine, (c.) Selenite and (d.) Selenate

SELENIUM CONTAINING SPECIES ( $\mu\text{g Se g}^{-1}$ )						
Sample	1	2	3	4	SeMet	5 6
Selenized-yeast	57.6	289.4	38.9	12.0	1209	
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	
	9.0	82.5	4.3	3.2	77	

SELENIUM CONTAINING SPECIES ( $\mu\text{g Se tablet}^{-1}$ )						
Nutritional supplements	1	2	3	4	SeMet	5 6
A	1.68	8.02	0.64	0.59	23.1	0.39 0.30
B	1.02	5.35	0.56	0.63	24.3	
C	2.13	10.1	0.15	0.08	23.0	

Table 2. Quantitative standard addition data for SeMet and semi-quantitative data for uncharacterised species in selenized-yeast and nutritional supplements.

The concentration of SeMet determined by standard addition in the selenized-yeast is  $1209 \mu\text{g Se g}^{-1}$ , which, is equivalent to  $3003 \mu\text{g SeMet g}^{-1}$  and approximately 60 % of the total Se present in the selenized-yeast. Although the selenized-yeast is not yet certified, this value agrees with values found using isotope dilution analysis with LC-MS, HPLC-ICP-MS and GC-MS techniques performed by the reference material supplier. The SeMet determinations in selenized-yeast therefore validate the method and demonstrate the suitability of the approach for the determination of SeMet in nutritional supplements. As the acid hydrolyzed nutritional supplements give a similar elution profile to the selenized-yeast it can be assumed that the source of selenium for the supplements was a selenized-yeast.

Further information on the selenium source used for

the nutritional supplements can be obtained by aqueous extraction of the nutritional supplements. Figure 5 shows a chromatogram of the aqueous extract of nutritional supplement A. A number of peaks can be observed, probably due to aqueous soluble selenopeptides and small selenoproteins. This information yields further evidence that selenized-yeast was used in the manufacture of the nutritional supplements.

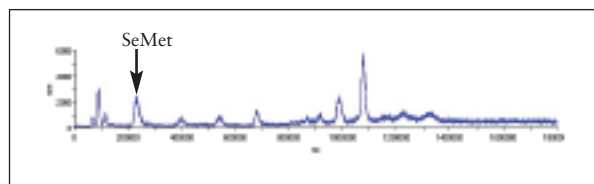


Figure 5. Aqueous extract of nutritional supplement A

Detection limits for the four selenium-containing standards were determined in accordance with the 3s model following fully quantitative analysis of the calibration blanks ( $n = 3$ ).

#### SELENIUM-CONTAINING SPECIES LIMITS OF DETECTION

	SeCy	SeMet	Se <sup>IV</sup>	Se <sup>VI</sup>
Instrument LOD (ng/g)	0.07	0.19	0.48	0.84

Table 3. Limits of detection for selenium-containing species

## Summary

The Thermo HPLC-ICP-MS instrument package offers a complete instrument solution for the sensitive and accurate determination of selenium-containing species in nutritional supplements. PlasmaLab software features allow rapid and automated integration, increasing productivity and in combination with the External Trigger Card permits failsafe automated instrument operation for routine speciation applications.

## References

1. Wrobel et al. Anal. Bioanal. Chem 2003, 375, p13-138

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