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Mapping of selenium metabolic pathway in yeast by LC Orbitrap mass spectrometry

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ABSTRACT

A high resolution mass spectrometric detection method is described for the identification of key metabolites in the *selenium pathway* in selenium enriched yeast. Iodoacetic acid (IAA) was used as the derivatizing reagent to stabilize the selenols. Oxidized forms of Selenocysteine (Se-Cys), selenohomocysteine (Se-HCys), selenogluthathione (Se-GSH), seleno- γ -glutamyl-cysteine (Se-Glu-Cys), N-(2,3-dihydroxy-1-oxopropyl)-Selenocysteine (Se-DOP-Cys), N-(2,3-dihydroxy-1-oxopropyl)-Selenohomocysteine (Se-DOP-HCys), selenomethionine (SeMet), seleno-s-adenosyl-homocysteine (Se-AdoHcy), the conjugate of glutathione and N-(2,3-dihydroxy-1-oxopropyl)-Selenocysteine (GSH-Se-DOP-Cys), and the conjugate of glutathione and N-(2,3-dihydroxy-1-oxopropyl)-Selenohomocysteine (GSH-Se-DOP-HCys) were found in the selenium enriched yeast certified reference material

(SELM-1). Selenols were also derivatized with a mercury tag, p-hydroxymercurybenzoate (PHMB). The selenol-PHMB complexes showed the overlapped isotopic patterns of selenium and mercury, which provided supporting information for the identification of selenols. Both methods showed good agreement (< 4 ppm difference) between the theoretical masses of the target compounds and the measured masses in the yeast matrix. The method using IAA as the derivatizing reagent was used to study the response of *Saccharomyces cerevisiae* to three forms of selenium, Se-Met, Na₂SeO₃ (Se (IV)), and Na₂SeO₄·10H₂O (Se (VI)) (concentration of Se: 100 mg/L). The production of selenocompounds observed over a six hour period was high in the Se-Met treated group compared to the groups treated with Se (IV), Se (VI).

KEY WORDS: Selenocompounds; derivatization; high resolution mass spectrometric identification; yeast.

INTRODUCTION

The trace mineral selenium (Se) has been recognized as an essential element since 1957.¹ Se plays important role in the formation and function of selenoproteins, including some important enzymes, such as glutathione peroxidase (GSHPx) and thioredoxin reductase.² It has been reported that, Se and GSHPx participate in epilepsy pathogenesis.³ The immuno-enhancing^{4,5}, anti-viral⁶⁻⁸, cancer prevention⁹⁻¹³ effects of Se, the protective role of Se against radiation,^{14,15} cardiovascular diseases,^{16,17} and reproductive disorders^{18,19} have also been demonstrated.

The important role of Se in human nutrition and in cancer prevention has triggered the development of foods supplemented with Se species. These nutritional supplements are particularly important in geographical regions where the levels of Se in soil and consequently in plants are relatively low.^{2,20,21}

One of the most economical means of supplementing selenium is selenium enriched yeast. After growing in Se-containing media, yeast (*Saccharomyces cerevisiae*, *S. cerevisiae*) can be harvested with a reproducibly high organic Se content. With currently available strains of *S.*

cerevisiae, up to 3000 ppm of Se level were obtained.²² Selenium enriched yeast is stable during storage, while inorganic Se could be reduced especially under humid condition, and Se-Met could be oxidized.²³ Se in the form of selenium enriched yeast has higher bioavailability than the Se from inorganic selenium sources.²⁴⁻²⁶ In both rat and mouse models, selenium enriched yeast has shown significantly lower acute oral toxicity than sodium selenite. It also showed lower subchronic toxicity (90 days) in the rat and beagle dog experiment.²⁷ In human studies, several doses of selenium enriched yeast (200, 300, 400, and even 800 µg per day), have shown no long-term toxic effects.²⁸⁻³¹

Besides safety, selenium enriched yeast showed effectiveness as well. Selenium enriched yeast as a feed additive has proven to be an effective form of Se supplementation, to improve the productive and reproductive performance of animals and produce quality.³² Long-term Se supplementation using selenium enriched yeast raised women's platelet GSHPx activity, and the levels of Se and GSHPx in whole blood, erythrocytes and plasma. It was found that, selenium enriched yeast was more effective in raising blood Se concentrations than brewer's yeast mixed with selenate.³³ The efficacy of selenium enriched yeast in cancer prevention has also been demonstrated.²⁸

Selenium enriched yeast is grown in a medium containing selenite (Se (IV)) or selenate (Se (VI)).³⁴ During the growth of the yeast, these two inorganic forms of Se can be converted to safer, bioactive organic species. It has been shown that in selenium enriched yeast, most of Se is incorporated into proteins.³⁵⁻³⁷ It has also been reported that, selenium methionine (Se-Met) incorporated into the yeast protein in place of methionine (Met), acts as a slow-release form of Se, avoiding the sudden change in the levels of Se during digestion and absorption.²³

While the major species of Se in selenium enriched yeast is proteinaceous Se-Met,³⁸ another important fraction of Se is found to be a mixture of low molecular weight selenocompounds. Whether they have different bioavailability, efficacy or toxicity in comparison with selenium enriched yeast or Se-Met remains unknown. Thus, it's important to characterize these minor Se species contained in the selenium enriched yeast and investigate their distributions, which may provide some information for pharmacokinetics, pharmacodynamics and toxicity studies.

The concentration of these compounds are however often three orders of magnitude lower

than their sulfur analogs.³⁹ Other difficulties arise from the complexity of the matrix, the lack of authentic standards for most of the selenocompounds for retention-based identification.

Mass spectrometry (MS) provides a useful tool for the identification of trace level selenocompounds, especially when these target analytes are associated with characteristic element-specific isotopic patterns. It was reported that Se-Met and γ -glutamyl-Se-methylselenocysteine in selenium enriched yeast were detected by inductively coupled plasma-mass spectrometry (ICP-MS) and electrospray tandem MS, but there were several unknown selenium species which couldn't be identified.⁴⁰ Se-Met and Se-methylselenocysteine (Se-MC) in selenium enriched yeast were identified also by ICP-MS and electrospray tandem MS detection.⁴¹ Se-Met and Se-adenosyl-selenohomocysteine (Se-AdoHcy) were found to be the principal selenocompounds in yeast using ICP-MS and electrospray ionization MS.⁴² Se-Met, selenocystine (Se-CySS) and selenoethionine were identified in an aqueous yeast extract using a quadrupole-time of flight (Q-TOF) tandem mass spectrometer.⁴³ Identification of some other selenocompounds yeast using MS detection have also been reported.⁴⁴⁻⁴⁷

Low molecular weight sulfur compounds in the *sulfur pathway* (as shown in Fig.1),^{48, 49} play important roles in the processes of metabolism, antioxidant defense and drug detoxification. There is close chemical similarity between selenium and sulfur, thus, when cultured in Se enriched medium, the replacement of the sulfur compounds in yeast with their corresponding seleno-compounds may occur. However, with the exception of Se-Met, Se-CySS and Se-AdoHcy, there is little published information about the identification of other Se substituted sulfur compounds in the *sulfur pathway* in selenium enriched yeast. In *Brassica juncea* (Indian mustard), two of the Se substituted sulfur compounds in the *sulfur pathway* (selenium-homocysteine (Se-HCys) and selenium-cystathionine (Se-Cysta)) were characterized using Q-TOF MS.⁵⁰ Whether the fact that these two selenocompounds haven't been reported in yeast is due to the species difference of organisms or due to the lack of an analytical method needs to be investigated.

For the extraction of nonproteinaceous selenocompounds, water extraction is widely used.^{43, 45, 47, 51} When extracting thiols in yeast (in our previous study), it was found that, more cysteine (Cys) and Met (both are protein amino acids) were obtained when using both the

cold water extraction in an ultrasonic bath and hot water extraction, compared with boiling 75% ethanol, and chloroform-methanol methods. This might be due to protein hydrolysis in the presence of water. To avoid the hydrolysis of Se-Met and selenocysteine (Se-Cys) from protein, in this study, 75% ethanol was used instead of water as the extracting solvent.

The main objective of the work described here was to identify the selenocompounds in the *selenium pathway using* high resolution MS. Orbitrap MS provides mass measurements accurate to within 2-5 ppm.⁵² This is often sufficient to identify a selenium compound which has a characteristic isotopic signature. Orbitrap MS offers not only high resolution, but also good sensitivity, which, coupled with chromatographic separation, allows the detection of trace amounts of compounds in biological samples. Besides identification of the selenocompounds, another objective of this work is to measure the effects of three forms of selenium, i.e. Se-Met, Na₂SeO₃ (Se (IV)), Na₂SeO₄·10H₂O (Se (VI)) on the biosynthesis of sulfur- and seleno-compounds in yeast (*S. cerevisiae*).

Figure 1. Flow diagram of sulfur metabolic pathways in yeast (adopted from ^{48, 49})

EXPERIMENTAL SECTION

Materials

Selenocystine, Se-Met, Met, cysteine (Cys), homocysteine (HCys), glutathione (GSH), cysteinyl-glycine (Cys-Gly), γ -glutamyl-cysteine (Glu-Cys), s-adenosyl-methionine (AdoMet), s-adenosyl-homocysteine (AdoHcy), cystathionine (Cysta), p-(hydroxymercuric) benzoic acid sodium salt (PHMB), DL-Dithiothreitol (DTT), iodoacetic acid (IAA), 5-Sulfosalicylic acid dehydrate (SSA), ammonium bicarbonate, sodium selenite, sodium selenate, and Sabouraud dextrose broth were purchased from Sigma–Aldrich Chemical Co. (Oakville, ON, Canada).

HPLC grade methanol was obtained from EMD Chemicals Inc. (Darmstadt, Germany). Formic acid and ammonium hydroxide were purchased from Anachemia Canada Inc. (Montreal, QC, Canada). High purity water was generated using a MILLI-Q-ADVANTAGE (A10) system from Millipore Corporation (Saverne, Alsace, France).

A solution of DTT (3 mmol/L) was prepared in water. A solution of IAA (3 mmol/L) was prepared in ammonium bicarbonate solution (10 mmol/L, with 0.5% $\text{NH}_4\cdot\text{OH}$, pH 9.5). A stock solution of PHMB (10 mmol/L) was prepared by dissolving the sodium salt in 0.1 % $\text{NH}_4\cdot\text{OH}$, and the working solution of PHMB (3.5 mmol/L) was diluted with water.

The selenium enriched yeast certified reference material (SELM-1) was provided by the National Research Council Canada.

Sample preparation

About one mg of the yeast sample was accurately weighed. One mL of 75 % ethanol was added, and the sample was vortex-mixed for three seconds, and heated to 95 °C for three min. After extraction, the sample was centrifuged (5000g, 3 min), and the supernatant was removed. The extraction was repeated twice. The supernatants from each extraction were combined, and evaporated under a gentle flow of nitrogen.

For the metabolic study, replicate samples of yeast (*S. cerevisiae*) cells were aseptically transferred from the parent culture into flasks containing fresh medium. The cultures were

incubated overnight at 25 °C/150 rpm. A control culture was treated with one mL of water. Experimental cultures were treated with either one mL of Se-Met, Se (IV), or Se (VI) solutions (Final concentration of Se: 100 mg/L). Samples of the yeast, 1.5 mL each, were collected at times 0 min, 15 min, 40 min, 68 min, 2 h, 4 h, and 6 h, after the addition of the selenium solutions and put in 8 mL of cold methanol (-80 °C) immediately to arrest enzyme activity. Prior to analysis, the samples were centrifuged and the methanol was discarded. The samples were extracted with one mL of 75% ethanol twice. The liquid extracts were evaporated under a gentle flow of nitrogen and 500 µL of IAA (3 mmol/L) was added. Aliquots of the extract, 200 µL each, were treated with 50 µL of water for the determination of free selenols or with 50 µL of DTT (3 mmol/L) for the determination of total selenols (free selenols plus oxidized selenols). The derivatization was allowed to proceed for one hour before 50 µL of cold SSA (10 %) was added to each vial. The samples were then centrifuged and the supernatants were removed and analyzed.

Similar protocols were implemented for derivatization with PHMB.⁵³

High performance liquid chromatography and mass spectrometry

Chromatographic separation was performed using an Agilent series 1100 HPLC system. A Zorbax eclipse XDB-C18 analytical column (4.6 x 150 mm, 5 µm) and a guard column of the same material were used. Mobile phase consisted of A: water containing 0.1% formic acid and B: methanol containing 0.1% formic acid. The gradient was as follows: 5 % solvent B for 1 min; 9 min linear increase up to 40 % solvent B; 10 min linear increase up to 90% solvent B; 7 min linear decrease to 5 % solvent B; 8 min hold at 5% solvent B. Flow rate was 0.2 mL/min. Injection volume was 10 µL.

Detection of positive ion was carried out on a LTQ-Orbitrap mass spectrometer manufactured by Thermo Fisher Scientific, Inc. (Bremen, Germany) at a resolution of 30,000. In the full scan mode, spectra over the m/z range 100-1000 were monitored with the electrospray voltage set to 3.5 kV. The capillary temperature was set to 300 °C. The sheath and auxiliary gases were nitrogen at flow rates of 20 and 10 arbitrary units, respectively. The tube lens voltage and capillary voltage were set to 63 and 14 V.

Identification of selenocompounds

The analytical strategy for the detection of trace levels of selenium analogs of sulfur compounds in the Met/Cys metabolic pathway was based on a directed search. In our earlier works, it was found that the key sulfur metabolites of this pathway could be identified, using IAA or PHMB⁵³ as the derivatizing reagent. This time after a full scan data acquisition, extracted ion chromatograms were plotted at the theoretical mass/charge ratio (m/z) of the sulfur compound + 48, which corresponds to the mass difference of the most intense isotopes of S and Se. The m/z of Se substituted disulfides, including symmetric disulfides and mixed disulfides, and other selenocompounds reported in the literature were searched as well. Then the spectra were inspected to confirm the presence of the characteristic Se isotopic patterns for selenol-IAA complexes and other underivatized selenocompounds, and the presence of the overlapped Se and mercury (Hg) isotopic patterns for the selenol-PHMB complexes. For the identification of Se-Cys and Se-Met, whose standards are commercially available, retention times were also matched with the standards. The “Elemental Composition” calculation tool supplied with the Orbitrap instrument was used for the comparison of the theoretical mass and the measured value. The elements chosen for inclusion in the calculation were set as follows: C [1-50], H [1-80], N [0-10], O [0-20], S [0-2], Se [1-2], Hg [0-1], and mass error [<10 ppm].

RESULTS AND DISCUSSION

Selenocompounds found in the selenium enriched yeast

The LTQ-Orbitrap mass spectrometer employed in this study combines a resolving power of up to 100,000 with good sensitivity. For this study, a resolution of 30,000 was deemed sufficient to provide appropriately accurate m/z and adequate sensitivity.

Figure S-1 shows the chemical structures of selenocompounds detected in selenium enriched yeast. There were six selenocompounds in the *selenium pathway*, including Se-Cys, Se-HCys, selenium-glutamyl-cysteine (Se-Glu-Cys), selenium-glutathione (Se-GSH), Se-Met, and Se-AdoHcy. Four other selenocompounds were also detected, including

N-(2,3-dihydroxy-1-oxopropyl)-Selenocysteine (Se-DOP-Cys), N-(2,3-dihydroxy-1-oxopropyl)-Selenohomocysteine (Se-DOP-HCys), the conjugate of glutathione and N-(2,3-dihydroxy-1-oxopropyl)-Selenocysteine (GSH-Se-DOP-Cys), and the conjugate of glutathione and N-(2,3-dihydroxy-1-oxopropyl)-Selenohomocysteine (GSH-Se-DOP-HCys).

Figure 2 shows the mass spectra of IAA derivatized thiols (Cys-IAA, HCys-IAA, GSH-IAA, Glu-Cys-IAA, Cys-Gly-IAA), IAA derivatized selenols (Se-Cys-IAA, Se-HCys-IAA, Se-GSH-IAA, Se-Glu-Cys-IAA) and PHMB derivatized selenols (Se-Cys-PHMB, Se-HCys-PHMB, Se-GSH-PHMB, Se-Glu-Cys-PHMB) analyzed at resolution 30,000. Selenol-IAA complexes were tentatively identified by the presence of the six-isotope pattern associated with Se, and selenol-PHMB complexes were associated with the overlapping ten-isotope pattern produced by combining Se and Hg. Identification of the individual compounds was confirmed by comparing the theoretical m/z with the accurately measured m/z in the yeast spectrum. It's quite interesting that four Se substituted thiols in the GSH cycle could be detected with the exception of Se-Cys-Gly. This is somewhat surprising considering the key role played by Cys-Gly in the catabolism of GSH due to GGT (gamma glutamyl transferase) activity, but is likely due to low concentrations. In our earlier studies (data not shown) the concentration levels for Cys-Gly were about an order of magnitude below the concentration of GSH.

Figure 3 shows the mass spectra of non-thiolic sulfur compounds in the *sulfur pathway*, Se-Met, and Se-AdoHcy analyzed at resolution 30,000. Se-AdoMet and Se-Cysta were not detected likely because of the combination of stability of these species and very low natural concentrations. Figure 4 shows the mass spectra of Se-DOP-Cys-IAA, Se-DOP-Cys-PHMB, Se-DOP-HCys-IAA, GSH-Se-DOP-Cys, and GSH-Se-DOP-HCys using the Orbitrap mass spectrometer at resolution 30,000. Se-DOP-HCys-PHMB couldn't be detected, possibly due to the low concentration of Se-DOP-HCys.

To the best of our knowledge, this is the first time that Se-HCys has been identified and measured in yeast, and the first time that selenocompounds in the *sulfur pathway* have been reported. As well, free selenols have been reported as a percentage of total selenols (this will be discussed later). Studies of this kind may eventually provide some perspective on the

relationship between the biological effect and the chemical forms of Se involved in Se metabolism.

Figure 2. Mass spectra of a, IAA derivatized thiols (Cys-IAA, HCys-IAA, GSH-IAA, Glu-Cys-IAA, Cys-Gly-IAA); b, IAA derivatized selenols (Se-Cys-IAA, Se-HCys-IAA, Se-GSH-IAA, Se-Glu-Cys-IAA) and c, PHMB derivatized selenols (Se-Cys-PHMB, Se-HCys-PHMB, Se-GSH-PHMB, Se-Glu-Cys-PHMB), using the Orbitrap mass spectrometer at resolution 30,000.

Figure 3. Mass spectra of a, non-thiolic sulfur compounds in the *sulfur pathway* (Met, AdoHcy, AdoMet, Cysta); b, Se-Met and Se-AdoHcy, using the Orbitrap mass spectrometer at resolution 30,000.

Figure 4. Mass spectra of a, IAA derivatized Se-DOP-Cys (Se-DOP-Cys-IAA), IAA derivatized Se-DOP-HCys (Se-DOP-HCys-IAA), GSH-Se-DOP-Cys, and GSH-Se-DOP-HCys; b, PHMB derivatized Se-DOP-Cys (Se-DOP-Cys-PHMB), using the Orbitrap mass spectrometer at resolution 30,000.

Table 1 shows the difference between the theoretical mass and the observed mass for these selenocompounds in yeast. Both the data from IAA derivatization and PHMB derivatization provided good agreement (< 4 ppm) with theoretical values. This is better agreement than reported by McSheehy *et al* using a quadrupole-time of flight (Q-TOF) tandem mass spectrometer.⁴³ The characteristic isotopic profile of the selenium-containing or mercury-containing species, together with accurate mass measurements provided strong evidence for the identification of these ten selenocompounds in yeast.

Since authentic standards for most of the selenocompounds are not commercially available, therefore no absolute quantitation is feasible. Seleno-compounds are chemical similar to their corresponding sulfur analogs. Therefore it was assumed that, the MS responses of selenocompounds were the same as their sulfur analogs, which has been validated by comparing Se-Cys (for which standard is available) and Cys responses. Based on the LODs of the sulfur species, LODs of the selenocompounds were estimated as shown in Table S-1.

Table 1 mass accuracy and integration ranges

Selenocompounds		Observed mass (m/z)	Accuracy (ppm)	Integration range (m/z)
IAA complexes	Se-Cys-IAA	227.97727	3.351	227.97-227.99
	Se-HCys-IAA	241.99327	3.280	241.98-242.01
	Se-GSH-IAA	414.04220	2.022	414.02-414.07
	Se-Glu-Cys-IAA	357.01909	1.823	357.00-357.04
	Se-DOP-Cys-IAA	315.99394	2.975	315.98-316.01
	Se-DOP-HCys-IAA	330.00951	2.606	329.99-330.03
PHMB complexes	Se-Cys-PHMB	491.96408	1.723	491.94-491.99
	Se-HCys-PHMB	505.97979	1.794	505.95-506.01
	Se-GSH-PHMB	678.02912	2.701	677.99-678.07
	Se-Glu-Cys-PHMB	621.00761	2.874	620.97-621.04
	Se-DOP-Cys-PHMB	579.98070	1.424	579.94-580.01
Se-Met		198.00329	2.793	197.99-198.01
Se-AdoHcy		433.07583	3.704	433.05-433.10
GSH-Se-DOP-Cys		563.05701	1.333	563.03-563.09
GSH-Se-DOP-HCys		577.07249	1.163	577.04-577.11

Figure 5 shows representative LC/MS chromatograms of selenocompounds with the addition of DTT in the selenium enriched yeast sample. Six Se substituted sulfur compounds out of nine compounds involved in the sulfur metabolic pathway were found, which indicates that, the *sulfur pathway* is important for the assimilation of Se. It was found that, in the absence of reducing reagent, no selenols were detected, suggesting that, in the selenium enriched yeast, the six selenols (Se-Cys, Se-HCys, Se-GSH, Se-Glu-Cys, Se-DOP-Cys, and Se-DOP-HCys) were in the oxidized form.

It has been reported that, the most common selenocompound in Se enriched yeast is Se-Met, accounting for 60–85% of Se^{54, 55}. Most of Se-met is incorporated into the proteins in place of Met. Accordingly, in our study, very little non-proteinaceous Se-Met and trace amounts of Se-Cys were observed. Other fractions of Se-Met and Se-Cys may be incorporated into the proteins, as is the case for Met. It was noted that more Se-Met could be detected after the addition of the reducing reagent DTT, which might indicate the existence of oxidized forms of Se-Met. It has been reported that Se-Met can be oxidized to methionine selenoxide (MetSeO).^{56, 57}

GSH-Se-DOP-Cys and GSH-Se-DOP-HCys were also found. These are conjugates of glutathione and 2,3-dihydroxy-propionyl-DHP selenocysteine, and glutathione and 2,3-dihydroxy-propionylseleno-homocysteine respectively.⁴⁶ Using the reducing reagent DTT, the reduced forms of these two selenocompounds were released, derivatized with IAA, and Se-DOP-Cys-IAA, Se-DOP-HCys-IAA were detected in this study. Se-CySS, Se-Met, and Se-methyl-selenocysteine have been detected in selenium enriched yeast,⁵⁸ but in this study, Se-methyl-selenocysteine was not found.

In the absence of reducing reagent, Se substituted disulfides, including symmetric disulfides like Se-GSH dimer, and mixed disulfides, such as the conjugate of Se-Cys and GSH (Se-Cys-GSH) or the conjugate of Se-Cys and Se-GSH (Se-Cys-Se-GSH) were not detected.

An attempt was made to identify selenocompounds found in other biological matrices and reported in the literature. For example, it has been reported that most of the Se in selenium enriched garlic is in the form of γ -glutamyl-Se-methylselenocysteine.^{55, 59} This compound was not found in yeast in this study.

Figure 5. Representative chromatograms showing six selenocompounds in the *selenium pathway* (a), and other four selenocompounds (b) in selenium enriched yeast SELM-1, with the addition of DTT. The m/z of the ion being monitored in each channel is indicated in the figure.

Changes in concentration of selenocompounds when yeast was treated with Se-Met, Se (IV), Se (VI)

Figure 6 shows the kinetic responses of Se-Cys and Se-GSH in yeast when treated with Se-Met and two inorganic forms of selenium, Se (IV), Se (VI). The responses of other selenocompounds are presented in Figure S-2. There are some differences in the production of selenocompounds among these three groups. The responses to selenocompounds were much higher in the Se-Met treated group than in the Se (IV) and Se (VI) treated groups. When the yeast was supplemented with Se-Met, the Se-Met detected in the yeast sample peaked very quickly, at about 40 min. Four other selenocompounds (Se-HCys, Se-GSH, Se-Glu-Cys, and Se-AdoHcy) peaked at about two hours after the addition of Se-Met. The production of selenocompounds can result from the competition between selenium and sulfur in the sulfur pathway. In yeast, as shown in Figure 1, it takes three steps for sulfate (SO_4^{2-}) to convert to sulfite (SO_3^{2-}). It takes another two steps for SO_3^{2-} to convert to homocysteine, and get into the *sulfur pathway*. Met is a constituent of the Met cycle, suggesting that it may be much easier for Se-Met to enter the pathway than it is for the two inorganic forms of Se. Once Se gets into the sulfur pathway, other selenocompounds besides Se-Met can be produced. The observation that Se-Met is a more available metabolic source of Se has also been made by other researchers.⁶⁰ It has been shown that, Se-Met is more effective than Se (IV) in increasing the liver and muscle protein Se levels of Se-deficient rats, because of its non-specific incorporation into proteins, taking the place of methionine.⁶¹

Figure 6 Levels of Se-Cys and Se-GSH in yeast when treated with Se-Met, Se (IV) and Se (VI). Time resolved measurements of other selenocompounds are presented in Figure S-2.

Figure 7 shows the percentage of free selenols in total selenols (a), and free thiols in total thiols (b). As shown in Figure 7(a), two hours after the addition of Se-Met, Se (IV), Se (VI), most of the selenols detected are in the oxidized forms. In the case of Se (IV), free Se-HCys accounted for only 0.8 %, and Se-Glu-Cys was all in the oxidized form. No Se-Cys and Se-GSH were detected at this time point. The percentage of free selenols in total selenols is much lower than that of free thiols in total thiols as shown in Figure 7(b). This may be due to the instability of selenols.

Figure 7(b) also shows that, after the addition of Se (IV), the percentage of free thiols in total thiols decreases significantly. It is reported that Se (IV) reacts with thiols giving selenotrisulfides following the reaction.^{62, 63}

Selenotrisulfides tend to decompose giving elemental selenium and superoxide, their stability being related to identity of thiol.⁶⁴⁻⁶⁶ This reaction could explain (i) why selenols are not detected in samples spiked with Se (IV), (ii) why Se (IV) is more toxic than Se (VI). The major toxicity associated with Se (IV)⁶⁷ can be due, indeed, both to the depletion of reduced thiols and superoxide generation.

Figure 7. Percentage of free selenols in total selenols (a) and free thiols in total thiols (b), two hours after the addition of Se-Met, Se (IV) or Se (VI). Se-Cys and Se-GSH were not detected two hours after the addition of Se (IV).

The relative responses of analytes are presented in this study. No quantification was done, because of the lack of authentic standards for most of the selenocompounds. In order to estimate the absolute quantities of these Se compounds, it was assumed that, the MS responses of selenocompounds were the same as their sulfur analogs. This approach has been validated by comparing Se-Cys for which we have obtained standards and Cys responses. Selenocompounds were normalized and the percentage of each selenocompound two hours after the addition of Se-Met, Se (IV) or Se (VI) was calculated. The percentage of each sulfur compound in the *sulfur pathway* at that time point was also calculated. As shown in Table 2 and Table 3, the principal selenocompound two hours after the addition of the three forms of Se was Se-AdoHcy, while the major sulfur compound was GSH. Se-AdoHcy accounts for 70.1% of selenocompounds after the addition of Se-Met, 93.4% after the addition of Se (IV) and 91.8% after the addition of Se (VI). This indicates that the Se from Se-Met, Se (IV) or Se (VI) can enter the Met circle, but very little Se enters the GSH circle. This finding is consistent with the literature. In a water extract of a selenium enriched yeast sample, the major nonproteinaceous selenocompound found was Se-AdoHcy.⁶⁸

Table 4 shows the ratio between selenocompounds and their corresponding sulfur analogs, 2 h after the administration of Se-Met, Se (IV), Se (VI) to the yeast culture. There was a significant increase of Se-HCys after the addition of all the three forms of Se. The production of all these six selenocompounds was much higher in the Se-Met treated group than in the Se (IV) and Se (VI) treated groups. After the addition of Se-Met, there were more Se-HCys, Se-Glu-Cys, Se-Met, and Se-AdoHcy than their sulfur analogs.

Table 2 Distribution of selenocompounds 2 h after the addition of Se-Met, Se (IV), Se (VI).
(%)

	Total Se-Cys	Total Se-Hcys	Total Se-GSH	Total Se-Glu-Cys	Se-Met	Se-AdoHcy
Se-Met	1.0	8.5	1.9	1.4	17.0	70.1
Se (IV)	0.0	5.2	0.0	0.2	1.3	93.4
Se (VI)	0.2	3.8	1.4	0.7	2.1	91.8

Table 3 Distribution of sulfur compounds 2 h after the addition of Se-Met, Se (IV), and Se (VI). (%)

	Total Cys	Total Hcys	Total GSH	Total Cys-Gly	Total Glu-Cys	Met	AdoMet	AdoHcy	Cysta
Control	0.4	0.1	77.5	1.5	0.9	1.6	0.0	17.6	0.5
Se-Met	1.0	0.1	74.4	1.3	0.5	1.0	3.4	18.0	0.3
Se (IV)	0.9	0.1	68.3	1.2	0.8	1.0	0.0	26.8	0.7
Se (VI)	0.9	0.1	83.8	1.6	0.7	1.4	0.6	10.4	0.5

Table 4 Ratio between selenocompounds and their corresponding sulfur analogs, 2 h after the addition of Se-Met, Se (IV), Se (VI).

	Se-Cys/ Cys	Se-HCys/ HCys	Se-GSH/ GSH	Se-Glu-Cys/ Glu-Cys	Se-Met/ Met	Se-AdoHcy/ AdoHcy
Se-Met	0.7	75.8	0.02	1.7	10.8	2.6
Se (IV)	*	4.5	**	0.03	0.2	0.4
Se (VI)	0.02	3.5	0.001	0.1	0.1	0.8

* No Se-Cys was detected

** No Se-GSH was detected

CONCLUSION

LTQ-Orbitrap mass spectrometry offers a good method in terms of sensitivity and resolution for the identification of selenocompounds in yeast. Good agreement (< 4 ppm difference) between the theoretical masses of the target compounds and the measured masses in the yeast matrix was obtained, using either IAA or PHMB as the derivatizing reagent. Using accurate mass measurements combined with the characteristic isotopic patterns of selenocompounds and the overlapped isotopic patterns of selenol-PHMB complexes, six Se substituted compounds in the sulfur pathway in selenium enriched yeast were identified. The method using IAA as the derivatizing reagent was successfully used to study the effect of the chemical forms of Se, i.e. Se-Met, Se (IV), and Se (VI)) on the production of selenocompounds in *S. cerevisiae*. A higher yield of selenocompounds was observed in the Se-Met treated group than in the Se (IV) and Se (VI) treated groups.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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