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Comparison of extraction methods for quantitation of methionine and selenomethionine in yeast by species specific isotope dilution gas chromatography–mass spectrometry

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Abstract

Fourteen extraction methods commonly cited in the literature were evaluated for the quantitation of methionine (Met) and selenomethionine (SeMet) in a yeast candidate certified reference material (CRM). Species specific isotope dilution (ID) gas chromatography–mass spectrometry (GC–MS) was utilized to effectively compensate for potential errors, such as losses during derivatization and clean up steps. Despite different extraction methods, the same derivatization procedure using methyl chloroformate was applied with a single exception, which was based on digestion with cyanogen bromide with 2% $SnCl_2$ in 0.1 M HCl. Significant differences in measured Met and SeMet concentrations were obtained when different extraction methods were used. A 4 M methanesulfonic acid reflux digestion was found to be the most efficient for both analytes. Digestion with CNBr with 2% $SnCl_2$ in 0.1 M HCl for the determination of SeMet showed the second highest extraction efficiency. Despite frequent use of enzymatic hydrolysis for the extraction of SeMet from yeast, very low extraction efficiencies for both analytes were obtained for four of eight tested methods. Among these, the highest extraction efficiencies for both analytes were obtained using 20 mg pronase and 10 mg lipase with incubation at 37 °C for 24 h. However, recoveries remained nearly 30 and 50% lower for Met and SeMet, respectively, compared to extraction with methanesulfonic acid. Lowest extraction efficiencies for both analytes were obtained when HCl or tetramethylammonium hydroxide (TMAH) digestions were used. Efficient extraction was also achieved using 200 mg (or 400 mg) of protease XIV with incubation at 37 °C for 72 h (or 24 h). Concentrations of 3331 ± 45 and 3334 ± 39 $\mu g\,g^{-1}$ (mean and one standard deviation, n = 4) for SeMet were obtained using 200 mg (72 h incubation) and 400 mg (24 h incubation) of protease XIV, respectively, in agreement with a value of 3404 ± 38 $\mu g\,g^{-1}$ obtained using a methanesulfonic acid reflux.

Keywords: Methionine; Selenomethionine; Gas chromatography; GC–MS; Isotope dilution; Speciation; Se

1. Introduction

Consumption of Se enriched supplements has increased dramatically as a result of the numerous health benefits reported, including protection of cells against the effects of free radicals, the normal functioning of the immune system and thyroid gland [1–3] as well as protection against various forms of cancers [4–7]. Yeast is a popular supplement medium wherein selenomethionine (SeMet) is usually the dominant Se species, it possessing higher bioavailability and lower toxicity than inorganic selenium. Significant efforts have been made in the development of analytical methods for the speciation of Se in yeast in recent years. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are currently the most commonly used separation techniques for speciation of SeMet in combination with detection by flame photometry [8], atomic emission [9], mass spectrometry [9–14] and inductively coupled

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plasma mass spectrometry (ICP-MS) [15–24]. More recently, species specific isotope dilution has been applied to the determination of methionine (Met) [14] and SeMet in yeast [11,14,24] in attempts to achieve more accurate and precise results.

Equilibration between the added spike and the endogenous analyte in the sample is a prerequisite to achieve accurate results using isotope dilution calibration. Results would be biased low if equilibration between the spike and the sample was not achieved prior to ratio measurements; as would occur if quantitative extraction of analyte from sample matrix was not achieved. Therefore, extraction procedures used are of paramount importance for the accurate determination of Met and SeMet in yeast or other solid samples.

Numerous extraction techniques have been developed for the extraction of SeMet in yeast, the most frequently used being based on enzymatic hydrolysis [10,15–18,20,25–27] with protease, proteinase K or a mixture of proteolytic enzymes. Procedures utilizing HCl [12,22] and tetramethylammonium hydroxide (TMAH) [20,28] have also been reported. Digestion with methanesulfonic acid extracts SeMet from yeast [10,14] with much higher efficiency than enzymatic approaches based on proteinase K and protease XIV [10]. A different technique, using cyanogen bromide to liberate SeMet from yeast, was recently reported by Wolf et al. [11]. However, results from a recent study [24] show incomplete extraction occurs with this method.

The popularity of enzymatic digestion protocols for the analysis of a modified amino acid (i.e., SeMet) is quite surprising. Conventional approaches typically employ 6 M HCl vapor phase hydrolysis under vacuum and heat [29]. Despite this harsh conditions, the majority of the twenty naturally occurring amino acids can be quantitatively recovered. Protocols to deal with vulnerable amino acid functionalities have also been developed. For example, liquid phenol has been used as an additive to protect hydroxylated amino acids, such as tyrosine and threonine. However, it seems that, in general, within the 'speciation field', metal-containing organic compounds are deemed unstable and prone to decomposition during sample preparation. As such, no harsh extraction protocols have been used, which is the likely reason for the high number of papers employing enzymatic protocols instead of acid digestion.

Despite the high extraction efficiencies claimed for various extraction procedures, the real efficiency of these methods is generally unknown and results obtained are hardly comparable due to the absence of reference materials certified for SeMet content. The National Research Council Canada has recently undertaken a project to address the need for a yeast Certified Reference Material (CRM) for validation of measurements of Met, SeMet and total Se. The objective of this study was to compare the performance of various extraction procedures found in the literature for the determination of Met and SeMet in a yeast material. Evaluation of the extraction efficiency can be achieved in different ways [30]. In this study, a candidate yeast CRM was used for this purpose and species specific ID effectively compensated for potential errors in the determination of Met and SeMet, such as losses during derivatization and clean up steps. The same derivatization procedure, based on methyl chloroformate, along with quantitation by GC–MS [14] was applied to extracts generated using a number of digestion techniques. All sample preparation and analyses were conducted in the same laboratory by the same analyst, hence the differences in the performance of the various methods can be reasonably attributed to variations in the efficiencies of the individual extraction methods.

2. Experimental

2.1. Instrumentation

A Hewlett-Packard HP 6890 GC (Agilent Technologies Canada, Mississauga, Canada) fitted with a DB-5MS column (Iso-Mass Scientific, Calgary, Canada) was used for the separation of the Met and SeMet in yeast extract. Detection was achieved with an HP model 5973 mass-selective detector using single ion monitoring.

2.2. Reagents and solutions

Hydrochloric acid was purified in the laboratory in a quartz still prior to use by subboiling distillation of reagent grade feedstock. Environmental grade ammonium hydroxide was purchased from Anachemia Science (Montreal, Canada). OmniSolv methanol (glass-distilled) and chloroform were purchased from EM Science (Gibbstown, NJ, USA). High-purity deionized water (DIW) was obtained from a NanoPure mixed bed ion exchange system fed with reverse osmosis domestic feed water (Barnstead/Thermolyne, IA, USA). Certified grade chloroform, formic acid and tetramethylammonium hydroxide were sourced from Fisher Scientific (Ottawa, Canada). Methanesulfonic acid (98% purity), methyl chlorofomate (99% purity), 3 M cyanogen bromide in $CHCl_2$ as well as protease XIV, protease VIII (Subtilisin), proteinase K, lipase and aminopeptidase enzymes were obtained from Sigma–Aldrich Canada (Oakville, Canada).

Natural abundance high-purity SeMet and high-purity ^{13}C enriched Met and ^{13}C enriched SeMet compounds were purchased from Sigma–Aldrich Canada. Individual stock solutions of 1000–2500 $\mu g\,ml^{-1}$ were gravimetrically prepared in 1% HCl solution and kept refrigerated until used.

A ^{74}Se enriched SeMet ($^{74}SeMet$) was donated by Dr. W. Wolf (Food Composition Laboratory, US Department of Agriculture, Beltsville, MD, USA) and used to prepare a stock solution of approximately 450 $\mu g\,ml^{-1}$ in 1% HCl. The concentration of $^{74}SeMet$ spike was verified by reverse spike isotope dilution using volumetrically prepared natural abundance SeMet standards. Lalmin Se yeast was obtained from Rosell-Lallemand (Montreal, Canada) and used as the test sample for this study.

2.3. Safety considerations

Methyl chloroformate is a highly toxic and flammable substance. Material Safety Data Sheets must be consulted and essential safety precautions employed for all manipulations.

2.4. Sample preparation and analysis procedure

Fourteen extraction procedures (herein denoted A–N) were used. Three sample blanks and four subsamples of yeast were prepared in each case. For each set of samples, an 0.10 g mass was spiked with 0.150 ml each of 2185.6 μg ml^{-1} ^{13}C enriched SeMet and 4206.1 μg ml^{-1} ^{13}C enriched Met for methods A–M. For method N, 0.300 ml of 450 μg ml^{-1} ^{74}SeMet was added to 0.10 g of yeast. Samples were then subjected to the following:

(A) After addition of 16 ml of 4 M methanesulfonic acid, the contents were refluxed on a hot plate for 16 h [10,14].
(B) After addition of 10 ml of 0.1 M HCl, the contents were vortex mixed for 5 min [12,22].
(C) After addition of 10 ml of 0.1 M HCl, the contents were maintained at 50 °C with sonication for 1 h [22].
(D) After addition of 10 ml of 6.0 M HCl, the contents were sonicated for 10 min [22].
(E) After addition of 10 ml of TMAH, the contents were maintained at 60 °C for 4 h [20,28].
(F) After addition of 5 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing 20 mg of protease XIV, the contents were maintained at 37 °C for 24 h [17,20,26].
(G) After addition of 5 ml DI water containing 20 mg of protease XIV, the contents were maintained at 37 °C for 24 h [17,20,26].
(H) After addition of 5 ml DI water containing 20 mg of protease XIV and 10 mg lipase, the contents were maintained at 37 °C for 24 h [16].
(I) After addition of 5 ml of 0.1 M Tris–HCl buffer (pH 7.5) along with 20 mg of protease VIII, the contents were maintained at 37 °C for 24 h [26].
(J) After addition of 5 ml DI water with 20 mg of protease VIII, the contents were maintained at 37 °C for 24 h [26].
(K) After addition of 5 ml of 30 mM Tris–HCl buffer (pH 7.5) and 5 mM $CaCl_2$ containing 20 mg of pronase and 10 mg of lipase, the contents were maintained at 37 °C for 24 h.
(L) After addition of 5 ml of 30 mM Tris–HCl buffer (pH 7.5) and 5 mM $CaCl_2$ containing 10 mg of pronase and 0.25 mg of aminopeptidase, the contents were maintained at 37 °C for 24 h.
(M) After addition of 5 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 40 mg of proteinase K, the contents were incubated at 50 °C for 18 h. Following addition of 20 mg of protease XIV, the contents were finally heated at 37 °C for 6 h [10,18].
(N) After addition of 2 ml of 2% $SnCl_2$ in 0.1 M HCl, the vials were vortex mixed for 5 min and then heated in a water bath at 37 °C for 24 h. After addition of 0.50 ml of 3 M CNBr in $CHCl_2$, the vials were then maintained at 37 °C for a further 24 h [11].

The derivatization procedure used for methods A–M was similar to that reported earlier [14] and based on a 1 ml volume of extract. Following the digestion, samples were centrifuged at 2000 rpm for 5 min and a 1 ml extract was pippetted into a 10 ml glass vial. After addition of an appropriate amount of ammonium hydroxide or HCl to adjust the pH of sample to 2–4, 0.75 ml volume of methanol–pyridine (3:1, v/v) was added followed by the slow addition of 0.250 ml of methyl chloroformate. The vial was then shaken manually for 1 min with periodic venting. One milliliter of chloroform was then added and the vial was shaken manually for 1 min. The sample was then centrifuged at 2000 rpm for 5 min and the chloroform layer transferred to a 1-ml glass vial for subsequent analysis by GC–MS. For extraction method N, the above derivatization procedure was not required since the products of CH_3SCN (for Met) and CH_3SeCN (for SeMet) are volatile compounds, which were extracted into 1 ml of chloroform prior to GC–MS analysis.

3. Results and discussion

Optimization of the GC–MS system for the determination of derivatized Met and SeMet following their derivatization with methyl chloroformate was performed as described previously [14] and conditions used in this study are summarized in Table 1. GC–MS parameters for the determination of Met (CH_3SCN) and SeMet (CH_3SeCN) after digestion with CNBr with 2% $SnCl_2$ in 0.1 M HCl were optimized and similar to those described earlier [24]. These conditions are also presented in Table 1.

3.1. Results for extraction methods A–E

As shown in Fig. 1, good separation and peak profiles for Met and SeMet were obtained under optimized conditions. The derivatized Met molecular ion ($C_8H_{15}O_4NS^+$) and the derivatized SeMet molecular ion ($C_8H_{15}O_4NSe^+$) were selected for quantitation in this study, similar to that reported previously [12]. The increased abundance at *m*/*z* 222 and 270 in Fig. 1b and c reflects the contribution from added ^{13}C enriched spikes. Ions at *m*/*z* 221 and 222 were selected as reference and spike ions for ID analysis using a ^{13}C enriched Met to calculate the final concentration of Met in the yeast. Relative abundances of 85.793 and 8.706% for ions at *m*/*z* 221 and 222 in the sample and 8.67 and 85.932% in the spike, calculated earlier [12], were used for the quantitation. Similarly, ions at *m*/*z* 269 and 270 with relative abundances of 45.102 and 4.210% in the sample and 2.272 and 45.193% in the spike were selected for ID analysis using a ^{13}C enriched

Table 1
GC–MS operating conditions

With methyl chloroformate derivatization	
Column	DB-5MS 30 m × 0.25 mm i.d., 0.25 μm d_f
Injection system	Split/splitless injector–splitless mode
Injector temperature	280 °C
Oven temperature program	120–260 °C at 20 °C/min (hold 2 min)
Carrier gas; flow rate	Helium; 1.5 ml/min
Transfer line temperature	260 °C
MS	HP model 5973 mass-selective detector
SIM parameters	Measured ions: *m/z* = 221, 222, 269 and 270 Dwell times: 25 ms for each *m/z*
MS quad temperature	150 °C
MS source temperature	250 °C
With CNBr sample preparation	
Column	DB-5MS 30 m × 0.25 mm i.d., 0.25 μm d_f
Injection system	Split/splitless injector–splitless mode
Injector temperature	180 °C
Oven temperature program	35 °C (hold 4 min) to 120 °C at 15 °C/min to 250 °C at 50 °C/min.
Carrier gas; flow rate	Helium; 1.2 ml/min
Transfer line temperature	180 °C
MS	HP model 5973 mass selective detector
SIM parameters	Measured ions: *m/z* = 106 and 100 Dwell times: 25 ms for each *m/z*
MS quad temperature	150 °C
MS source temperature	250 °C

SeMet spike. All four ions were monitored under selective ion monitoring (SIM) mode. Peak areas were used to calculate the reference to spike ion ratios, from which the analyte concentrations were calculated. The following equation was used for the quantitation of Met and SeMet in yeast:

$$C_x = C_y \frac{v_y}{m_x} \frac{A_y - B_y R_n}{B_x R_n - A_x} \frac{AW_x}{AW_y} \quad (1)$$

where C_x is the analyte concentration (μg g^{-1}), C_y the concentration of enriched spike (μg ml^{-1}), v_y the volume (ml) of spike used to prepare the blend solution of sample and spike, m_x the mass (g) of yeast sample used, A_y the abundance of reference ion in the spike, B_y the abundance of spike ion in the spike, A_x the abundance of reference ion in the sample, B_x the abundance of spike ion in the sample, R_n the measured reference/spike ion ratio (mass bias corrected) in the blend solution of sample and spike, AW_x the atomic mass of analyte in the sample and AW_y is the atomic mass of analyte in the spike. As is evident from this equation, only the reference/spike ion ratios in the spiked samples need to be measured to derive the final analyte concentrations. The mass bias correction factor was calculated from the expected to measured ratio using a natural abundance Met and SeMet standard solution. Results obtained for methods A–M using methyl chloroformate derivatization are summarized in Table 2.

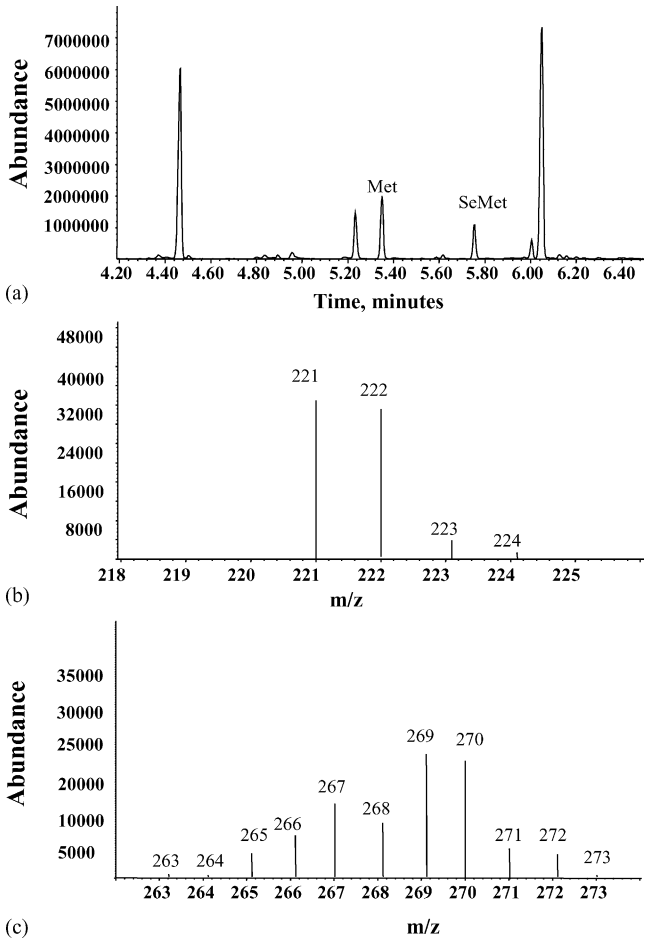

Fig. 1. (a) Total ion GC–MS chromatogram (*m/z* 50–300) of a spiked (^{13}C-enriched Met and SeMet) yeast extract derivatized with methyl chloroformate; (b) Met ion isotope pattern following derivatization with methyl chloroformate and (c) SeMet ion isotope pattern following derivatization with methyl chloroformate.

As is evident from Table 2, method A, based on a 4-M methanesulfonic acid reflux, provided the highest results for both Met and SeMet. The absence of degradation of either Met or SeMet during this prolonged digestion was experimentally confirmed, as reported previously [24]. Concentrations of Met and SeMet measured in standard solutions, which were refluxed for 16 h in 16 ml of 4 M methanesulfonic acid were in good agreement to those obtained in control samples not subjected to reflux, confirming absence of degradation of these compounds during digestion.

Both HCl and TMAH digestions (methods B–E) resulted in very low concentrations of Met and SeMet in this yeast sample compared to data generated using method A, contrary to the high extraction efficiencies reported by others [12,20,22,27].

3.2. Results for methods based on enzymatic hydrolysis

Methods based on enzymatic hydrolysis have been widely used to efficiently extract protein bound Se species for

Table 2
Results for determination of Met and SeMet in yeast following different extraction methods

Method used	Met ($\mu g\,g^{-1}$)	SeMet ($\mu g\,g^{-1}$)
A (4 M methanesulfonic acid for 16 h)	5947 ± 35	3404 ± 38
B (0.1 M HCl vortex for 5 min)	117 ± 1	100 ± 2
C (0.1 M HCl at 50 °C sonication for 1 h)	123 ± 2	95 ± 2
D (6.0 M HCl sonication for 10 min)	123 ± 1	99 ± 3
E (TMAH at 60 °C for 4 h)	143 ± 14	115 ± 12
F (20 mg protease XIV and buffer, at 37 °C for 24 h)	2179 ± 57	1434 ± 38
G (20 mg protease XIV and DIW, at 37 °C for 24 h)	2707 ± 28	1759 ± 35
H (20 mg protease XIV and 10 mg lipase, at 37 °C for 24 h)	2115 ± 51	1411 ± 36
I (20 mg subtilisin and buffer, at 37 °C for 24 h)	222 ± 10	109 ± 4
J (20 mg subtilisin and DIW, at 37 °C for 24 h)	212 ± 20	112 ± 3
K (20 mg pronase and 10 mg lipase, at 37 °C for 24 h)	4310 ± 130	1800 ± 28
L (10 mg pronase and 0.25 mg aminopeptidase, at 37 °C for 24 h)	620 ± 55	359 ± 28
M (40 mg proteinase K at 50 °C for 18 h and 20 mg protease XIV, at 37 °C for 6 h)	520 ± 73	410 ± 34
N (CNBr with 2% $SnCl_2$ in 0.1 M HCl)	ND	2260 ± 12

determination of SeMet in various yeast samples. However, results obtained in this study revealed large variations in measured concentrations of both Met and SeMet in this yeast using various enzymatic hydrolysis methods (F–M, shown in Table 2). Method K produced the highest concentrations of Met and SeMet among the tested enzymatic hydrolysis methods, but these results are still 30 and 50% lower than data obtained using method A for Met and SeMet, respectively.

Surprisingly very low concentrations of Met and SeMet were obtained using methods I and J, contrary to the generally high extraction efficiency obtained by others using the same enzymes [26]. Similar results were obtained using methods I and J when a different bottle of Subtilisin enzyme from a different lot number was used. A experiment was undertaken to determine whether the analytes still remained in the yeast matrix following digestion. Four samples used for method I were rinsed with 10 ml of DIW and the supernatants obtained by centrifuging at 2000 rpm for 5 min were discarded. This procedure was repeated two more times. After addition of ^{13}C enriched Met and SeMet spikes, yeast residues were then digested with 4 M methanesulfonic acid as prescribed in method A. Concentrations of 5608 ± 110 and 2840 ± 39 $\mu g\,g^{-1}$ were obtained for Met and SeMet, respectively, in the yeast residues, confirming inefficient hydrolysis by subtilisin enzyme.

Nevertheless, the above observations suggest that enzymatic hydrolysis methods used in previous publications are far from being quantitative and cannot be used to achieve accurate results for Met and SeMet in this yeast.

3.3. Optimization of methods based on enzymatic hydrolysis

Significant differences of 40 and 30% in extraction efficiencies for Met and SeMet in yeast, respectively, were obtained when the same amount of protease XIV (20 mg) taken from two different bottles originating from two batches were used. This suggests that a 24-h incubation time with use of 20 mg enzyme for 0.1 g of sample, (a sample-to-enzyme ratio of 5 or larger) may not be sufficient to achieve quantitative results for this yeast, despite these conditions being previously used by many other groups [15–17,26]. It is of interest to investigate the optimum extraction efficiency of enzymatic methods of hydrolysis for determination of Met and SeMet in this yeast. Protease XIV was chosen for this since it has been the most frequently used enzyme for the determination of SeMet in yeast. Several experiments were conducted to study the effects of amount of enzyme and incubation time. Sample preparation was similar to that described for method F but with various amounts of protease XIV and various incubation times.

It was noted that blank levels of Met increased significantly with increases in the amount of Protease XIV used, as well as increases in the incubation time, resulting in over correction for the blank, thereby prohibiting accurate determination of Met in this yeast. The high blank levels are probably due to the hydrolysis of the enzyme itself as it contains Met residues but no SeMet. Thus, only SeMet was measured in these studies; results are shown in Figs. 2 and 3. It is evident from Fig. 2 that concentrations of SeMet increased significantly as the mass of protease XIV used increased from 20 to 100 mg using an incubation time of 24 h at 37 °C. A slight increase in the concentrations of SeMet was obtained as the mass of protease XIV increased from 100 to 200 mg but no significant change in concentration of SeMet was observed as the mass of protease XIV was increased to 400 mg.

As shown in Fig. 3, the effect of incubation time on the extraction efficiency of SeMet is largely influenced by the amount of protease XIV used. No optimum was found, even after 108 h incubation with use of 20 mg of protease XIV for 0.1 g of subsample, the most frequent ratio (5:1) reported in the literature. A similar trend, but with a smaller slope, was found when 40 mg was used. Clearly, a constant efficiency of extraction of SeMet was achieved when an incubation time longer than 48 h is used with 200 mg of protease XIV. No significant difference in concentration of SeMet was noted when 300 or 400 mg of protease XIV was used under tested incubation times (not shown in Fig. 3). To achieve quantitative

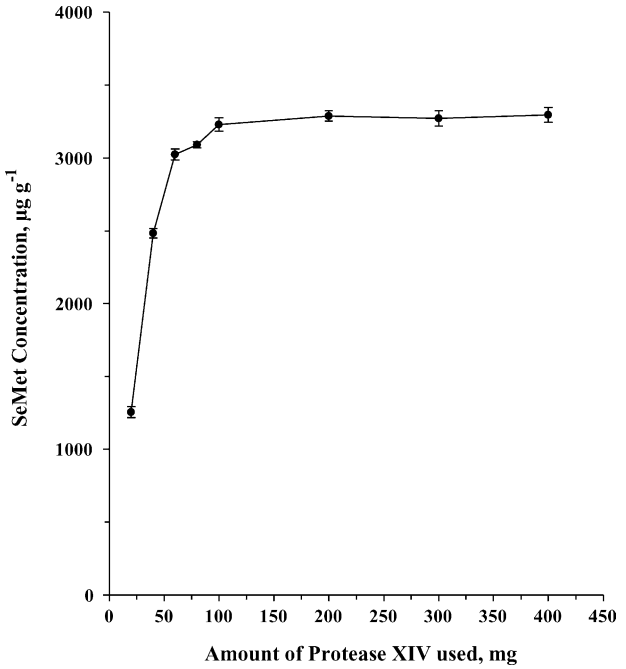

Fig. 2. Effect of amount of protease XIV on extraction efficiency of SeMet in yeast incubated at 37 °C for 24 h. Error bars represent one standard deviation based on four measurements.

extraction at lowest cost, a 72-h incubation at 37 °C and use of 200 mg of protease XIV for 0.1 g of subsample, was selected for the final quantitation of SeMet in this yeast sample. A concentration of 3331 ± 45 μg g^{-1} (mean and one standard deviation, n = 4) was obtained for SeMet, similar to the 3404 ± 38 μg g^{-1} generated using methanesulfonic acid (method A). It is interesting to note that quantitative results (3334 ± 39 μg g^{-1} for SeMet) can be obtained with a shorter incubation time (24 h) if a larger amount of protease XIV is used (400 mg).

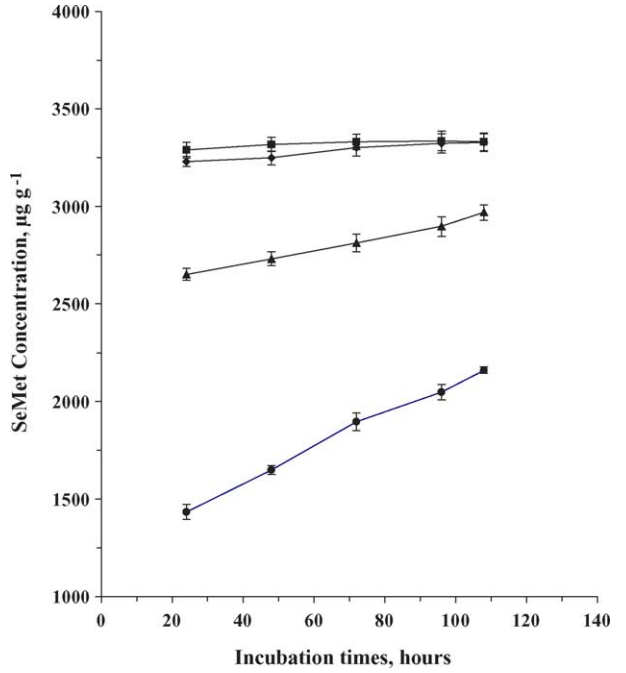

Fig. 3. Effect of incubation time on extraction efficiency of SeMet in yeast. Mass of protease XIV used: (●) 20 mg; (▲) 40 mg; (♦) 100 mg and (■) 200 mg. Error bars represent one standard deviation based on four measurements.

3.4. Extraction using digestion with CNBr

In order to perform ID analysis for the determination of Met and SeMet in yeast using digestion based on CNBr with 2% $SnCl_2$ in 0.1 M HCl (method N), the molecular ion of CH_3SeCN^+ and its CH_3Se^+ fragment ion were monitored for SeMet. The CH_3SCN^+ and CH_3S^+ ions characteristic of Met were selected as a result of the attached ^{13}C enriched methyl group, shown in Fig. 4. Unfortunately, skewed isotope patterns for CH_3SeCN^+ (m/z 115–123) and CH_3Se^+ (m/z 88–97) were observed. Similarly, isotope patterns for CH_3SCN^+ ion (m/z 70–75) and its CH_3S^+ fragment ion (m/z 44–49) were also skewed. These effects may be due to either

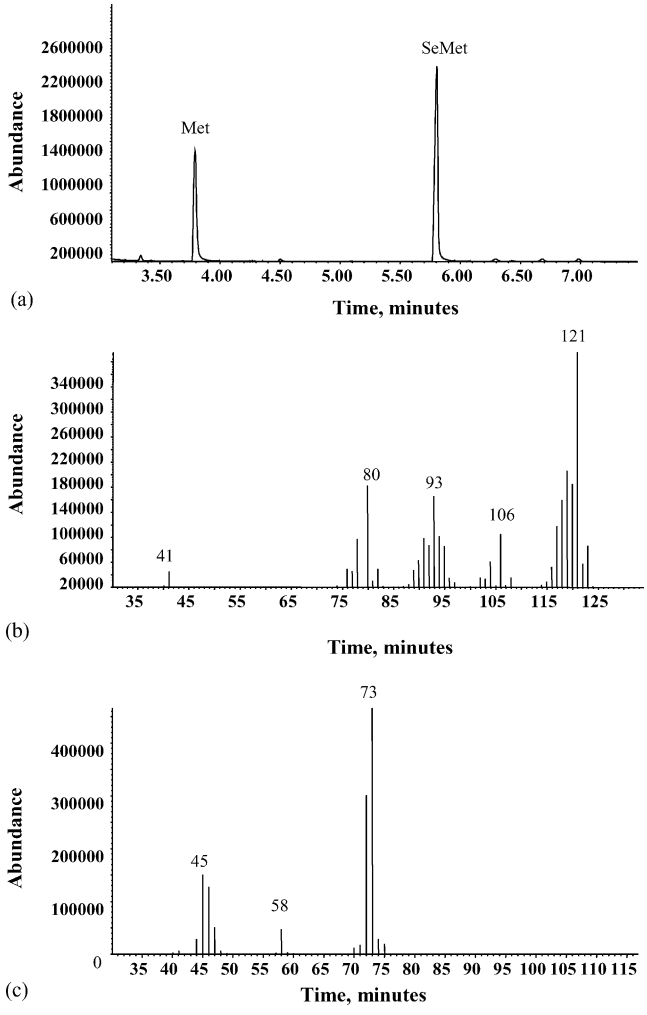

Fig. 4. (a) Total ion GC–MS chromatogram (m/z 50–300) of a mixed natural abundance standard solution following digestion with CNBr with 2% $SnCl_2$ in 0.1 M HCl; (b) spectrum of SeMet peak and (c) spectrum of Met peak.

isobaric interferences on these masses or as a result of some of these ions being deprotonated. As a result, ID analysis using ^{13}C enriched spikes for the accurate determination of Met and SeMet was prohibited.

Good isotope patterns for the fragment ion $SeCN^+$ (*m/z* 100–108) characterizing SeMet and SCN^+ (*m/z* 58–60) for Met were obtained. Thus, ions at *m/z* 106 and 100 were selected as reference and spike ions for ID analysis using a ^{74}Se enriched SeMet for the quantitation of SeMet in the yeast using the CNBr digestion method. Met was thus not measured due to lack of sulfur enriched Met. A measured ratio of 55.75 ± 0.14 (one standard deviation, $n = 4$) at *m/z* 106/100 in an unspiked yeast extract was not significantly different from the expected natural abundance ratio of 55.7426 (48.899%/0.8772%), confirming the absence of any significant spectroscopic interference on the selected ions arising from the sample matrix. As shown in Table 2, a concentration of 2260 ± 12 μg g^{-1} (one standard deviation, $n = 4$) was obtained using this method, significantly lower than that obtained using method A.

4. Conclusions

Fourteen different extraction methods applied to the same yeast sample produced significantly different results for Met and SeMet, highlighting the fact that the extraction step is of paramount importance of achieving accurate results. Use of 4 M methanesulfonic acid in a reflux digestion achieved the highest extraction efficiency for both Met and SeMet. Use of CNBr to form volatile CH_3SeCN for quantitation of SeMet produced results about 34% lower than the methanesulfonic acid extraction. Large variations in extraction efficiencies for both Met and SeMet were observed with different enzymatic methods of hydrolysis. Although pronase and lipase resulted in the highest extraction efficiencies among eight tested enzymatic methods based on a 24-h incubation time, results remained close to 30 and 50% lower than those obtained using the reflux with methanesulfonic acid for Met and SeMet, respectively.

It is important to optimize both the amount of enzyme used as well as incubation time prior to its use for quantitation. Possible differences in activities amongst different batches must be considered. To achieve efficient extraction, a method using 200 mg (or 400 mg) of protease XIV with incubation at 37 °C for 72 h (or 24 h) was developed. The concentration of SeMet measured in the candidate yeast CRM obtained using this method is in agreement with that obtained using the most efficient extraction method employing methanesulfonic acid. The enzymatic method developed in this study provides independent confirmation of the results obtained by acid digestion, suggesting that results obtained using these protocols are not method dependent or functionally defined. However, the amount of enzyme required for maximum recovery is high (hundreds of milligrams), making it a relatively expensive digestion procedure for routine analysis.

Results from this study clearly demonstrate that a yeast CRM certified for SeMet content is urgently needed to validate methodologies for such measurements and to enable comparison of results obtained by different research teams.

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