Anal Bioanal Chem (2008) 390:629–642
DOI 10.1007/s00216-007-1654-8

ORIGINAL PAPER

Total selenium and selenomethionine in pharmaceutical yeast tablets: assessment of the state of the art of measurement capabilities through international intercomparison CCQM-P86

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Received: 9 July 2007 / Revised: 16 September 2007 / Accepted: 19 September 2007 / Published online: 16 October 2007

Abstract Results of an international intercomparison study (CCQM-P86) to assess the analytical capabilities of national metrology institutes (NMIs) and selected expert laboratories worldwide to accurately quantitate the mass fraction of selenomethionine (SeMet) and total Se in pharmaceutical tablets of selenised-yeast supplements (produced by Pharma Nord, Denmark) are presented. The study, jointly coordinated by LGC Ltd., UK, and the Institute for National Measurement Standards, National Research Council of Canada (NRCC), was conducted under the auspices of the Comité Consultatif pour la

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Quantité de Matière (CCQM) Inorganic Analysis Working Group and involved 15 laboratories (from 12 countries), of which ten were NMIs. Apart from a protocol for determination of moisture content and the provision of the certified reference material (CRM) SELM-1 to be used as the quality control sample, no sample preparation/extraction method was prescribed. A variety of approaches was thus used, including single-step and multiple-step enzymatic hydrolysis, enzymatic probe sonication and hydrolysis with methanesulfonic acid for SeMet, as well as microwave-assisted acid digestion and enzymatic probe sonication for total Se. For total Se, detection techniques included inductively coupled plasma (ICP) mass spectrometry (MS) with external calibration, standard additions or isotope dilution MS (IDMS), inductively coupled plasma optical emission spectrometry , flame atomic absorption spectrometry and instrumental neutron activation analysis. For determination of SeMet in the tablets, five NMIs and three academic/institute laboratories (of a total of five) relied upon measurements using IDMS. For species-specific IDMS measurements, an isotopically enriched standard of SeMet (^{76}Se-enriched SeMet) was made available. A novel aspect of this study relies on the approach used to distinguish any errors which arise during analysis of a SeMet calibration solution from those which occur during analysis of the matrix. To help those participants undertaking SeMet analysis to do this, a blind sample in the form of a standard solution of natural abundance SeMet in 0.1 M HCl (with an expected value of 956 mg kg^{-1} SeMet) was provided. Both high-performance liquid chromatography (HPLC)–ICP-MS or gas chromatography (GC)–ICP-MS and GC-MS techniques were used for quantitation of SeMet. Several advances in analytical methods for determination of SeMet were identified, including the combined use of double IDMS with HPLC-ICP-MS following extraction with methanesulfonic acid and simplified two-step enzymatic hydrolysis with protease/lipase/driselase followed by HPLC-ICP-IDMS, both using a species-specific IDMS approach. Overall, satisfactory agreement amongst participants was achieved; results averaged 337.6 mg kg^{-1} (n=13, with a standard deviation of 9.7 mg kg^{-1}) and 561.5 mg kg^{-1}(n=11, with a standard deviation of 44.3 mg kg^{-1}) with median values of 337.6 and 575.0 mg kg^{-1} for total Se and SeMet, respectively. Recovery of SeMet from SELM-1 averaged 95.0% (n=9). The ability of NMIs and expert laboratories worldwide to deliver accurate results for total Se and SeMet in such materials (selensied-yeast tablets containing approximately 300 mg kg^{-1} Se) with 10% expanded uncertainty was demonstrated. The problems addressed in achieving accurate quantitation of SeMet in this product are representative of those encountered with a wide range of organometallic species in a number of common matrices.

Keywords Pilot study CCQM-P86 · Pharmaceutical tablets · Selenised-yeast supplements · Selenomethionine · Total selenium · Isotope dilution mass spectrometry

Introduction

The addition of Se, an essential nutrient [1], to the diet in the form of enrichment, dietary supplements or fortified food is becoming increasingly common owing to its often-insufficient content in the standard diet in many countries. Selenised yeast (Se-yeast) is attractive as a supplementary source of Se owing to its low cost, ability to act as a precursor for selenoprotein synthesis and its high content of selenomethionine (SeMet), a beneficial form of Se found in most foods. However, there are a variability of Se-yeast supplements with respect to Se content and speciation. This, combined with a lack of knowledge of the identity and concentration of the multiple Se species present, has made Se-yeast the most widely investigated food supplement containing selenium [2–21].

Accurate measurement of SeMet concentration in raw yeast (with elevated milligrams per kilograms concentrations of total Se) has presented a significant analytical challenge during the last decade [2, 6–10, 12]. The highest extraction efficiencies for this vital Se species have been achieved using hydrolysis with proteolytic enzymes and methanesulfonic acid [2, 6–10,

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12, 13, 22], the former approach being the most widely used and, for the most part, reliant on proteinase K and protease XIV. The extraction efficiency with enzymatic hydrolysis critically depends on the type of enzyme and the extraction conditions [7]. Furthermore, the SeMet content/total Se value (used for calculation of the extraction efficiency) seems to be dependent on the source of yeast analysed and can be affected by the degree of oxidation of SeMet, which may occur during the entire analytical procedure. Consequently, large variations in extraction efficiencies for SeMet with different enzymatic protocols have been reported for yeast-based materials [2, 7, 12, 13, 19, 21–23].

Most quantification approaches have been based on calculations of percentage Se distributions, as determined from relative Se response (area) of the peaks corresponding to the substances eluted [2, 15]. However, accurate results for SeMet in Se-yeast based on use of species-unspecific isotope dilution (ID) in combination with inductively coupled plasma (ICP) mass spectrometry (MS) coupled with high-performance liquid chromatography (HPLC), and species-specific ID (using ^{13}C-enriched SeMet available from Sigma) with gas chromatography (GC)–ICP-MS and GC-MS have recently been reported [2, 6–10, 12]. Despite such advances in quantitative speciation methodology, the lack of commercially available isotopically enriched SeMet species with which to perform species-specific IDMS calibration with GC-ICP-MS or HPLC-ICP-MS, of simplified sample preparation procedures, and of "speciated" yeast certified reference materials (CRMs) for validation of measurements in such complex samples are considered remaining problems.

Recently, a Se-yeast CRM (SELM-1), certified for total Se (2,059±64 mg kg^{-1}) and SeMet (3,389±173 mg kg^{-1}) content has been made commercially available by the National Research Council of Canada (NRCC). An international intercomparison exercise, piloted by NRCC, was carried out in parallel to the certification of this material in order to assess the state of the art of SeMet measurement capabilities. This study concluded that the determination of SeMet in yeast requires significant sample preparation to ensure exhaustive extraction of the analyte without decomposition. The extraction efficiency obtained for SeMet appeared to be critically dependent on the method of extraction used [10], which was in agreement with results recently reported on the comparison of different extraction methods for determination of SeMet [7].

This CCQM-P86 pilot study was undertaken to assess the ability of laboratories to accurately quantify the mass fractions of total Se and SeMet (of relevance to health) in pharmaceutical supplements. It broadens the scope and degree of difficulty of earlier measurements in this field since the material examined was Se-yeast mixed with excipients normally incorporated into commercially available pharmaceutical tablets; the sample matrix is thus more complex and contains much lower concentrations of Se than those encountered in neat Se-yeast (e.g., 6-fold lower than CRM SELM-1). A call for participation was sent out in November 2005, with response from 15 laboratories representing 12 countries (ten national metrology institutes, with the remainder being invited expert universities/institutes). Two blister packs of pharmaceutical tablets, formulated from one batch produced by Pharma Nord, Denmark, were sent to each participant. Between-tablet homogeneity for total Se and SeMet mass fractions was tested at LGC and NRCC, respectively, prior to their release. The NRCC CRM SELM-1 (3,389±173 mg kg^{-1}) to be used as the quality control sample and a protocol for determination of moisture content were sent to each

Table 1 CCQM-P86 participants and affiliations

Institute/organisation	Country	Contact
Corvinus University of Budapest (BUD)	Hungary	Péter Fodor
Complutense University of Madrid (MAD)	Spain	Carmen Cámara
National Institute of Metrology P. R. China (NIM)	China	Wang Jun
US Department of Agriculture (USDA)	USA	Wayne Wolf
National Measurement Institute, Australia (NMIA)	Australia	Lindsey Mackay
Institute for National Measurement Standards, National Research Council of Canada (CNRC)	Canada	Ralph Sturgeon
LGC Ltd. (LGC)	UK	Heidi Goenaga-Infante
Centre National de la Recherche Scientifique (PAU)	France	Joanna Szpunar
Oviedo University (OVI)	Spain	Alfredo Sanz-Medel
National Institute of Standards and Technology (NIST)	USA	Gregory Turk
CSIR-National Metrology Laboratory (CSIR)	South Africa	Alex Barzev
Laboratorio Tecnológico del Uruguay (LATU)	Uruguay	Raquel Huertas
Istituto Nazionale di Ricerca Metrologica (INRIM)	Italy	Enrico Rizzio
Laboratoire National d'Essais (LNE)	France	Guillaume Labarraque
Instituto Nacional de Tecnología Industrial (INTI)	Argentina	Liliana Valiente

participant along with a standard solution of natural-abundance SeMet in 0.1 M HCl (having an expected value of 956 mg kg^{-1} SeMet, which was unknown to participants). For species-specific IDMS measurements, an isotopically enriched standard of SeMet (^{76}Se-enriched SeMet) was provided. Participants were requested to report results on a dry weight basis for at least three replicate analyses along with uncertainty budgets [24]. This report details the results obtained for the determination of total Se and SeMet content in the CCQM-P86 Se-yeast tablets and discusses remaining challenges for the quantification of SeMet in complex food/supplement samples.

Experimental

Sample and calibration standards

The CCQM-P86 test sample was a yeast-based material incorporated into a pharmaceutical formulation (Pharma Nord, Vejle, Denmark) containing additional excipients such as cellulose, dicalcium phosphate, silicon dioxide, magnesium salts and titanium dioxide. The sample was in the form of a 300 mg tablet, packaged in a foil blister pack containing 15 tablets. The tablets were sealed in the pack under nitrogen and stored below 20°C prior to distribution to participants. Two foil packs were sent to each of the participants summarised in Table 1.

A solution of natural-abundance SeMet standard (with an expected value of 956 mg kg^{-1} SeMet) was provided by LGC in the form of a blind sample which served to help delineate any errors in the participant's results arising from analysis of this simple calibration solution from those which occur during analysis of the matrix sample. By providing the participants with the expected value for the SeMet mass fraction in this solution after their results for the CCQM-P86 sample had been submitted, resolution of any differences facilitated interpretation of results. The solid SeMet standard was produced and characterised for purity by Mikromol (99.57%; Luckenwalde, Germany). In order to ensure stability, the aqueous solution of SeMet was prepared by dissolving the solid standard in 0.1 M HCl. It was stored in the dark at 4°C prior to being sent to the participants under dry-ice conditions.

CRM SELM-1 (Se-yeast, NRCC, Ottawa, Canada) was distributed to all participants to assist with method evaluation.

Participants who intended to use ID for the determination of SeMet were provided with approximately 1 mg of an isotopically enriched ^{76}Se standard of SeMet (Isosciences, King of Prussia, USA). This material was not certified for isotopic composition, which, if required, had to be determined by each user. The isotopic enrichment of the inorganic ^{76}Se standard used to synthesise the ^{76}Se-enriched SeMet was 99.8%, as indicated by the manufacturer.

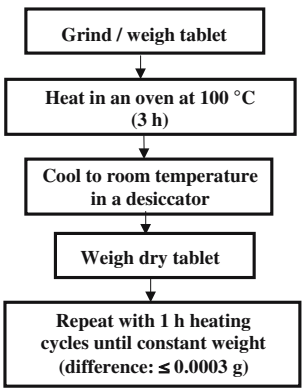

Fig. 1 The method for determination of tablet moisture content

Measurement method

As noted earlier, apart from recommending a procedure for the determination of moisture content in the tablets, as outlined in Fig. 1, no sample preparation/extraction method was prescribed by the coordinating laboratories. As a consequence, participants were free to develop and validate their own approaches, as summarised in Tables 2 and 3.

Determination of total Se

As evident from Table 2, most CCQM-P86 participants used ICP-MS with ID, external calibration or the standard additions technique for quantification of total Se. Exceptions were National Institute of Standards and Technology (NIST), Istituto Nazionale di Ricerca Metrologica (INRIM), US Department of Agriculture (USDA), Laboratorio Tecnológico del Uruguay (LATU) and Instituto Nacional de Tecnología Industrial (INTI), which used instrumental neutron activation analysis (INAA), GC-MS (after derivatisation of the acid digest), ICP optical emission spectrometry (OES) and flame atomic absorption spectrometry (FAAS), respectively. As digestion methods, microwave-assisted acid digestion and enzymatic probe sonication were used.

Determination of SeMet

For the determination of SeMet in the CCQM-P86 tablets and in the standard solution, the majority of the participants used HPLC-ICP-MS with species-specific ID, external calibration or standard additions, except for USDA and NRCC, which used GC-MS, and NIST, which used GC-ICP-MS. National Measurement Institute, Australia (NMIA) reported two independent results obtained by species-specific ID and standard additions. Oviedo University (OVI) used postcolumn ID analysis, as described for the first time by Heumann et al. [25], as the method of calibration. Different extraction techniques, including mul-

Table 2 Analytical methods and instrumental techniques (total Se)

Laboratory	Digestion method	Instrumentation	Calibration
BUD	Acid digestion, laboratory pressure cooker	ICP-MS (CC)	EC, SA
MAD	Enzymatic probe sonication	ICP-MS (CC)	EC
NIM	Microwave-assisted acid digestion	ICP-MS (CC)	ID (^{78}Se spike)
USDA	Acid digestion/chelation with 4-(3-trifluoromethyl)phenyldiazirine	GC-MS	ID (^{82}Se spike)
NMIA	Microwave-assisted acid digestion	ICP-MS (CC)	ID (^{74}Se spike)
LGC	Microwave-assisted acid digestion	ICP-MS (CC)	ID (^{77}Se spike)
PAU	Microwave-assisted acid digestion	ICP-MS (CC)	SA, IS (Rh)
OVI	Microwave assisted acid digestion	ICP-MS (CC), interference correction	ID (^{77}Se spike)
NIST	None	INAA	EC (one point)
CSIR	Microwave-assisted acid digestion	Hydride generation–ICP-MS (magnetic sector)	ID (^{82}Se spike)
LATU	Microwave-assisted acid digestion	ICP-OES	SA
IMRIM	None	INAA	EC
LNE	Microwave-assisted acid digestion	ICP-MS (CC)	ID (^{78}Se spike)
INTI	Microwave-assisted acid digestion	FAAS	EC (multipoint)

ICP inductively coupled plasma, *MS* mass spectrometry, *CC* collision cell, *GC* gas chromatography, *INAA* instrumental neutron activation analysis, *OES* optical emission spectrometry, *FAAS* flame atomic absorption spectrometry, *EC* external calibration, *SA* standard additions, *ID* isotope dilution, *IS* internal standard

tiple-step and single-step enzymatic hydrolysis, hydrolysis with methanesulfonic acid and derivatisation with CNBr [8] or methylchloroformate for GC-ICP-MS or GC-MS [6] were used for the quantification of SeMet in the solid sample. Table 3 gives an overview of the methods applied and the instrumentation used by each participant.

Since the main challenges of the study deal with the accurate quantification of SeMet in the tablets, the digestion procedure, instrumentation and calibration method used by each participant are briefly summarised below.

Corvinus University of Budapest

For speciation analysis, 200 mg of ground tablet was placed in a 15-mL polyethylene vial followed by the addition of 30 mg protease XIV dissolved in 4.5 mL 50 mmol L^{-1} potassium phosphate buffer (pH 7.4). The samples were stirred at 200 rpm at 37°C for 24 h. After proteolysis, samples were centrifuged at 4,100 *g* for 25 min at 15°C. The supernatants were filtered through a 0.45-μm cellulose nitrate syringe filter (Millipore, Tullagreen, USA). Freshly prepared digests were appropriately diluted before analysis. Quantification of SeMet was performed by HPLC with collision-cell ICP-MS (using H_2as the collision gas and monitoring the isotopes ^{78}Se and ^{80}Se) and external calibration with SeMet (Sigma Chemicals, St. Louis, MO, USA) standards. The separation was carried out on a Hamilton PRP-X100 anion-exchange column using a mobile phase of 200 mmol L^{-1} ammonium dihydrogen phosphate at pH 6.0 with isocratic elution [22].

Complutense University of Madrid

Enzymatic treatment and ultrasound probe sonication [21] was used for sample preparation of the yeast CRM and

Table 3 Analytical methods and instrumental techniques (selenomethionine, *SeMet*)

Laboratory	Extraction/derivatisation	Instrumentation	Calibration
BUD	Protease XIV, 24 h at 37°C + shaking (1 step)	HPLC-ICP-MS (CC)	EC, SA (multipoint)
MAD	Protease XIV + ultrasound probe sonication, 2 min (1 step)	HPLC-ICP-MS (CC)	ID (^{76}SeMet spike)
NIM	Protease + lipase, 18 h at 37°C (1 step)	HPLC-ICP-MS (CC)	ID (^{76}SeMet spike)
USDA	Methanesulfonic acid, 16 h, reflux, CNBr derivatisation	GC-MS	ID (^{76}SeMet spike)
NMIA	Methanesulfonic acid, 16 h, reflux	HPLC-ICP-MS (CC)	ID (^{76}SeMet spike)
NMIA	Methanesulfonic acid, 16 h, reflux	HPLC-ICP-MS (CC)	SA (multipoint)
NRCC	Methanesulfonic acid, 16 h, reflux, methyl chloroformate derivatisation	GC-MS	ID (^{13}C-SeMet spike)
LGC	Protease + lipase + driselase, 20 h at 37°C + mixing (repeated 2 times)	HPLC-ICP-MS (CC)	ID (^{76}SeMet spike)
PAU	Protease + lipase, 17 h at 37°C + stirring (repeated 3 times)	HPLC-ICP-MS (CC)	SA (multipoint)
OVI	Protease, 16 h at 37°C (1 step)	HPLC-ICP-MS (CC)	Postcolumn IDA (^{77}Se spike)
NIST	Methanesulfonic acid, 16 h, reflux, CNBr derivatisation	GC-ICP-MS (CC)	ID (^{76}SeMet spike)

HPLC high-performance liquid chromatography

tablets. For this, 20 mg protease XIV and 5 mL ultrapure water were added to approximately 50 mg of sample (yeast or ground tablets). The extracts obtained after 2 min of sonication were centrifuged at 7,500 *g* for 30 min and the supernatants were filtered. In order to avoid oxidation, β-mercaptoethanol (0.1%) was added to the hydrolysed samples, which were appropriately diluted before analysis. Samples were stored at 4°C for no longer than 2 h until they were analysed. Separation of selenocompounds was achieved using an anion-exchange column (Hamilton PRP-X100) with a mobile phase consisting of 10 mmol L^{-1} citric acid in 2% (v/v) methanol, adjusted to pH 5.0 with ammonium hydroxide. SeMet content in yeast, tablets and SeMet solution was determined by collision-cell ICP-MS using species-specific single ID analysis (IDA) by monitoring the isotope ratios $^{80}Se/^{76}Se$ and $^{78}Se/^{76}Se$.

National Institute of Metrology P. R. China

One-step enzymatic hydrolysis was performed by adding 3 mL of a 75 mmol L^{-1} tris(hydroxmethyl)aminomethane hydrochloride (Tris-HCl) buffer solution (pH 7.5) containing 40 mg protease and 20 mg lipase to approximately 0.2 g of the Se-yeast or ground tablet. Incubation at 37°C was carried out in the dark for 18 h. The hydrolysed samples were centrifuged at 3,000 rpm for 30 min and the supernatants were filtered and diluted appropriately before analysis. HPLC-ICP-MS measurements were performed using an Agilent Technologies 1100 HPLC system for chromatographic separations and an Agilent 7500c ICP-MS instrument (Agilent Technologies, Palo Alto, CA, USA) for element-specific detection. Double species-specific IDMS was achieved using the $^{80}Se/^{76}Se$ isotope ratio for calibration. Species separation was carried out on an Agilent Zorbax Rx-C_8 column (250 mm×4.6-mm inner diameter) with a particle size of 5 μm.

US Department of Agriculture

A ^{76}Se-enriched SeMet spike was added to one ground tablet (approximately 0.29 g) at the time of weighing. The samples were boiled overnight under reflux with 4 mol L^{-1} methanesulfonic acid and the digests were treated overnight at 37°C with CNBr and 2% (w/v) $SnCl_2$. The reaction product, CH_3SeCN, was extracted with chloroform, with the extracts stored at 4°C for no longer than 1 day prior to analysis [9]. Determination of SeMet was performed by GC-MS using an Agilent 6890 GC instrument with an Agilent 5973 MSD instrument and single species-specific IDA. For GC-MS measurements, negative chemical ionisation with methane reagent gas was used. The GC column was a 15-m Agilent HP-5MS. Ions at *m/z* 106 and *m/z* 102 for ^{80}SeCN and ^{76}SeCN, respectively, were monitored in the single ion monitoring mode. ^{76}Se-enriched SeMet solutions were calibrated against a natural abundance standard of SeMet (US Pharmacopeia, Rockville, MD, USA) by reverse IDMS.

NMIA using ID

Individual tablets were removed from the blister pack and crushed using a mortar and pestle. Approximately 0.3 g of the resulting powder was accurately weighed into glass round-bottom flasks and the SeMet spike solution (approximately 0.4–0.5 g) was added gravimetrically. The spike material used was ^{76}SeMet. Reflux extraction was performed with 25 mL of 4 M methanesulfonic acid at 110°C under nitrogen for 16 h. After extraction, the samples were diluted to 50 mL with Milli-Q water prior to analysis. Determination of SeMet was performed by HPLC-ICP-MS using exact matching double IDMS. Duplicate calibration blends were prepared using independently prepared working standards and the same spike solution as used for the sample blends. The calibration blends were prepared and diluted to 30 mL with 2 mol L^{-1} methanesulfonic acid. HPLC-ICP-MS measurements were performed using an Agilent 1100 HPLC instrument coupled to an Agilent 7500c ICP-MS instrument fitted with a collision cell. The collision cell was operated with no gas. The isotope ratios $^{78}Se/^{76}Se$, $^{77}Se/^{76}Se$ and $^{82}Se/^{76}Se$ were monitored. Confirmation analyses were also conducted with H_2 gas in the collision cell at 2 mL min^{-1} by monitoring the same isotope ratios as without collision gas. Reversed-phase ion-pairing HPLC separations were performed on a Phenomenex Luna C_{18} column (150 mm×2.0 mm) with 5-μm particle size, using water/methanol/heptafluorobutyric acid (85:15:0.1 v/v) as a mobile phase at a flow rate of 0.2 mL min^{-1}. Samples were analysed in three replicates, each bracketed by a mole-matched calibration blend.

NMIA using standard additions

The extraction of SeMet from the solid tablet was performed using the procedure described in the previous section but without the addition of the ^{76}SeMet spike. After extraction, the samples were diluted to 50 mL with Milli-Q water. A subsample of approximately 5 mL was removed and accurately weighed into a polypropylene container, to which 0.25 mL of selenocysteine (125 μg g^{-1} Se), as the internal standard, was introduced. A standard solution of SeMet was then added (approximately 0.2 g). The standard additions method used was a two-point calibration. Three replicates of each spiked sample were analysed in the sequence bracketed by the unspiked solution. The samples were analysed using an Agilent 1100 HPLC instrument

coupled to an Agilent 7500c ICP-MS instrument using the conditions described in the previous section.

National Research Council of Canada

Six sample blanks, nine subsamples of yeast tablets and three quality control samples of SELM-1 were prepared. Approximately 0.30 g each of the yeast tablet and SELM-1 was spiked with a suitable amount of ^{13}C-enriched SeMet. After addition of deionised water and methanesulfonic acid (resulting in a concentration of 4 mol L^{-1} for methanesulfonic acid and 30-mL volume in total), the contents were refluxed on a hot plate for 16 h. A 6-mL volume of extract was used for the derivatisation. After addition of 2.64 mL ammonium hydroxide and 4.50 mL methanol/pyridine (3:1 v:v) to a 24-mL glass vial containing 6 mL extract, 0.750 mL methyl chloroformate was slowly added. The vial was shaken manually for 1 min with venting. Chloroform (1 mL) was then added to the vial and the vial was capped. The vial was shaken manually for 10 s and vented. This step was repeated a second time. The vial was then shaken manually for 1 min. The chloroform layer was then transferred to a 1.8-mL glass vial for subsequent GC-MS analysis. Determination of SeMet was performed using GC-MS with IDA [6, 10]. The GC-MS instrument used for this work was a Hewlett-Packard HP 6890 gas chromatograph and a HP 5973 mass spectrometer (Agilent Technologies Canada, Mississauga, ON, Canada) fitted with a DB-5MS column (Iso-Mass Scientific, Calgary AL, Canada). Peak areas were used to generate ratios of selected ions, from which the analyte concentrations in the yeast tablets were calculated.

LGC

An appropriate quantity of ^{76}SeMet solution was added to a whole tablet (approximately 0.3 g), both accurately weighed to give a $^{78}Se/^{76}Se$ ratio of approximately 0.3. This was followed by the addition of 60 mg protease and 30 mg lipase in 10 mL of a previously degassed 30 mmol L^{-1} Tris-HCl buffer solution (pH 7.5). Incubation at 37°C was then carried out in the dark for 20 h. During enzymolysis, the sample slurries were constantly and gently homogenised, using a rotary shaker set at 60 rpm. Hydrolysed samples were centrifuged at 3,000 rpm for 30 min and the supernatants were filtered and stored at −20°C. The residue was then subjected to proteolytic digestion with one more fresh buffered enzymatic solution also containing 100 mg driselase (used to release cell-wall-bound components). Finally, the two supernatants were pooled, filtered and diluted 100-fold prior to their analysis for Se speciation. Determination of SeMet was performed using HPLC-ICP-MS with double species-specific IDMS (using the isotope ratios $^{78}Se/^{76}Se$ and $^{82}Se/^{76}Se$). HPLC-ICP-MS measurements were performed using an Agilent Technologies 1200 HPLC system (Palo Alto, CA, USA) for chromatographic separations and an Agilent 7500ce ICP-MS instrument for element-specific detection. Reversed-phase ion-pairing HPLC was performed on an Agilent Zorbax Rx-C_8column (250 mm×4.6-mm inner diameter, 5-μm particle size) using a 98:2 water/methanol mixture containing 0.1% (v/v) trifluoroacetic acid (as ion-pairing agent) as the mobile phase at a flow rate of 0.9 mL min^{-1}.

Centre National de la Recherche Scientifique

The whole tablet (approximately 0.29 g) was incubated (20 h, 37°C) with 5 mL lipase/protease (10/20 mg) in 30 mmol L^{-1} Tris-HCl containing dithiothreitol. After centrifugation (2,500 rpm, 5 min), the procedure was repeated on the residue; in total, three consecutive incubations were carried out. The combined supernatants were analysed (after suitable dilution) by anion-exchange HPLC coupled with collision-cell ICP-MS (in H_2 mode) and quantification was performed using the standard additions technique at three spike levels in the presence of a 10 ppb Rh internal standard. HPLC separations were performed on a Hamilton PRP-X100 anion-exchange column using an Agilent Technologies 1100 HPLC system. A gradient elution of acetic acid and triethylamine at pH 4.7 was used [26]. Se-specific detection of the compounds separated by HPLC was performed using an Agilent 7500ce ICP-MS instrument.

Oviedo University

Tablets were ground immediately before extraction using an agate grinding vessel and a ball mill grinder. For extraction, 20 mg protease and 5 mL of Milli-Q water were added to 0.2 g of sample and the mixture was then incubated for 16 h at 37°C. Hydrolysed samples were further centrifuged and filtered (0.45 μm). Extracts were diluted approximately 1:20 with ultrapure water before injection into the HPLC system. Those samples were analysed on the same day as the extraction. Chromatographic separation of Se species from extracted tablets and also from the 0.1 M HCl solution of SeMet (which was diluted 1:1,000 by weight) was performed using an anion-exchange HPLC column (Hamilton PRP-X100) coupled to an Agilent 7500c ICP-MS instrument (in H_2 mode). Ammonium citrate (0.5 mol L^{-1}, pH 5.0) was used as the mobile phase at a flow rate of 0.9 mL min^{-1}. To quantify species, postcolumn ID was used [12]. A ^{77}Se-enriched standard solution of the appropriate concentration was continuously introduced using a peristaltic pump (at a flow rate of 0.1 mL min^{-1}) at the end of the column through a T-piece. The ^{76}Se, ^{77}Se,

^{78}Se and ^{82}Se isotope intensities were continuously monitored over the whole chromatogram. Intensity chromatograms were converted, after adequate mathematical treatment, into mass flow chromatograms. The integration of the peak corresponding to SeMet (using Origin® software) provided the amount of Se present in that peak.

National Institute of Standards and Technology

The method used for extraction and derivatisation of SeMet was based on previous work by Yang et. al. [9]. Briefly, three sample blanks, four subsamples of the SELM-1 control material and ten CCQM-P86 tablets were processed by accurately weighing approximately 0.25 g of SELM-1 or one whole CCQM-P86 tablet (about 0.3 g) into a precleaned 50-mL digestion tube and combined with an accurately weighed aliquot of the isotopically enriched ^{76}SeMeth solution spike. The spiked samples were then diluted to 18 mL with Milli-Q water and then 6 mL methanesulfonic acid was added. The contents were then placed in an Environmental Express HotBlock™ digestion system and refluxed at 90°C for 16 h. The extracts were then cooled to room temperature and centrifuged at 2,000 rpm for 7 min. Approximately 1-mL volume of the supernatant was transferred to a 7-mL amber glass vial and 0.475 mL ammonia hydroxide, 1.48 mL of 4% (w/v) $SnCl_2$ in 0.2 mol L^{-1} HCl and 0.50 mL of 3 mol L^{-1} CNBr in $CHCl_2$ were added. The vials were capped and shaken on a vortex mixer for 5 min and maintained at 37°C for 24 h. The derivatised analyte from the blanks, SELM-1 and tablets was extracted into 1, 6 and 4 mL of chloroform, respectively, for GC-ICP-MS with IDA. In addition, a stock solution of 3,616 ug g^{-1} natural SeMet was prepared by accurately weighing dl-SeMet (Sigma-Aldrich) crystals (stated purity 99%) and dissolving the sample in 1% HCl, after which they were stored at 20°C. A natural-abundance SeMet sample was also prepared from this stock solution in order to determine the instrumental mass bias. For GC-ICP-MS measurements, a Thermo Fisher Scientific TRACE GC Ultra™ instrument (Thermo Fisher, Waltham, MA, USA) and a Thermo Fisher Scientific X7 ICP-MS instrument were used. GC separations were performed on a 30-m J & W Scientific DB-5MS+DG column (J & W Scientific, Folsom, CA, USA) with a 250-μm inner diameter and a stationary phase of 5% diphenyl/95% dimethylpolysiloxane (df=250 μm).

Results and discussion

The CCQM-P86 sample, NRCC CRM SELM-1 and any requested standards were distributed to all participants several days after an accompanying letter containing the protocol explaining the work to be conducted and a 'results report' form for submission of data had been sent. General instructions for handling and preservation of sample and calibration standards, instructions for determination of tablet moisture content, a request to report results for at least three replicate analyses on a dry weight basis (in milligrams per kilogram) and a request for a full description of the extraction and measurement procedures as well as an evaluation of an uncertainty budget in accordance with the principles of the *Guide to the expression of uncertainty in measurement* [24] was made.

Prior to the distribution of the test samples, an assessment of their homogeneity was conducted to ensure that this

Table 4 Moisture content and methods

Participant laboratory	Moisture content (%)	Method
BUD	5.90; 4.05; 4.93; 9.38; 5.33; 7.14 (mean 6.12, n=6)	Recommended method
MAD	4.42; 4.00; 4.94; 4.41; 4.44 (mean 4.44, n=5)	Recommended method
USDA	3.36; 3.46; 3.25; 3.06 (mean 3.28, n=4)	Recommended method
NMIA	3.0; 2.9; 2.8; 3.3 (mean 3.0, n=4)	Recommended method
LGC	3.76; 3.57; 3.70; 3.59; 3.66; 3.66 (mean 3.66, n=6)	Recommended method
PAU	3.46; 2.71; 3.92; 3.13 (mean 3.31, n=4)	Recommended method
OVI	8.38; 8.98; 8.33; 8.89 (mean 8.64, n=4)	Recommended method
NIST	1.88; 3.98; 3.39 (mean 3.08, n=3)	Recommended method
CSIR	3.61; 3.55 (mean 3.58, n=2)	Recommended method
LATU	5.0 (n=1)	Modified method (4-h heating at 105°C)
INRIM	1.49 (n=1)	Modified method (0.6 g, drying over P_2O_5 until constant weight)
LNE	3.9; 3.8 (mean 3.85, n=2)	Recommended method
INTI	2.2; 2.47 (mean 2.34, n=2)	Modified method (2-h heating with subsequent 1-h intervals)
NIM	Mean 4.32 (n=4)	Recommended method
NRCC	Mean 4.16 (n=4)	Modified method (4-h heating with subsequent 1-h intervals)

Table 5 Results for the determination of total Se in CCQM-P86 tablets

Participant	Reported result (mg kg^{-1})	Expanded uncertainty (k=2) (mg kg^{-1})	Relative uncertainty (%)
INRIM	306.3	1.3	0.4
USDA	316.6	26.6	8.4
LATU	320	25	7.8
NIST	335.7	5.1	1.5
BUD	337	16	4.7
NMIA	337	13	3.8
INTI	337	29	8.6
MAD	337.6	7.1	2.1
CSIR	338.7	10	3.0
LGC	342	3.2	0.9
NIM	345	7	2.0
OVI	347	8	2.3
LNE	347	9	2.6
PAU	348.3	4.0[a]	1.1

[a] Standard deviation (1s, n=3)

would not be a limiting characteristic of the study. Between-tablet homogeneity for total Se and SeMet mass fractions was undertaken at coordinating laboratories of LGC and NRCC, respectively. For total Se, double IDMS measurements by collision-cell ICP-MS were performed on solutions arising from microwave-assisted acid digestion of the whole tablet. The relative standard deviation (RSD; 1s, n=20) was 1.3%. For SeMet, hydrolysis with methanesulfonic acid prior to derivatisation and quantitation using GC-IDMS was used. The RSD (1s, n=11) was 3.6%. These levels of variability were deemed sufficiently small (fit for purpose) that this factor would not compromise the results of this study.

A method for determination of tablet moisture content, summarised in Fig. 1, was recommended for all participants to follow. The majority utilised this approach, except for LATU, INRIM, INTI and NRCC, which used their own method or the proposed method with minor modifications. Table 4 summarises methods and results submitted for moisture content by all participants. Typically, a minor contribution to the overall uncertainty budget (0.1% or less) was derived from correction for moisture. Average moisture content based on measurements from 14 laboratories (except INRIM, which reported a moisture value 2.8-fold lower than the average value) was 4.19%.

Determination of total Se in the tablets

Results for total Se were acceptable for the majority of the participants and are summarised in Table 5 (dry-weight basis) and are graphically displayed in Fig. 2. All uncertainties reported are expanded uncertainties with a

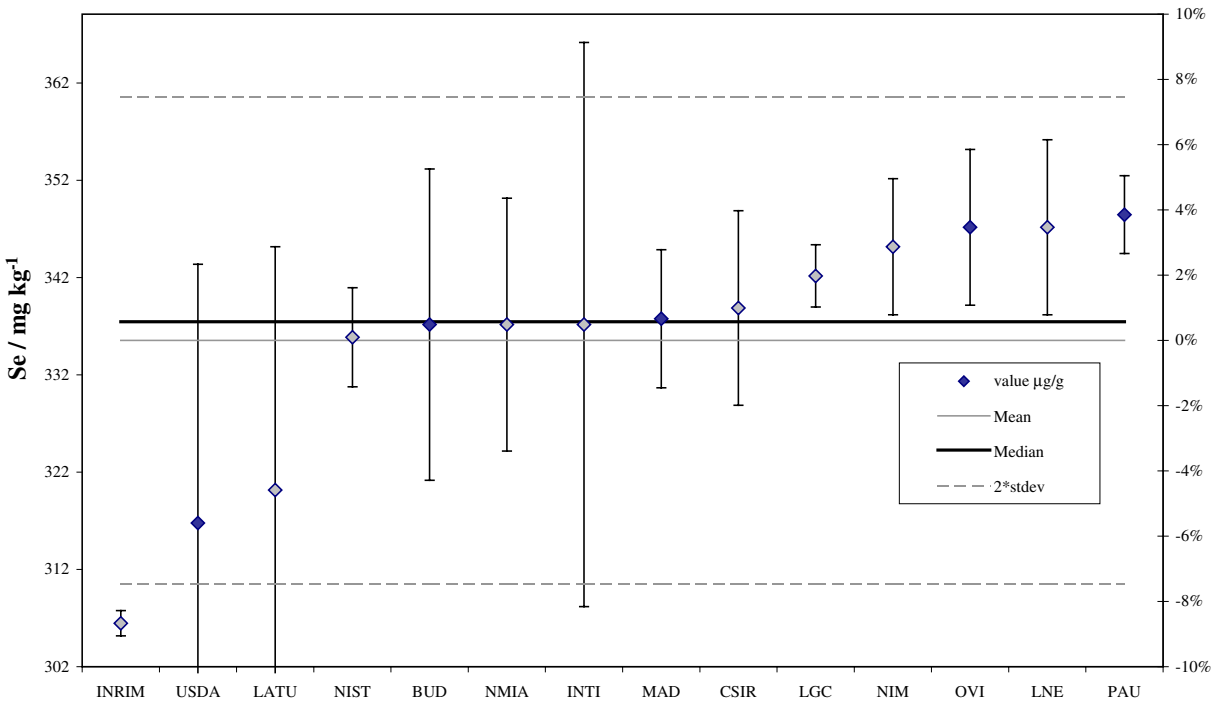

Fig. 2 Results for total Se in CCQM-P86 yeast tablets. Data presented in *light grey* depict results reported by national metrology institutes (NMIs). *Error bars* depict combined expanded uncertainties, except for PAU [standard deviation (1s), n=3]. The *grey line* and the *black line* represent the mean and the median of all data, respectively. The *dashed lines* are 2 times the standard deviation (1s) of all data. See Table 1 for an explanation of the acronyms for the participants

coverage factor (k) of 2, except for Centre National de la Recherche Scientifique (PAU), which reported their standard deviation (1s, n=3).

The data submitted by INRIM are biased approximately 10% lower than the median value. Their reported moisture content was also unusually low (1.49 %) in comparison with the mean of other participants (4.19%). INRIM subsequently reported that problems had occurred during determination of moisture content using their in-house method and, when repeating the measurement using the method proposed in the CCQM-P86 protocol, a value of 2.91% was achieved, which resulted in a total Se concentration of 315.5±10.2 mg kg^{-1}, in reasonable agreement with the results reported by the other participants.

Table 5 also shows that the relative expanded uncertainty was lower than 5% for the majority of participants, except for LATU (7.8%), USDA (8.4%) and INTI (8.6%). Although USDA used IDA with GC-MS after derivatisation of the acidic digest, their results for total Se in the tablets show a relatively large expanded uncertainty which arises, in part, from one of the individual results that could be considered an outlier; however, this result was included by the participant as there was no known experimental reason to justify its exclusion. LATU and INTI used ICP-OES and FAAS, respectively (Table 3), for instrumental analysis. The remainder of the participants used INAA or ICP-MS after microwave-assisted acid digestion; the majority used ID for quantitation.

Determination of SeMet

Control sample SELM-1

As noted earlier, the existing NRC CRMSELM-1 (Se-yeast) was sent to all participants and was intended to assist with method evaluation. The coordinating laboratories did not indicate a need to report results for this material. However, nine participants reported ten results for SeMet in SELM-1; these are summarised in Table 6. Standard deviations for PAU and NMIA were not calculated since standard deviation data for so few data points (n=2) are almost meaningless.

The certified value for SeMet in SELM-1 (which was 3,431±157 mg kg^{-1}) was recently revised by the NRCC to 3,389±173 mg kg^{-1} SeMet. This was done completely independently of this CCQM-P86 intercomparison and as part of the NRCC's quality system, which specifies periodic examination of the stability of their CRMs so as to be able to assign shelf life and account for any degradation. Recovery data were thus calculated on the basis of the revised certified value of 3,389±173 mg kg^{-1} SeMet.

For the majority of the participants, the results for the determination of SeMet in CRM SELM-1 presented in Table 6 are within the window defined by the revised certified value and its associated expanded uncertainty. It is interesting to note that, when competently used, a standard additions calibration technique also yields acceptable data (compared with IDMS). This is the case with the NMIA data, which are based on sample hydrolysis with methanesulfonic acid prior to determination by HPLC-ICP-MS. The mean results submitted by the USDA and Complutense University of Madrid (MAD) were both outside the certified range but biased only approximately 1% or less lower than the certified range. The results submitted by Corvinus University of Budapest (BUD) are biased significantly low (23 %) with respect to the certified value. This is likely because standard additions calibration coupled with single-step enzymatic hydrolysis for extraction of SeMet was

Table 6 Results for determination of SeMet in certified reference material SELM-1

Participant	Mean of results (mg kg^{-1})	Standard deviation (1s) (mg kg^{-1})	n	Recovery of certified value (%)
BUD	2,602	38	3	76.8
USDA	3,179	70	4	93.8
MAD	3,215	122	5	94.9
LGC	3,235	34	3	95.4
NMIA (ID)	3,311	Not calculated	2	97.7
NMIA (SA)	3,390	Not calculated	2	100.0
NRCC	3,314	14	6	97.8
OVI	3,329	44	3	98.2
PAU	3,353	Not calculated	2	98.9
NIST	3,396	141	4	100.2

Table 7 Results for the determination of SeMet in CCQM-P86 tablets

Participant	Reported result (mg kg^{-1})	Expanded uncertainty (k=2) (mg kg^{-1})	Relative uncertainty (%)
BUD	483	10	2.1
MAD	502	11	2.2
NIM	521	17	3.3
USDA	540.4	15.6	2.9
NMIA-1 (ID)	575	53	9.2
NMIA-2 (SA)	564	56	9.9
NRCC	580	41	7.1
LGC	588	27	4.6
PAU	596	33[a]	5.5
OVI	612	28	4.6
NIST	615	24	3.9

[a] Standard deviation (1s, n=3)

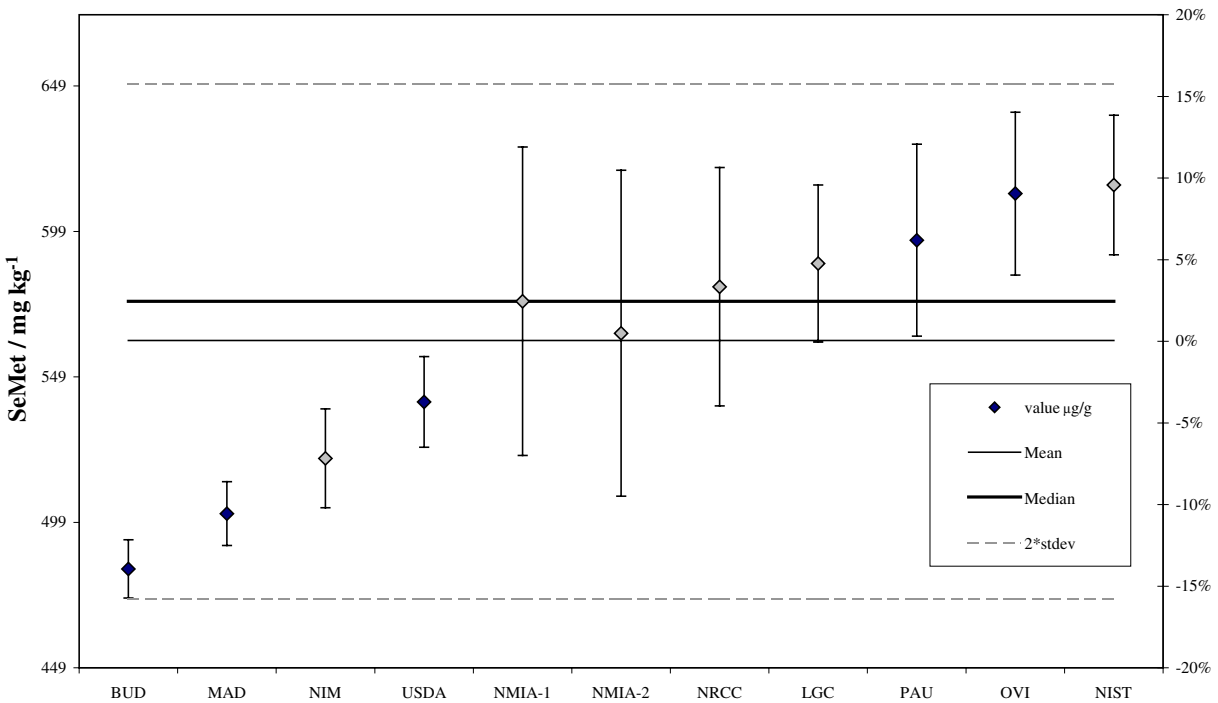

Fig. 3 Results for selenomethionine (SeMet) in yeast tablets. Data presented in *light grey* depict results reported by NMIs. *Error bars* depict combined expanded uncertainties (k=2), except for PAU [standard deviation (1s), n=3]. The *grey line* and the *black line* represent the mean and the median of all data, respectively. *The dashed line* are 2 times the standard deviation (1s) of all data. NMIA-1: ID; NMIA-2: SA

used. Such an approach has been reported to provide relatively poor recoveries of SeMet from Se-yeast [10].

CCQM-P86 tablets and SeMet solution

Ten participants reported 11 results for SeMet in the CCQM-P86 tablets. The results are summarised in Table 7 (dry-weight basis) and are graphically displayed in Fig. 3; most participants used ID. All uncertainties reported are expanded uncertainties with a coverage factor (k) of 2, except for PAU, which reported the standard deviation of the data (1s, n=3). The data presented in Table 7 show that the relative expanded uncertainties range from 2.1 to 9.9%; quite respectable considering that there is a sixfold decrease in the total concentration of Se in the CCQM-P86 sample compared with the SELM-1 control [10]. It appears that acid digestion with methanesulfonic acid used by NRCC, NMIA (using both, ID and standard additions), USDA and NIST as well as the multistep enzymatic extraction methods (used by PAU and LGC) provided higher extraction efficiencies for SeMet, in agreement with conclusions reported previously [2, 7, 10]. Earlier studies have demonstrated that the extraction efficiency achieved with enzymatic methods critically depends on the extraction conditions. However, the use of multistep enzymatic procedures and/or the use of large quantities of enzymes have resulted in extraction efficiencies similar to those achieved with the acid hydrolysis method. In comparison with previously developed protocols, that used by LGC wherein a combination of proteolytic and cell-wall-degrading enzymes (e.g. driselase) was used in reasonable amounts and with relatively larger solvent volumes enabled consistent results to be obtained with a two-step enzymatic digestion.

The single-step enzymatic extraction methods used by OVI, NIM and BUD were quite similar. They also all used HPLC-ICP-MS for quantitation. Again, likely because BUD used standard additions for calibration, their result is 16% lower than the median of all results and their recovery of SeMet from CRM SELM-1 is relatively poor. Although OVI and NIM used ID calibration, their reported values differed

Table 8 Results for the determination of SeMet in the standard solution (expected value 956 mg kg^{-1} SeMet)

Participant	Reported result (mg kg^{-1})	Expanded uncertainty (k=2) (mg kg^{-1})	Relative uncertainty (%)
NIM	753	8	1.1
PAU	777	57[a]	7.3
MAD	856	19	2.2
NRCC	953	25	2.6
USDA	961.8	16.2	1.7
NMIA	964	36	3.7
OVI	1,019	31	3.0
BUD	1,138	102	9.0

[a] Standard deviation (1s, n=3)

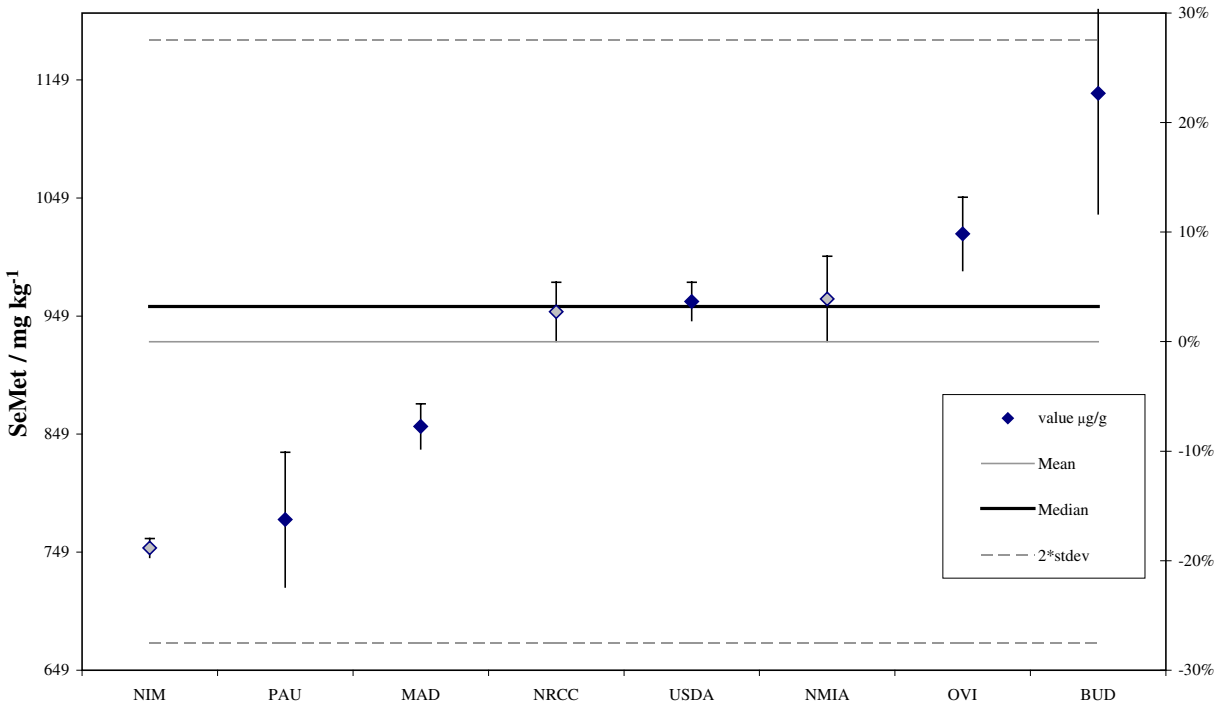

Fig. 4 Results for SeMet in standard solution. Data presented in *light grey* depict results reported by NMIs. *Error bars* depict combined expanded uncertainties, except for PAU [standard deviation (1*s*), $n=3$]. The *grey line* and the *black line* represent the mean and the median of all data, respectively; the median overlaps the expected value. The *dashed lines* are 2 times the standard deviation (1*s*) of all data

by 17%. Similar results obtained in a previous study [10] attributed this to a large potential variation in the efficiency of digestion when enzymes of different activities were used.

As noted earlier, a standard solution of SeMet was made available in the form of a blind sample which served to help delineate any errors in participant's results arising from analysis of this simple calibration solution from those which occur during analysis of the matrix sample. Results for SeMet in the standard solution are summarised in Table 8 and are graphically displayed in Fig. 4. LGC and NIST, although participating in the determination of SeMet, did not report results for the standard solution. Difficulties were encountered getting this solution through USA Customs, so NIST never received it. Unfortunately, LGC sent the standard solution a second time to participants that requested it, without keeping record of the number of available vials, with the consequence that there was no solution left for analysis by LGC when this process was completed. All uncertainties reported in Table 8 are expanded uncertainties having a coverage factor (k) of 2, except for PAU, which reported the standard deviation of the data (1*s*, $n=3$).

Table 8 and Fig. 4 show that the results from OVI (using postcolumn ID) and from NIM (using species-specific ID) for the analysis of the SeMet standard solution differ by about 30%, suggesting that the disparity in their SeMet results for the tablets likely arises owing to the use of their different ID calibration approaches (rather than a consequence of undertaking digestion with enzymes of different activities). No explanation for this disagreement can be offered at this time, as both ID approaches to quantitation have been shown to be fundamentally sound [12, 25].

The results reported for SeMet in the tablets by MAD based on single-step enzymatic probe sonication and ID are approximately 13% lower than the median. Concurrently, their result for the SeMet solution is 10.5% lower than the expected value. These observations suggest a problem with quantitation of the isotopic spike, preparation of the reverse spike, or inaccuracy in the dosing of the spike to the samples. As evident in Table 8 and Fig. 4, results for SeMet in the standard solution reported by NRCC, USDA and NMIA agree very well with the median, which also overlaps the expected value of 956 mg kg^{-1} for this solution.

Table 9 Summary statistics for CCQM-P86

	Total Se in CCQM-P86 tablets[a]	SeMet in CCQM-P86 tablets
Mean (mg kg^{-1})	337.6	561.5
Median (mg kg^{-1})	337.6	575.0
Standard deviation (mg kg^{-1})	9.7 (13)	44.3 (11)
Relative standard deviation (%)	2.9	7.9
Standard deviation of the mean (mg kg^{-1})	0.74	4.0

[a] Excluding the result reported by INRIM

Summary statistics for this study are presented in Table 9. In view of the differences amongst the approaches used to calculate the expanded uncertainties, the median is the preferred statistic characterising these data [27]. For most participants using ID calibration for determination of SeMet, measurement of the reference-to-spike ratio in the sample blend contributed most to the overall uncertainty.

The results for total Se presented in Table 9 exclude those reported by INRIM for reasons given earlier. For SeMet, no result was excluded in an effort to preserve the integrity of the data and the overall robustness of the study.

Conclusions

The results presented in this study have demonstrated that the determination of SeMet in pharmaceutical yeast tablets is a challenging exercise, since the recovery of this analyte critically depends on both the extraction procedure and the calibration procedure used. Either hydrolysis with methanesulfonic acid or a simplified two-step enzymatic extraction (combining proteolytic and cell-wall-degrading enzymes) of the whole tablet can be recommended with quantitation by ID using GC-ICP-MS or HPLC-ICP-MS. The use of species-specific ID has proven to minimise the effects of any potential species transformation on the accuracy of the resultant data. However, using ID, one can achieve high-quality data only if a natural-abundance SeMet primary standard, well characterised for purity and content, is available and if isotopic equilibration between the isotopically enriched SeMet (spike) and the natural-abundance SeMet in the sample is assured.

Quantitative speciation data obtained for a SeMet standard solution (with an expected concentration value) by the measurement method of those participants undertaking SeMet analysis of the tablets have proven, for the first time, to be a very useful tool to distinguish any errors arising from calibration from those occurring during extraction of SeMet from the yeast matrix.

The performance of the majority of the participants was very good, illustrating their ability to obtain accurate results for total Se and SeMet in the pharmaceutically formulated tablets of a complex matrix (containing approximately 300 μg kg^{-1} Se) with 10% expanded uncertainty.

As noted in earlier studies [28], not only the major Se species (SeMet), but also minor species (e.g. selenomethionine selenoxide hydrate, selenomethyl selenocysteine and γ-glutamyl selenomethyl selenocysteine) must also be considered. Further efforts on the characterisation of Se-yeast reference materials for speciation validation should, therefore, be pursued. In this vein, the quality of the results achieved in this study should also be realised for the determination of other Se species (and total Se) in foods or supplement matrices provided due diligence is taken to ensure an appropriate extraction process is achieved and species-specific spikes are available for quantitation by ID. Indeed, having accepted such conditions, application to quantitation of other organometallic species in similar matrices should be possible with the same level of performance. Application to biological matrices in general (e.g. tissue, serum), however, cannot be confidently extrapolated because of the potentially significantly lower mass fractions of such species in these matrices and the potential impact of the matrix on the extraction efficiency.

Acknowledgements LGC particularly acknowledges the efforts of E. Warburton and the Reference Materials Team, especially D. Curtis for help with sending the samples and standards to the participants. Special thanks go to S. Moesgaard from Pharma Nord, Vejle, Denmark, for providing the CCQM-P86 tablets. The pilot laboratories acknowledge, in particular, the efforts of R. Harte for sending the relevant documents to the participants and for collecting the data. The work described in this paper was supported by the UK Department for Innovation, Universities & Skills as part of the National Measurement System Chemical & Biological Metrology Knowledge Base Programme.

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