Certification of a Reference Material (CRM) of Selenium enriched yeast

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Abstract. Certified reference materials (CRMs) are essential tools for ensuring food safety, playing a critical role in methods development, validation, as well as internal and external quality control. CRMs are also used in the verification of the accuracy of analytical methods, for the measurement uncertainty estimation, to assess the traceably of the analytical results or the calibration of analytical instruments. In this work, we present the production and certification process of a reference material (selenium-enriched yeast). The studies for the certification of reference material (CRM 8969.0001) were realized by the Inorganic Analysis Laboratory (Labin) of the National Institute of Metrology, Quality and Technology (Inmetro). The batch preparation (lot), the homogeneity, stability, characterization studies are presented. The certified values includes total selenium (total Se) content (2637 ± 139) mg/kg and selenomethionine (SeMet) content (3248 ± 325) mg/kg.

1. Introduction

CRMs contribute decisively to increasing the confidence of measurement results, with the aim of providing metrological traceability. These materials are used to calibrate instruments, assign value to physical/chemical properties of materials, validate methods of measurement and ensure the quality of processes [1].

Dietary supplements formulated with Se and its species have been increasingly consumed due to beneficial health effects. However, it is crucial to avoid poorly formulated supplements that exceed the recommended concentrations set forth by Government Agencies, as they may lead to adverse health effects, as has been observed in various countries [2]. Yeasts are the most studied and used as base for supplements, they are the most acceptable by consumers, have high protein content, low cost, are rarely toxic and can be used in their raw state [3]. Some yeast materials contain more than 60 species of Se [4], with SeMet being the most abundant.

The bioavailability and potential toxicity of an element are closely tied to its chemical species. Obtaining relevant information on these species is essential for accurately evaluating the risks and benefits associated with dietary supplements.

As a National Institute of Metrology, Inmetro holds the authority and responsibility for implementing metrology in critical sectors of the country, including health preservation, safety, environmental protection, consumer safeguarding, and prevention of deceptive trade practices. Within this framework, Inmetro has developed a non-existent (in Brazil) Certified Reference Material (CRM) to aid food supplement manufacturers and testing laboratories in evaluating the quality of their products.

In this regard, the CRM, total Se and SeMet in yeast, main raw material used for the manufacture of dietary supplements enriched with selenium, will be a fundamental tool to guarantee the reliability of the total Se and of SeMet measurement results in dietary supplements.

The production planning and subsequent certification studies were carried out according to the requirements of ISO 17034:2016 [5], ISO Guide 35:2017 [6].

2. Production and Certification Methodology

CRM production requires careful planning, selection of subcontractors, assignment of property values and their uncertainties etc., according to ISO 17034:2016 [5]. Some information regarding the lot, its production and certification are described below:

2.1. Name and description of the material

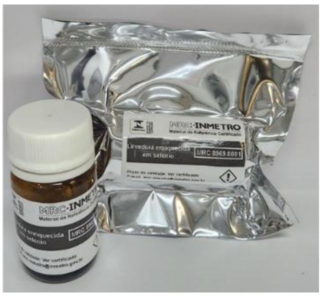

Figure 1. CRM 8969.0001

The CRM 8969.0001 "Selenium-enriched yeast" consists of an amber-type glass bottles with a capacity of 30 mL containing approximately 8 g of *Saccharomyces cerevisiae yeast*.
Certified available at: *http://www.inmetro.gov.br/metcientifica/mrc-descricao/mrc-8969.asp*

2.2. Intended use

The CRM is intended to validate methods and guarantee the quality of the results of the mass fraction of total Se and SeMet in *Saccharomyces cerevisiae yeast.* This CRM can be used as a calibrant for a closely similar sample matrix. If the measurement of SeMet mass fraction does not utilize a calibrant with a closely similar matrix, the appropriate method extraction and moisture corrections should be employed. It is important to note that this material is not intended for nutritional, medical, or diagnostic purposes, and its commutativity has not been assessed.

2.3. Batch Preparation (lot)

Planning and certification studies were conducted following the requirements of ISO 17034:2016 [5], ISO Guide 35:2017 [6], and the Technical Standards of Inmetro. Stringent safety procedures and good laboratory practices were implemented during the material preparation process. For the production of material lot, about 2kg of selenized yeast obtained from a local producer was manually homogenized and bottled (8 g) in a 30 mL amber-type glass bottles. The units were bottled under argon atmosphere and subsequently irradiated using 25 kGy of gama radiation by exposure to cobalt (^{60}Co) source for sterilization purpose. The bottles were protected from light in aluminium package.

2.4. Certification and metrological traceability

The digestion and extraction procedures, along with the validation of methods, and can be found in previous work [7-9]. Inductively coupled plasma optical emission spectrometry (ICP OES) was employed for the measurement of total Se, for homogeneity study, transport, and storage stability studies, as well as for the characterization studies, utilizing external calibration. Additionally, for the characterization, the ICP OES technique was used with one-point calibration strategy as second independent methodology [5]. For the measurement of SeMet, homogeneity and transport stability studies were conducted using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) with a C_{18} chromatographic column and external calibration. Storage stability and characterization studies were performed using ultraperformance liquid chromatography in combination with triple quadrupole mass spectrometry (UPLC-MS/MS) with a C_{18} column and external calibration. Additionally, for the characterization process, the UPLC-MS/MS technique with one-point calibration was employed. Metrological traceability to the SI was established with the use certified reference material SRM 3149 (National Institute of Standards and Technology-NIST) and CRM Selm-1 (National Research Council Canada-NRCC) for the total Se and SeMet, respectively.

3. Results and discussion

3.1. Homogeneity study

For this study, twelve randomly selected bottles from the batch were analized. Figure 2 shows the mass fraction of total Se and SeMet versus the bottle filling order.

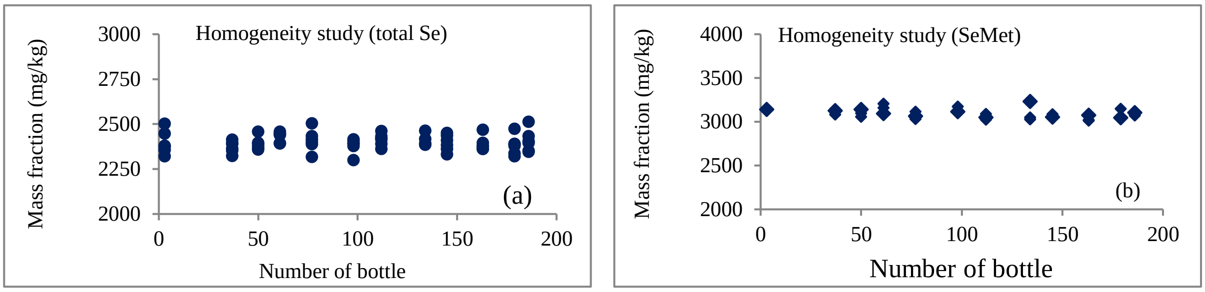

Figure 2. Homogeneity study for total Se (a) and SeMet (b)

The mass fraction of total Se and SeMet showed a visually random behavior, indicating that further studies could proceed to evaluate the standard uncertainty associated with the heterogeneity/homogeneity (u_{hom}) between bottles. The ANOVA test was conducted to assess u_{hom}. MQ_{within} (variation within units) > $MQ_{between}$ (variation between units) then the equation 1 was utilized to calculate u_{hom}. The results are presented in table 1.

Table 1. Standard uncertainty associated with heterogeneity/homogeneity.

Standard uncertainty associated to heterogeneity between bottles (total Se mass fraction)	Standard uncertainty associated to heterogeneity between bottles (SeMet mass fraction)
u_{bb} = 8.0 mg/kg u_{bb} = 0.33%	u_{bb} = 15 mg/kg u_{bb} = 0.48%

$$u_{hom} = \sqrt{\frac{MQ_{within}}{n_0}}\sqrt[4]{\frac{2}{\nu_{MQwithin}}} \tag{1}$$

Where: u_{hom} is the uncertainty of heterogeneity/homogeneity between bottles; MQ_{within} is the variation within units; n_0 is the number of replicas; $\nu_{MQwithin}$ are the degrees of freedom

3.2. *Stability study*

The stability study aims to determine the degree of instability of a reference material or confirm its stability. The stability of the reference material was studied over a short period (transport stability study) and another for long period (storage stability study).

3.2.1. Transport stability study (20 and 35) °C. The objective of this study is to simulate and determine the transport conditions of the sample, employing the isochronous model. Two units (bottles) were extracted from the freezer (-22 ± 7) °C every week and subjected to the study temperatures (20 and 35) °C. After a duration of 6 weeks, the samples were analyzed under repeatability conditions. To assess the lot's behaviour over time, a graph illustrating the total Se mass and SeMet fraction as a function of study time at a temperature of 20°C was generated (figure 3).

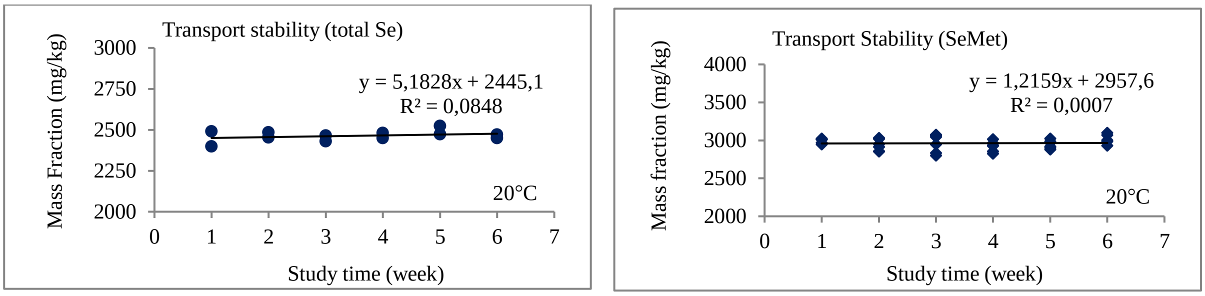

Figure 3. Transport stability study (20 ºC) for total Se and SeMet

The significance of the regression slope was evaluated using the criterion: $|\alpha| < t_{\alpha,n-2} \cdot S_a$, where $|\alpha|$ represents the slope, $t_{\alpha,n-2}$ denotes Student's t value for the 95% significance level and degrees of freedom (n-2), and S_a stands for the standard error associated with the regression slope. The mass fraction of total Se remained stable throughout the 6-week study at 20 °C, with p=0.35 (greater than 0.05), and 5.18 < 11.99. Similarly, the mass fraction of SeMet was considered stable, with p=0.88 (greater than 0.05), and 1.21 < 16.06. The standard uncertainty due to instability/stability at the transport temperature of 20 °C was estimated using equation 2, and the results are presented in table 2.

Table 2. Standard uncertainty associated with the transport stability of the material (20 °C)

Standard uncertainty associated with the transport stability (total Se mass fraction)	Standard uncertainty associated with the transport stability (SeMet mass fraction).
u_{trn} = 27 mg/kg u_{trn} = 1.1 %	u_{trn} = 47 mg/kg u_{trn} = 1.6 %

$$u_{trn} = s_b . t \quad (2)$$

Where: u_{trn} is the standard uncertainty associated with the transport stability; S_b is the standard error for the estimated slope; t is time.

The mass fraction of total Se was found to be stable during the 6-week at 35 ºC but, the data revealed a concerning degradation of SeMet at this temperature. These results are consistent with the observations reported in the CRM Selm-1 certificate, where it was shown that at 44 °C, the mass fraction of SeMet also decreased with time.

3.2.2. Storage stability study.

Long-term stability studies are conducted to evaluate the material's stability under specific storage conditions throughout its product lifetime. For this study, two units (bottles) were extracted from the freezer (-22 ± 7) °C and subjected to analysis. In the classic stability study, the samples are prepared simultaneously (same batch) under identical conditions but analyzed separately at different times, characterizing a reproducibility condition. The stability of the material was assessed using regression analysis, similar to the approach employed in the transport stability study. To examine the sample's behaviour over time, graphs illustrating the mass fraction of total Se and SeMet versus the study time were created (see Figure 4).

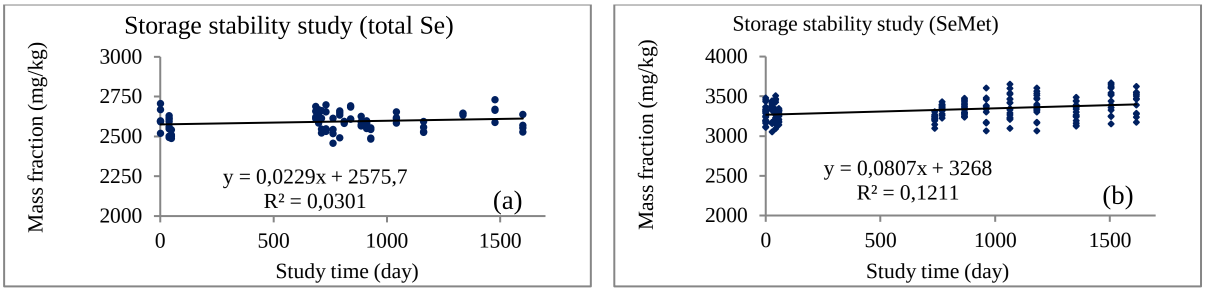

Figure 4. Storage stability study for total Se (a) and SeMet (b)

Total Se: Throughout the study period, the mass fraction of total Se was determined to be stable, with p=0.12 (greater than 0.05) and 0.022 < 0.029. The standard uncertainty related to storage instability/stability was calculated using equation 3, and the results are presented in table 3.

Table 3. Standard uncertainty associated with the storage stability of the material.

Standard uncertainty associated with the storage stability: total Se mass fraction.
u_{lts} = 23 mg/kg ; u_{lts} = 0.9%

$$u_{lts} = s_b . t \quad (3)$$

Where: u_{lts} is the standard uncertainty associated with the transport stability; s_b is the standard error for the estimated slope; t is time.

SeMet: The mass fraction of SeMet was not considered stable; the data displayed a statistically significant trend, with $p=3.38 \times 10^{-07}$ (less than 0.05), and 0.08 > 0.03. The statistically significant slope in the data may have been influenced by factors such as time lag, equipment changes, and the recertification of the CRM Selm-1. In this scenario, the observed data aligns with the guidelines outlined in item 8.7.4 "Assessment of stability uncertainties in the case of a known significant trend" of ISO Guide 35:2017 [6]. Equation 4 takes into account the uncertainty calculated in item 8.7.3 and incorporates an additional increase in uncertainty using the rectangular distribution method. Table 4 provides the standard uncertainty associated with the storage stability of the material.

Table 4. Standard uncertainty associated with the storage stability of the material

Standard uncertainty associated with the storage stability: SeMet mass fraction
$u_{(lts)tend}$ = 44 mg/kg; $u_{(lts)tend}$ = 1.3 %

$$u_{(lts)tend} = \sqrt{\left(\frac{V_{t0}-V_{tpred}}{2\sqrt{3}}\right)^2 + \left(S(b_1).(t_{m1}+t_{cert})\right)^2} \quad (4)$$

Where: $u_{(lts)tend}$ is the standard uncertainty associated with long term stability in the presence of significant trend; V_{t0} is the value of the quantity monitored in the stability study at time $t_{0;}$ V_{tpred} is the value of the quantity monitored in the stability study over time t_{pred} (estimated time for degradation); s_{b1} is the standard error for the estimated slope; t_{m1} is the time interval between assigning value and starting point of stability monitoring; t_{cert} is the certificate validity period 2 years after material is released.

3.3. Repeated use study

Experimental studies were handled in accordance with the guidelines ISO Guide 35:2017 [6], particularly when reusing the same CRM unit (bottle). In this instance, the same bottle was repeatedly analyzed until its contents were depleted. Subsequently, regression analysis was conducted, and the obtained data are illustrated in figure 5.

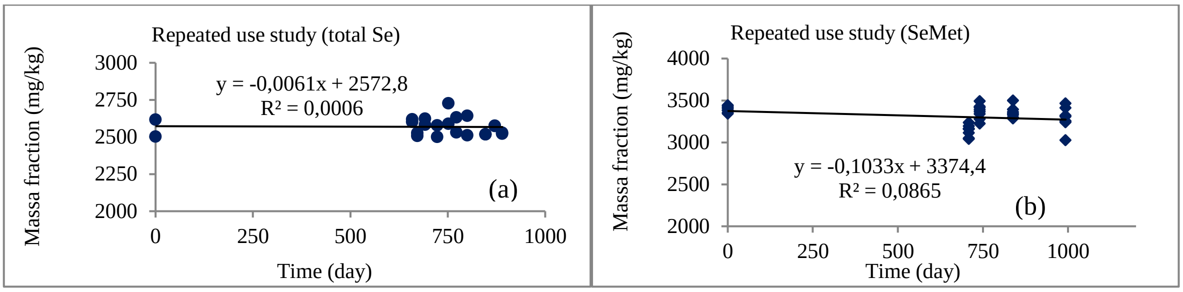

Figure 5. Repeated use study for total Se (a) and SeMet (b)

Throughout the studied period (890 days), the mass fraction of total Se was determined to be stable, with $p=0.91$ (greater than 0.05), and $0.006 < 0.115$. Similarly, the SeMet mass fraction was considered stable during the period studied (933 days), with $p=0.065$ (greater than 0.05), and $0.103 < 0.110$.

3.4. Characterization

The mass fraction of total Se was determined using 6 units (bottles). Table 5 presents the measured values for the mass fraction and the expanded uncertainty estimated from the characterization study of total Se. The analysis was conducted using both the ordinary least squares (OLS) calibration curve and value transfer with one-point calibration.

Table 5. Total Se characterization by (OLS) calibration curve and by one point calibration

	(OLS) calibration curve	one-point calibration
Arithmetic average (mg/kg)	2587	2686
Expanded uncertainty (mg/kg)	59	95

The uncertainty value obtained through one-point calibration was higher than one achieved through the calibration curve, mainly due to the uncertainty value of Selm-1 used as calibrant for one-point calibration, which is approximately 3.4 % (2031 ± 70) mg/kg. The equation 5 was employed to assess the agreement between the mass fraction value of total Se obtained through the OLS calibration curve and one-point calibration.

$$\left|x_{cal\ curve} - x_{one\ point\ cal}\right| < k\sqrt{{u_{cal\ curve}}^2 + {u_{one\ point\ cal}}^2}: 99\ \text{mg/kg} < 111\ \text{mg/kg} \quad (5)$$

Hence, there was consistency between the total Se mass fraction values obtained through the OLS calibration curve and one-point calibration.

For the determination of the SeMet mass fraction, 6 units (bottles) were used. Table 6 displays the measured values for the mass fraction and measurement uncertainty pertaining to the SeMet characterization study. The analysis was performed using the GLS calibration curve and value transfer with one-point calibration.

Table 6. SeMet characterization by calibration curve (GLS) and by one point calibration.

	Calibration curve (GLS)	One point calibraton
Arithmetic average (mg/kg)	3256	3239
Expanded uncertainty (mg/kg)	255	318

The uncertainty value obtained through one-point calibration was higher than that obtained through the GLS calibration curve due to the uncertainty value of Selm-1 used for calibration, which is approximately 8 % (3190 + 260) mg/kg. This directly impacts the combined uncertainty value. Equation 5 was utilized to assess the agreement between the mass fraction value of SeMet obtained through the GLS calibration curve and one-point calibration, and the comparison showed consistency of the results.

3.5. Certification

The values assigned to the mass fraction of Se and SeMet parameters were determined by the arithmetic mean of the values obtained from the calibration curve and one-point calibration. The uncertainty was then calculated using the quadratic sum of the uncertainties obtained from both methods. Assuming independence of the variables, the uncertainty associated with a property value was computed using equation 6.

$$u_{comb\ CRM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{trn}^2 + u_{lts}^2} \qquad (6)$$

The certified values of CRM are shown in table 7.

Table 7. Mass fraction and expanded uncertainty of CRM 8969.0001

	Total Se	SeMet
(w ± U) mg/kg	2637 ± 110	3248 ± 319

3.6. Post-certification stability monitoring

The mass fractions of total Se and SeMet in CRM 8969.0001 were measured during storage and are currently being monitored in ongoing studies. The certified values have exhibited stability and remained within the expanded uncertainty limits, without any indication of trend, meeting the acceptance criteria of the normalized error equation for both property values.

3.7. Extension MRC validity period.

The validity period of CRM 8969.0001 has been extended until 2028, based on studies and the extension of validity of CRM Selm-1, which has been extended until 2032. Both CRM Selm-1 and CRM 8969.0001 consist of *Saccharomyces cerevisiae* yeasts with closely similar matrices. They were cultivated in selenium-enriched medium at the same concentrations and under identical culture conditions [10]. Additionally, both materials underwent irradiation using 25 kGy of ^{60}Co and were packaged in amber-type glass bottles. To ensure the stability of SeMet, the material units (bottles) were stored at -20 °C. The storage stability uncertainty was reevaluated, considering the validity period of 6 years in accordance with item 8.7.3 of ISO Guide 35:2017 [6]. New expanded uncertainty values were determined and are presented in table 8.

Table 8. Mass fraction and new expanded uncertainty of CRM 8969.0001.

	Total Se	SeMet
(w ± U) mg/kg	2637 ± 139	3248 ± 325

3.8. Information on transport and storage

The material is stable at a temperature of 20 °C for a maximum of 6 weeks. However, due to temperature variations that can occur during transport, the material must be transported with ice or gel. The responsibility for transporting, as well as maintaining these conditions, is the customer's. All related transport and security information are contained in the MSDS (Material Safety Data Sheet), available at the website (http://www.inmetro.gov.br/metcientifica/formularios/form_mrc.asp).

4. Conclusions

The CRM 8969.0001 was produced, comprising 200 amber-type glass bottles with a 30 mL capacity, each containing approximately 8 g of *Saccharomyces cerevisiae* yeast.

The lot was found to be homogeneous for total Se and SeMet. The stability studies, which simulated transport conditions, revealed that the lot remained stable for both parameters throughout the 6-week study period. In the study of storage stability, total Se and SeMet parameters were also found to be stable.

The recalculations were made considering a validity period of 6 years, based on studies of the closely similar material, Selm-1. The risks associated with these changes were deemed to be low in comparison to the benefits gained. This extension will allow customers to have a longer period to use the material.

The validity of CRM 8969.0001 was extended to August 31, 2028.

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