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Production and certification of a reference material of selenium enriched yeast

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ABSTRACT
Certified Reference Materials (CRMs) are indispensable for ensuring food safety, playing a fundamental role in method development, validation, and both internal and external quality assurance. Given the growing consumption of selenium-based dietary supplements and the associated health risks of improper dosage, the availability of specific CRMs is crucial for regulatory compliance and consumer protection. This study presents the production and certification process of a selenium-enriched yeast reference material. The certification of this CRM (CRM 8969.0001) was conducted by the Inorganic Analysis Laboratory (Labin) of the National Institute of Metrology, Quality and Technology (Inmetro). The work comprises the procedures for batch preparation, homogeneity assessment, stability studies, and material characterisation. The certified values are: total selenium (Se) at (2637 ± 139) mg/kg and selenomethionine (SeMet) at (3248 ± 325) mg/kg. This CRM provides a critical tool for Brazilian manufacturers and analytical laboratories to ensure the quality and safety of selenium supplements.

Section: RESEARCH PAPER

Keywords: selenium; selenomethionine; enriched yeast; CRM production

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1. INTRODUCTION

Certified Reference Materials (CRMs) play a crucial role in enhancing the reliability of measurement results by ensuring metrological traceability. They are employed to calibrate instruments, assign values to physical and chemical properties of materials, validate measurement methods, and guarantee process quality [1].

Dietary supplements containing selenium (Se) and its various species have seen increasing consumption due to their recognised health benefits. However, it is essential to avoid poorly formulated products that exceed the recommended concentrations established by government agencies, as such excess may cause adverse health effects, as reported in several countries [2]. Yeasts are among the most studied and commonly used bases for supplements, as they are well accepted by consumers, rich in protein, low-cost, rarely toxic, and usable in their raw state [3]. Some yeast materials contain more than 60 selenium species [4], with SeMet being the most abundant. The bioavailability and potential toxicity of selenium are closely linked to its chemical species.

Metrology is fundamental for ensuring that measurements remain stable, comparable, and accurate, providing confidence [5]. As Brazil's National Metrology Institute, Inmetro is responsible for implementing metrology in critical sectors such as public health, safety, environmental protection, consumer safeguarding, and the prevention of unfair trade practices. Within this context, Inmetro has developed, for the first time in Brazil, a CRM to assist food supplement manufacturers and testing laboratories in assessing product quality. Currently, only two selenium-enriched yeast CRMs are listed in the COMAR Database (Selm-1 – NRCC and MRC 8969 – Inmetro), while the CRM SEEY-1 (yeast isotopically enriched in ^{82}Se) is available from the NRCC website. The availability of a Brazilian CRM is therefore crucial to advancing scientific excellence, fostering economic development, reducing dependence on foreign standards, and improving accessibility for users across Brazil and

Latin America. This contributes to regional sustainability, self-sufficiency, and technological sovereignty.

Production planning and subsequent certification studies were conducted in accordance with ISO 17034:2016 [6] and ISO Guide 35:2017 [7].

2. EXPERIMENTAL

2.1. Reagents, materials, consumables, and instrumentation

Reagents: Methanol (MeOH, HPLC grade ≥ 99.9 %); acetonitrile (ACN, HPLC/spectroscopic grade ≥ 99.9 %); tetrabutylammonium hydroxide (TBAOH, ≥ 97 %); ammonium dihydrogen phosphate ($NH_4H_2PO_4$, ≥ 99.5 %); trifluoroacetic acid (TFA); protease type XIV (*Streptomyces griseus*); lipase type VII (*Candida rugosa*); nitric acid (HNO_3, 65 %, sub-boiling distillation); and Type I water (resistivity 18 MΩ·cm).

Materials: Certified Reference Material (CRM Selm-1); Standard Reference Material (SRM 3149); yeast raw material supplied by a national manufacturer (*Saccharomyces cerevisiae* strain enriched with selenium to a target mass fraction of 2000 mg/kg) [8].

Consumables: PVDF syringe filters, pore size 0.45 µm; C_{18} Luna column, 150 mm × 2 mm × 3 µm (Phenomenex); C_{18} column, 50 mm × 2.1 mm × 1.7 µm (Waters).

Instrumentation: Analytical balance (Sartorius, ME 235S); pH meter MP 230 (Mettler Toledo); vortex mixer AP 56 (Phoenix); thermostat shaker NT 712 (Nova Ética); centrifuge Z300K (Hermle); air oven (Nova Ética); high-pressure asher HPA-S (Anton Paar); high-performance liquid chromatography (HPLC) system (PerkinElmer, Flexar) coupled to an inductively coupled plasma mass spectrometer (ICP-MS, Elan DRC II, PerkinElmer); ultra-performance liquid chromatography (UPLC) system (Waters, Acquity); mass spectrometer (MS, Waters Xevo™ TQ MS); inductively coupled plasma optical emission spectrometer (ICP-OES, Jobin Yvon-Horiba, ULTIMA 2).

3. A PRODUCTION AND CERTIFICATION METHODOLOGY

3.1. Certification protocols

The production of a CRM requires meticulous planning, including the selection of subcontractors, the assignment of property values, and the evaluation of associated uncertainties. Certification studies, which include homogeneity, stability, and characterisation assessments, were conducted in accordance with ISO 17034:2016 [6], ISO Guide 35:2017 [7], and Inmetro's Technical Standards.

3.2. Batch preparation

Strict safety procedures and good laboratory practices were observed throughout the preparation process. For batch production, approximately 25 kg of selenised yeast, obtained from a local supplier, were manually homogenised and dispensed in 8 g portions into 30 mL amber-type glass bottles. The units were filled under an argon atmosphere and subsequently sterilised by exposure to gamma radiation (25 kGy) from a cobalt-60 source. The bottles were then protected from light by being vacuum-sealed in aluminium packaging (Figure 1).

3.3. Name and description of the material

Figure 2 shows CRM 8969.0001, "Selenium-Enriched Yeast", presented in 30 mL amber-type glass bottles, each containing approximately 8 g of *Saccharomyces cerevisiae* yeast.

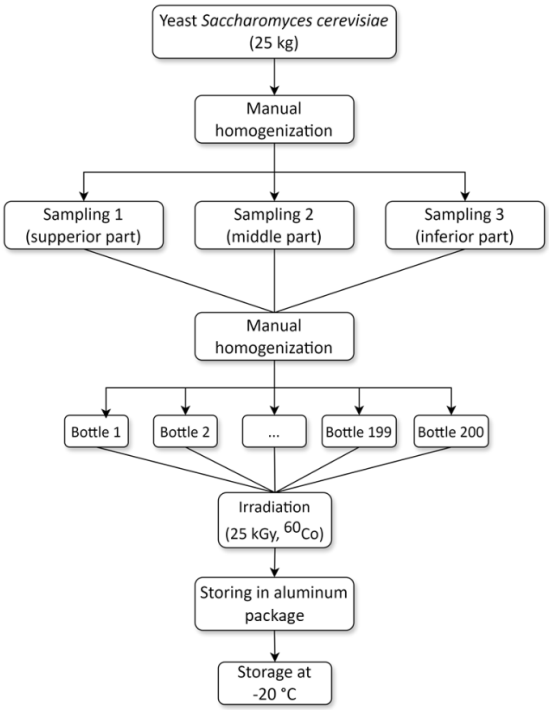

Figure 1. Main production steps of the yeast CRM batch.

Figure 2. CRM 8969.0001. Information available at: http://www.inmetro.gov.br/metcientifica/mrc-descricao/mrc-8969.asp

3.4. Intended use

This CRM is intended to validate analytical methods and to ensure the quality of results for the mass fractions of total selenium (Se) and selenomethionine (SeMet) in *Saccharomyces cerevisiae* yeast. It can also be used as a calibrant for samples with a closely comparable matrix. When measuring the SeMet mass fraction without such a matrix-matched calibrant, appropriate extraction procedures and moisture corrections must be applied.

It should be emphasised that this material is not intended for nutritional, medical, or diagnostic purposes, and its commutability has not been evaluated.

3.5. Analytical methods and metrological traceability

The digestion and extraction procedures, along with method validation, are detailed in previous studies [9], [10]. The operational conditions for the determination of total Se and SeMet are summarised in Table 1.

Table 1. Operational conditions for Se total and SeMet determination.

Operational condition for Se total determination by ICP OES	
Radio frequency potency (W)	1400
Principal gas flow rate (L/min)	16
Auxiliary gas flow rate (L/min)	0.4
Nebulizer gas flow rate (L/min)	0.8
Sample flow rate (mL/min)	1.3
Wavelength (nm)	196
Operational condition for SeMet determination by HPLC-ICPMS	
HPLC	
Column C_{18} Luna; Phenomenex (mm)	150 × 2 × 0.003
Pre-column; Phenomenex (mm)	4 × 2
Mobile phase	TBAOH (0.05 mM); $NH_4H_2PO_4$ (0.5 mM); ACN (1 %)
ICP-MS	
Radio frequency potency (W)	1350
Principal gas flow rate (L/min)	15
Auxiliary gas flow rate (L/min)	1
Nebulizer gas flow rate (L/min)	0.96
Isotope	^{82}Se
Operational condition for SeMet determination by UPLC-MS/MS	
UPLC	
Column C_{18}; Waters, USA (mm)	50 × 2.1 × 0.0017
Mobile phase	0.05 % TFA in MeOH:H_2O
ESI-MS/MS	
Collision energy m/z 109; 181 (V)	22; 10
Cone tension (V)	15
Capillary tension (kV)	3
MS/MS transitions (m/z):	198>109; 198>181

ICP-OES with external calibration was employed for the measurement of total Se in homogeneity, transport and storage stability studies, as well as in characterisation assessments. In addition, during characterisation, ICP-OES was applied using a one-point calibration strategy as an independent calibration method [6].

For SeMet measurement, homogeneity and transport stability studies were conducted using HPLC-ICP-MS with a C_{18} chromatographic column and external calibration. Storage stability and characterisation studies were performed using UPLC-MS/MS with a C_{18} column and external calibration. Furthermore, in characterisation, UPLC-MS/MS was also applied with a one-point calibration strategy.

Metrological traceability to the International System of Units (SI) was established through the use of the Certified Reference Materials SRM 3149 for total Se and CRM Selm-1 for SeMet.

4. RESULTS AND DISCUSSION

The results of the certification studies for the mass fraction of total Se and SeMet are presented in Figure 3 to Figure 6. In all graphs, the ordinate axis corresponds to the mass fraction values (mg/kg), while the abscissa axis represents the number of units investigated in the homogeneity study (Figure 3), the study time in weeks for transport stability (Figure 4), the study time in days for storage stability (Figure 5), and the study time in days for repeated-use (Figure 6).

4.1. Homogeneity study

Twelve bottles were randomly selected from the batch and analysed. Figure 3 shows the mass fraction of total Se and SeMet plotted against the bottle filling order. Both analytes exhibited a visually random distribution, suggesting sufficient homogeneity of the material and allowing the estimation of the standard uncertainty associated with between-bottle variation (u_{hom}).

The ANOVA test was applied to assess u_{hom}. Since the within-unit variance exceeded the between-unit variance ($MQ_{\mathrm{within}} > MQ_{\mathrm{between}}$), equation (1) was used for the calculation:

$$u_{\mathrm{hom}} = \sqrt{\frac{MQ_{\mathrm{within}}}{n_0}} \sqrt[4]{\frac{2}{\nu_{\mathrm{MQwithin}}}}, \quad (1)$$

where u_{hom} is the standard uncertainty of between-bottle heterogeneity, MQ_{within} is the variance within units, n_0 is the number of replicates, and ν_{MQwithin} is the associated degrees of freedom. The results are summarised in Table 2.

4.2. Stability study

The stability studies were conducted to assess potential degradation of the CRM, both under transport and long-term storage conditions.

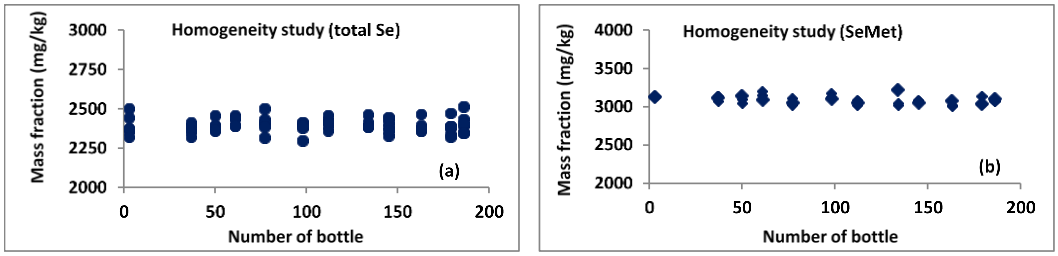

Figure 3. Homogeneity study for total Se (*left*) and SeMet (*right*).

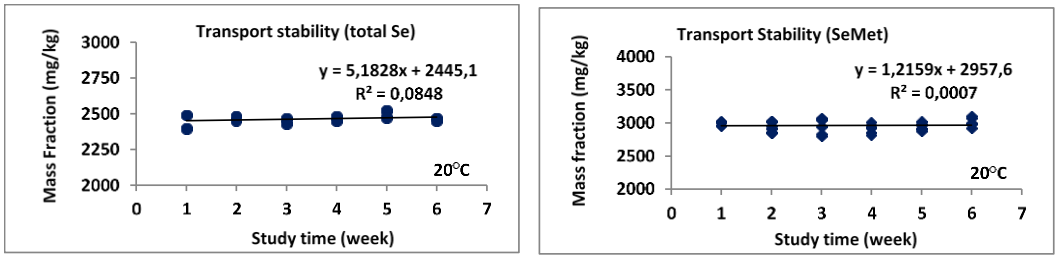

Figure 4. Transport stability study (20°C) for total Se (*left*) and SeMet (*right*).

4.2.1. Transport stability study (20 and 35) °C

The objective of this study was to simulate and determine the transport conditions. The transport stability was evaluated using the isochronous model. Two bottles were removed weekly from the freezer (–22 ± 7 °C) and exposed to the study temperatures (20 °and 35 °C) over six weeks. At the end of this period, the samples were analysed under repeatability conditions. Figure 4 shows the mass fraction of total Se and SeMet plotted against the study

Table 2. Results of certification studies CRM 8969.0001.

Homogeneity		
	u_{hom} (mg/kg)	u_{hom} (%)
Total Se mass fraction	8.0	0.33
SeMet mass fraction	15	0.48
Transport stability		
	u_{trn} (mg/kg)	u_{trn} (%)
Total Se mass fraction	27	1.1
SeMet mass fraction	47	1.6
Storage stability		
	u_{lts} (mg/kg)	u_{lts} (%)
Total Se mass fraction	23	0.9
SeMet mass fraction	44	1.3
Characterisation		
mg/kg	**Arithmetic average**	**Expanded uncertainty**
Calibration curve (OLS) – total Se	2587	59
One-point calibration – total Se	2686	95
Calibration curve (GLS) - SeMet	3256	255
One-point calibration - SeMet	3239	318
Certified values		
mg/kg	**Certification**	**Certification new uncertainty extended**
Total Se mass fraction	2637 ± 110	2637 ± 139
SeMet mass fraction	3248 ± 319	3248 ± 325

time at 20 °C. The significance of the regression slope was evaluated using the criterion: $|\alpha| < t_{\alpha,n-2}\, S_a$, where $|\alpha|$ is the slope, $t_{\alpha,n-2}$ is the Student's t value (95 % confidence level, n - 2 degrees of freedom), and S_a is the standard error of the slope.

At 20 °C, both total Se (5.18 < 11.99 and p = 0.35 > 0.05) and SeMet (1.21 < 16.06 and p = 0.88 > 0.05) were considered stable. The standard uncertainty due to transport stability (u_{trn}) was calculated using equation (2):

$$u_{trn} = s_b \cdot t, \tag{2}$$

where u_{trn} is the standard uncertainty associated with transport stability, s_b is the standard error of the slope, and t is the study time. The results are presented in Table 2.

At 35 °C, total Se remained stable, but a notable degradation of SeMet was observed. This finding aligns with the CRM Selm 1 certificate, which reported a decline in SeMet at 44 °C over time.

4.2.2. Storage stability study

Long-term storage stability was assessed to evaluate the material under recommended conditions throughout its certified lifetime. Two bottles were periodically removed from the freezer (–22 °C ± 7 °C) and analysed. The assessment followed the classic model (samples analysed at different times) and the regression approach applied in the transport stability study. Figure 5 shows the mass fraction of total Se and SeMet plotted against the study time. Over the study period, total Se was found to be stable (0.022 < 0.029 and p = 0.12 > 0.05). The corresponding uncertainty was calculated using equation (3):

$$u_{lts} = s_b \cdot t, \tag{3}$$

where u_{lts} is the standard uncertainty related to storage stability, s_b is the standard error of the slope, and t is the study time. The results are presented in Table 2.

In contrast, SeMet displayed a statistically significant trend (0.08 > 0.03 and $p = 3.38 \times 10^{-7}$ < 0.05). This trend may have been influenced by factors such as time lag, equipment changes, or the recertification of CRM Selm-1. In line with ISO Guide 35:2017 [7], section 8.7.4 ("Assessment of stability uncertainties in the case of a known significant trend"), equation (4) was applied to incorporate an additional uncertainty component using the rectangular distribution:

$$u_{(lts)tend} = \sqrt{\left(\frac{V_{t0} - V_{tpred}}{2\sqrt{3}}\right)^2 + [s(b_1) \cdot (t_{m1} + t_{cert})]^2}\,, \tag{4}$$

where $u_{(lts)tend}$ is the standard uncertainty associated with long-term stability in the presence of a 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} (the estimated time for degradation), $s(b_1)$ is the standard error for the estimated slope, t_{m1} is the time interval between assigning a value and the starting point of stability monitoring, and t_{cert} is the certificate validity period, 2 years after the material is released. The calculated uncertainties for storage stability are given in Table 2.

4.3. Repeated use study

The repeated use study was conducted with the recommendations of ISO Guide 35:2017 [7], which addresses the reuse of the same CRM unit. In this study, a single bottle was repeatedly analysed until its contents were exhausted. Regression analysis was then applied to evaluate potential trends, and the results are presented in Figure 6.

Over the monitored period of 890 days, the mass fraction of total Se was shown to be stable (0.006 < 0.115 and p = 0.91 > 0.05). Similarly, the mass fraction of SeMet was also considered stable over the 933-day period (0.103 < 0.110 and p = 0.065 > 0.05). These findings confirm that the repeated opening and use of a single CRM unit do not compromise the stability of either total Se or SeMet within the evaluated timeframe.

4.4. Characterisation

The mass fraction of total Se was determined using six units (bottles). The measured values and corresponding expanded uncertainties obtained from the characterisation study are summarised in Table 2. Two calibration strategies were employed: (i) an ordinary least

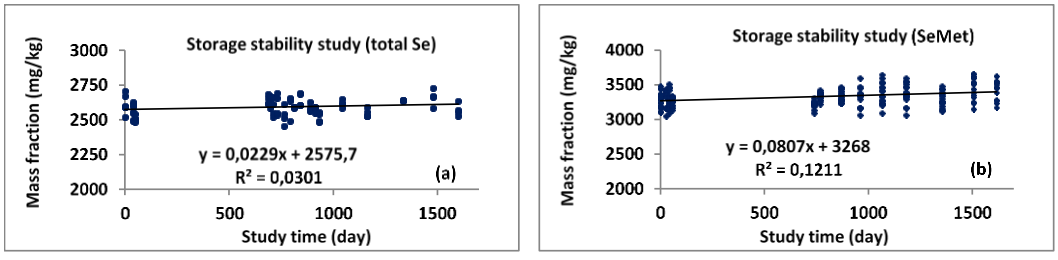

Figure 5. Storage stability study for total Se (*left*) and SeMet (*right*).

squares (OLS) calibration curve, and (ii) value transfer using one-point calibration.

The expanded uncertainty associated with one-point calibration was higher than that obtained with the calibration curve. This difference is mainly attributed to the uncertainty of Selm-1, used as the calibrant in the one-point calibration, which has a certified value of (2031 ± 70) mg/kg, corresponding to a relative uncertainty of approximately 3.4 %.

To evaluate the agreement between the mass fraction values of total Se obtained by the OLS calibration curve and by one-point calibration, equation (5) was applied.

$$\left|x_{\text{cal curve}} - x_{\text{one-point cal}}\right| < k \cdot \sqrt{u_{\text{cal curve}}{}^2 + u_{\text{one-point cal}}{}^2}, \quad (5)$$

where $x_{\text{cal curve}}$ is the mass fraction of total Se obtained by the calibration curve, $x_{\text{one-point cal}}$ is the mass fraction of total Se obtained by one-point calibration, k is the coverage factor, $u_{\text{cal curve}}$ is the uncertainty of total Se obtained by the calibration curve, and $u_{\text{one-point cal}}$ is the uncertainty of total Se obtained by one-point calibration.

Hence, the comparison demonstrated consistency between the mass fraction values of total Se obtained by the OLS calibration curve and one-point calibration, 99 mg/kg < 111 mg/kg.

For the determination of the SeMet mass fraction, six units (bottles) were analysed. The measured values and associated uncertainties are presented in Table 2. Two calibration strategies were employed: (i) a generalised least squares (GLS) calibration curve, and (ii) value transfer using one-point calibration.

The comparison demonstrated consistency between the mass fraction values of SeMet obtained through the GLS calibration curve and one-point calibration, 17 mg/kg < 412 mg/kg.

4.5. Certification

The certified values for the mass fraction of total Se and SeMet were established as the arithmetic mean of the results obtained from both the calibration curve and one-point calibration approaches. The associate uncertainty was calculated using the quadratic sum of the uncertainties obtained from both methods. Assuming independence of the contributing sources, the combined standard uncertainty ($u_{\text{comb CRM}}$) for a certified property value was calculated according to equation (6):

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

where u_{char} is the uncertainty associated with characterisation, u_{hom} is the uncertainty associated with homogeneity, u_{trm} is the uncertainty associated with transport stability, and u_{lts} is the uncertainty associated with storage stability. The final certified values, along with their expanded uncertainties, are summarised in Table 2.

The relative contributions of each source of uncertainty are illustrated in Figure 7. As observed, characterisation uncertainty accounted for the largest share of the overall certified value uncertainty, most likely due to the greater number of contributing factors, including instrumental repeatability, gravimetric preparation of samples and standards, and the mathematical model of the calibration curve. Further details on the uncertainty components related to characterisation are available in previous studies [9], [10], [11]. In contrast, owing to the high batch homogeneity, the contribution of homogeneity uncertainty was the smallest among all sources.

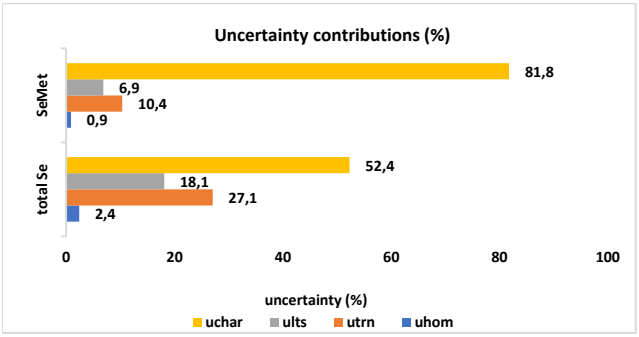

Figure 7. Uncertainties contributions.

4.6. 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.

4.7. Extension of the CRM validity period

The validity of CRM 8969.0001 was extended until 2028, following the extension of CRM Selm-1 to 2032. Both CRMs are based on *Saccharomyces cerevisiae* yeasts with highly comparable matrices [8], cultivated in selenium-enriched media at the same concentrations and under identical culture conditions [12]. In addition, both materials were irradiated with 25 kGy of ^{60}Co and packaged in amber-type glass bottles. To preserve SeMet stability, units were stored at –20 °C. The storage stability uncertainty was re-evaluated considering a validity period of six years, in accordance with ISO Guide 35:2017 [7]. The updated expanded uncertainty values are presented in Table 2.

4.8. Information on transport and storage

The CRM remains stable at 20 °C for up to six weeks. Nevertheless, considering possible temperature fluctuations during transport, shipments must be performed under cooling conditions, using ice or gel packs. The responsibility for ensuring appropriate transport and maintenance of these conditions lies with the customer. Detailed information regarding transport, handling, and safety requirements is provided in the Material Safety Data Sheet (MSDS), available on the Inmetro website [1].

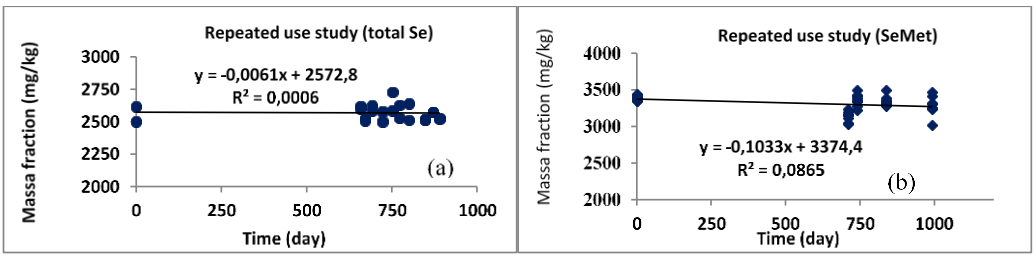

Figure 6. Repeated use study for total Se (*left*) and SeMet (*right*)

5. CONCLUSIONS

CRM 8969.0001 was produced as a batch of 200 amber-type glass bottles, each with a capacity of 30 mL and containing approximately 8 g of *Saccharomyces cerevisiae* yeast. The batch was shown to be homogeneous with respect to total Se and SeMet. Stability assessments under simulated transport conditions confirmed that both parameters remained stable for six weeks at 20 °C, while storage stability studies demonstrated consistent results.

Based on data obtained from the closely comparable material Selm-1, a validity period of six years was established. The associated risks were considered negligible compared to the benefits, as this extension allows for a longer timeframe for use.

Accordingly, the validity of CRM 8969.0001 has been extended until 31 August 2028, providing users with a reliable and stable reference material for the determination of total Se and SeMet.

AUTHORS' CONTRIBUTION

Márcia S. da Rocha, Lilian da Silva, Jefferson R. Souza, Lucas J. Carvalho, and Rodrigo V. P. Leal contributed to the methodology, formal analysis, investigation, validation, and visualization. Márcia S. da Rocha contributed to the conceptualization, project administration, and writing—original draft. Lilian da Silva, Jefferson R. Souza, Lucas J. Carvalho, Rodrigo V. P. Leal, Thiago O. Araujo, Marcelo D. Almeida, Rodrigo C. Sena, and Janaína M. R. Caixeiro contributed to the writing—review & editing. Thiago O. Araujo, Marcelo D. Almeida, Rodrigo C. Sena, and Janaína M. R. Caixeiro contributed to resources and supervision.

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