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Research Article

Development of reference materials for the determination of calcium, iron, selenium, and zinc in dietary supplements

Tran Thanh Son[1*], Van Ket Dang[1], Cuong Dang Huu[1], Trang Tran Thi Thu[2], Nguyen Nguyen Thi Thao[2], Oanh Dang Thi[1]

[1]National Institute for Food Control (NIFC), Hanoi, Viet Nam,
[2]University of Science, Vietnam National University, Hanoi, Vietnam

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Abstract

Food supplements are increasingly popular among consumers because of their convenience and nutritional value. Among the ingredients, essential minerals are added a lot because of their effects on health at all ages especially calcium (Ca), iron (Fe), selenium (Se), and zinc (Zn). To ensure the quality of products meets the requirements of consumers, the strengthening of control methods through testing is necessary. To ensure the quality of testing results, reference material/certified reference material is an effective tool for each laboratory. Nowadays, the source of reference material/certified reference material is highly dependent on overseas suppliers with expensive costs and inconvenient supply. This research was carried out in order to successfully produce a certified reference material of Ca, Zn, Fe and Se dietary supplements that can be supplied to domestic laboratories. Research results have produced certified reference material with characteristic values for Ca, Zn, Fe and Se content as follows: 786 ± 19.8 (mg/100g); 6.35 ± 0.40 (mg/100g); 7.42 ± 0.30 (mg/100g); and 18.4 ± 1.10 (µg/100g), respectively. The estimation of product shelf life is up to 1197 days.

Keywords: *reference material, certified reference material, dietary supplements, minerals.*

1. INTRODUCTION

The utilization of reference materials to ensure the precision and reliability of analytical results is experiencing heightened demand across various disciplines, particularly within the field of food analysis [1]. Reference materials play a critical role in establishing the comparability and traceability of analytical results across laboratories and over time. Their application in quality control, proficiency testing, and method validation is essential for laboratories to demonstrate technical competence to regulatory authorities and stakeholders. However, no single reference material can accommodate all analytes and matrix types; each reference material is characterized by specific matrix attributes and necessitates analyte-specific analytical methods. Consequently, the development of reference materials must be tailored to targeted matrices and analytes to support accurate and reliable measurements.

Powdered milk-based supplements have become increasingly popular in Viet Nam due to their convenience and perceived health benefits, including disease prevention. These products serve as sources of essential vitamins and minerals, particularly for individuals whose dietary habits may limit their intake of such nutrients or who are unwilling to consume certain nutrient-rich foods. However, maintaining a balanced intake of nutrients from natural food sources and conducting regular health monitoring are critical. Excessive consumption of minerals and vitamins through supplementation can result in toxicity and, in severe cases, pose serious health risks.

*Corresponding author: Tran Thanh Son (E-mail: ttson194@gmail.com)
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Table 1. *Various food reference materials have been investigated through both domestic and international studies*

No.	Matrix	Analyte	Year	References
1.	Health supplement	Vitamin B1, B2, B6	2020	[2]
2.	Water	As, Cd, Pb, Hg	2021	[3]
3.	Dairy products	*Escherichia coli*	2021	[4]
4.	Food Simulation	Vitamins and organic nutrient group	2000	[5]
5.	Chocolate	Caffeine Theobromine Theophylline	2004	[6]
6.	Fish oil and vegetable oil	Fatty acids	2013	[7]
7.	Formula milk	Vitamins and fatty acids	2019	[8]
8.	Animal tissue (herring, cod, cormorant)	Mercury Methyl mercury	2020	[9]

Food safety control of these products is based on the product self-declaration by the facility/manufacturer to the management agency and regulatory documents such as QCVN 8-2:2011/BYT National technical regulation on the limits of heavy metals contamination in food [10]; QCVN 8-1:2011/BYT National technical regulation on the limits of mycotoxins contamination in food [11]. In addition, minerals (Ca, K, Fe, Zn, Se, ...) are often added by manufacturers to increase the nutritional value of the product and the content of these minerals is specified in Circular 43/2014/TT-BYT according to the Table of recommended nutritional requirements for Vietnamese people and Maximum tolerance thresholds [12]. The recommended dietary allowances and maximum tolerance levels of some metals are shown in **Table 2** below. Food testing is essential for supporting food management by providing evidence that informs regulatory compliance decisions. Therefore, the testing results must ensure accuracy and objectivity.

Table 2. *Table of recommended nutrient intakes and maximum tolerance threshold [12, 13]*

Age group, gender	Calcium (mg/day)	Selenium[1] (µg/day)	Iron[2] (mg/day)	Zinc[3] (mg/day)
I. Recommended nutrient intake				
Adult male	700	34	18,3	7,0
Adult female	700	26	39.2	4.9
II. Maximum tolerance threshold				
Adult male	2500	400	45	40
Adult female	2500	400	45	40

[1] *Recommended nutrient requirements are calculated from the mean requirement value +2 SD.*
[2] *Moderate iron bioavailability (about 10% of iron is absorbed): a serving of meat or fish from 30g - 90g/day or vitamin C from 25 mg - 75 mg/day.*
[3] *Moderate absorption*

Milk and dairy products are widely consumed and accepted across various age groups and cultural contexts. Fortified food products are frequently derived from milk powder, owing to milk's naturally high content of essential nutrients such as calcium (Ca), zinc (Zn), iron (Fe), selenium (Se), vitamins, and other bioactive compounds. The bioavailability of nutrients in milk is generally higher compared to other food sources. Consequently, the powdered milk-based supplements with minerals such as Ca, Zn, Fe, and Se is common practice, aimed at enhancing their nutritional value and promoting consumer health [14]. This strategy ensures broader accessibility to these essential nutrients across diverse populations.

The use of reference materials throughout analytical processes is critical to ensuring the accuracy and reliability of testing methods. Reference materials also empower laboratories to proactively participate in interlaboratory comparisons when needed. Currently, the domestic capacity for reference materials provide is limited, while demand among testing laboratories remains high. Although experienced foreign suppliers of reference materials are available, elevated costs, prolonged procurement times, and limited matrix relevance to Vietnamese food products present notable barriers.

Powdered milk-based supplements, characterized by their complex matrices of proteins, fats, and carbohydrates, are among the most representative yet analytically challenging food samples. Successfully developing reference materials from these supplements would support method standardization, enhance accuracy assessment, and strengthen analyst proficiency. In turn, this would enable laboratories to detect methodological discrepancies and implement corrective or improvement measures, thereby elevating overall test quality.

In response to the urgent need for quality assurance in testing results of powdered milk supplements, this research was conducted to develop reference materials for the determination of the essential trace elements Ca, Fe, Se, and Zn in these products.

2. MATERIALS AND METHODS

2.1. Research object/materials

Reference material for metals calcium, iron, selenium and zinc was in powdered milk-based supplements.

2.2. Chemicals and standards

2.2.1. Equipment and tools for prototyping

The equipment utilized in the production of reference materials includes a cube mixer, a glove box for sample subdivision, a seal welding machine, and other laboratory instruments.

2.2.2. Equipment, tools, chemicals and solvents for analysis

The analysis of Ca, Fe, Zn, and Se was conducted using an inductively coupled plasma optical emission spectrometer (ICP-OES) and an inductively coupled plasma mass spectrometer (ICP-MS), both manufactured by Perkin Elmer. Sample preparation employed auxiliary equipment, including a Vortex shaker and a furnace, alongside standard laboratory apparatus. Analytical-grade reagents were procured from reputable suppliers and met the required purity specifications: nitric acid (HNO_3, 65%, Merck), hydrochloric acid (HCl, 37%, Merck), hydrogen peroxide (H_2O_2, Merck), and argon gas (Messer). Quantification of Ca, Fe, and Zn was carried out using ICP-OES at emission wavelengths of 317.933 nm, 238.204 nm, and 206.200 nm, respectively. Selenium was determined using the Se^{78} isotope on the ICP-MS system.

2.3. Preparation of milk-based reference material

The reference material was developed using a powdered milk matrix. Initially, a survey was conducted to select natural powdered milk sources that met the required mineral content criteria. Once suitable sources were identified, samples were procured and adjusted to a consistent moisture level. Homogenization of the sample was performed using a drum mixer to ensure uniformity. The homogenized material was subsequently subdivided into tin-lined pouches using an automated powder packaging system. Prior to storage, each unit was subject to visual inspection and labeled with a unique product code. Following packaging, the reference material underwent homogeneity and short-term stability testing to determine batch qualification. Batches deemed non-compliant were discarded and regenerated, while those meeting the acceptance criteria advanced to subsequent stages of development, as shown in **Figure 1**.

2.4. Research methods

2.4.1. Analysis methods

The quantification of Ca, Fe, and Zn in the samples was carried out in accordance with the AOAC Official Method 2011.14, utilizing inductively coupled plasma optical emission spectrometry (ICP-OES) [15]. Se was determined following AOAC Official Method 2015.06, employing inductively coupled plasma mass spectrometry (ICP-MS) [16]. All analyses were conducted in a laboratory accredited TCVN ISO/IEC 17025:2017 standard, ensuring compliance with recognized quality and technical competence requirements.

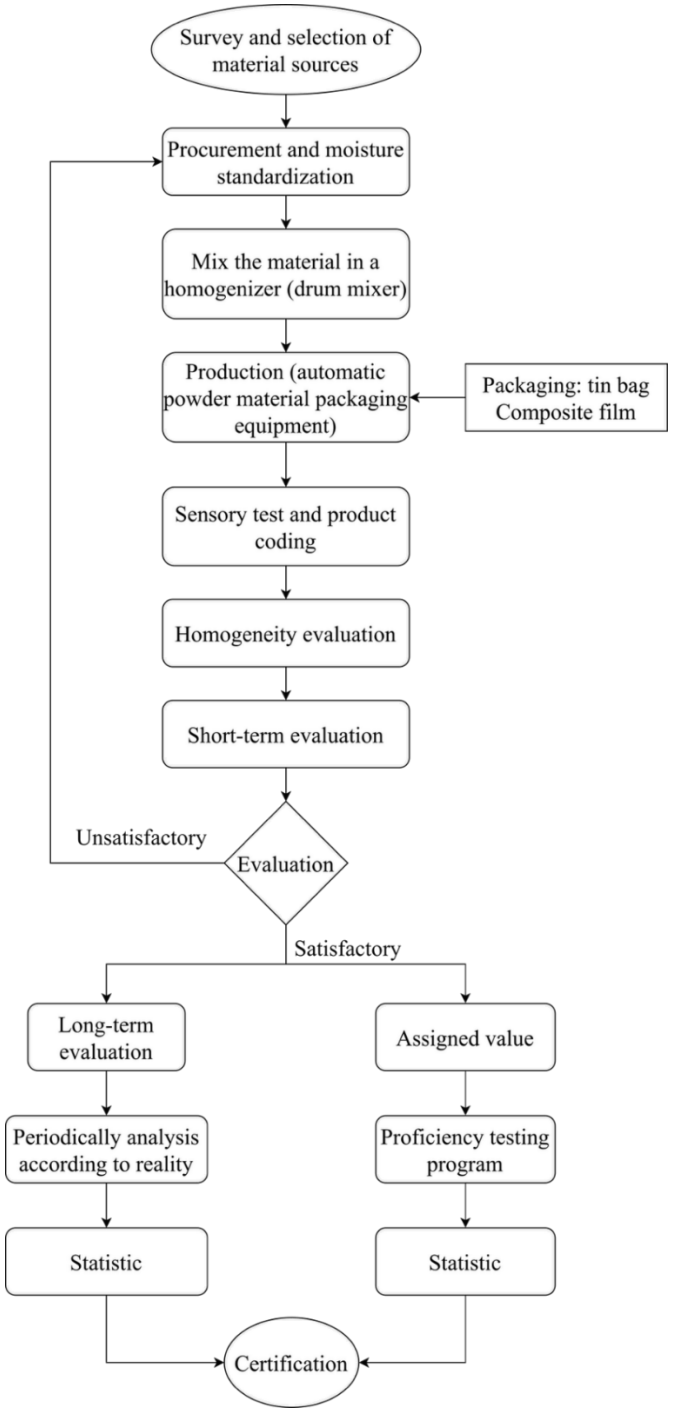

Figure 1. *Experiment designation*

2.4.2. Homogeneity assessment

Homogeneity assessment was conducted in accordance with ISO 13528:2022 [17] as follows:

A total of ten reference material units were randomly selected, and each unit was analyzed in duplicate. The standard deviation between units was evaluated using one-way analysis of variance (ANOVA) to determine whether statistically significant variation existed among the samples following the preparation process.

2.4.3. Evaluation of stability, assigned value and measurement uncertainty

2.4.3.1. Stability

Stability evaluation was conducted in accordance with ISO 13528:2022 [17].

The stability of the reference material was assessed at three distinct time intervals: 51, 113, and 236 days following production. At each time point, five units were randomly selected for analysis. To evaluate potential temporal variation in the material, one-way analysis of variance (ANOVA) was applied to assess the standard deviation among the sample groups. This statistical approach determined whether any significant differences in analyte concentration emerged across the designated stability study time points.

2.4.3.2. Assigned value and measurement uncertainty

The assigned value of the reference material was calculated using the following formula:

$$x_{RM} = y_{char} = \frac{\sum_{i=1}^{p} y_i}{p} \quad (1)$$

The measurement uncertainty of the reference material was calculated using the following formula:

$$u_{RM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{lts}^2} \quad (2)$$

The measurement uncertainty associated with sample heterogeneity was calculated using the following formula:

$$u_{hom} = \sqrt{u_r^2 + u_{bb}^2} \quad (3)$$

The measurement uncertainty associated with laboratory analysis was calculated using the following formula:

$$u_{char} = \frac{s_y}{\sqrt{p}} \quad (4)$$

The long-term measurement uncertainty was calculated using the following formula:

$$u_{lts} = s_{b_1}(t_{m_1} + t_{cert}) \quad (5)$$

In which:

x_{RM}: assigned value of the reference material;

y_i: value reported by selecte laboratory participate in PT;

p: number of accepted results post-outlier elimination according to Grubb's test;

u_{RM}: standard uncertainty of the assigned value;

u_{char}: uncertainty associated with a value assigned in a characterization study;

u_{hom}: uncertainty associated with heterogeneity;

u_{lts}: uncertainty associated with long-term stability.

2.4.3.3. Statistical method

R software with one-way analysis of variance (ANOVA) was applied to calculate homogeneity, F.test and T.test to evaluate short-term stability. Microsoft Excel software is used to evaluate long-term stability and determine the assigned value and measurement uncertainty of the assigned value.

3. RESULTS AND DISCUSSION

3.1. Results of the homogeneity evaluation

The research team randomly selected 10 items for testing. The test results for Ca, Zn, Fe, and Se are shown in **Table 3**.

Table 3. *Analytical results for the homogeneity evaluation of Ca, Zn, Fe, and Se content*

No.	Results							
	Ca (mg/100g)		Zn (mg/100g)		Se (µg/100g)		Fe (mg/100g)	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	772	820	5.23	5.16	21.0	20.2	7.75	7.65
2	793	810	5.36	5.99	19.4	19.9	7.90	7.90
3	774	774	5.82	5.36	21.0	20.2	7.74	7.68
4	764	794	5.26	5.06	20.5	20.3	7.49	8.15
5	767	792	5.74	5.69	18.9	19.4	7.70	7.70
6	817	747	5.05	5.06	21.8	19.5	8.09	8.05
7	791	802	5.89	4.99	20.4	18.9	7.62	7.98
8	813	813	5.67	5.83	18.5	18.8	7.98	7.81
9	830	814	5.16	5.49	22.1	19.0	8.28	7.88
10	864	837	5.20	5.42	20.2	18.4	8.27	7.71

The obtained analytical results were further entered and processed for one-factor ANOVA analysis using R software, obtaining the P-values of Ca, Zn, Fe and Se as 0.91; 0.82; 0.64 and 0.97, respectively. It can be seen that the P-values obtained after one-factor ANOVA analysis are all greater than 0.05. Therefore, the difference between the samples is not statistically significant. Thus, the production items achieved homogeneity in Ca, Zn, Fe and Se between the items. From the results of the homogeneity evaluation, the measurement uncertainty related to the material homogeneity (uhom) of the Ca, Zn, Fe and Se content was calculated according to formula (3) and the results were 14.8 (mg/100g); 0.07 (mg/100g); 0.165 (mg/100g) and 0.267 (µg/100g), respectively.

3.2. Results of the long-term stability evaluation

Five items were randomly selected after 51, 113 and 236 days to conduct long-term stability eveluation of Ca, Zn, Fe and Se. The average results of two analyses are shown in **Table 4** below.

Table 4. *Mean values from dual time-point analyses*

Time	Ca (mg/100g)	Zn (mg/100g)	Se (µg/100g)	Fe (mg/100g)
	786	6.07	18.6	8.00
	793	6.00	18.5	7.41
51st day	810	6.03	18.6	7.72
	808	6.16	18.6	7.60
	802	6.12	18.6	7.53
	801	6.08	18.5	7.64
	798	6.02	18.4	7.82
113rd day	793	6.14	18.4	7.58
	804	6.18	18.7	7.56
	797	6.14	18.5	7.64
	794	6.22	18.6	7.41
	792	6.17	18.5	7.67
236nd day	801	6.07	18.4	7.40
	792	6.19	18.6	7.55
	791	6.05	18.4	7.59

From the results obtained, one-way analysis of variance (ANOVA) was used to determine whether there was a significant change between sample groups at different study times. The P-value of one-way analysis of variance for Ca, Zn, Fe and Se contents was 0.379; 0.050; 0.231; and 0.055, respectively. Thus, the calculated P-values were greater than or equal to 0.05, from which it can be concluded that the Ca, Zn, Fe and Se contents in sample groups at different study times were not significantly different. The study sample had long-term stability after 236 days.

3.3. Calculation of assigned value and measurement uncertainty

3.3.1. Assigned value

The research team conducted a proficiency testing program and selected laboratories whose reported results in the program all had z-scores < 1 to describe the characteristics of the created reference materials [18]. In this program, 06 laboratories participated in the program and reported results. However, the laboratories did not fully report the indicators according to the designed program. The results reported by the laboratories that participated in the program and had z-scores < 1 are shown in **Table 5**.

In this program, 06 laboratories participated in the **program** and reported the results. However, the laboratories did not fully report the indicators according to the designed program. The results reported by the laboratories that participated in the program and had z-score < 1 are shown in **Table 5**.

***Table 5**. Analytical results of Ca, Zn, Fe and Se concentrations reported by selected laboratories*

Lab code	Results			
	Ca (mg/100g)	Zn (mg/100g)	Fe (mg/100g)	Se (µg/100g)
4	786	5.50	7.68	16.5
37	785	6.40	7.49	17.3
45	—	6.50	—	—
47	790	—	7.40	20.6
55	—	6.99	7.11	19.1

The results obtained from the selected laboratories were used to calculate the assigned value (xRM) of the reference material according to formula (1), from which the results for the parameters of Ca, Zn, Fe and Se were drawn as 786 (mg/100g); 6.35 (mg/100g); 7.42 (mg/100g); 18.4 (µg/100g), respectively.

3.3.2. Measurement uncertainty of the assigned value

The measurement uncertainties of the laboratories (uchar) for the Ca, Zn, Fe and Se contents were calculated according to formula (4) and the results were: 0.50 (mg/100g); 0.31 (mg/100g); 0.12 (mg/100g); and 0.92 (µg/100g), respectively. The upper limit (Lup) and lower limit (Llow) of the reference material for the parameters were also calculated. The control chart of the values obtained at the study times is shown in **Figure 2**.

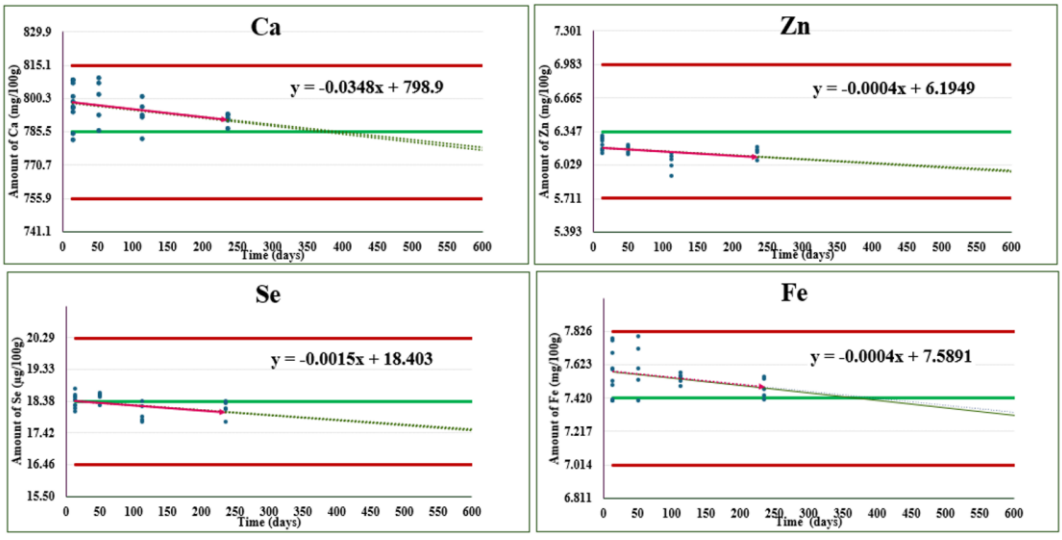

***Figure 2.** Control chart of Ca, Zn, Fe and Se*

3.3.3. Long-term measurement uncertainty and combined measurement uncertainty

The long-term measurement uncertainty for the Ca, Zn, Fe and Se contents was calculated according to formula (5) and the results were: 13.1 (mg/100g); 0.131 (mg/100g); 0.194 (mg/100g); 0.410 (µg/100g), respectively. The measurement uncertainty of the reference material for the Ca, Zn, Fe and Se contents was

calculated according to formula (2) and the results were: 19.8 (mg/100g); 0.40 (mg/100g); 0.30 (mg/100g); and 1.10 (µg/100g), respectively. Thus, the percentage of measurement uncertainty for the Ca, Zn, Fe and Se contents in the reference material were 5.04%; 12.6%; 8.09% and 11.97%, respectively.

3.3.4. Shelf life estimation

The research results show that the linear regression formula of the sample for the Ca, Zn, Fe and Se contents are respectively: y = -0.0348x + 798.9; y = -0.0004x + 6.1949; y = -0.0004x + 7.5891 and y = -0.0015x + 18.403. The tb1 of the Ca, Zn, Fe and Se contents (1.84250; 1.98734; 1.56652 and 2.51225) is larger than the t(95,n-2) from the theory (0.06339) at n - 2 degrees of freedom (n is the number of observation points) so the non-zero slope coefficient of the formulas is statistically significant at 95%.

With the results obtained and following the instructions in GUIDE 35 [19], the research team calculated the estimated shelf life of the product. The calculated shelf life of the sample for Ca, Zn, Fe and Se content is: 1197, 1234, 1263 and 1270 days respectively. For the calculated shelf life, the estimated shelf life of the sample will be the minimum time for each individual index of the sample, so the shelf life of the sample is 1197 days. The shelf life evaluated in this study is similar to the shelf life of some reference materials with similar sample matrix published by the National Institute of Standards and Technology [20, 21].

4. CONCLUSIONS

After the research, evaluation and implementation process, the research team produced a batch of reference materials of powdered milk supplements with an estimated shelf life of 1197 days. The samples were studied, evaluated for stability under room conditions and sent to qualified laboratories for testing. From the results obtained, the reference materials had assigned values and measurement uncertainties for Ca, Zn, Fe, and Se contents as follows: 786 ± 19.8 (mg/100g); 6.35 ± 0.40 (mg/100g); 7.42 ± 0.30 (mg/100g); and 18.4 ± 1.10 (µg/100g).

The research still needs to continue to monitor the samples in real time to have more data to accurately evaluate the shelf life of the samples. The study produced a quality control reference material for analyzing mineral indicators in food with stability similar to the international reference material.

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