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# Development of a certified reference material for the analysis of vitamins in multivitamin tablets

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## Abstract

Multivitamin tablet certified reference material (CRM, 108-10-019) was developed for the analysis of seven water-soluble vitamins, including thiamine, riboflavin, nicotinamide, pantothenic acid, pyridoxine, biotin, and folic acid. The CRM was prepared in powder form by grinding multivitamin tablets and then mixing, sieving, and bottling the powder. For the certification of each water-soluble vitamin, the isotope dilution mass spectrometry based on the liquid chromatography was applied. The methods for each analyte were validated by confirming the repeatability and reproducibility and by comparing with other CRMs. The property values and uncertainties for the vitamins were determined with 10 units from sample stored at − 20 °C. The homogeneity of each certified component was also examined in the range of 0.48–2.2%. All certified values for the seven water-soluble vitamins were stable for 3 or 6 years after the initial certification under storage conditions at − 20 °C. For fat-soluble vitamins, including retinol, α-tocopherol, cholecalciferol, and phylloquinone, two expert laboratories participated in analyses based on official methods, and the mean values of the reported results were assigned as reference values. The multivitamin tablet CRM (108-10-019) will be useful for validating analytical methods and for ensuring the quality of results for vitamin analysis in multivitamin tablets or similar products.

**Keywords** Water-soluble vitamins, Isotope dilution method, Multivitamin tablets, Certified reference materials, Fat-soluble vitamins

## Introduction

Vitamins are essential micronutrients and are generally classified as water-soluble vitamins (WSVs) or fat-soluble vitamins (FSVs) according to their solubility. WSVs include vitamin C and B-complex vitamins such as thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and cobalamin (B12). WSVs function as precursors of coenzyme and enzyme cofactors in energy metabolism and participate in the maintenance of healthy muscles, skin, eyes, hair, and liver (Huskisson 2007). FSVs include vitamins A, D, E, and K, with retinol, cholecalciferol, tocopherol, and pyroquinone being the most representative forms, respectively, and play an important role in eye and bone health and antioxidant activity in the body (Gregory

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1996). Due to their nutritional significance, accurate measurements of vitamins in foods, biological fluids, or tablets represent an important research area. Vitamin measurements have been performed via chromatography combined with various detection methods such as diode array detector (DAD) (Wongyai 2000; Markopoulou 2002; Jin et al. 2012), fluorescence detector (FLD) (Nojiri 1998; Li 2000), flame ionization detector (FID) (Reto et al. 2007; Kadioglu et al. 2009), or mass spectrometry (MS) (Chen et al. 2007; Chen and Wolf 2007), according to the characteristics of each vitamin. In addition, studies on the development and application of new analytical techniques have been actively pursued, with the aim of simultaneously and rapidly analyzing various vitamins with high accuracy and precision. Porada et al. (2022) introduced an electroanalytical method with the use of an environmentally friendly sensor to determine levels of vitamins B2, B9, B12, and B3 in different types of food samples. Bakhsh et al. (2022) reported on a simultaneous voltametric determination of vitamins B6 and C using modified electrodes in food samples.

In the development of analytical methods for food analysis, the quality of a method is usually assessed by several parameters such as the repeatability, reproducibility, limit of detection, or limit of quantification. Additionally, certified reference material (CRM) based on similar matrixes can be utilized to objectively evaluate the accuracy and precision of an analysis. Matrix CRMs are generally real-world samples such as foods or environmental or clinical substances that contain the analytes of interest (Walker 1999). A certificate for the analytes of interest is provided along with the materials, and the CRM user can utilize the materials to test and validate their analytical methods. The Korea Research Institute of Standards and Science (KRISS) has developed various food matrix CRMs, including CRMs of infant formula for organic nutrients (Lee et al. 2019a, b), kimchi cabbage for pesticides residue (Ahn et al. 2011), soybean paste for ochratoxin (Ahn et al. 2016), potato chips for acrylamide (Kim et al. 2010). As the National Metrology Institute (NMI) of Korea, KRISS conducts research on metrologically valid method development, uncertainty evaluation, and traceability (Kim et al. 2013; Lee et al. 2013, 2017, 2019a, b; Lee and Kim 2014; Hyung et al. 2018; Ju et al. 2020). These efforts ensure that the property values of developed CRMs have internationally equivalency and are traceable to SI units. In addition, the NMI complies with international standards such as ISO 17034 (2016) and ISO Guides 30 (2015) and 35 (2017) and confirms that CRMs produced in KRISS meet the requirements for reference materials.

Multivitamin tablets are a relatively simple matrix containing various WSVs and FSVs; thus, multivitamin tablets represent a good matrix CRM for verifying and validating a vitamin analysis system. KRISS developed the first batch of multivitamin tablet CRM (108-10-019) for analysis of water-soluble vitamins including riboflavin, nicotinamide, folic acid, pantothenic acid and pyridoxine. However, this CRM was issued and disseminated only in limited period due to lack of stability study. Another CRM batch (108-10-019) was developed as an alternative reference material. In this study, the entire process of preparation of standard materials, assignment of certified values, evaluation of homogeneity, evaluation of long-term stability, and assignment of standard values for this multivitamin certified reference substance was explained.

## Experimental

### Chemicals and reagents

Calibration standard materials and isotope labeled standard materials were purchased from a commercial producer as listed in Table 1. In the case of WSVs, purity of each vitamin was determined via purity assessment based on the mass balance method (Kim et al. 2013; Lee and Kim 2014).

All the organic solvents used in this study were HPLC-grade obtained from Burdick & Jackson (Muskegon, MI, USA). Ammonium formate, formic acid, pyrogarllol, KOH, anhydrous sodium sulfate, lipase, potassium carbonate were purchased from Sigma-Aldrich (St. Louis, MO. USA). Filter cartridges were obtained from Whatman (Clifton, NJ, USA).

### Preparation of candidate reference material

Commercial multivitamin tablets were utilized for the preparation of a candidate reference material. A bulk amount (100 bottles, 90 tablets/bottle) of multivitamin was purchased from a local retail dealer. Approximately 17.9 kg of multivitamin tablets was employed as starting materials to prepare the reference material. To ensure good homogeneity of the analytes in the reference materials, the tablets were first ground with a food processor and then pulverized using a laboratory mill (FRITSCH, Model No. Pulverisette 14, Germany) with a 500 μm sieve ring. The pulverized sample was passed through 100 μm nylon sieve cloth to collect powder particles smaller than the sieve size. To homogenize the collected sample, the pulverized sample was mixed with a V-blender for over 10 h. The prepared homogenized sample (14.3 kg) was bottled at 10 g per unit in 30-mL amber bottles stored in a freezer (− 20 °C) after remaining in an argon chamber for 20 min.

**Table 1** Information of calibration standard and isotope labeled materials applied in the current study

| Analyte | Calibration standard material | Purity (%)[a] | Isotope labeled material[b] |
|---|---|---|---|
| Thiamine | Thiamine HCl (Dr. Ehrenstofer GmbH) | 95.1 ± 0.1 | $^{13}C_4$-Thiamine HCl (IsoSciences LLC.) |
| Riboflavin | Riboflavin (Dr. Ehrenstofer GmbH) | 99.8 ± 0.1 | $^{13}C_4$, $^{15}N_2$-Riboflavin (IsoSciences LLC.) |
| Nicotinamide | Nicotinamide (Chromadex) | 99.4 ± 0.3 | Nicotindamide-$d_4$ (CDN Isotope) |
| Pantothenic acid | Calcium Pantothenate (Chromadex) | 99.6 ± 0.2 | $^{13}C_3$, $^{15}N_1$-Calcium Pantothenate (Cambridge Isotope Laboratories) |
| Pyridoxine | Pyridoxine HCl (Chromadex) | 99.5 ± 0.7 | $^{13}C_4$-Pyridxoen HCl (Cambridge Isotope Laboratories) |
| Biotin | Biotin (Dr. Ehrenstofer GmbH) | 99.7 ± 0.1 | Biotin- $d_2$ (IsoSciences LLC.) |
| Folic acid | Folic acid (Chromadex) | 89.2 ± 0.1 | $^{13}C_5$-Folic acid (Merck Eprova AG) |
| Retinol | Retinol (Sigma-Aldrich) | ≥ 95 | – |
| Tocopherols | α-Tocopherol (Sigma-Aldrich) | ≥ 95 | – |
| Cholecalciferol | Cholecalciferol (Sigma-Aldrich) | ≥ 95 | – |
| Phylloquinone | Phylloquinone (Sigma-Aldrich) | ≥ 95 | – |

[a] Purity assay was performed by mass balance method for 7 WSVs including thiamine HCl, riboflavin, nicotinamide, calcium pantothenate, pyridoxine HCl, biotin and folic acid. Purities of retinol, α-tocopherol, cholecalciferol and phylloquinone were based on the manufacturer's certificates

[b] Isotope labeled materials were utilized an internal standard for IDMS method for certification of 7 WSVs

## Assignment of certified values

### *Calibration standard solutions*

All standard solutions were prepared gravimetrically in multiples and used to verify the consistency of preparation, as described in our previous studies (Lee et al. 2016). Briefly, each WSVs calibration standard material except folic acid was accurately weighed and dissolved in water to prepare an appropriate concentration for each analysis. Considering the solubility of folic acid, 1 mg of accurately weighed folic acid is first dissolved in 10 mL of 10 mM ammonium acetate solution, which was added 0.1% 2-mercaptoethanol and was adjusted to pH 10 with ammonia solution. Thereafter, the folic acid standard solution was prepared by diluting it to a target concentration by adding a mixture of acetonitrile, methanol and water (26:14:60, v/v). Corresponding isotope standard solutions were also prepared in the same way. For each of multiple standard solutions, two isotope ratio standard solutions were prepared by gravimetrically mixing calibration standard and isotope standard solution to make a 1:1 isotope ratio. The multiple isotope ratio standard solutions were run by LC/MS with the same conditions of each analytical instrumental method, and one of the ratio standard solution was selected as a calibrating standard for IDMS method.

### *Analysis of pantothenic acid and pyridoxine*

A sample of ground multivitamin tablets (0.1 g) was placed in a glass bottle, and 35 mL of distilled water was added to the bottle. The exact amounts of sample and extraction solvents were determined by weighing the bottle before and after the addition of each component. After being vortexed for 30 min, the sample solution was kept at 4 °C for 3 h. Then, 1 mL of the sample extract was transferred to a vial and spiked with an appropriate amount of the isotope standard solution to generate an isotope ratio close to 1:1. The sample was passed through a filter cartridge and then diluted with water to an appropriate concentration for LC/MS analysis.

LC/MS analysis was performed using an Agilent 6410 Triple Quadrupole LC/MS system (Santa Clara, CA, USA) connected to an Agilent 1200 Series LC system (Waldbronn, Germany). Chromatographic separation was performed using a Waters X-Bridge C18 column (i.d.: 4.6 mm, length: 150 mm, particle size: 3.5 µm) connected to a C18 guard column. The mobile phases were water (phase A) and methanol (phase B), which both contained 0.1% formic acid, and the flow rate was 0.3 mL/min. Gradient elution started with 100% A for 5 min and changed linearly to 50% B over 5 min. The mobile phase was then maintained in isocratic mode for 10 min, returned to 100% A over 1 min, and maintained at 100% A for 19 min (total: 40 min). The injection volume was 10 µL. MS analysis was conducted using electrospray ionization (ESI) in the positive ion mode. The optimized MS conditions for analyte detection were as follows: capillary voltage: 4000 V, nebulizer gas ($N_2$) pressure: 40 psi, nebulizer gas ($N_2$) temperature: 350 °C, drying gas flow: 10 L/min, fragmentor voltage (applied to the extraction skimmer): 150 V. Detection was performed in selected reaction monitoring (SRM) mode. The SRM channels were $m/z$ 220 → $m/z$ 90 for pantothenic acid, $m/z$ 224 → $m/z$ 94 for $^{13}C_3$,$^{15}N_1$-pantothenic acid, $m/z$ 170 → $m/z$ 152 for pyridoxine, and $m/z$ 174 → $m/z$ 156 for $^{13}C_4$-pyridoxine. The collision energy was set to 12 and 9 eV for pantothenic acid and pyridoxine, respectively.

#### *Analysis of biotin*

A sample of ground multivitamin tablets (1 g) was placed in a glass bottle, and then, the sample was spiked with biotin-$d_2$ solution to generate an isotope ratio close to 1:1. Extraction solvent, i.e., 10 mmol/L ammonium formate in $H_2O$ (pH 3.8), was added to the bottle. After being vortexed for 30 min, the sample solution was maintained at 4 °C for 3 h. Then, the sample solution was passed through a 0.22 µm filter (PURDISC NYL 25 FILTER, 25 mm) and diluted with water for instrumental analysis. LC/MS analysis was performed using an Agilent 6410 Triple Quadrupole LC/MS system (Santa Clara, CA, USA) connected to an Agilent 1200 Series LC system (Waldbronn, Germany). Chromatographic separation was performed using a Waters X-Bridge C18 column (i.d.: 4.6 mm, length: 150 mm, particle size: 3.5 µm) connected to a C18 guard column. The mobile phases were 10 mmol/L ammonium formate in $H_2O$ (pH 3.5) (phase A) and methanol (phase B), and the flow rate was 0.3 mL/min. The separation was conducted by isocratic elution with 45% A and 55% B for 15 min. The injection volume was 10 µL. MS analysis was conducted using ESI in the positive ion mode. The optimized MS conditions for analyte detection were as follows: capillary voltage: 3000 V, nebulizer gas ($N_2$) pressure: 35 psi, nebulizer gas ($N_2$) temperature: 350 °C, drying gas flow: 11 L/min, fragmentor voltage (applied to the extraction skimmer): 65 V. Detection was performed in SRM mode, with SRM channels set to $m/z$ 245 → $m/z$ 227 for biotin and $m/z$ 247 → $m/z$ 229 for biotin-$d_2$. The collision energy was set to 10 eV.

#### *Analysis of folic acid*

Folic acid was characterized using a dedicated ID–LC/MS method, with modifications to the previously published method (Jung et al. 2007). In brief, a sample (0.1 g) was spiked with an appropriate concentration of $^{13}C_5$-folic acid solution and mixed with 20 mL of extraction solution, i.e., acetonitrile:methanol:water (26:14:60). After extraction for 3 h at 4 °C, the sample was filtered with a 0.22 µm filter (PURDISC NYL 25 FILTER, 25 mm) and diluted with water for instrumental analysis. LC/MS analysis was performed using a ThermoElectron TSQ Quantum mass spectrometer (San Jose, CA, USA) coupled with an ESI interface and a Waters ACQUITY UPLC system (Milford, MA, USA). The LC column was a Phenomenex Luna C18 (i.d.: 4.6 mm, length: 250 mm, particle size: 5 µm) connected to a C18 guard column. Isocratic elution was utilized with 10% $H_2O$ (0.1% formic acid) and 90% organic mobile phase, corresponding to acetonitrile:methanol:water (26:14:60 v/v). The MS conditions were optimized for folic acid as follows: ionspray voltage: 4900 V, temperature: 350 °C, sheath gas pressure: 40 µL/min, auxiliary gas pressure: 10 µL/min. Detection was performed in SRM mode, and a collision energy of 25 eV was applied to the collision cell. Dissociation channels of $m/z$ 442 → 295 and $m/z$ 447 → 295 were chosen for folic acid and $^{13}C_5$-folic acid, respectively.

#### *Analyses of thiamine, riboflavin, and nicotinamide*

Thiamine (Joo 2020), riboflavin (Lee et al. 2016), and nicotinamide (Shin et al. 2013) were analyzed using specific ID–LC/MS methods established and maintained in this laboratory as a higher-order reference method. Each method has been sufficiently validated to accurately quantify each vitamin and each cited paper describes in detail the verification results of the analysis method.

#### *Characterization and homogeneity test*

The characterization and homogeneity test were established in accordance with ISO Guide 35 (2017), as described in detail in a previous paper (Lee et al. 2019a, b). The characterization and homogeneity assessment were simultaneously performed by analyzing 10 units of prepared sample. The mean value of the 10 units was assigned as the property value, and the standard deviation of the measurement results of 10 units indicated the homogeneity of the analyte in the CRM. Uncertainties of property values were estimated by combining the uncertainties associated with the measurement of the property values and the unit homogeneity of the analytes in the CRM.

#### *Stability monitoring*

The long-term stability of the certified analytes was monitored with the CRMs stored under storage conditions (− 20 °C) for 3 or 6 years. Each analysis was performed for four CRM units with the same method employed in the certification procedure.

### Assignment of reference values

FSVs, including retinol, α-tocopherol, cholecalciferol, and phylloquinone, were analyzed by two expert laboratories based on the Korea Food Code (MFDS 2021). The reference materials were shipped to each laboratory, and each laboratory carried out the experiments independently and reported the results to KIRSS. The averages of the participants' results were assigned as reference values, and the uncertainties reported by each laboratory were combined to determine the uncertainty of the final assigned values.

#### *Analysis of retinol and tocopherol*

The participating laboratories analyzed retinol and tocopherol based on the Korea Food Code (MFDS 2021). A sample (0.4–1 g) was placed in a round-bottom flask.

Saponification was performed with ethanol (30 mL), 10% pyrogallol ethanol solution (1 mL), and 90% KOH (3 mL) and 30 min of heating in a boiling water bath with a refluxing cooler. After the sample was rapidly cooled to room temperature, the mixture was transferred to a brown separatory funnel to extract analytes with petroleum ether (30 mL). After two rounds of extraction, the collected petroleum ether was washed with water (10 mL) until it was free of alkali, as determined by a lack of color change with phenolphthalein. The organic solvent was sufficiently dehydrated with anhydrous sodium sulfate and evaporated to dryness under reduced pressure at 40–50 °C. For retinol analysis, the sample was reconstituted with isopropanol and analyzed with an LC–FLD, with the excitation set to 340 nm and emission set to 460 nm. For tocopherol, the excitation was set to 298 nm, and the emission was set to 325 nm.

#### *Analysis of cholecalciferol*

Both participating laboratories used an LC/UVD to analyze cholecalciferol based on the Korea Food Code (2021). Here 1 g of a sample was weighed and placed in a round-bottom flask. For saponification of the sample, 40 mL of 10% pyrogallol ethanol solution and 10 mL of 90% potassium hydroxide were added to the flask, which was placed in a boiling water bath for 30 min with a refluxing cooler. After the sample had rapidly cooled to room temperature, it was transferred to a brown separatory funnel. The sample in the funnel was vigorously mixed for 10 min after the addition of 50 mL of hexane. The sample was then left to stand, and the hexane layer was then transferred to a separate brown separatory funnel. This hexane extraction step was performed three times. Next, 100 mL of potassium hydroxide (1 N) was added to the collected hexane solution, and the sample was vigorously mixed for 15 s. After being left to stand, the water layer was discarded. Then, 40 mL of potassium hydroxide (0.5 N) was added to the hexane layer, and the water layer was discarded again after mixing until no color appeared with phenolphthalein. The hexane layer that has been sufficiently dehydrated by the addition of anhydrous sodium sulfate was placed in a brown flask and dried under reduced pressure at 40–50 °C. The sample was reconstituted with methanol and analyzed with an analytical column (Phenomenex, Capcellpak C18 UG120, i.d.: 4.6 mm, length: 250 mm, particle size: 5 μm) and UV detector (254 nm).

#### *Analysis of phylloquinone*

Both laboratories used an LC/UVD for analyzing cholecalciferol based on the Korea Food Code (2021). For the analysis, 1 g of sample was weighed in a test tube, and 15 mL of distilled water (approximately 38 °C) was added. Next, 5 mL of 0.8 M phosphate buffer solution and 1 g of lipase were added, and the sample was dissolved using a stirrer. The sample test tube, which was closed with a stopper, was vigorously shaken so that the lipase was sufficiently dispersed. Enzyme treatment was applied for 120 min at 38 °C. To stop the enzyme reaction, 10 mL of an alcohol mixture and 1 g of potassium carbonate were sequentially added, and the sample was shaken. Then, 30 mL of hexane was added to the test tube, and the test tube was shaken vigorously by a shaker for at least 10 min. The sample was left in a cool dark place for at least 10 min to confirm that it had separated into two layers (if the layers were not completely separated, the sample was then centrifuged at 1000 rpm for 10 min). Next, 1 mL of the supernatant was transferred to a separate glass test tube, and a nitrogen concentrator was used. After the sample had dried, 1 mL of methanol was added again, and the sample was dissolved with a shaker and filtered through a 0.45-μm nylon membrane filter for use as a test solution.

## Results and discussion

According to ISO Guide 30 (2015), the certified value is defined as an assigned value for a property of a reference material that is accompanied by an uncertainty statement and statement of metrological traceability in the RM certificate. In order to state clear uncertainty budget, a primary method based on isotope dilution was developed and validated for the analytes to be certified. To demonstrate that certified values are traceable to the definition of SI units, calibration standard materials are subjected to purity assay which is internationally equivalent. In this study, 7 WSVs were assigned as certified value by meeting these requirements. On the other hand, four FSVs were assigned as reference values based on the results by using the official method in expert laboratories. The reference values for FSVs are lack of sufficient uncertainty evaluation and traceability statement.

### Certification study for water soluble vitamins

#### *Method validation*

Dedicated ID–LC/MS methods were developed to determine the levels of seven WSVs in the candidate multivitamin tablet reference material. For analyzing pantothenic acid and pyridoxine, an ID–LC/MS method was developed with $^{13}C_3$,$^{15}N_1$-calcium pantothenic acid and $^{13}C_4$-pyridoxine–HCl. The applied extraction and instrumental conditions, as described in the above section, were optimized for only pantothenic acid and pyridoxine analysis. In order to validate the method, the repeatability and reproducibility tests were carried out. For the repeatability test, three sub-samples were taken from a bottle of the multivitamin reference material and analyzed by the

developed ID–LC/MS method. The average and standard deviation of the measurement results were evaluated and repeatability was determined with relative standard deviation of results. As shown in Table 2, the relative standard deviation for both analytes in each period was in the range of 0.28–0.55%, indicating high repeatability for this method. An identical repeatability test was performed 1 month later, and the results confirmed the reproducibility of the method. The overall relative standard deviations (%) for the measurement results of pantothenic acid and pyridoxine over the two periods are 2.36% and 0.77% in the multivitamin tablets, respectively. These results strongly indicate that the developed method can produce reliable results for pantothenic acid and pyridoxine.

In the case of biotin, the sample was spiked with biotin-$d_2$ solution as an internal standard for quantification. For the extraction of biotin from the sample, 10 mmol/L ammonium formate buffer (pH 3.8) was used and held at 4 °C for 3 h. Based on the change in biotin content in the sample, it was confirmed that the biotin content reached equilibrium after 3 h of extraction. Therefore, the extraction time should not be shorter than 3 h. The same sample was analyzed at two different times using the developed ID–LC/MS method for biotin analysis. The results showed a repeatability of 0.17%—0.94% and a reproducibility of 0.63% (Table 2).

For the determination of folic acid, the ID–LC/MS method for infant formula (Jung et al. 2007) was adopted and modified for a multivitamin tablet sample. $^{13}C_5$-folic acid solution was added to the sample as an internal standard, and the target analytes were extracted with organic mobile phase, i.e., acetonitrile:methanol:water (26:14:60), after comparison with water and potassium phosphate buffer solution. For the instrumental analysis method, the effectiveness has already been confirmed through the certification process of folic acid in infant formula; thus, this approach was applied here. When three subsamples of homogenized sample were analyzed by the developed method, the relative standard deviation was less than 1%. When the analysis was conducted independently after a period of time, the RSD of the two measurements was 0.45%.

To objectively verify the developed methods, NIST SRM3280 multivitamin/multielement tablets were analyzed with the developed ID–LC/MS methods. Comparative results for thiamine, riboflavin, and nicotinamide have already been published in our previous papers (Shin et al. 2013; Lee et al. 2016; Joo 2020), and the results of pantothenic acid, pyridoxine, biotin, and folic acid are summarized in Table 3. SRM 3280 was analyzed by each developed method, and the measurement results were in good agreement with the results presented in the certificate of analysis (NIST 2012) within their uncertainties.

The ID–LC/MS methods for thiamine, riboflavin, and nicotinamide have been described in previous papers, and each validated method was applied to the current candidate material for assignment of these property values.

Consequently, the developed ID–LC/MS methods were validated to assign property values for the seven WSVs in the multivitamin tablet samples.

**Table 2** Repeatability and reproducibility results for pantothenic acid, pyridoxine, biotin and folic acid analysis in homogenized multivitamin tablet samples, obtained by the developed ID–LC/MS method

| Period | Subsample No | Measurement results | | | |
|---|---|---|---|---|---|
| | | Pantothenic acid (g/kg) | Pyridoxine (g/kg) | Biotin (mg/kg) | Folic acid (mg/kg) |
| 1st | Subsample 1–1 | 24.85 ± 0.22[a] | 21.84 ± 0.47 | 25.41 ± 0.45 | 108.4 ± 1.9 |
| | Subsample 1–2 | 25.11 ± 0.42 | 21.64 ± 0.46 | 25.80 ± 0.18 | 108.0 ± 2.1 |
| | Subsample 1–3 | 24.99 ± 0.29 | 21.61 ± 0.45 | 25.86 ± 0.39 | 109.7 ± 2.2 |
| | Average | 24.98 ± 0.29 | 21.70 | 25.69 ± 0.28 | 108.7 ± 2.1 |
| | Standard deviation | 0.13 (0.52 rel%[b]) | 0.12 (0.55 rel%) | 0.24 (0.94 rel%) | 0.91 (0.84 rel%) |
| 2nd | Subsample 2–1 | 25.90 ± 0.98 | 21.39 ± 0.79 | 25.91 ± 0.55 | 109.9 ± 3.3 |
| | Subsample 2–2 | 25.76 ± 1.05 | 21.47 ± 0.78 | 25.96 ± 0.52 | 108.4 ± 3.4 |
| | Subsample 2–3 | 25.82 ± 0.96 | 21.55 ± 0.78 | 25.89 ± 0.54 | 109.9 ± 2.8 |
| | Average | 25.83 ± 0.98 | 21.47 ± 0.78 | 25.92 ± 0.53 | 109.4 ± 3.0 |
| | Standard deviation | 0.07 (0.28 rel%) | 0.08 (0.37 rel%) | 0.04 (0.17 rel%) | 0.86 (0.78 rel%) |
| Average | | 25.40 | 21.59 | 25.81 | 109.1 |
| Standard deviation among period | | 0.60 (2.36 rel%) | 0.17 (0.77 rel%) | 0.17 (0.63 rel%) | 0.49 (0.45 rel%) |

[a] The values following "±" are the expanded uncertainties of the preceding values at the 95% level of confidence

[b] The unit "rel%" indicates the relative percentage of the standard deviation in comparison with the corresponding mean value

**Table 3** Results for pantothenic acid, pyridoxine, and biotin obtained by the developed ID–LC/MS method in a SRM 3280 (multivitamin/multielement tablets)

| Compound | Units | Certified value | Measured value[a] |
|---|---|---|---|
| Pantothenic acid | g/kg | 7.3 ± 0.96[b] | 7.9 ± 0.30 |
| Pyridoxine | g/kg | 1.48 ± 0.14[c] | 1.47 ± 0.15 |
| Biotin | mg/kg | 0.0234 ± 0.0032 | 0.0234 ± 0.0002 |
| Folic acid | mg/kg | 394 ± 22 | 398 ± 32 |

[a] The values are obtained by each of the developed ID–LC/MS method ($n = 3$)

[b] The values following "±" are the expanded uncertainties of the preceding values at the 95% level of confidence

[c] The values were converted as pyridoxine contents from pyridoxine HCl in the certificates, (1.81 ± 0.17) g/kg in SRM 3280

### *Characterization and homogeneity test*

To characterize the prepared candidate multivitamin tablet reference material, property value assignment, homogeneity tests, and uncertainty evaluations were performed for seven WSVs according to the ISO Guide 35 (2017). As reported in previous studies (Ahn et al. 2011; Ahn et al. 2016; Kim et al. 2010), our laboratory has established protocols for assigning the certified value and evaluating its uncertainty, and mathematical details of the statistical evaluation process also reported.

For each analyte, the validated IDMS method was applied to single sample taken from 10 units of material, and the mean value ($C_{\text{mean}}$) of the results was assigned as the property value. The standard deviation ($SD_{bb}$) of the measurement results of the ten units is considered as the between unit homogeneity of the analyte in the CRM, and this homogeneity includes the repeatability of the measurement method. For uncertainty budgeting, each uncertainty factor related with property value was categorized and evaluated as random ($u^2_{\text{char, ran}}$) and systematic ($u^2_{\text{char,sys}}$) uncertainties (Lee et al. 2019a, b). For example, Table 4 shows the uncertainty sources in the ID–LC/MS method for the determination of biotin in the multivitamin tablet CRM.

In our measurement scheme, the uncertainty of mean value ($u(C_{\text{mean}})$) can be associated with the measurement of the property value $\left(u^2_{\text{char}}\right)$ and the between-unit homogeneity $\left(u^2_{bb}\right)$ as denoted Eq. (1). $u^2_{\text{char}}$ can be divided to two parts, systematic effects ($u^2_{\text{char, sys}}$) and random effects $\left(u^2_{\text{char, ran}}\right)$, and random effects are related with the repeatability of the measurement method ($s_r$). As mentioned above, the standard deviation ($SD_{bb}$) is results of contribution of between-unit homogeneity and repeatability of the measurement method. Finally, uncertainty of mean value can be evaluated as combining the uncertainties from systematic effects in IDMS measurement method and between-unit homogeneity.

$$\begin{aligned} u(C_{\text{mean}}) &= \sqrt{u^2_{\text{char}} + u^2_{bb}} = \sqrt{u^2_{\text{char, sys}} + u^2_{\text{char, ran}} + u^2_{bb}} \\ &= \sqrt{u^2_{\text{char, sys}} + s^2_r + u^2_{bb}} = \sqrt{u^2_{\text{char, sys}} + SD_{bb}} \end{aligned} \quad (1)$$

According to the ISO Guide 35, the uncertainty of the certified value ($u_{\text{CRM}}$) is determined by combining the uncertainties of measurement of the property

**Table 4** Uncertainty sources in the ID–LC/MS method for the determination of biotin in multivitamins

| Group | Uncertainty components | Sources (evaluation methods) | Typical value[a] (relative %) |
|---|---|---|---|
| I[b] | Standard solution | Purity of the reference material (from the KRISS purity analysis) | 0.1 |
| | | Gravimetric preparation (from cross-check of independent sets of calibration solutions) | 0.2 |
| | Isotope ratio standard | Gravimetric mixing (from cross-check of multiple isotope ratio standards from each individual standard solution) | 0.2 |
| | Peak area ratio of biotin and $^2H_2$-biotin from LC/MS measurements of isotope ratio standard | Repeatability of multiple measurements | 0.3 |
| II[c] | Mass of sample taken for analysis | Readability and linearity of the balance used (from the certificate of the balance) | < 0.01 |
| | Mass of $^2H_2$-biotin solution spiked into sample taken for analysis | Readability and linearity of the balance used (from the certificate of the balance) | < 0.01 |
| | Peak area ratio of biotin and $^2H_2$-biotin from LC/MS measurements of sample extract | Repeatability of multiple measurements | 0.1–0.3 |

[a] Typical standard uncertainty of each source is based on the measurement protocol used in this study

[b] Group I includes the uncertainty components common to the measurement of all bottles (Systematic effect, $u_{\text{sys}}$)

[c] Group II includes the uncertainty components that are unique to each bottle (Random effect, $u_{\text{ran}}$)

value $(u^2_{\text{char}})$, the between-unit homogeneity $(u^2_{bb})$ and stabilities including short $(u^2_{\text{sts}})$ and long-term $(u^2_{\text{lts}})$ as expressed in Eq. (2). Stability uncertainties were set to zero based on the confirmation that each analyte was stable in the CRM after stability monitoring for certain period of time.

$$u_{\text{CRM}} = \sqrt{u^2_{\text{char}} + u^2_{bb} + u^2_{\text{sts}} + u^2_{\text{lts}}} \quad (2)$$

In this way, $C_{\text{mean}}$ and $u(C_{\text{mean}})$ are assigned as the certified value and the between-unit homogeneity was also determined for thiamine, riboflavin, nicotinamide, pantothenic acid, pyridoxine, biotin, and folic acid as listed in Table 5. The relative standard deviations of the measurement results of the 10 units ranged from 0.48 to 2.2%, indicating a homogeneity that is acceptable for a CRM. Figure 1 is the example of homogeneity test results for thiamine and pantothenic acid in this multivitamin tablet CRM. For the assignment of certified values, the purities of the calibration standard materials for individual target analytes were determined by the mass balance method, which included LC/UV analysis for structurally related impurities, thermo-gravimetric analysis for non-volatile impurities, Karl–Fischer coulometry for water content, and headspace GC/MS for residual solvents. This procedure was applied to thiamine HCl, riboflavin, nicotinamide, calcium pantothenate, pyridoxine HCl, biotin, and folic acid as listed in Table 1. These purities were applied to characterize each vitamin and to confirm that the certified values were traceable to the definition of SI units.

**Table 5** Certification results for multivitamin tablet CRM 108-10-019

| Compound | Units | Certified value[a] | *k* | Relative standard deviation (%)[b] |
|---|---|---|---|---|
| Thiamine | g/kg | 23.38 ± 0.31 | 2.1 | 0.48 |
| Riboflavin | g/kg | 22.8 ± 1.7 | 2.1 | 2.2 |
| Nicotinamide | g/kg | 15.19 ± 0.44 | 2.1 | 0.93 |
| Pantothenic acid | g/kg | 25.09 ± 0.40 | 2.1 | 0.64 |
| Pyridoxine | g/kg | 21.62 ± 0.73 | 2.1 | 1.3 |
| Biotin | mg/kg | 25.65 ± 0.46 | 2.3 | 0.76 |
| Folic acid | mg/kg | 109.9 ± 6.0 | 2.2 | 2.2 |

[a] The number following "±" is the expanded uncertainty of the property value for the 95% level of confidence

[b] The relative standard deviation is based on measurement results among 10 or more units. This represents the homogeneity of each compound and is associated as one of the uncertainty factors of the certified values

### *Stability monitoring*

For the long-term stability monitoring, the certified analytes were analyzed at 3 and/or 6 years after the initial certification. Four units stored at − 20 °C were analyzed by the IDMS method used for the assignment of property values. The monitoring results are presented in Fig. 2, which shows that the measurement results and initial certification results agree within their uncertainties. Therefore, the certified values of seven WSVs in CRM 108-10-019 are reliable to be used as long as stored in − 20 °C freezer.

## Assignment of reference values

Four FSVs including retinol, α-tocopherol, cholecalciferol, and phylloquinone were assigned as reference values by the measurement results from two expert laboratories. They have been accredited by KOLAS (Korea Laboratory Accreditation Scheme), and already participated in the campaign for assignment of reference values of nutrients in infant formula CRM 108-02-003.

These laboratories measured 4 FSVs by the official methods based on Korea Food Code (MFDS 2021), which are sufficiently reliable method to assay the FSVs in multivitamin tablets. Each laboratory verifies their measurement capability with various ways including reproducibility test and comparison with other reference materials. The participants were provided 2 bottles

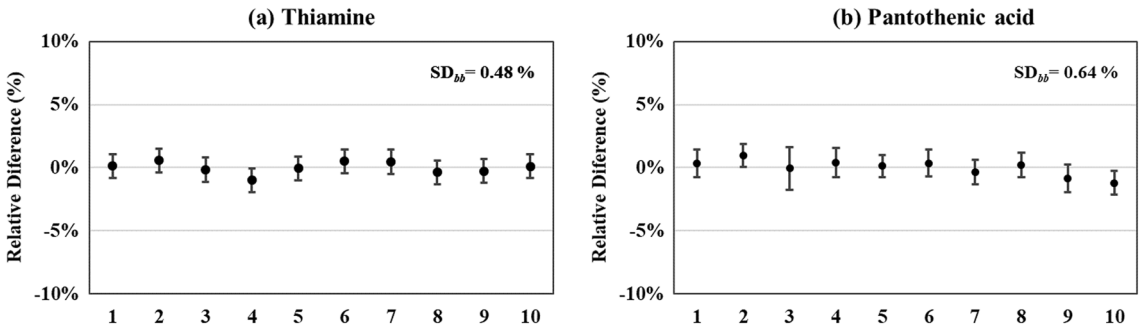


**Fig. 1** Example of homogeneities of thiamine (**a**) and pantothenic acid (**b**) in multivitamin tablet CRM 108-10-019. The error bars are the relative expanded uncertainties of the measurement values of the corresponding units with a 95% level of confidence

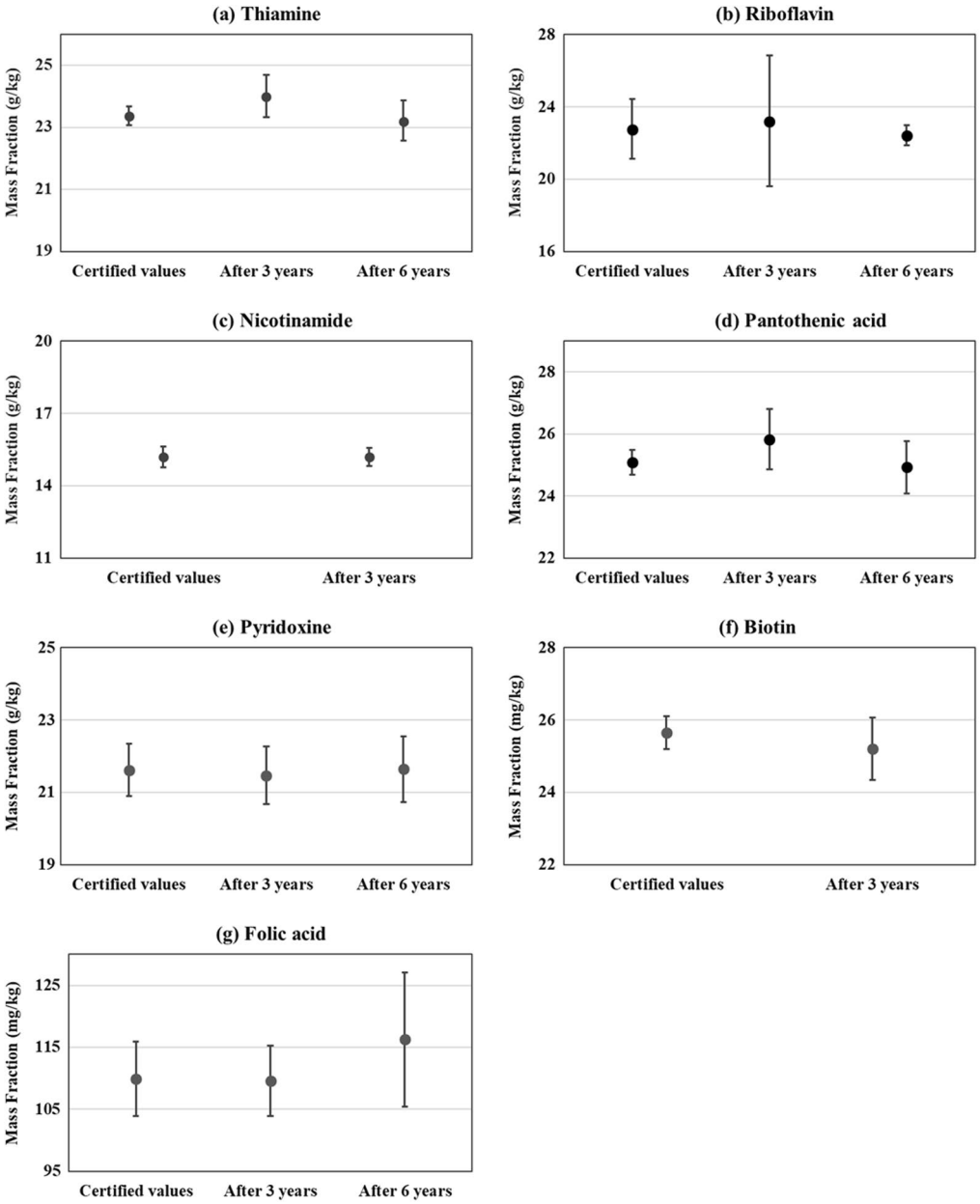


**Fig. 2** Results of long-term stability monitoring for the certified values in multivitamin tablet CRM 108-10-019 stored at − 20 °C

of multivitamin tablet CRM and asked to analyze each bottle along with commercial certified reference materials at different time period. The participants reported the results of 3 sub-samples and mean of these values at each period, and the measurement uncertainties related with the calibration solution, calibration curve and sample preparation. The result of each laboratory for analyte was set as the mean value and combined uncertainty from both periods as listed in Table 6. The mean value of two reported values from the participants was determined as reference values of 4 FSVs. Uncertainty was evaluated by considering the measurement uncertainty of each laboratory and the difference in results between the two participants (Table 6). The homogeneity and stability of the reference values were not considered. Although not certified values, these values sufficiently serve as a guide for each FSV analysis.

**Table 6** Reference values for multivitamin tablet CRM 108-10-019

| Compound | Units | Reference Value[a] | Lab 1 | Lab 2 |
|---|---|---|---|---|
| Retinol (Vitamin A)[b] | mg/kg | 14.3 ± 2.6 | 13.3 ± 0.77 | 15.4 ± 1.39 |
| α-Tocopherol (Vitamin E)[b] | mg/kg | 14.3 ± 3.4 | 17.2 ± 0.40 | 11.4 ± 0.39 |
| Cholecalciferol (Vitamin $D_3$)[c] | mg/kg | 0.125 ± 0.003 | 0.104 ± 0.010 | 0.145 ± 0.006 |
| Phylloquinone (Vitamin $K_1$)[b,c] | mg/kg | 16.7 ± 3.0 | 14.8 ± 1.4 | 18.57 ± 0.53 |

[a] The number following "±" is the expanded uncertainty of the property value at the 95% level of confidence ($k = 2$)

[b] LC–FL

[c] LC–UV

## Conclusions

In this study, a multivitamin tablet CRM (108-10-019) was developed for the analysis of selected vitamins. The candidate material was homogeneously prepared, bottled, and stored at − 20 °C. Validated isotope dilution mass spectrometry methods were applied for the certification of thiamine, riboflavin, nicotinamide, pantothenic acid, pyridoxine, biotin, and folic acid. These seven WSVs were assigned certified values, including property values and appropriate uncertainties. Based on homogeneity testing and stability monitoring, the candidate material was found to be sufficient for use as a CRM. In addition, reference values were assigned for four FSVs, including retinol, α-tocopherol, cholecalciferol, and phylloquinone, based on results from participating expert labs. This multivitamin tablet CRM (108-10-019) is intended for use in calibrating instruments and evaluating the reliability of analytical methods for determining organic nutrients in multivitamin tablets or similar products.

#### Abbreviations

| | |
|---|---|
| WSV | Water-soluble vitamin |
| FSV | Fat-soluble vitamin |
| IDMS | Isotope dilution mass spectrometry |
| LC/MS | Liquid chromatography/mass spectrometry |
| ID–LC/MS | Isotope dilution–liquid chromatography/mass spectrometry |
| ESI | Electrospray ionization |
| SRM | Single reaction monitoring |
| CRM | Certified reference material |
| LC–FLD | Liquide chromatography–fluorescence detector |
| LC–UVD | Liquide chromatography–UV/Vis detector |
| RSD | Relative standard deviation |
| NIST | National Institute of Standards and Technology |
| SRM | Standard reference material |
| GC/MS | Gas chromatography/mass spectrometry |

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#### Author contributions

JL contributed to conceptualization, methodology, investigation, data curation, formal analysis, writing, original draft, writing—review and editing. HS contributed to methodology, investigation, data curation. DS contributed to methodology, investigation, data curation. BMK contributed to methodology, investigation, data curation. JW contributed to methodology, investigation, data curation. BK contributed to data curation. SL contributed to methodology, investigation, data curation, writing—review. SYB contributed to methodology, investigation, data curation, writing—review. JH contributed to methodology, investigation, data curation. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

Not applicable.

### Declarations

#### Competing interests

The authors declare that they have no competing interests.

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