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RESEARCH PAPER

Development of a certified reference material for the accurate analysis of the acrylamide content in infant formula

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Abstract

A certified reference material (CRM), KRISS CRM 108–02-006, was developed for the accurate analysis of low levels of acrylamide in infant formula matrices. The CRM is an infant formula fortified with acrylamide at a similar level as that stipulated by the European Union regulation for baby food. Commercially available infant formulas were processed by freeze-drying, and the subsequent homogenization of the fortified material to produce 961 bottles of the CRM in one batch. The CRM bottles containing approximately 15 g of the material in each unit were stored in a storage room at − 70 ℃. High-purity acrylamide was used as the primary reference material, and its purity was assessed using an in-house mass-balance method to obtain results metrologically traceable to the International System of Units. The acrylamide content of the infant formula CRM was evaluated using isotope dilution–liquid chromatography/mass spectrometry as a reference method, which was established by our research group. An acrylamide content of 55.7 ± 2.1 µg/kg was assigned as the certified value of the CRM with the expanded uncertainty at a 95% confidence level. The homogeneity study showed good uniformity of the acrylamide content among units, providing a relative standard deviation of 1.2% of the mean value. A stability study was also performed by monitoring the CRM under different temperature conditions and periods. The stability results indicated that the acrylamide content in the CRM under the storage conditions of − 70 ℃ remained stable for up to 10 months.

Keywords Acrylamide · Certified reference material · Isotope dilution–liquid chromatography/mass spectrometry · Infant formula · Homogeneity and stability of the infant formula CRM

Introduction

Acrylamide is a useful chemical for many industries related to paper, cosmetics, and petroleum. However, as it is a food contaminant and a probable carcinogen, it adversely affects humans [1, 2]. Acrylamide forms in starchy foods during cooking at high temperatures above 120 ℃. Since this food-related issue was first raised in 2002 by Swedish researchers, acrylamide levels in food have been monitored worldwide [3–6]. The Commission of the European Union (EU) established regulations to mitigate the presence of acrylamide in food in 2017, and legislations on benchmark levels are expected to be renewed in 2023 [7]. The Korean government has implemented regulations to reduce the presence of acrylamide in food since 2021. Accordingly, there is an increasing demand for the reliable measurement of acrylamide content in foods. Additionally, the accurate analysis of acrylamide in daily food is required to alleviate the safety concerns associated with dietary exposure.

We have previously developed a potato chip paste certified reference material (CRM) for analyzing acrylamide in representative carbohydrate-rich fried foods [8, 9]. The European reference material (ERM; crispbread) BD272 is also available for acrylamide analysis. The mass fractions of acrylamide in both CRMs are several hundreds of µg/kg in starchy food matrices. However, no other CRMs are available for the analysis of low acrylamide contents in infant food matrices. Infants and young children are vulnerable to acrylamide exposure from food. The EU has set the lowest benchmark level of acrylamide as 40 µg/kg for infants and young children. Infant formula is a representative baby food consumed daily by infants under 12 months

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of age. Therefore, we developed a new infant formula CRM in accordance with the EU regulations for acrylamide in baby foods.

Isotope dilution–liquid chromatography/mass spectrometry (ID-LC/MS) has been widely used to analyze acrylamide [3, 8–12]. In this study, an optimized analytical procedure for infant formula sample analysis, based on our previously established ID-LC/MS method, was applied to characterize the low levels of acrylamide content. Prior to sample analysis, ^{13}C-labeled acrylamide was added as an internal standard (IS) to account for recovery during sample treatment and instrumental sensitivity. Simple sample extraction using a chloroform–methanol solution and solid-phase extraction (SPE) clean-up were sequentially conducted for the LC/MS instrumental analysis. The developed ID-LC/MS method was validated based on its repeatability and intermediate precision. Finally, the infant formula CRM was characterized using the developed method to obtain metrologically traceable measurement results. Herein, we describe the complete procedure for the development of the infant formula CRM and the analysis of its acrylamide content, including the evaluation of the associated uncertainties. In addition, the homogeneity was assessed to evaluate the uniformity of the acrylamide content throughout the CRM batch, and the stability was monitored to verify the maintenance of the acrylamide levels under specific storage conditions.

Materials and methods

Materials and chemicals

Acrylamide was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as the primary reference material for CRM characterization. The purity of the acrylamide was determined via the following procedures developed in-house based on a mass-balance approach. The purity was 99.9% with the standard uncertainty of 0.1%, which was applied to correct the concentration of the standard solution. The mass-balance approach using various analytical methods has been widely used to determine the purity of the reference material and detailed descriptions of the purity assay are provided elsewhere [13–15]. Briefly, the structurally related impurities in the analyte were analyzed using liquid chromatography–ultraviolet detection (LC-UV), and the water content was determined via Karl Fischer coulometry using an oven-drying method. Nonvolatile inorganic impurities were evaluated using thermogravimetric analysis. The residual solvent was quantified with headspace–gas chromatography MS. Isotope-labeled acrylamide ($^{13}C_3$-acrylamide) that was used as the IS was obtained from Cambridge Isotope Laboratories Inc. (Andover, USA). Acetic acid, methanol, and chloroform were purchased from Burdick and Jackson (Muskegon, NJ, USA). The infant formula used as the raw material to produce the CRM was purchased from local markets.

CRM production procedure

Commercial infant formulas were used to produce the CRM that contained low levels of acrylamide, ranging from 5 to 8 µg/kg. A spiking solution was prepared to produce the CRM with acrylamide levels based on EU regulations. An acrylamide solution was prepared in water at a concentration of 750 µg/kg. Approximately 16 kg of infant formula as the raw material was fortified with 1 L of the acrylamide solution. The fortified infant formula paste was mixed for 4 h and freeze-dried overnight. The dried material was ground using a rotor mill (Pulverisette 14: Fritsch, Idar-Oberstein, Germany) at 13,000 rpm and subsequently sifted to prepare samples with a particle size ranging from 50 to 250 µm. The final homogenization process was conducted using a three-dimensional mixer (JT3DM-300; JT, Kyunggido, South Korea) at 15 rpm for 8 h. Subsequently, 15 g of the homogenized sample was bottled and sealed in an argon atmosphere. A total of 961 bottles of the infant formula CRM were produced and maintained at − 70 ℃ in a storage room.

Sample preparation procedure

Four standard solutions (1 mg/kg) containing purity-assigned acrylamide were gravimetrically prepared in water. $^{13}C_3$-acrylamide was dissolved in water to obtain an isotope-labeled standard solution with the same concentration as that of the aforementioned standard solutions. Each standard solution was diluted ten times and gravimetrically mixed with the isotope-labeled standard solution to prepare two standard solutions at an isotope ratio of 1:1. Acrylamide and $^{13}C_3$-acrylamide in standard solutions with eight isotope ratios were analyzed using LC/MS to select one standard solution as the calibration standard for the determination of the acrylamide content in the samples.

Approximately 1 g of the infant formula was placed in a 50-mL conical flask and spiked with the isotope-labeled standard solution to obtain a 1:1 isotope ratio. The sample was then mixed with 10 mL of water for 10 min. Next, 10 mL of a chloroform–methanol (2:1, v:v) solvent mixture was added and mixed for 10 min to remove lipids. After centrifugation at 5000 rpm for 10 min, the upper aqueous layer was passed through a polyvinylidene fluoride syringe filter. The extract was purified using Oasis PRiME HLB (6 cc, 200 mg) and Oasis PRiME MCX (6 cc, 150 mg) SPE cartridges (Waters, Manchester, UK). Each SPE cartridge was washed with 3 mL of methanol followed by elution of the loaded extract with 0.5 mL of water. Ten microliters of the final extract was injected into the LC/MS system.

ID-LC/MS measurements and quantification of acrylamide

A QTRAP 6500 + LC/MS system (SCIEX, Framingham, MA, USA) was used for ID-LC/MS measurements. The optimized conditions for LC analysis were obtained using an Acquity HSS C18 column (1.8 µm, 2.1 × 100 mm) from Waters (Manchester, UK). The mobile phase was a mixture solution with 0.2% acetic acid and 0.5% methanol in water. The separation was performed at a flow rate of 0.2 mL/min under isocratic conditions. The sample from the LC system was ionized using a positive electrospray at 5500 V. Acrylamide and $^{13}C_3$-acrylamide were monitored in selected reaction monitoring (SRM) modes at *m/z* 72 → 55 and *m/z* 75 → 58, respectively.

The property value of the infant formula CRM is the acrylamide concentration measured using the ID-LC/MS method. The acrylamide content (C_{sample}), expressed as a mass fraction (µg/kg), was calculated using Eq. 1 and the ID-LC/MS measurements. The exact matching of the isotope ratio with single-point calibration was performed in this study, and a detailed explanation can be found in previous reports [8, 9, 16–20].

$$C_{sample} = \frac{m_{is-sol,spiked} \cdot AR_{sample} \cdot m_{s-sol,std.mix.} \cdot C_{s-sol}}{m_{sample} \cdot AR_{std.mix.} \cdot m_{is-sol,std.mix.}} \quad (1)$$

where $m_{is\text{-}sol,\ spiked}$ is the mass of the $^{13}C_3$-acrylamide solution added to the sample; AR_{sample} indicates the area ratio of the acrylamide and $^{13}C_3$-acrylamide peaks in the LC/MS results of the infant formula sample; $m_{s\text{-}sol,std.mix.}$ and $m_{is\text{-}sol,std.mix.}$ represent the masses of the acrylamide standard solution and isotope-labeled acrylamide solution, respectively, which are added to the isotope-ratio standard solution; $C_{s\text{-}sol}$ corresponds to the concentration of acrylamide in the standard solution; m_{sample} is the mass of the sample used for the analysis; and $AR_{std.mix.}$ presents the area ratios of the acrylamide and $^{13}C_3$-acrylamide peaks observed in the LC/MS chromatogram of the isotope-ratio standard solution. The uncertainty evaluation of the measurement values was performed in our laboratory according to the Guide to the Expression of Uncertainty in Measurement [21].

The infant formula CRM contained a certain amount of water. Therefore, the final content of acrylamide in the infant formula samples was determined using dry-mass correction. Three separate aliquots were collected from the same CRM units, which were also selected for the ID-LC/MS measurements. The recommended mass of the aliquot for dry-mass correction is ≥ 1 g. The aliquot was weighed before and after drying for 2 weeks in a desiccator with an adequate amount of P_2O_5 desiccant. The mass difference between each sample was used to correct the acrylamide content in the sample.

Homogeneity study and certified value assignment

Ten units of the infant formula CRM were chosen for homogeneity tests of the acrylamide content. A single aliquot taken from each unit was analyzed following the homogeneity study described in Sect. 7.5.2 of the ISO Guide 35:2017 [22]. Four subsamples from two units were analyzed to determine the certified acrylamide content in the infant formula CRM. The day before the measurement, the units, which were stored at − 70 ℃, were taken from the storage room and were subsequently maintained at room temperature prior to the sample preparation.

The uncertainty of the certified value (u_{CRM}) was evaluated using Eq. 2. A comprehensive explanation of the associated uncertainties can be found in previous reports [8, 16–20].

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

where u_{char} is the uncertainty associated with characterization, including the purity assay, gravimetric preparation of the sample and standard solution, and instrumental analysis; u_{hom} is the uncertainty associated with between-unit homogeneity; and u_{lts} is the uncertainty associated with long-term stability, which is estimated to be zero when the material is stable.

Stability evaluations

The scheme of the stability studies was designed based on Sect. 8.2 of the ISO Guide 35:2017 [22]. The long-term stability of acrylamide in the infant formula CRM was monitored after storage at − 70 ℃ for 5 and 10 months. The stability of the CRM stored at − 20 ℃ for 1 month was evaluated as that under the storage conditions of a typical user. Additionally, CRM samples were maintained at room temperature and 40 ℃ for 1 month and then analyzed for their stability under transport conditions. Two units were randomly selected for each stability test and stored at different temperatures for specific periods.

Results and discussion

Acrylamide analysis using ID-LC/MS

The ID-LC/MS method used herein was improved and validated based on the results of our previous study [8, 9]. The extraction procedure with the solvent mixture was applied to reduce the sample size and remove the matrix for the accurate determination of the acrylamide content in the infant formula CRM. In our previous study, only water was used for the extraction. However, in this study, the sample

treatment with a binary solvent lowered the baseline in the LC/MS analysis and facilitated the quantification of the low levels of acrylamide. Figure 1 presents the SRM chromatograms of acrylamide and $^{13}C_3$-acrylamide extracted from the CRM using ID-LC/MS. The fragment ions at $m/z = 55$ and $m/z = 58$ for the $[M + H\text{-}NH_3]^+$ ions of acrylamide and $^{13}C_3$-acrylamide, respectively, were monitored for quantitative analysis. These product ions were both observed at a retention time of 3.2 min without any interfering peaks from the sample matrix. As described above, the acrylamide content was determined using the area ratio of the observed acrylamide and its isotope-labeled peaks in the LC/MS results of the CRM and standard solutions. The limit of detection (LOD), indicating a signal-to-noise ratio (S/N) of 3, was evaluated to be 0.03 µg/kg using the standard solution. The limit of quantification (S/N = 10) for acrylamide was estimated to be 0.1 µg/kg.

In this study, the ID-LC/MS method was validated as a high-order reference method based on its repeatability and intermediate precision, including the associated uncertainties of acrylamide measurements. The repeatability of the ID-LC/MS analysis was evaluated using the three subsampling measurements at the same period of time. The intermediate precision of the method was assessed using the same sample set for the repeatability test, employing a newly prepared standard solution at different time points. As listed in Table 1, the repeatability and the intermediate precision values were less than 1.1% and 1.4%, respectively. These repeatability and intermediate precision results indicate that the ID-LC/MS method is reliable and reproducible as a high-order reference method for the accurate analysis of the acrylamide content.

A previously developed potato chip CRM, KRISS 108-10-003, was analyzed to verify the modified ID-LC/MS method. The certified value of the potato chip CRM was estimated as 0.455 ± 0.012 mg/kg using the previously designed ID-LC/MS method. The acrylamide content in the potato chip CRM was evaluated using the ID-LC/MS method adopted in this study, employing the newly optimized sample treatment and instrumental conditions. Additionally, the sample size used for the analysis was reduced from 3 to 1 g. The measurement result, 0.445 ± 0.012 mg/kg, obtained using the current ID-LC/

Table 1 Repeatability and intermediate precision of ID-LC/MS measurement results for homogenized infant formula samples

Periods	Subsample No	Results obtained by ID-LC/MS (µg/kg)[a]
		Infant formula
		Acrylamide
1	1	56.1 ± 1.3
	2	55.9 ± 1.3
	3	55.6 ± 1.4
	Mean	55.9
	SD[b]	0.3 (0.47%)
2	1	57.9 ± 1.5
	2	57.5 ± 1.4
	3	56.8 ± 1.5
	Mean	57.4
	SD[b]	0.5 (0.92%)
3	1	56.2 ± 1.1
	2	57.4 ± 0.9
	3	56.5 ± 0.9
	Mean	56.7
	SD[b]	0.6 (1.11%)
Overall mean		56.6
SD among periods[c]		0.8 (1.36%)

[a]The number following "±" is the expanded uncertainty with a 95% confidence level for the measurement result

[b]The standard deviation (SD) was obtained using three values measured within a day

[c]The SD was obtained using three mean values measured at different time points

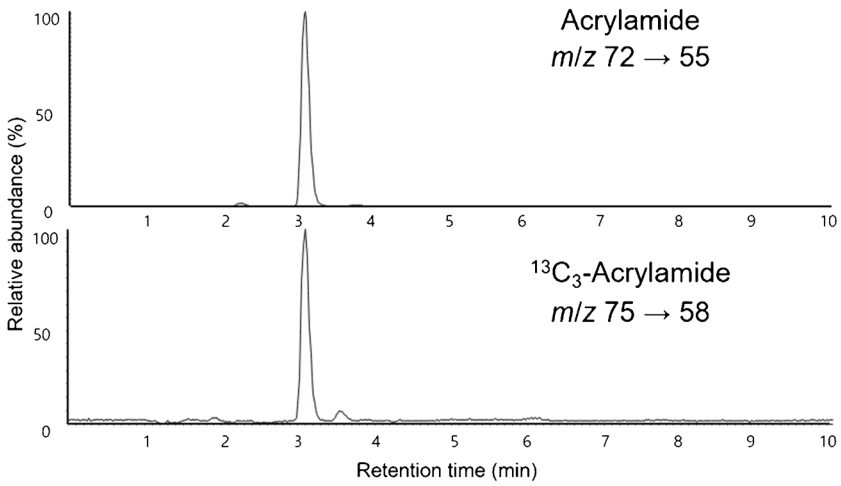

Fig. 1 SRM chromatograms of acrylamide and $^{13}C_3$-acrylamide extracted from the infant formula CRM

MS method was comparable to the certified value within the uncertainty.

Homogeneity test and characterization of the infant formula CRM

The recommended number of units for the homogeneity study is $\sqrt[3]{N_{prod}}$ (the total number of units produced, N_{prod}) according to Sect. 7.4.1 of the ISO Guide 35:2017 [22]. Therefore, ten units from a total of 961 bottles of the infant formula CRM were randomly chosen for the between-unit homogeneity test. A single subsample from each unit was analyzed using ID-LC/MS. As per the homogeneity study illustrated in Fig. 2, the standard deviation (SD) among the units is 0.7 µg/kg (1.2% of the mean value), indicating that the infant formula CRM shows sufficient between-unit homogeneity.

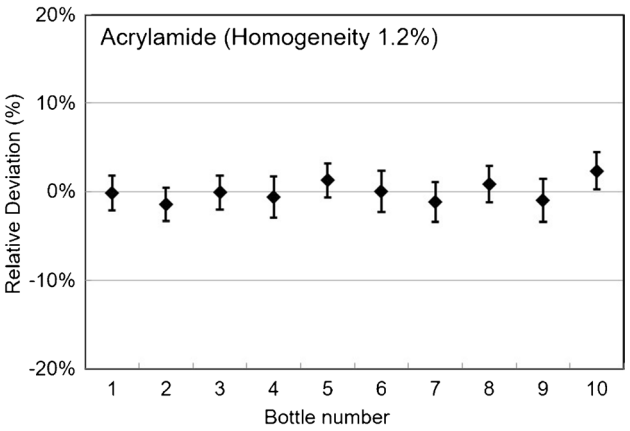

Fig. 2 Homogeneity assessment of the infant formula CRM. Error bars correspond to the expanded measurement uncertainties of each unit with a confidence level of 95%

To characterize the CRM, the purity assignment of the primary reference material is an essential step in establishing the metrological traceability of the certified value. By assigning purity to high-purity acrylamide in the mass fraction, based on the mass-balance method, allows the results to be metrologically traceable to the International System of Units (SI units). As mentioned above, the concentration of the standard solution was corrected based on the assigned purity of acrylamide using in-house protocols. Additionally, the uncertainty of the assigned purity values contributes to the final measurement uncertainty of the acrylamide content.

Four subsamples from two units and the standard solutions were gravimetrically prepared for instrumental analysis, as described in "Sample preparation procedure." The ID-LC/MS results with the associated uncertainties are summarized in Table 2. The mean of the measured values obtained using the ID-LC/MS method was 55.7 µg/kg, and the SD was 0.5 µg/kg (0.8% of the mean value). The final measured values of acrylamide were calculated by applying the dry-mass correction. The mean value of the dry-mass correction factor was 1.016, which was multiplied by C_{sample} in Eq. 1. The associated uncertainty of the certified value for the infant formula CRM (u_{CRM}) was a result of both the systematic and random effects. Table 3 presents the uncertainty budget for the ID-LC/MS measurement of the acrylamide content in the infant formula CRM. As described above, the value of u_{CRM} was calculated using Eq. 2 with the uncertainty components of the measurements. u_{CRM} presents a combination of the uncertainty related to characterization (u_{char}) and the uncertainty arising from the between-bottle inhomogeneity (u_{hom}). u_{char} can be calculated from Eq. 3.

$$u_{char} = \sqrt{\left(\frac{SD}{\sqrt{n}}\right)^2 + u_{c,sys}^2} \quad (3)$$

Table 2 ID-LC/MS measurement results for the certification of the acrylamide content in the infant formula CRM

Subsample no	Measurement result (µg/kg)	Uncertainty component	Value of uncertainty (µg/kg)
1	56.0	$u_{c,sys}$ [a]	0.7 (1.2%, $\nu = 5$)
2	56.2	u_{char} [b]	0.7 (1.3%, $\nu = 6$)
3	55.5	$u_{c,ran}$ [c]	0.3 (0.5%, $\nu = 20$)
4	55.2	SD_{bb} [d]	0.7 (1.2%, $\nu = 9$)
Mean	55.7	u_{hom} [e]	0.6 (1.1%, $\nu = 6$)
SD	0.5 (0.8%, $\nu = 3$)	u_{CRM} [f]	0.9 (1.7%, $\nu = 11$)

[a]The combined uncertainty from the systematic effect ($u_{c,sys}$) during characterization

[b]The uncertainty associated with characterization

[c]The combined uncertainty from the random effect ($u_{c,ran}$) during characterization

[d]The standard deviation between the values of bottles (SD_{bb}) from homogeneity study

[e]The uncertainty associated with between-bottle variability by the subtraction of $u_{c,ran}$ from SD_{bb}

[f]The combined uncertainty associated with the certified value of CRM

Table 3 Uncertainty budget for the characterization of acrylamide in infant formula CRM using the ID-LC/MS method

Systematic effects	u_{sys} [a] (rel. %)	Degree of freedom	Random effects	u_{ran} [b] (rel. %)	Degree of freedom
Purity assay of the primary reference material	0.08	10	Gravimetric preparation of sampling and spiking for each unit analysis	< 0.02	∞
Gravimetric preparation of the standard solutions	1.03	3	Dry-mass measurement of subsample from each unit	< 0.04	2
Gravimetric preparation of the isotope-ratio standard solutions	0.54	4	Determination of the area ratio of the acrylamide and $^{13}C_3$-acrylamide peaks from the instrumental analysis of each sample extract	< 0.9	2
Determination of the area ratio of the acrylamide and $^{13}C_3$-acrylamide peaks from the instrumental analysis of the isotope-ratio standard solution	0.34	2			

[a]The standard uncertainty values from the systematic effect that are uniform among units (u_{sys})

[b]The standard uncertainty values from the random effect that are different between units (u_{ran})

where $u_{c,sys}$ is the combined uncertainty of the systematic effect obtained through characterization.

The SD between the bottles from the homogeneity test (SD_{bb}) included uncertainty due to random effects. Therefore, u_{hom} was estimated by subtracting the combined uncertainty related to the random effect ($u_{c,ran}$) from that of SD_{bb}. Finally, the certified value of the acrylamide content for the CRM was assigned to be 55.7 ± 2.1 µg/kg, where the expanded uncertainty was at a confidence level of 95% ($k = 2.2$).

Stability monitoring of the infant formula CRM

Four subsamples from two unopened units were assessed following the same analytical procedure used for the ID-LC/MS measurement, to monitor the stability of the infant formula CRM. The infant formula CRM bottles were maintained in the storage room at −70 ℃ for long-term storage. After storage at −70 ℃ for 5 and 10 months, the acrylamide contents of the infant formula CRM samples were analyzed for their long-term stability. The CRM units were stored at −20 ℃, room temperature, and 40 ℃ for 1 month to ensure stability during the user's storage and transport conditions. A graphical representation of the stability results is shown in Fig. 3. The measured values from the stability tests except the result obtained after storage at 40 ℃ are in good agreement with the certified value, within the measurement uncertainties in accordance with Sect. 8.10.3.2 of the ISO Guide 35:2017 [22]. Therefore, the stability study revealed that the infant formula CRM maintained a stable acrylamide level under storage conditions with a temperature lower than room temperature. However, the acrylamide level after storage at 40 ℃ decreased to 39.4 ± 2.9 µg/kg, which indicated the instability of the CRM under high-temperature storage conditions. Microorganisms are known to degrade

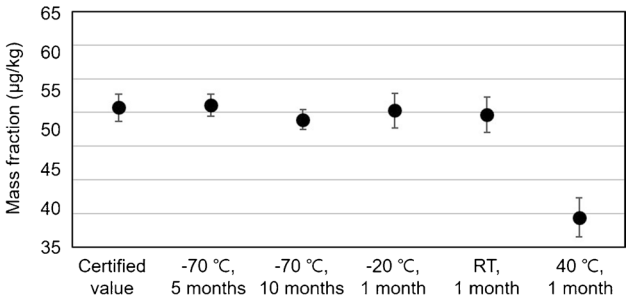

Fig. 3 Stability assessment of the infant formula CRM under different storage conditions. Error bars correspond to the expanded measurement uncertainties of the measured values with a confidence level of 95%

acrylamide [23, 24]. The infant formula CRM was produced without a sterilization process; therefore, the degradation of acrylamide occurred under high-temperature storage conditions. Additionally, thermal effects could facilitate reactions between acrylamide and other ingredients of the infant formula CRM, resulting in a decreased acrylamide content [25]. The infant formula CRM requires low-temperature storage and refrigerated transport conditions to maintain the stability of its acrylamide content.

Conclusions

An infant formula, CRM (108-02-006), was developed for the analysis of the acrylamide content at a level similar to that stipulated by the EU regulations for baby food. To the best of our knowledge, no other CRM is currently available for evaluating low levels of acrylamide in the matrices of baby food. Thus, the infant formula CRM could help to control the acrylamide levels in food by verifying the

acrylamide content with CRM. The ID-LC/MS method was established as a laboratory protocol at the National Metrology Institute of Korea to determine low levels of acrylamide in infant formula matrices. The infant formula CRM was characterized using the ID-LC/MS method, obtaining an acrylamide content of 55.7 ± 2.1 µg/kg as the certified value of the CRM with the expanded uncertainty at a 95% confidence level. The CRM exhibited a sufficient homogeneity of the acrylamide content at 1.2% of the relative SD among the units. The stability evaluation of the CRM under different storage conditions (− 70 ℃, − 20 ℃, room temperature, and 40 ℃) was performed to confirm the consistency of the certified value under the studied conditions. The stability results showed that acrylamide contents were stable within the associated uncertainties for up to 10 months under the CRM storage conditions at − 70 ℃. The stability of the acrylamide content in the CRM will be assessed periodically to check that the certified value remains acceptable for use. The CRM developed for the accurate analysis of acrylamide in infant formulas can be used for the calibration and quality control of the analytical procedure as well as to establish the metrological traceability of the measurement results to the SI units.

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Declarations

Competing interests The authors declare no competing interests.

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