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Development of Methyl 2-aminobenzoate Reference Material in a Biocidal Product Matrix

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Abstract : The utilization of methyl 2-aminobenzoate as a biocide and pesticide has raised concerns regarding its potential toxicity. To assess its safety, it is crucial to determine its quantity and related toxicity using reference materials (RMs) or certified reference materials (CRMs). As an RM and CRM containing methyl 2-aminobenzoate within a biocidal product matrix is currently unavailable, this study aimed to produce a high-quality RM containing methyl 2-aminobenzoate, ensuring its homogeneity and stability, following the ISO Guide 35 and ISO 17034. The study determined that the produced RM exhibited homogeneity, as indicated by a calculated F-value (1.91) smaller than the critical F-value (3.02). In the assessment of isochronous short-term stability, the slope of the linear regression for the RM showed no statistically significant difference from zero when stored at temperatures of 4, 18, and 60 °C for 4 weeks. Regarding classical long-term stability, the RM demonstrated sustained stability over the course of one year when stored at 4 °C. This study has successfully developed an RM for monitoring methyl 2-aminobenzoate in biocides and pesticides. Its quality underwent rigorous evaluation, confirming both homogeneity and stability.

Keywords : Methyl 2-aminobenzoate, Reference Material, Stability, Homogeneity, Biocides, GC-MS

Introduction

Pesticides and biocides are subject to strict regulations worldwide due to their potential impact on human health and the environment. One such regulation is the EU Biocidal Product Regulation (BPR).[1] In Korea, public concern arose regarding chemical safety following the humidifier disinfectant incidents, wherein the misuse of biocides such as polyhexamethylene guanidine (PHMG) and 5-chloro-2-methylisothiazol-3(2H)- one/2 methylisothiazol-3(2H)-one (CMIT/MIT) led to severe lung injuries and fatalities through inhalation.[2,3] This prompted the Korean government to enact the Consumer Chemical Products (CCPs) and Biocides Safety Management Act (K-BPR) in 2018, mandating that all CCPs and biocides must meet safety standards before being distributed in the market. Under the K-BPR, the content and quantity of biocides in CCPs and biocidal products are subject to regular monitoring and regulation.

Reference materials (RMs) are substances characterized by homogeneity and stability in specific properties, making them suitable for use in specific measurement procedures. Numerous studies have been conducted to develop RMs for various applications.[4-6] For instance, researchers explored the potential of creating an RM for pesticide residue analysis in a cucumber matrix. Fifteen specific pesticides were spiked into the cucumber at 0.075 mg/kg, and their homogeneity between units and stability at different temperatures were rigorously examined over 9 weeks.[4] Another example involves the development of an RM for hair analysis of methamphetamine and its primary metabolites amphetamine. This RM displayed concentrations of 4.86±0.69 ng/mg for methamphetamine and 4.63±0.44 ng/mg for amphetamine, demonstrating its potential for quality assurance in forensic laboratories.[5] Our laboratory also established a chloroform RM with between-unit and within-unit standard deviations of 0.84% and 2.24%, respectively, which remained stable for 50 days.[6] Furthermore, various certified reference materials (CRMs), which are reference materials with a certified value, accompanied by an uncertainty and metrological traceability, have been developed with more rigorous validation processes. These RMs and CRMs play pivotal roles in regulating biocidal substances and products, serving purposes such as calibration, quality control, method valida-

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tion, and proficiency testing (PT).[7-9]

The objective of this study is to develop an RM for methyl 2-aminobenzoate, also known as methyl anthranilate, a chemical commonly added to various products for its grape flavor, under conditions that simulate the matrix of biocidal products.[10] This chemical has been shown to have relatively low toxicities to mammals, including humans, as evidenced by its oral lethal dose 50% (LD50) value for rats and dermal LD50 value for rabbits, both of which are in the g/kg range.[11-12] It is utilized in a variety of products, such as candy, beverages, gum, air fresheners, biocides, and even as a bird repellent due to its ability to activate pain receptors in birds related to taste and smell.[10-13] However, caution must be exercised when using methyl 2-aminobenzoate in aquatic environments due to its potential toxicity to aquatic organisms, as evidenced by its LD50 for fish, which falls in the mg/L range.[11,14] When young fish are exposed to high levels of methyl 2-aminobenzoate, they exhibit symptoms such as a cyanotic condition and rapid grill movements, suggesting possible oxygen deficiency. Methyl 2-aminobenzoate, which is more soluble in fats than water, can be absorbed into the fish's gill membranes, affecting their respiration.[14] Anthranilates can readily undergo metabolic hydrolysis.[15] While specific assays for hydrolysis products in young fish were not conducted, anthranilic acid is a likely hydrolytic product. This acid might alter the pH of the plasma, disrupting oxygen transport.[14] Methyl 2-aminobenzoate exhibits poor biodegradability, low volatility, and a slow rate of hydrolysis, making it challenging to remove once released into the environment.[16] Therefore, monitoring its toxicity and ensuring the quantity of products containing methyl 2-aminobenzoate are of paramount importance.

Currently, there is no RM or CRM available for methyl 2-aminobenzoate that is suitable for PT involving biocidal products. In light of this, we developed an RM by dissolving it in a solvent composition designed to mimic biocidal product matrices, making it suitable for PT of biocides. Ethanol was chosen as the matrix for the RM because of its common use in biocidal products containing methyl 2-aminobenzoate with a high ratio. To create this RM for methyl 2-aminobenzoate, we conducted assessments for homogeneity, monitored its stability, carried out method validation, and calculated uncertainties, all of which confirmed its suitability for quality assurance. All these processes were performed using gas chromatography-mass spectrometry (GC-MS), which is commonly used for the analysis of biocides and pesticides, as many of these substances exhibit high volatility, providing precise and accurate results.[17-18] These analytical procedures and interpretations were conducted in accordance with ISO Guide 35[7] and ISO 17034.[8] Adhering to the ISO guidelines ensures the highest quality, global acceptance, and consistent outcomes across various applications.

Experimental

Materials

Methyl 2-aminobenzoate (99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and methyl N-methyl-2-aminobenzoate (also known as methyl N-methylanthranilate, used as an internal standard, > 95.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethanol with HPLC grade purity was purchased from Daejung Chemicals & Metals (Siheung, Korea).

Preparation of the solutions to construct the calibration curves

To prepare the 100 μg/mL stock solutions of methyl 2-aminobenzoate and methyl N-methyl-2-aminobenzoate, 1 mg of each compound was added to separate 10 mL volumetric flasks containing ethanol, and then filled to the marked line. Calibration curves for five points (0.02, 0.5, 1, 2, and 3 mg/L) were established by adding different volumes (2, 50, 100, 200, and 300 μL) of the methyl 2-aminobenzoate stock solution to separate 10 mL volumetric flasks containing 100 μL of the methyl N-methyl-2-aminobenzoate stock solution.

Preparation of methyl 2-aminobenzoate RM

Ethanol was selected as the matrix for the RM due to its widespread use in air freshener products that contain methyl 2-aminobenzoate, with a ratio of 85–90% ethanol. At the National Institute of Environmental Research (NIER), proficiency tests, administered twice every five years for licensing purposes, are typically conducted within the range of 10 mg/kg to 100 mg/kg . Therefore, the concentration of methyl 2-aminobenzoate in the RM was selected to be 20 mg/kg, falling within this range and positioned below the midpoint. To create a 4 kg solution of 20 mg/kg RM, 80 mg of methyl 2-aminobenzoate was added to a 100 mL volumetric flask and filled with 100 g of ethanol. The sample was sonicated for more than one hour to ensure homogenization, then scaled up. The 800 mg/kg solution was transferred to a large stainless container, diluted with ethanol to 4 kg, and stirred for 1–2 hours using a homogenizer. The final RM was then divided into 200 separate bottles.

GC-MS analysis

Analysis of methyl 2-aminobenzoate was conducted using a Shimadzu GC-MS instrument (GCMS-TQ8050 NX, Kyoto, Japan) at the Advanced Bio-Interface Core Research Facility, Sogang University, equipped with a DB-5MS column (30 m × 0.25 mm, 0.25 μm, Agilent, Santa Clara, CA, USA). High-purity helium (99.999%, Ewha Industrial Gas Co., Ltd., Seoul, Korea) served as the carrier gas, with a flow rate of 1.0 mL/min. The temperature of the sample inlet, transfer line, and ion source was set at 250, 250, and 220°C, respectively. An injection was performed

in split mode (10:1). The temperature was maintained at 50°C for 2 min before being increased to 150°C at a rate of 8°C/min. The temperature was held at 150°C for 5 min and then increased by 10°C/min to 250°C and held for an additional 3 min. The MS conditions were as follows: a 2.5 min solvent delay; SIM (Selective-ion monitoring) mode with *m/z* 119.0 and 92.0 selected for methyl 2-aminobenzoate and with *m/z* 165.0 and 105.0 selected for methyl N-methyl-2-aminobenzoate. Chromatographic peaks were identified, and area integrations were performed using the GCMS solution software (Shimadzu Corporation, Kyoto, Japan). The concentration of the RM was calculated based on the relative area with respect to that of methyl N-methyl-2-aminobenzoate in the standard calibration curve.

Validation

The linearity of the calibration plot was determined by calculating the coefficient of determination (R^2). The limits of detection (LOD) and limits of quantitation (LOQ), which provide critical criteria for assay sensitivity, were established based on signal-to-noise ratios of 3 and 10, respectively. Furthermore, the recovery, indicating its alignment with true values, and precision, reflecting the consistency of repeated measurements, were systematically evaluated.

Homogeneity test

The measurement of homogeneity was performed by randomly selecting ten bottles of RM. Two portions from each of the ten bottles were extracted and tested in triplicate after 10-fold dilution. To create the diluted solution, 1 g of each portion of the RM and 100 μL of the methyl N-methyl-2-aminobenzoate stock solution were added to a 10 mL volumetric flask, followed by the addition of ethanol to fill the flask to the marked line. To estimate homogeneity, an F-test using analysis of variation (ANOVA) was employed, and the uncertainty due to heterogeneity (u_{bb}) was computed using the following equations based on ISO Guide 35[7] and ISO 17034:[8]

$$S_{bb} = u_{bb} = \sqrt{\frac{(M_{\text{between}} - M_{\text{within}})}{n}} \quad (M_{\text{between}} > M_{\text{within}}) \quad (1)$$

$$u_{bb} = \sqrt{\frac{M_{\text{within}}}{n}} \sqrt[4]{\frac{2}{df_{\text{within}}}} \quad (M_{\text{between}} < M_{\text{within}}) \quad (2)$$

where S_{bb} is the standard deviation of between-bottle homogeneity; M_{between} and M_{within} are, respectively, the mean squares of between-group and within-group; n is the number of observations; df_{within} is the within-group degrees of freedom. If M_{between} is larger than M_{within}, both Equation (1) and (2) can be used to calculate the uncertainty due to heterogeneity, and the greatest result should be considered as the level of uncertainty. However, if M_{within} is greater than M_{between}, only Equation (2) should be used to determine the uncertainty due to heterogeneity.[19]

Stability test

Both short-term stability over 4 weeks and long-term stability over a year were assessed. For the short-term stability evaluation, bottles were placed under storage conditions (4 , 18, and 60°C). After each planned exposure time (0, 1, 2, and 4 weeks), two randomly chosen bottles were transferred to a reference condition (–20°C), where degradation is considered highly improbable until analysis. Upon completion of the exposure periods for all units, simultaneous measurements were conducted for the entire set of bottles. For the long-term stability assessment, all vials were stored at 4°C and two randomly selected bottles were subsequently analyzed at intervals of 0, 3, 6, 9, and 12 months. In both stability test, two portions were extracted from each bottle, diluted ten times using the same procedure as in the homogeneity test, and measured in triplicate.

The stability of the RM was monitored using a linear regression plot, and the significance of the slopes was tested using a t-test. The calculation of the uncertainty due to instability, represented by u_s, was also carried out using Equation (3).

$$u_s = s(b_1) \times t \quad (3)$$

where $s(b_1)$ is the standard deviation of slope; t is the time of monitoring period. All the analytical and statistical procedures were carried out following the guidelines outlined in ISO Guide 35[7] and ISO.[8] The instability uncertainties for short-term (u_{sts}) and long-term (u_{lts}) were individually calculated.

The relative standard uncertainties for heterogeneity ($u_{bb,rel}$) and instability for short-term ($u_{sts,rel}$) and long-term ($u_{lts,rel}$) were determined by dividing each respective uncertainty value by the mean, followed by multiplication by 100.[19]

Results and Discussion

GC-MS chromatogram of methyl 2-aminobenzoate

Figure 1 illustrates the chemical structures of (a) methyl 2-aminobenzoate and (b) methyl N-methyl-2-aminobenzoate, with a weighted molecular mass 151.2 and 165.2, respectively. Since there is no commercially available isotopically labeled 2-aminobenzoate, a structurally comparable compound, methyl N-methyl-2-aminobenzoate, was utilized as an internal standard. During the GC-MS analysis in the selected ion monitoring (SIM) mode, the peaks were identified by their respective characteristic *m/z* values: for methyl 2-aminobenzoate, *m/z* values of 119.0 and 92.0; for methyl N-methyl-2-aminobenzoate, *m/z* values of 165.0 and 105.0.

The GC-MS chromatogram for the 10-fold diluted RM containing methyl 2-aminobenzoate and methyl N-methyl-2-aminobenzoate is presented in Figure 2. The chromatographic analysis revealed two peaks with distinct retention

times. The first peak, detected at 14.70 min, was identified as methyl 2-aminobenzoate, while the second peak, observed at 16.08 min, was recognized as methyl N-methyl-2-aminobenzoate. Through repeated measurements, it was established that the retention times and baseline remained consistent and stable.

Validation

Figure 3 represents the calibration curve for methyl 2-aminobenzoate standard solutions. The graph depicts the relationship between the relative peak area values of methyl 2-aminobenzoate and its concentrations (0.02, 0.5, 1, 2, and 3 mg/L). The linearity of the calibration curve is indicated by the coefficient of determination (R^2), which is reported in Table 1. With an R^2 value exceeding 0.999, the calibration curve exhibits a strong linear relationship. Table 1 presents a comprehensive overview of the validation results, including the LOD, LOQ, as well as recovery and precision values. These results demonstrate that the analytical method employed in this study is highly sensitive and well-suitable for the precise quantification of methyl 2-aminobenzoate.

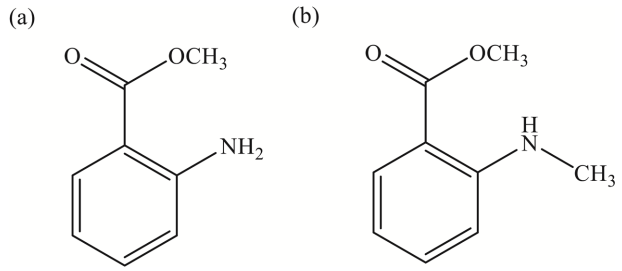

Figure 1. Structure of (a) methyl 2-aminobenzoate and (b) methyl N-methyl-2-aminobenzoate.

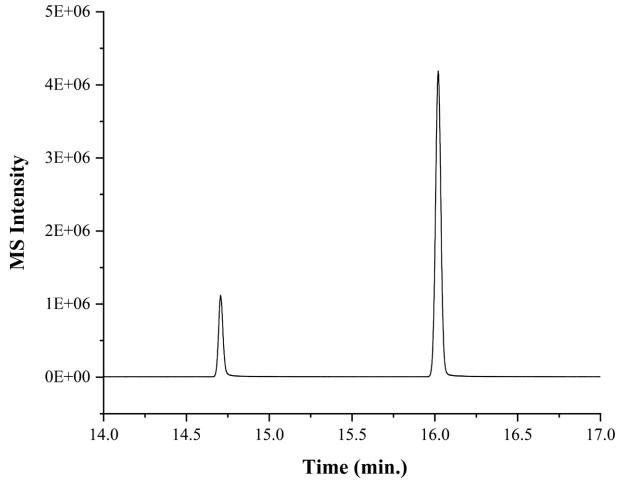

Figure 2. GC-MS chromatogram of the 10-fold diluted RM for methyl 2-aminobenzoate at m/z 119.0 and 92.0, and for methyl N-methyl-2-aminobenzoate at m/z 165.0 and 105.0. The peaks corresponding to methyl 2-aminobenzoate and methyl N-methyl-2-aminobenzoate were observed at 14.70 min and 16.08 min, respectively.

Homogeneity Assessment

The results of the homogeneity test are presented in Table 2. The *P*-value of 0.16 obtained from the test was greater than 0.05, and the calculated F value ($F_{calculated}$) of 1.91 was lower than the critical F value ($F_{critical}$) of 3.02 at 95 % confidence interval. These findings suggest that there is no significant difference between the bottles, confirming the homogeneity of the RM. Since $M_{between}$ (0.21 mg/kg) is larger than M_{within} (0.11 mg/kg), the uncertainty due to heterogeneity was estimated by using both Equation (1) and Equation (2). The larger value was selected as the final uncertainty due to heterogeneity, which was determined to be 0.23 mg/kg (1.14 %).

Stability Assessment

The stability assessment can be divided into two categories: short-term stability (often referred to as "Transportation stability") and long-term stability.[7,8] Short-term stability focuses on consistency under potential transporta-

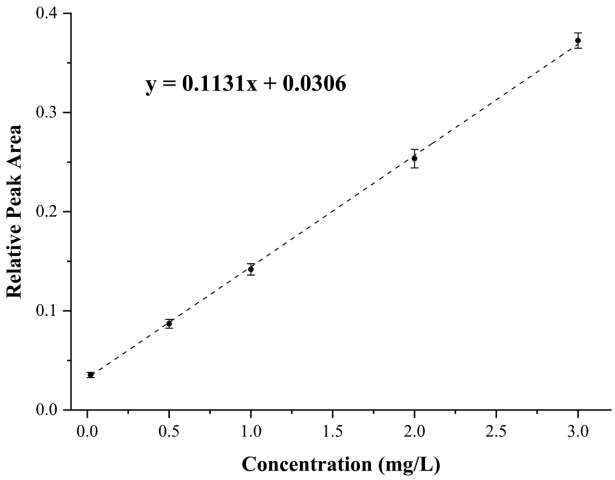

Figure 3. Calibration curve of methyl 2-aminobenzoate for quantitative analysis of the developed RM. The curve was constructed at five different concentrations, i.e., 0.02, 0.5, 1, 2, and 3 mg/L with the addition of the methyl N-methyl-2-aminobenzoate, i.e., 1 mg/L as an internal standard.

Table 1. Calibration results (linearity (R^2), LOD, LOQ, recovery (%), and precision (RSD, %)).

Linearity (R^2)	LOD (mg/kg)	LOQ (mg/kg)	Recovery (%)	Precision (RSD, %)
0.9996	0.02	0.09	98.3	2.0

tion conditions, which could impact its certified values. Considering reasonable temperatures during transportation, assessments were conducted at 4, 18 and 60°C. Long-term stability pertains to the consistency of an RM under specific storage conditions over an extended period. In this study, we evaluated its stability at a representative storage temperature of 4°C for one year.

For the short-term stability test, we utilized the isochronous approach, a method in which samples were placed at the storage conditions for a planned exposure time and then moved to reference conditions. They were remained reference conditions until the end of all exposure times, with all samples being measured simultaneously at the end of the study.[20-22] This approach offers the advantage of conducting studies under conditions of repeatability similar to those used in homogeneity studies.[21,22] In contrast, since the long-term evaluation measure stability at the temperature at which it is stored, we employed a classical approach, where measurements were taken based on elapsed time.

To evaluate the stability of the produced RM, linear regression analysis was employed. A stable RM is characterized by a slope close to zero, indicating minimal change in concentration over time. If the absolute value of the slope ($|b_1|$) is smaller than the multiplicity of the product of the critical t-value at a 95 % confidence interval with n-2 degree of freedom ($t_{0.95,n-2}$) and the standard deviation of the slope $s(b_1)$, then the slope can be considered statistically not different from zero.

Table 2. Homogeneity assessment for methyl 2-aminobenzoate RM.

Sample	Concentration (mean ± S.D. (mg/kg), n=2)
1	19.57 ± 0.18
2	19.40 ± 0.14
3	19.55 ± 0.21
4	19.80 ± 0.85
5	19.20 ± 0.00
6	19.90 ± 0.14
7	19.40 ± 0.28
8	19.50 ± 0.42
9	20.30 ± 0.14
10	19.95 ± 0.07
$M_{between}$ (mg/kg)	0.21
M_{within} (mg/kg)	0.11
$F_{calculated}$	1.91
$F_{critical}$	3.02
u_{bb} (mg/kg)	0.23
$u_{bb,rel}$ (%)	1.14
P-value	0.16

Short-term stability test

The isochronous short-term stability was assessed over a 4 week period at temperatures of 4, 18, and 60°C, with the results presented in Figure 4. As the storage temperature increased, there was a noticeable trend of greater deviation in concentration, attributed to increased transportation instability at higher temperatures. Detailed numerical results of regression analysis can be found in Table 3.

For all storage temperatures, the stability assessment indicated that the value of $|b_1|$ was lower than the value of $t_{0.95,n-2} \times s(b_1)$, and the *P*-value exceeded the significance level of 0.05. Calculations using Equation (3) for 4 weeks yielded an uncertainty due to instability of 0.20 mg/kg (1.04%), 0.74 mg/kg (3.79%), and 0.85 mg/kg (4.20%) for the respective temperatures. These results suggest that transportation should be carried out at temperature below 60°C for duration up to 4 weeks, with lower temperatures providing increased stability.

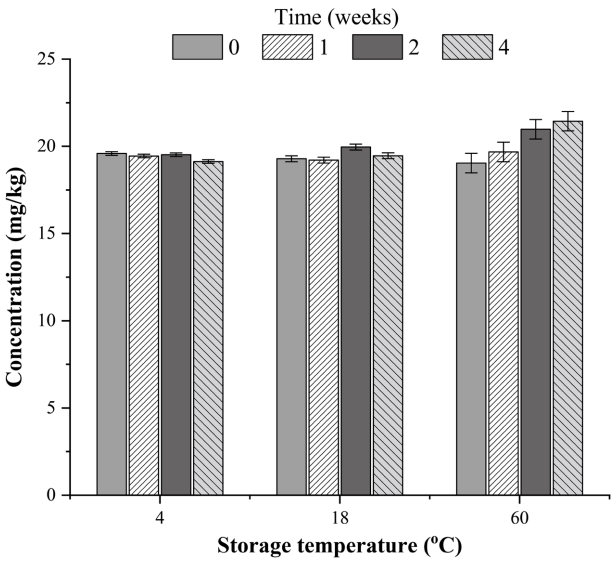

Figure 4. Result of the isochronous short-term stability monitoring of the RM over 4 weeks at temperatures of 4, 18, and 60°C.

Table 3. Short-term stability assessment for methyl 2-aminobenzoate RM.

	Storge temperature		
	4°C	18°C	60°C
b_1	-0.11	0.071	0.62
$s(b_1)$	0.05	0.18	0.21
$t_{0.95,n-2}$	4.30	4.30	4.30
u_{sts} (mg/kg)	0.20	0.74	0.85
$u_{sts,rel}$ (%)	1.04	3.79	4.20
P-value	0.068	0.16	0.051
Statistical significance	No	No	No

Table 4. Long-term stability assessment for methyl 2-aminobenzoate RM.

b_1	$s(b_1)$	$t_{0.95,n-2}$	u_{lts} (mg/kg)	$u_{lts,rel}$ (%)	*P*-value	Statistical significance
-0.11	0.06	3.18	0.67	3.40	0.38	No

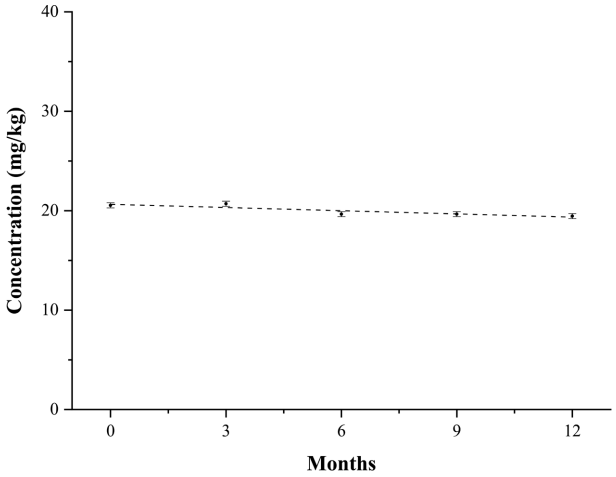

Figure 5. Result of long-term stability monitoring over one year at 4°C.

Long-term stability test

Figure 5 represents the stability of the RM over one year at 4°C. The slope of the regression line (-0.11, shown in Table 4) is close to zero, further supporting the stability of the RM. The results of the stability monitoring are presented in Table 4. The $|b_1|$ value was found to be less than $t_{0.95,n-2} \times s(b_1)$, leading to the conclusion that the RM remained stable throughout the monitoring period. The *P*-value obtained from the test was 0.38, which exceeded the significance level of 0.05. The estimated uncertainty due to instability was 0.67 mg/kg (3.40 %) when the t-value in Equation (3) was calculated for one year.

Evaluations of stability and homogeneity verified the consistency of the RM, demonstrating its potential for CRM development. Further interlaboratory characterization experiments using various methods or laboratories are necessary to determine its suitability as a CRM. Combining the third uncertainty component from these experiments will provide a more comprehensive estimation of the expanded uncertainty of the produced RM.

Conclusions

The RM for monitoring methyl 2-aminobenzoate in pesticides and biocides was successfully developed and validated through GC-MS analysis. Its suitability for quality assurance was confirmed through an assessment of its homogeneity and stability in accordance with ISO guidelines. The homogeneity test showed that the manufactured RM was sufficiently homogeneous, as the calculated F value, *i.e.*, $F_{calculated}$, of 1.91 was smaller than the critical F value, *i.e.*, $F_{critical}$, of 3.02. In the short-term stability test, the RM was stable for up to 4 weeks when stored at 4, 18, and 60°C. In the long-term test, it remained stable for one year at 4°C. Further characterization through supplementary experiments across multiple laboratories and methods are needed to fully understand the expanded uncertainty and determine the RM's suitability as a CRM.

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Author contributions

All authors contributed to the study conception and design. So Yeon Lee was involved in conducting statistical analysis, and preparing the manuscript. Kyungmin Kim performed the experiment, while Han Bin Oh served as the principal investigator and directed the entire research effort. Junghyun Kim ensured that the RM follows the K-BPR regulations, and the work was supervised by Wooil Kim.

Declarations

Conflict Interests

The authors declare that they have no conflicts of interest.

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