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Development of a Certified Reference Material from Caffeine Solution for Assuring the Quality of Food and Drug Measurements

Adel B. Shehata*, Abdulrahman R. AlAskar, Mohammed A. AlRasheed, Abdulrahman M. AlZahrany, Fahd A. AlKharraa, Sowailem A. AlSowailem

Chemistry Department/National Measurement and Calibration Center (NMCC), Saudi Standards, Metrology and Quality Organization (SASO), Riyadh, Kingdom of Saudi Arabia
Email: *adelshehata63@yahoo.com

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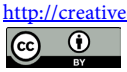


Abstract

Caffeine intake by pregnant women, adults and children can be harmful to the health of all particularly fetuses if the intake exceeds the permissible limits. Therefore, it is of fundamental importance to measure its concentration accurately using certified reference materials (CRMs). In the literature, no scientific details are published about the certification of caffeine standard solutions, and therefore, the present article covers this gap. A batch of caffeine solution was prepared in concentration of 1000 mg/kg and bottled. Homogeneity and stability of the candidate reference material were assessed by HPLC-UV and the results showed that the material is homogenous and stable enough. Characterization of the caffeine reference material was performed by HPLC-UV, LC-MS/MS and UV-VIS-NIR spectrophotometer in three different days and the characterization uncertainty was estimated in accordance with the requirements of ISO GUM. The certified value (999.86 ± 8.54 mg/kg) was derived as a weighted mean from the gravimetry and the three characterization methods and the certified uncertainty was calculated according to ISO Guide 35. The produced CRM is of strong interest to the food and drug analytical laboratories for the validity and credibility of their caffeine measurement results.

Keywords

Caffeine, Reference Material, Homogeneity, Stability, Characterization, Certification

1. Introduction

Caffeine is a stimulant found naturally in the leaves, fruits, or seeds of more than

60 plants in the world and is also manufactured and added to a number of foods, beverages and medicines [1]. It is considered as the main component of coffee, which contributes partially to the bitterness of the beverage [2]. In addition, it increases concentration and alertness, provides the body with energy, and improves the physical performance [3] [4] [5]. Official regulatory bodies around the world have regulated the addition of caffeine to some beverages in which it is not naturally occurring. A level of 350 mg was generally approved, which is comparable to that provided by coffee and yerba mate. Also, there are some regulators who have authorized the level of 450 mg for adults. However, for a pregnant woman, caffeine consumption should not exceed 200 mg per day, and for adolescents, consumption should not exceed 100 mg [6]. This means that measuring the concentration of caffeine in food and medicinal products to which it is added is an important issue to ensure that the added doses do not exceed the permissible limits. Published research shows that caffeine can be analyzed in food and drug products using HPLC-UV [6] [7]-[12]. It can also be analyzed by LC-MS/MS and UV-VIS-NIR spectrophotometry techniques [13] [14] [15]. The confidence in the measurement results by all methods depends on the metrological traceability to the SI units. Traceability is defined as a property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributes to the measurement uncertainty [16]. Certified reference materials (CRMs) are the measurement standards used to provide traceability to the SI units in chemical analysis through the calibration of measuring equipment [17]. They include several types, among which are the standard solutions, which are prepared by gravimetry and then characterized by analytical techniques [18]. By reviewing the literature for caffeine CRMs, it has been found that Lane Sander *et al.* at NIST have certified a suite of reference materials which represent the first green tea-containing reference materials with certified values for catechins and alkaloids including caffeine [19]. Shehata *et al.* extracted caffeine from roasted and ground coffee and measured its purity by HPLC-UV and UV-VIS-NIR spectrophotometer [20]. However, no scientific details were published about the preparation and certification of a reference material from caffeine standard solution. Therefore, the present work has been focused on the development of such a certified reference material and for that, a batch has been prepared as 1000 mg/kg by dissolving a certain mass of highly pure caffeine in ultrapure water. The target uncertainty is 1% and the intended use of the RM is for calibration, quality control, and proficiency testing. The prepared solution was homogenized and bottled into 50 HDPE bottles each is 125 mL. The homogeneity and stability of the candidate reference material was assessed by HPLC-UV and the material was found homogeneous and stable enough. The characterization of the caffeine concentration was carried out by HPLC-UV, LC-MS/MS and the UV-VIS-NIR spectrophotometer in three different days in accordance with requirements of ISO 17034 and ISO guide 35 [21] [22]. The certified value was assigned as a weighted mean by compiling the results obtained from gravimetry and the three analytical methods and all the de-

tails are reported in this paper.

2. Materials and Methods

2.1. Reagents and Solvents

Methanol and formic acid (HPLC grade) were obtained from Merck, (Darmstadt, Germany). Pure caffeine (100%) and para-amino acetophenone (>99%) were purchased from Sigma-Aldresh (St. Louis, Missouri, USA). The caffeine CRM, NMIA M724c (99.8% ± 0.6%) was obtained from the national metrology institute of Australia, NMIA. Ultrapure water was obtained from Millipore Milli-Q RG, USA. The plastic container and the HDPE bottles were purchased from a local supplier.

2.2. Equipment

The HPLC-UV system used was of the model Ulti Mate 3000 equipped with an auto-sampler, quaternary pump and a UV detector of the same model produced by Thermoscientic (Wathham, Massachusetts, USA). The column used for separation was of the type hypersll gold (50 mm × 2.1 mm × 1.9 μm) and the software was Chromeleon 6. The mobile phase was assembled from 75% water and 25% methanol (v:v). The flow rate was 0.25 mL/min, the injection volume was 10 μL and the column temperature was kept at 21˚C. The UV-VIS-NIR spectrophotometer was Hitachi UH4150, Japan with an automatic wavelength correction and a pair of 1 cm matched quartz cells. The spectral bandwidth was 1 nm and the wave length accuracy was 0.3 nm and the caffeine solution samples were measured at 275 nm. The LC-MS/MS used for caffeine characterization was of model UltiMate 3000 equipped with a quaternary pump, an autosampler of the same model and a mass detector of model TSQ Quantum produced by Thermoscientic (Wathham, Massachusetts, USA). Chremelone and Xcalibur software packages were used to run the instrument. The chromatographic separation was performed, using a hypersll gold HPLC column (50 mm × 2.1 mm × 1.9 μm) and the mobile phase was assembled from water acidified with 0.1% formic acid and methanol (85%:15%, v/v). The injection volume was 10 μL, flow rate was 0.35 mL/min and the column temperature was kept at 21˚C. The ion source (ESI) parameters were: spray voltage, 3500 V, vaporizer temperature, 200˚C, the sheath gas pressure, 40 psi, the aux gas pressure, 10 psi and the capillary temperature was 270˚C.

2.3. Preparation of the Candidate RM Batch

The RM plastic container, 15 L and the 50 HDPE bottles were carefully washed with ultrapure water acidified with 5% nitric acid then rinsed with water and dried. The batch of caffeine solution was prepared gravimetrically as 1000 mg/kg by weighing 7290 mg of pure caffeine and dissolving it in 7283240 mg (7.283240 kg) of ultrapure water. Weighing of caffeine was done using a Mettler Toledo calibrated analytical balance with capacity of 220 g and readability

of 0.01 mg. Meanwhile, weighing the mass of solution (candidate RM batch) was done by a Mittler Toledo calibrated balance of capacity 64100 g and readability of 10 mg. The container was closed and swirled to homogenize the solution and left for one night on a mechanical shaker for complete homogenization. The prepared concentration was calculated by equation 1 and was found 999.93 mg/kg.

$$C = \frac{m \times p}{m_{soln}} \tag{1}$$

where

C—concentration (mg/kg);

m—mass of caffeine powder (mg);

p—purity of caffeine powder (mass fraction);

m_{soln}—mass of caffeine solution (kg).

The RM batch solution was bottled into 50 HDPE bottles and systematic selection was applied to select bottles for homogeneity, stability and characterization studies. The bottles were tightly closed, sealed and kept in a refrigerator.

2.4. Preparation of the Calibration Solutions

A stock solution of caffeine CRM purchased from NMIA was prepared as 200 mg/kg in a 100 mL flask. Five calibration solutions were prepared by gravimetric dilution as 10, 20, 30, 40 and 50 mg/kg for the external calibration of HPLC-UV and the UV-VIS-NIR spectrophotometer. Meanwhile, for the calibration of LC-MS/MS, a stock solution of 1000 µg/kg was prepared from the CRM purchased from NMIA in 100 mL flask and 5 calibration solutions were gravimetrically diluted from it as 100, 150, 200, 250 and 300 µg/kg. To each of these solutions, a concentration of 250 µg/kg from the IS (para-amino acetophenone) was added.

2.5. Sample Preparation

A sample of concentration 25 mg/kg was gravimetrically diluted from the candidate RM (999.93 mg/kg) for measurements by HPLC-UV and the UV-VIS-NIR spectrophotometer. However, for measurements by LC-MS/MS, a sample of 250 µg/kg was gravimetrically prepared and an IS concentration of 250 µg/kg was added to it.

2.6. Homogeneity Study

The number of bottles required to study the RM homogeneity was taken as 10% (5 bottles) of the produced number of bottles and the systematic selection approach was used to select these 5 bottles. This is to ensure that all parts of the candidate RM batch are represented in the study and the statistical significance of the results is strongly indicating the material homogeneity. Bottle 1 (B1) was selected to represent the lower part of the batch, B25 to represent the middle, and B50 to represent the upper part of the batch. Moreover, B12 was selected to

represent the region between the middle and bottom, and B37 to represent the region between middle and the upper part of the batch. Each of the selected bottles was dived into 3 portions. For study of the between and the within bottle homogeneity, a sample from each portion was diluted gravimetrically with ultrapure water to 25 mg/kg. The analytical strategy was the simple randomized design in which, a single run with all units observed in duplicate in random order to avoid any trend that might have occurred due to the filling order of the bottles.

2.7. Stability Study

A short-term stability study of 4 weeks storage at 4˚C and 40˚C was carried out in accordance with ISO Guide 35 [22]. For storage at 4˚C, we have selected 4 bottles and at 40˚C, we selected other 4 bottles. At each temperature one bottle was stored for 4 weeks, one for 3 weeks, one for 2 weeks and one for 1 week. Moreover, we selected one bottle and measured its concentration at room temperature before the storage begins (0 time). The selection was made systematically as follows: B2, B7, B13, B18, B23, B28, B33, B38 and B43. Bottles 2, 13, 28 and 38 were stored at 4˚C. Meanwhile, bottles 7, 18, 33 and 43 were stored at 40˚C. After the storage period was over, the 8 bottles were stored at 4˚C a reference temperature for one night. This temperature was selected because the candidate RM is a water solution which if stored at 0˚C or below, it will become ice. After the storage period of the selected samples was over, the samples were then conditioned to room temperate and a sample from each bottle was diluted to 25 mg/kg and measured 3 times by the isochronous approach under repeatability conditions by calibrated HPLC-UV [23]. For the long-term stability, three bottles were systematically selected (B4, B26, B48) and were stored at room temperature for 6 months. The measurements were carried out by HPLC-UV at 4 time points: 0, 1, 3, 6 and 12 months by the classical approach [22]. At each time point, each bottle was measured three times so that the total number of measurements is nine and the average was calculated.

2.8. Characterization Study

Three bottles (B8, B27, B42) were systematically selected for the characterization study so that the upper, middle and bottom of the RM batch are represented. The measurements by each method were carried out in three different days (D1-D3) to ensure reproducibility of the results. In each day, a sample from each bottle was gravimetrically diluted to 25 mg/kg and measured by HPLC-UV (Method 1) and the UV-VIS-NIR Spectrophotometer (Method 2). In case of LC-MS/MS (Method 3), measurements were carried out using the ESI in the (+) mode according to the transitions shown in **Table 1**.

A sample from each bottle was gravimetrically diluted to 250 µg/kg and measured. The number of measurements from each bottle was 3 so that the total number of measurements by each equipment per day was 9.

Table 1. Transitions used for quantification of the candidate caffeine RM by LC-MS/MS.

Analyte	Parent ion (m/z)	Fragment ion (m/z)	Collision Energy (V)
Caffeine	195.1	138.1	30
IS	136.1	94.2	20

3. Results and Discussion

3.1. Traceability of the Measurement Results

The traceability link of the mass fraction value of the gravimetrically prepared candidate RM batch and that of the internal standard (IS) to the SI units was established by weighing the mass of caffeine, the mass of caffeine solution and the mass of IS using calibrated balances. In addition, the traceability of the mass fraction values measured by the three methods of analysis was achieved by calibration of each equipment with the NMIA CRM of purity (99.8% ± 0.6%) and by the gravimetric preparations and dilutions using a calibrated analytical balance.

3.2. Homogeneity and Short-Term Stability

The homogeneity measurements were carried out by HPLC-UV calibrated in the range of 10 - 50 mg/kg since this method showed very good repeatability. Each portion was measured 2 times so that the total number of measurements per bottle is 6 [22]. The obtained concentration values were multiplied by the dilution factor of each bottle and the results are shown in **Table 2**.

These results were tested for outliers by Grubbs test and no outlier was detected. The distribution of the results of each bottle was examined by the Q-Q plot as shown in **Figure 1**.

The figure is an example Q-Q plot of bottles 1 and 50, which bracket bottles of the whole batch and it shows the theoretical z-score (x) plotted against the actual z-score of data (y). It can be seen that the homogeneity data is distributed around the predicted line indicating that it is normally distributed. The results in **Table 2** were statistically analyzed by ANOVA-single factor in order to know if there are significant differences between bottles or not. The obtained ANOVA results are recorded in **Table 3**.

The table shows that F (0.16999) is less than F_{crit} (2.75871) and the p-value is 0.952 which is >0.05 indicating that there are no significant differences between bottles [22] [24]. This means that the candidate RM is homogeneous and can be characterized as a reference material. The uncertainty (σ_h) of the material heterogeneity was calculated using Equation (2), in which MS_{within} and $\sqrt{MS_{within}}$ are the mean square within bottles and the degrees of freedom of MS_{within} respectively and, n is the number of measurements per bottle [22].

$$\sigma_h = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{v\left(MS_{within}\right)}} \tag{2}$$

The uncertainty was found 0.79 mg/kg, which is a satisfactory figure when compared with the fit-for-purpose heterogeneity uncertainty set as 1 ppm.

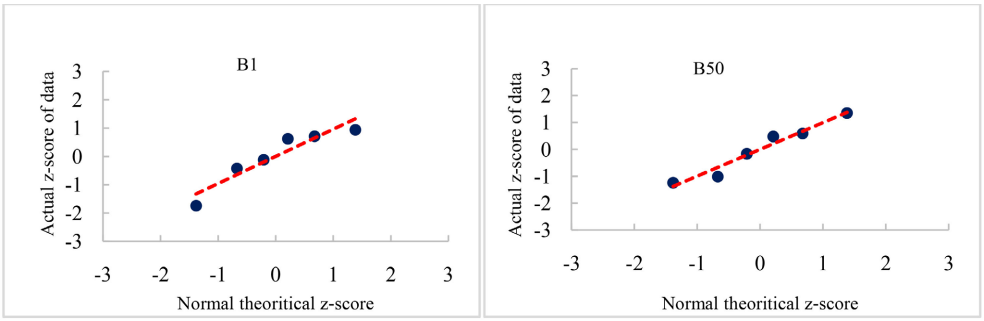


Figure 1. Q-Q plots for the homogeneity results of B1 and B50.

Table 2. Concentrations of the diluted Candidate RM samples for homogeneity study.

Concentration (mg/kg)				
B1	B12	B25	B37	B50
996.52	1004.44	997.37	993.55	996.93
998.54	996.81	997.58	999.93	998.79
995.69	993.97	993.81	985.94	995.06
992.10	992.34	995.92	997.87	996.64
999.40	996.32	998.50	1000.59	992.41
998.78	996.77	998.24	999.32	992.99

Table 3. Single factor ANOVA of the candidate RM homogeneity results.

Source of Variation	*SS*	*df*	*MS*	*F*	P-value	F_{crit}
Between Groups	8.906848	4	2.22671	0.16999	0.952	2.75871
Within Groups	327.4766	25	13.09906			
Total	336.3835	29				

For the short stability measurements carried out by the HPLC-UV, the measured concentration of each sample was multiplied by the corresponding dilution factor and the average at each time point was calculated. The results were reported in Table 4 and Table 5 at 4˚C and 40˚C respectively.

To assess these short stability results in both tables, regression analysis was carried out. The t-statistic was calculated by dividing the absolute value of the slope, $|b_1|$ of regression by the standard error $s(b_1)$ and was found smaller than the t-critic obtained from the t-table at $df = 4$. Hence, no trend was detected indicating good stability of the candidate RM under shipment conditions. For more explanation of the stability results, the concentration values in Table 4 and Table 5 were plotted against the storage time points within limits of the certified uncertainty as it can be seen in Figure 2.

The solid line in the figure represents the certified value (999.86 mg/kg) and the dashed lines represent the certified uncertainty limits. It is evident that the concentration of the RM is quite stable during the storage period and did not show any trend. This means that the candidate RM when certified can remain

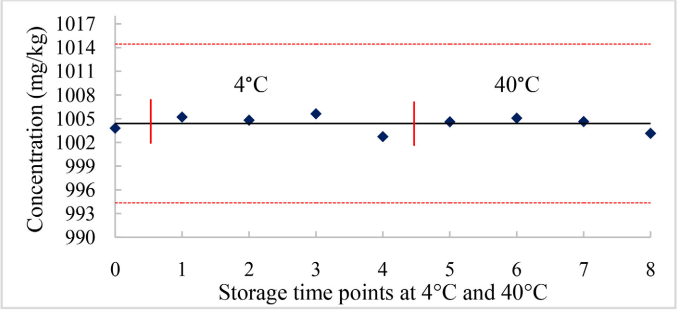


Figure 2. The RM concentration within the certified uncertainty limits at 4˚C and at 40˚C.

Table 4. The short-term stability results of the candidate RM stored for 1, 2, 3 and 4 weeks at 4˚C.

Temperature, ˚C	Storage time (weeks)	Concentration (mg/kg)
4˚C	0	1003.79
	1	1005.21
	2	1004.80
	3	1005.61
	4	1002.74

Table 5. The short-term stability results of the candidate RM stored for 1, 2, 3 and 4 weeks at 40˚C.

Temperature, ˚C	Storage time (weeks)	Concentration (mg/kg)
40˚C	0	1003.79
	1	1003.79
	2	1004.60
	3	1005.06
	4	1004.63

stable if shipped to customers at 25˚C within a period of 4 weeks. The uncertainty in the concentration results arising from the material instability during transportation has been calculated using Equation (3) [25].

$$u_{Sts} = \frac{SD}{\sqrt{\sum_{i=1}^{n}\left(t_i - \bar{t}\right)^2}} t \tag{3}$$

The t_i is the storage time point, $\bar{t}$ is the average of the time points, t is the number of storage weeks and the SD was calculated for the mean of the concentration data in Table 4 and Table 5. The uncertainty was found 0.39, which is fit-for-the purpose since it did not exceed the set limit, 0.5 mg/kg.

3.3. Characterization of the Candidate RM

The characterization measurements of caffeine RM concentration were per-

formed to assign the certified value (y_{char}). Since this concentration is a non-operationally defined measurand, the measurements were carried out using three independent methods in one laboratory in accordance with ISO guide 35 [22] [26] [27] [28] [29]. A typical calibration curve by the CRM solutions for each equipment is given in **Figure 3**.

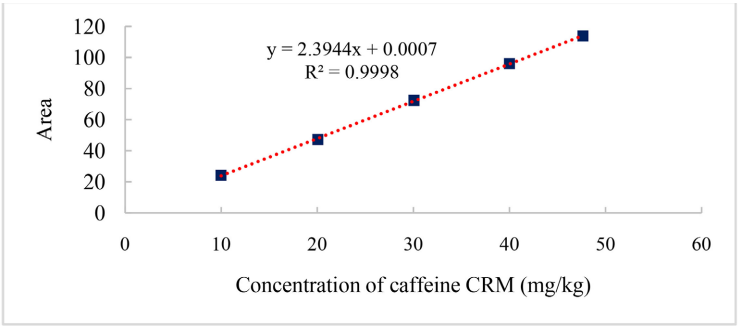
From this figure, one can notice that R^2 is near to 1 giving rise to the good quality of the calibration. The concentration of caffeine measured by HPLC-UV and UV-VIS-NIR in which external calibration was used has been calculated by Equation (4).

$$C_x = \frac{A-b}{a} \tag{4}$$

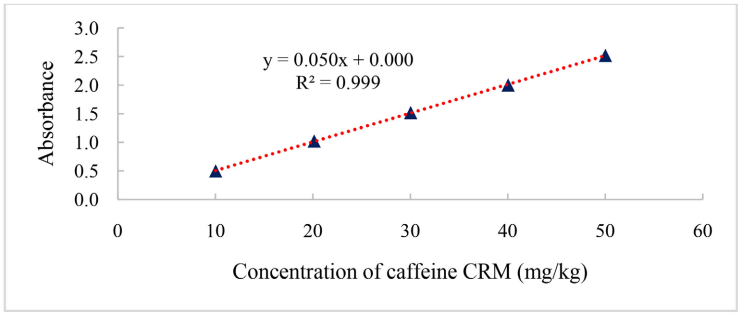
where

C_x—concentration of unknown (mg/kg)

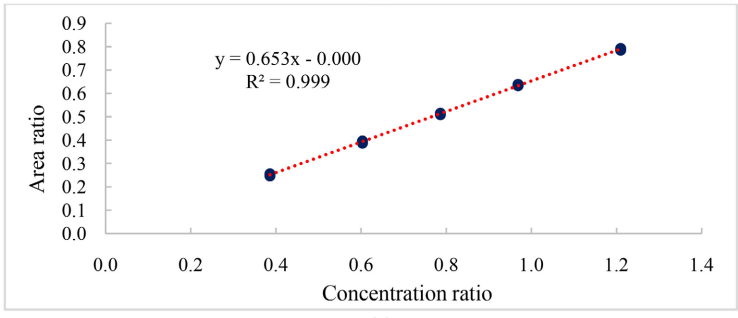
A—area of unknown in case of HPLC-UV and absorbance in case of UV-VIS-NIR



(a)



(b)



(c)

Figure 3. Typical calibration curves of: (a) HPLC-UV; (b) UV-VIS-NIR and (c) LC-MS/MS.

b—intercept

a—slope

Meanwhile, the concentration measured by LC-MS/MS method in which internal standard calibration was used has been calculated by Equation (5).

$$C_x = \frac{\left(\frac{A_x}{A_{IS}} - b\right) \times C_{IS}}{a} \quad (5)$$

where

C_x—concentration of unknown (mg/kg)

A_x—area of unknown

A_{IS}—area of IS

b—intercept

C_{IS}—concentration of IS added to the sample

a—slope

Typical chromatograms of caffeine produced by HPLC-UV and LC-MS/MS are shown in Figure 4.

It is clear that caffeine was retained at 1.827 min in case of HPLC-UV, while it was retained at 9.17 min and the IS at 4.26 min in case of LC-MS/MS. The concentration values measured by the three methods were multiplied by the corresponding dilution factors and the obtained results in D1-D3 were reported in Table 6.

Table 6. Concentration of candidate RM measured by the three methods in D1-D3.

	Concentration (mg/kg)								
	HPLC-UV			UV-VIS-NIR			LC-MS/MS		
	D1	D2	D3	D1	D2	D3	D1	D2	D3
	999.88	997.70	1000.66	996.73	1000.22	1002.70	988.35	983.47	1022.82
	1000.46	998.45	1001.02	997.52	1001.01	1003.48	1016.48	1003.17	974.00
	999.32	999.08	1000.75	998.32	1001.01	1001.92	967.05	987.53	1020.50
	1000.66	998.21	1000.56	997.52	1001.79	1002.70	972.64	1018.69	984.01
	999.98	997.92	1000.22	997.52	999.43	1002.70	1007.71	1009.05	1005.50
	1000.23	997.58	1001.29	997.52	1000.22	1002.70	1004.42	1004.29	1019.20
	1000.97	998.60	1000.46	997.52	999.43	1002.70	1010.63	1012.16	995.54
	999.49	998.05	1000.31	998.32	999.43	1002.70	971.32	982.94	994.05
	999.72	997.44	1001.51	997.52	1000.22	1002.70	997.79	977.99	1023.50
Ave	1000.08	998.11	1000.75	997.61	1000.31	1002.70	992.93	997.70	1004.35
SD	0.55	0.53	0.44	0.45	0.78	0.37	18.75	14.85	18.40
RSD%	0.05	0.05	0.04	0.05	0.08	0.04	1.89	1.49	1.83
Grand mean		999.65			1000.21			998.33	

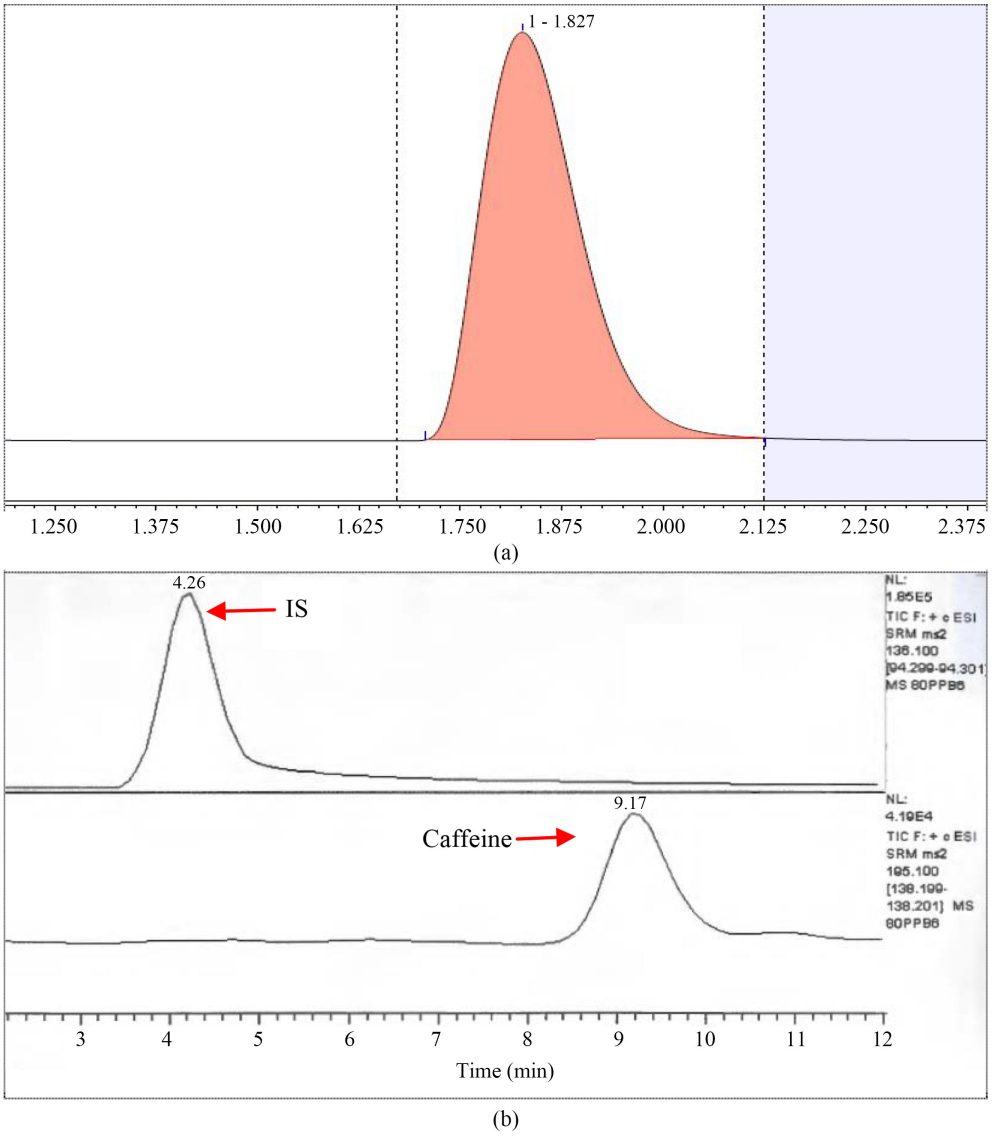


Figure 4. Typical chromatograms of caffeine: (a) for HPLC-UV and (b) for LC-MS/MS.

These results were tested for outliers by Grubbs test and no outlier values were detected. Since the nine average values obtained in the three days by the three methods contribute to the certified value, they were tested for distribution by establishing the Q-Q plot as shown in **Figure 5**.

It can be seen that the values are distributed well around the predicted line, which indicates that these values follow the normal distribution model. On the other hand, it was noticed that the RSD% in case of HPLC-UV and UV-VIS-NIR spectrophotometer is smaller than that in case of LC-MS/MS. This indicates that the precision of both methods is better than precision of the LC-MS/MS method. Moreover, the agreement of the three methods results in **Table 6** has been tested by ANOVA-single factor [22]. The null hypothesis H_0: is that, there is no significant difference between results of the methods along the three days. The obtained ANOVA results were recorded in **Table 7**.

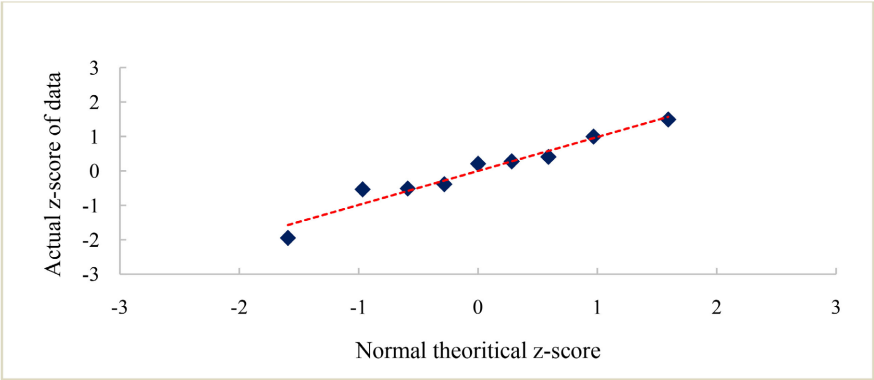


Figure 5. The Q-Q plot of the averages concentrations of candidate RM in D1-D3 by the three analytical methods.

Table 7. ANOVA for agreement of the three methods results of the candidate RM.

Source of Variation	*SS*	*df*	*MS*	*F*	P-value	F_{crit}
Between Groups	791.12504	8	98.8906	0.975	0.462	2.069
Within Groups	7300.03624	72	101.3894			
Total	8091.16129	80				

The ANOVA revealed that $F < F_{crit}$ and the p-value > 0.05, which means that the null hypothesis is valid, *i.e.* the agreement between the three methods is very good.

3.4. The Characterization Uncertainty

3.4.1. Uncertainty of Measurements by HPLC-UV and UV-VIS-NIR Spectrophotometer

Estimation of the uncertainty in the measurement results by the externally calibrated HPLC-UV and UV-VIS-NIR spectrophotometer is based on the mathematical model in Equation (4) and the calculations were performed according to ISO GUM [30]. From this equation, the explicit sources of uncertainty are the measured area/absorbance, the slope and intercept of the calibration lines. In addition to these sources, there are implicit sources of uncertainty, namely: the CRM concentration and the sample preparation. They were represented by the term ∂C_{CRM} in condition that its concentration equals zero. Hence, the mathematical model in Equation (4) was modified by adding the term ∂C_{CRM} as it can be seen in Equation (6).

$$C_x = \frac{A-b}{a} + \partial C_{CRM} \tag{6}$$

The uncertainty resulting from the repeatability of the measured area, absorbance, slope and intercept has been estimated as described elsewhere [31]. Meanwhile, the uncertainty of the mass of sample was calculated by Equation (7), where c_1 and c_2 are sensitivity coefficients. Each of them equals 1 since the uncertainties were expressed in mg.

$$u_c(m) = \sqrt{\left(c_1 \cdot u_{m_{Un}}\right)^2 + \left(c_2 \cdot u_{m_{H_2O}}\right)^2} \quad (7)$$

In addition, uncertainty of the CRM stock solution was calculated based on the mathematical model in Equation (1) and uncertainty of the largest CRM calibration solution (50 mg/kg) was calculated using Equation (8) and was taken to represent uncertainty of the CRM concentration.

$$u_{C_{cal\ sol}} = C\sqrt{\left(\frac{u_{C_{Stock}}}{C_{stock}}\right)^2 + \left(\frac{u_{m_{stock}}}{m_{stock}}\right)^2 + \left(\frac{u_{m_{cal\ sol}}}{m_{cal\ sol}}\right)^2} \quad (8)$$

To combine the two components of the term δC_{CRM}, the uncertainty of mass was divided by the value of mass (u_m/m_{sample}) and uncertainty of the largest calibration solution was divided by the solution concentration ($u_{cal\ sol}/C_{cal\ sol}$). Hence, their u_c was calculated according to Equation (9).

$$u_c(\partial C_{CRM}) = C\sqrt{\left(\frac{u_m}{m_{sample}}\right)^2 + \left(\frac{u_{cal\ sol}}{C_{cal\ sol}}\right)^2} \quad (9)$$

The sensitivity coefficients, c_i were calculated by differentiating Equation (6) and were used to calculate the combined standard uncertainty according to Equation (10) [30] [31].

$$u_c = \sqrt{\left(\frac{\partial c}{\partial A} \cdot u_A\right)^2 + \left(\frac{\partial c}{\partial b} \cdot u_b\right)^2 + \left(\frac{\partial c}{\partial a} \cdot u_a\right)^2 + \left(\frac{\partial c}{\partial C_{CRM}} \cdot u_{\partial C\ CRM}\right)^2} \quad (10)$$

The results of the characterization uncertainty calculation by HPLC-UV and by the UV-VIS-NIR are shown in **Table 8**.

Table 8. The characterization uncertainty of the candidate RM.

Method	Source of uncertainty	±Value	Distribution	Sensitivity coefficient, c_i
HPLC-UV	Area	0.016	Normal	19.72
	Slope	0.0041	Normal	−19.72
	Intercept	0.0011	Normal	−494.23
	δC_{CRM}	0.14323	Normal	1
	The u_c	0.15	Normal	
	Average dil Factor	39.81		
	$u_c \times$ dil Factor	±5.97 mg/kg		
UV-VIS-NIR	Absorbance	0.0003	Normal	19.72
	Slope	0.0001	Normal	−19.72
	Intercept	0.00004	Normal	−494.22
	δC_{CRM}	0.15033	Normal	1
	The u_c	0.156	Normal	
	Average dil Factor	39.94		
	$u_c \times$ dil Factor	±6.23 mg/kg		

3.4.2. Uncertainty of Measurements by LC-MS/MS

The mathematical model used for calculation of th candidate RM concentration measured by LC-MS/MS is shown in equation 6. From this equation, the explicit sources of uncertainty are area of unknown, area of IS, intercept, slope and concentration of the IS added to the unknown sample. In addition, the CRM concentration and the sample mass are implicit sources of uncertainty. The uncertainty of the repeatability of the measured area of unknown and IS, slope and intercept has been estimated as described elsewhere [31]. The uncertainty of sample mass was calculated using Equation (11) and uncertainty of the largest CRM concentration was calculated using Equation (9).

$$u_c(m) = \sqrt{\left(c_1 \cdot u_{m_{Un}}\right)^2 + \left(c_2 \cdot u_{m_{IS}}\right)^2 + \left(c_3 \cdot u_{m_{H_2O}}\right)^2} \tag{11}$$

The uncertainty of the mass of sample was divided by the value of mass (u_m/m_{sample}) and uncertainty of the largest calibration solution was divided by the solution concentration ($u_{cal\ sol}/C_{cal\ sol}$). These two contributions were squared and added to the uncertainty of the IS in the unknown sample according to Equation (12) [32].

$$u_{C_{IS}} = C_{IS}\sqrt{\left(\frac{u_{IS\ stock}}{C_{IS\ stock}}\right)^2 + \left(\frac{u_{m_{IS}}}{m_{IS}}\right)^2 + \left(\frac{u_{m_{H_2O}}}{m_{H_2O}}\right)^2 + \left(\frac{u_m}{m_{sample}}\right)^2 + \left(\frac{u_{cal\ sol}}{C_{cal\ sol}}\right)^2} \tag{12}$$

The sensitivity coefficients were calculated by differentiation of Equation (5) and were used to calculate the combined standard uncertainty u_c according to Equation (13) [32].

$$u_c = \sqrt{\left(\frac{\partial c}{\partial A_{Un}} \cdot u_{A_{Un}}\right)^2 + \left(\frac{\partial c}{\partial A_{IS}} \cdot u_{A_{IS}}\right)^2 + \left(\frac{\partial c}{\partial a} \cdot u_a\right)^2 + \left(\frac{\partial c}{\partial b} \cdot u_b\right)^2 + \left(\frac{\partial c}{\partial C_{IS}} \cdot u_{IS}\right)^2} \tag{13}$$

The uncertainty calculation results in case of LC-MS/MS were shown in **Table 9**.

Table 9. The characterization uncertainty of the candidate RM concentration by LC-MS/MS.

Method	Source of uncertainty	±Value	distribution	Sensitivity coefficient, c_i
Method 3	Area of unknown	34159.42	Normal	9.66308×10^{-5}
	Area of IS	57175.05	Normal	-3.00321×10^{-5}
	Slope	0.0026	Normal	−377.87827
	Intercept	0.0015	Normal	−383.308
	Concentration of IS	1.64	Normal	0.99
	The u_c	3.07		
	Average dil factor	4040.23		
	u_c × dil Factor/1000	12.40 mg/kg		

3.4.3. Uncertainty of the Gravimetric Concentration of the Candidate RM

The uncertainty of the mass of caffeine powder, $u_{m\ caff}$ and of the mass of solution, $u_{m\ soln}$ has been calculated using Equation (14) in which the max error of the balance used was obtained from its OIML specification and the calibration factor was obtained from the calibration certificate.

$$u_m = \sqrt{\left(\frac{\text{max error}}{\sqrt{3}}\right)^2 + 2\left(m \times \text{cal factor}\right)^2} \tag{14}$$

Secondly, the uncertainty of the gravimetric concentration, u_{Grav} was calculated by Equation (15) based on the mathematical model in Equation (1). The obtained value was found 999.93 ± 3.00 mg/kg.

$$u_{Grav} = C\sqrt{\left(\frac{u_p}{p}\right)^2 + \left(\frac{u_{m_{caff}}}{m_{caff}}\right)^2 + \left(\frac{u_{m_{soln}}}{m_{soln}}\right)^2} \tag{15}$$

3.4.4. Agreement between Results of the Three Characterization Methods

The agreement between the caffeine RM concentration values measured by the three methods and the concentration value from the gravimetric preparation was studied. These values and their associated expanded uncertainties are shown in Table 10.

The concentration values of the three methods are close to each other and to the gravimetric value as it can be seen from their plot in Figure 6.

Table 10. The gravimetric value and the average concentration of each characterization method.

Method	Grand mean (mg/kg)	U_{exp} (mg/kg)
Gravimetry	999.93	3.00
HPLC-UV	999.65	11.84
UV-VIS-NIR	1000.21	12.45
LC-MS MS	998.33	24.81

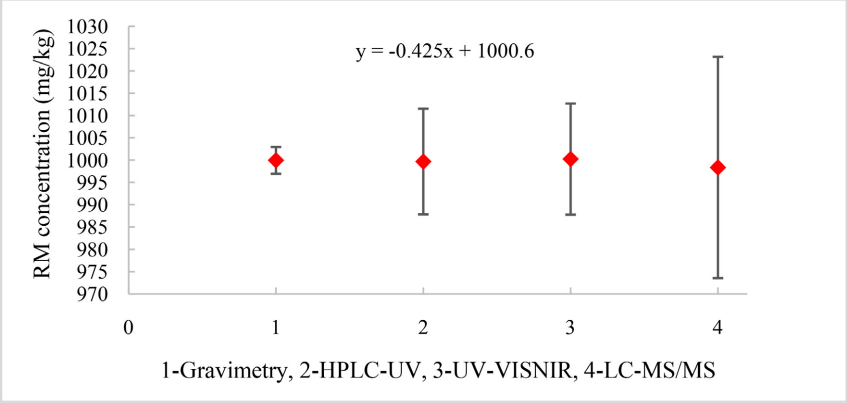


Figure 6. Graphical representation of the methods results: 1) Gravimetry; 2) HPLC-UV; 3) UV-VIS-NIR; 4) LC-MS/MS.

To confirm agreement between the four values, regression analysis was performed. The slope of the regression line (b_1) and its standard error $s(b_1)$ were found −0.425 and −1.239 respectively and agreement of the methods was tested by Equation (16) [22].

$$|b_1|/s(b_1) < t_{0.95,n-2} \tag{16}$$

The $|b_1|/s(b_1)$ (0.343) was found smaller than the $t_{0.95,n-2}$ (3.182) using $df = 3$ at 95% level of confidence. In addition, the P-value, 0.34 was found larger than 0.05 indicating that there is no significant difference between average concentrations of the methods and the gravimetric concentration value.

3.5. The Long-Term Stability

The long-term stability was assessed by the classical approach measurements of samples stored at real time for 12 months. The measurements were carried out at 0, 1, 3, 6 and 12 month time points using the HPLC-UV method and the obtained results were recorded in **Table 11**.

The regression line of these results was plotted in **Figure 7**.

The slope of the line (b_1), the standard error, $s(b_1)$ of the slope and the P-value were found −0.0680, 0.355 and 0.860 respectively. The t-statistic, $|b_1|/s(b_1)$ was found 0.192 which is less than the t-critc, 2.776 at $df = 4$ and 95% confidence level. This means that the slope of regression line does not deviate significantly from zero indicating that the reference material can remain stable throughout the validity period. The uncertainty due to the long-term stability was calculated using Equation (17) [20] [22].

Table 11. The results of the RM concentration in the long-term stability.

Time (M)	Concentration (mg/kg)
0	1000.57
1	996.87
3	1004.98
6	1000.38
12	998.93

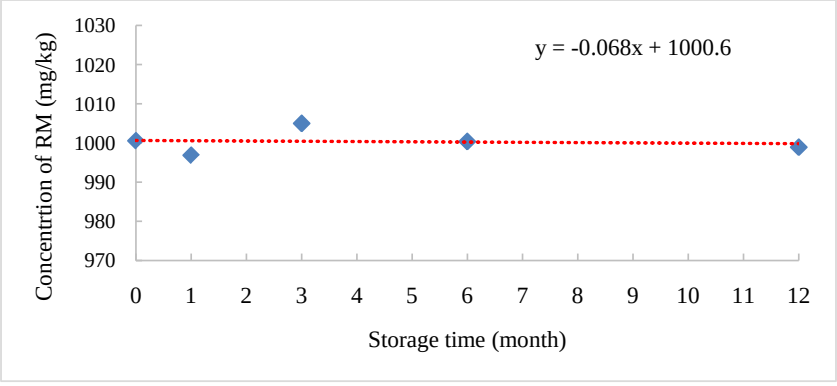


Figure 7. The regression line of the long-term stability study of the candidate RM.

$$u_{lts} = \text{slope} \times t_{Cert} \tag{17}$$

This equation can be defined as follows:

u_{lts}—uncertainty of the long term stability

slope—slope of the regression line

t_{Cert}—time of CRM validity (36 M)

The long-term uncertainty was found 2.45 mg/kg. Moreover, the results of the long-term stability recorded in **Table 8** were plotted against the storage time as shown in **Figure 8**.

The solid line in the figure represents the certified concentration value (999.86 mg/kg) and the dashed lines represent the certified uncertainty limits. It is clear that the RM concentration values measured along 12 months did not deviate out of the uncertainty limits during the storage period confirming the conclusion reached from the trend analysis that the material is stable.

3.6. The Value Assignment

The grand means of the characterization results by the HPLC-UV, UV-VIS-NIR spectrophotometer and LC-MS/MS shown in **Table 6** were used to derive the certified value as a weighted mean since the uncertainty of each method result was well estimated in the SI units as required by ISO Guide 35 [22]. Each method weight, W_i was calculated as inverse of the standard uncertainty by Equation (18).

$$W_i = \frac{1}{u_i^2} \tag{18}$$

The weighing factor, w_i of each method grand mean was calculated using Equation (19).

$$w_i = \frac{W_i}{\sum_{i=1}^{p} W_i} \tag{19}$$

The weighted mean of each method was calculated by multiplying the grand mean, X_i by the method weight, W_i and hence, the certified value of the caffeine concentration was calculated by Equation (20) [22].

$$y_{char} = \frac{\sum_{i=1}^{p} W_i X_i}{\sum_{i=1}^{p} W_i} \tag{20}$$

The weighted characterization uncertainty, u_{char} associated with each method mean has been calculated by Equation (21).

$$u_{char} = \sum w_i^2 u_{ci}^2 \tag{21}$$

The certified uncertainty, U_{CRM} was calculated by Equation (22) using $k = 2$ at confidence level of approximately 95%. The calulation results were reported in **Table 12**.

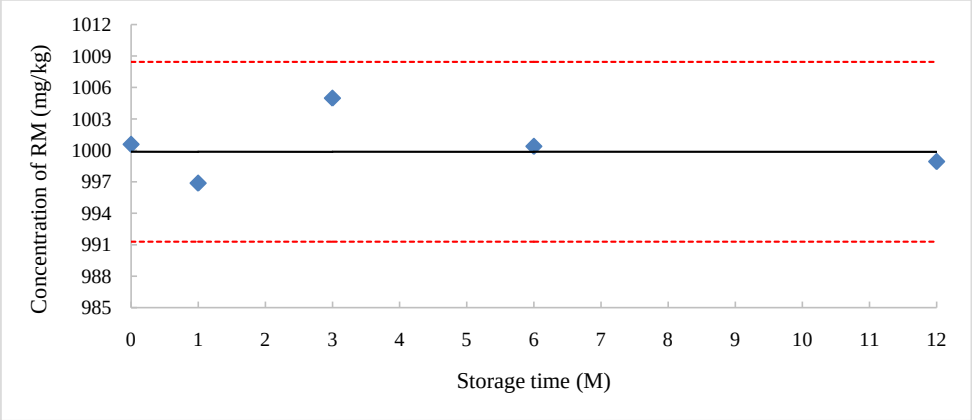


Figure 8. Results of the certified value of the candidate RM within the certified uncertainty limits in 12 months.

Table 12. Derivation of the certified value and its uncertainty of the caffeine RM.

Method (M)		M1	M2	M3
	Gravimetry	HPLC-UV	UV-VIS-NIR	LC-MS/MS
Mean, X_i	999.93	999.65	1000.15	998.33
Combined standard Uncertainty, u_c	3	5.97	6.23	12.40
Weight of the method mean, W_i	0.11	0.03	0.03	0.01
$\sum W_i$			0.17	
Weighing factor, w_i	0.65	0.16	0.15	0.04
W_1X_1	111.103			
W_2X_2		28.03		
W_3X_3			25.77	
W_4X_4				6.50
Weighted mean, y_{char}		999.86		
$w_1^2u_c^2$ (Gravimetry)	9.42			
$w_1^2u_c^2$ (HPLC)		0.95		
$w_2^2u_c^2$ (UV-VIS-NIR)			0.88	
$w_3^2u_c^2$ (LC-MS/MS)				0.22
$\sum w_i^2u_c^2$			11.47	
$u_{char} = \sqrt{\sum w_i^2u_c^2}$			3.39	
σ homogeneity (σ_h)			0.79	
Short-term stability (sts)			0.39	
Long-term stability (lts)			2.45	
k			2	
U_{CRM}			8.54 mg/kg	
U_{CRM} %			0.85	

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{homo}^2 + u_{sts}^2 + u_{lts}^2} \quad (22)$$

The certified value derived from gravimetry and the three analytical methods was found 999.86 mg/kg and the certified uncertainty was found ±8.54 mg/kg (*i.e.* 0.85%) which is fit-for-the purpose when compared with the target uncertainty set as 1%.

4. Conclusion

The preparation and certification of a reference material from caffeine solution has been described. The homogeneity and stability studies revealed that the material is homogeneous and stable enough and the characterization results by the three analytical methods were in very good agreement. The certified value was derived as a weighted mean from the results of gravimetry and the three methods and was found 999.86 ± 8.54 mg/kg. This CRM will be very useful for food and drug testing laboratories for calibration, quality control and PT for the laboratory accreditation schemes.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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