CRM REPORT

A C G publications *J. Chem. Metrol.* XX:X (202X) X-XX

journal of chemical metrology

Patulin reference material certified by RP-HPLC-UV and gravimetry for food safety analysis

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

Chemistry Department, National Measurement and Calibration Center (NMCC), Saudi Standards, Metrology and Quality Organization (SASO), Riyadh, Kingdom of Saudi Arabia

(Received August 20, 2024; Revised September 23, 2024; Accepted September 30, 2024)

Abstract: Patulin, a mycotoxin commonly found in contaminated food products, is a significant health concern and its accurate determination is of fundamental importance. The availability of a patulin reference material (RM) is a key tool for the confidence in analytical results. In this paper, a batch of patulin in acetonitrile acidified with 0.1% formic acid RM was gravimetrically prepared. The prepared RM was ampouled and subjected to homogeneity, short-term, long-term stability and characterization studies by high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The chromatographic separation was carried on a C-18 column using a mobile phase of ultra-pure water and acetonitrile (80:20) % and the detection was made at 276 nm. These studies were carried out in accordance with ISO 17034 and ISO 33405. The prepared patulin reference material was found to be homogeneous and stable enough as demonstrated by the results of the analysis of variance (ANOVA) and the regression analysis. The value assignment was based on combing data from gravimetry and HPLC-UV as weighted mean. The certified uncertainty was calculated from four contributions: characterization, homogeneity, short- and long-term stability. The preparation and the assigned value of the patulin RM (26.78±1.66 mg/kg) were validated by successful participation of SASO/NMMC in the key comparison CCQM-K154. d. This produced patulin certified reference material (CRM) will be useful as a calibrant and as quality control material in food safety analysis.

Key words: Patulin; reference material; HPLC-UV, homogeneity; stability; certified value.

1. Introduction

Patulin is a toxin produced by certain types of fungi, including Penicillium, Aspergillus, and Bysochlamys. Penicillium expansum is the primary reason behind apple rot and is the most common source of Patulin [1-2]. Patulin has been mainly found in apple and apple products and occasionally in pears, grapes, apricots, strawberries, blueberries and peaches [3-4]. Patulin contamination is a serious concern due to its potential for causing severe acute and chronic health problems [5]. This toxin is believed to harm the body by binding to essential cellular components containing sulfur such as glutathione and proteins leading to disruptions in protein and DNA production, cellular organelle malfunction and ultimately cell death [6-9]. As a result, chronic exposure to patulin has been linked to neurological, immune, genetic, developmental and carcinogenic effects. Therefore, accurate and precise measurements

[*] Corresponding author: E-Mail: adelshehata63@yahoo.com ; a.shehata@saso.gov.sa

The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology Month-Month 202X EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.115.24.08.3313
Available online: October 09, 2024

of patulin in the different matrices is of fundamental importance for food safety. Numerous methods have been developed for quantifying patulin in some fruits, including thin-layer chromatography (TLC) [10], mass spectrometry [11], calorimetry [12] and gas chromatography-mass spectrometry (GC-MS) [13]. Currently, high-performance liquid chromatography with ultraviolet light detection is the most widely used technique for determination of patulin. Iqbal et al extracted patulin from fruit samples including oranges, apples, apricots, lemons, and guava. They have measured the concentration of the extracted patulin by HPLC-UV at 276 nm using a mobile phase of water and acetonitrile (90:10) % at a flow rate of 1.5 mL/min [17]. The EU has established maximum patulin levels of 50 µg/kg for apple juice and its ingredients, 25 µg/kg for solid apple products and a stricter limit of 10 µg/kg for products intended for infants [18-19]. These limits must be rigorously monitored using precise and accurate measurement methods to prevent consumers from being exposed to the health risks associated with patulin if it is present in food products at levels higher than permitted. Certified reference materials (CRMs) are a crucial tool in the reliability of analytical method results. They are consistently homogeneous and stable, characterized by a rigorous metrological process [20-21]. Calibration solutions from CRMs are essential for establishing the metrological traceability of the measurement results to the SI units, validation of analytical methods and achieving the highest accuracy in many analytical fields, among them the global food safety analysis [22]. The aim of this paper is to prepare and certify a reference material from patulin in acetonitrile acidified with 0.1% formic acid. This reference material was prepared gravimetrically and characterized by a validated HPLC-UV method at 276 nm. Homogeneity, short- and long-term stability were also studied by HPLC-UV according to ISO 33405 requirements. The preparation and value assignment of the RM has been validated by participation in the CCQM key comparison CCQM-K154. d. The comparison results showed that the assigned value and its associated uncertainty produced by SASO/NMCC were in good agreement with the key comparison reference value (KCRV) and its uncertainty. The details of the preparation and certification of the patulin reference material are illustrated in this article.

2. Experimental

2.1. Reagents and Solvents

The patulin reference material (97%) used for preparing the RM batch was obtained from LGC, UK. Acetonitrile (HPLC grade) and formic acid (98-100%) were purchased from Merck, Darmstadt, Germany. Ultrapure water was produced using a Millipore Milli-Q RG system (USA). The patulin CRM (99.30 ± 0.35 %) used for calibration of HPLC-UV was obtained from LGC, UK.

2.2. Equipment

Patulin measurements were performed using a Thermo Scientific Ultimate 3000 ultra-high-performance liquid chromatography (HPLC) system equipped with an autosampler, quaternary pump and UV detector (Waltham, Massachusetts, USA). The chromatographic separation of patulin was achieved using a Hypersil Gold HPLC column (150 mm *x* 4.6 mm, 5 µm particle size) at a flow rate of 0.8 mL/min. The column temperature was maintained at 21 °C, the injection volume was 10 µL and the UV detection wavelength was 276 nm. The HPLC system was controlled by Chromeleon 6 software. The elution was isocratic using a mobile phase consisting of ultra-pure water and acetonitrile (80:20) %. The patulin reference material (RM) was prepared in a 250 mL class A glass round flask. It was mechanically shacked for one night then divided into 100 dark glass ampoules each is 2 mL. The machine used for ampouling the candidate reference material was of the model Ampulmatic®-10 Laboratory Ampule Sealer, manufactured by Bioscience inc, Pennsylvania, USA. The calibrated analytical balance of 220 g capacity and 0.01 mg readability used in the gravimetric preparation was purchased from Mittler Toledo.

2.3. Preparation of the patulin batch RM

A mass of 5.24 mg from the patulin RM (97%) was weighed into a 250 mL volumetric flask. A mass of 192849.5 mg of acetonitrile acidified with 0.1% formic acid was added to the volumetric flask until the total mass of solution reached 0.19285 kg. The flask was capped, sealed and wrapped in an aluminum foil to protect the patulin solution from light. Then, the flask was gently shacked for 2 minutes and left on a mechanical shaker overnight for homogenization of the candidate RM solution. The homogenized RM was ampouled in 100 dark brown glass ampoules each containing 2 mL. The unit selection for the homogeneity, stability and characterization studies was carried out and the all units were kept in dark.

2.4. Homogeneity Study

The systematic selection approach was used to select 10 ampoules (10%) for the homogeneity study [24]. The selected ampoules were of numbers: 1-12-22-33-44-55-66-77-88-99. The analysis was carried out by the HPLC-UV validated method and the analytical strategy was the simple randomized design in a single run. Each of the 10 ampoules was observed in 6 injections in random order to avoid any trend that might have occurred due to the ampouling order. The ANOVA single factor was used for the statistical analysis of the homogeneity measurement results.

2.5. Stability study

The short-term stability study was carried out at 4, 23 and 40°C. A group of 4 ampoules were selected for study at each temperature. One ampoule of each group was measured at 0 storage time and the rest of ampoules were stored for 3 weeks. After that, the all ampules were stored at a reference temperature of -20 °C for 24 hours. The samples were then conditioned at room temperature and the mass fraction of patulin in each ampoule was measured 3 times by HPLC-UV using the isochronous approach. For the long-term stability, 10 ampoules were selected and stored in a refrigerator at 4 °C. Two ampules were measured at 0-month storage and then two ampoules were measured at each time point (3, 6, 9 and 12 M) in 5 observations each. The measurements of the short and long-term stability were also carried out by the validated HPLC-UV.

2.6. The Characterization Study

The characterization study of the patulin candidate RM was carried out in three different days (D1-D3). Six ampoules were selected for that study where two ampoules were measured 5 times in each day. The measurements were carried out using the validated HPLC-UV method.

2.7. The Calibration Solutions

A stock solution was prepared by dissolving 4.450 mg of the patulin CRM (99.30%) in a 50 mL flask containing 25027.07 mg of 0.1% acidified acetonitrile. The total mass of the resulting stock solution was determined to be 25031.52 mg (0.025032 kg). The mass fraction of patulin in the stock solution was calculated by equation 1 and was found to be 176.53 mg/kg [25].

$$x = \frac{m_{x}\ p}{m_{soln}} \qquad (1)$$

where,

x	- mass fraction (mg/kg)
m	- mass of patulin CRM (mg)
p	- purity of patulin CRM (%)
m_{soln}	- mass of stock solution (kg)

The stock solution was kept in dark in a refrigerator and five calibration solutions namely 10, 20, 25, 30, and 50 mg/kg were then prepared by gravimetric dilution from the stock solution. The dilution was carried out in the injection vials used for measurements.

3. Results and discussion

3.1. The Gravimetric Value of Patulin RM Mass Fraction

The mass fraction of patulin in the candidate RM is a non-operationally defined measurand and was characterized by two methods, gravimetry and HPLC-UV [24]. The mass fraction resulting from the gravimetric preparation of the RM batch was calculated using equation 1 and was found to be 26.36 mg/kg.

3.1.1. The Uncertainty of the Gravimetric Mass Fraction

The sources of uncertainty of the mass fraction determined gravimetrically were identified from equation 1 as: mass of CRM, purity of CRM and mass of ACN solution. The uncertainty of the mass of CRM and mass of ACN solution (u_m) was estimated by equation 2 which incorporates the maximum error of the balance used in weighing, the mass of sample, m and the calibration factor quoted from the calibration certificate [26].

$$u_m = \sqrt{\left(\frac{Max error}{\sqrt{3}}\right)^2 + 2\left(m \times calb\ factor\right)^2} \qquad (2)$$

The uncertainty of the gravimetric mass fraction, u_{Grav} was calculated by equation 3, which incorporates the three contributions in equation 1 and was found to be 0.413 mg/kg [27].

$$u_{Grav} = x\sqrt{\left(\frac{u_p}{p}\right)^2 + \left(\frac{u_m}{m_{PAT}}\right)^2 + \left(\frac{u_m}{m_{ACN}}\right)^2} \qquad (3)$$

3.2. The Homogeneity of the Patulin RM

The homogeneity of the patulin candidate RM was assessed by HPLC-UV and the results obtained were presented in Table 1 and graphically in Figure 1. The figure shows the mean of 6 measuremnets of the mass fraction in each of the 10 ampoules. The error bars represent the standard deviation of the mean.

Table 1. The patulin mass fraction (mg/kg) of the 10 ampules selected for homogeneity study

S1	S12	S22	S33	S44	S55	S66	S77	S88	S99
26.53	26.50	26.42	26.51	26.51	26.45	26.65	26.48	26.51	26.50
26.48	26.51	26.55	26.46	26.57	26.58	26.55	26.51	26.55	26.50
26.56	26.50	26.56	26.47	26.53	26.49	26.42	26.45	26.46	26.58
26.50	26.50	26.50	26.58	26.49	26.47	26.48	26.40	26.57	26.49
26.51	26.50	26.48	26.48	26.44	26.49	26.44	26.41	26.58	26.47
26.52	26.46	26.53	26.42	26.53	26.54	26.51	26.51	26.55	26.48

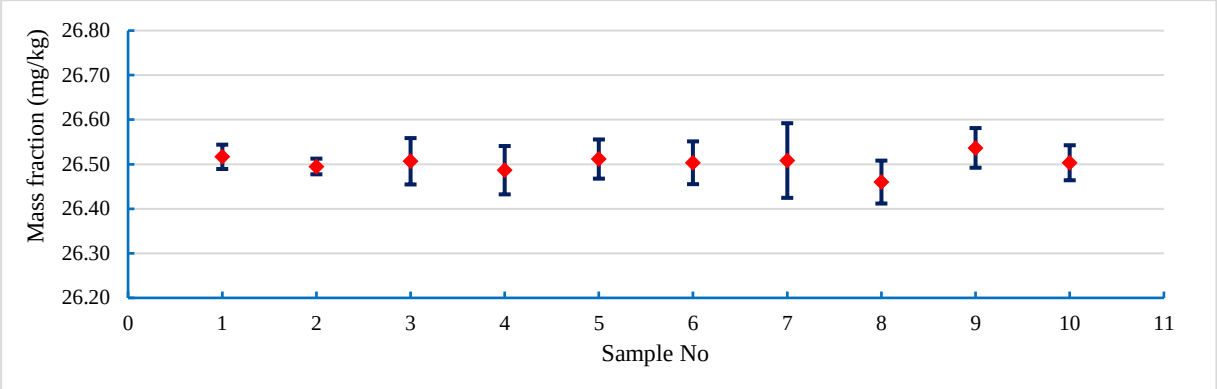

Figure 1. A graphical representation of the averages mass fraction of the 10 homogeneity samples.

From Figure 1, it is clear that the mass fractions of the 10 samples are close to each other. The normality of the homogeneity data was tested by the Q-Q plot, which compares the theoretical quantiles (z-scores) from a normal distribution to the observed quantiles of the data. Figure 2 shows three Q-Q plots for three representative samples. These plots show that the data points closely align with the theoretical line, confirming normality of the homogeneity data [24,28].

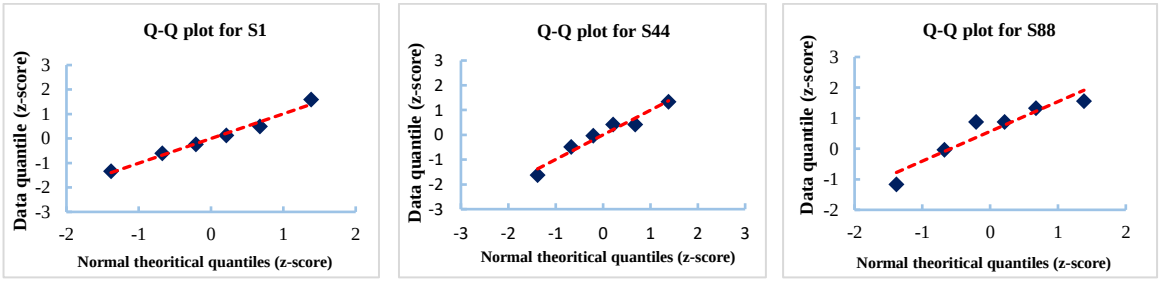

Figure 2. The Q-Q plot of three samples indicating normality of the homogeneity data

The homogeneity of the data presented in Table 1 was assessed by one-way ANOVA. The results of the ANOVA summarized in Table 2, indicate that F (0.976) is less than $F_{critcal}$ (2.073) and the p-value, 0.47 is greater than 0.05. These findings fulfill the criteria for homogeneity of variances, confirming that the patulin RM samples are homogeneous. Consequently, this RM was subjected to short-term, long-term stability and characterization studies [24,28].

Table 2. the ANOVA-single factor results of the RM samples tested for homogeneity.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.02090688	9	0.00232298	0.976	0.47	2.073
Within Groups	0.11891012	50	0.00237820			
Total	0.13981700	59				

The uncertainty, σ_h of the material inhomogeneity was calculated by equation 4 and was found to be 0.089 ppm [24,28].

$$\sigma_h = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{\nu MS_{within}}} \qquad (4)$$

3.3. The Short-term Stability

The average mass fraction values of the short-term stability at 4°C, 23°C and 40°C after storage for 0, 1, 2 and 3 weeks are presented in Table 3. At 0-storage, the average mass fraction for three ampoules was found to be 26.60 mg/kg. This value and the average mass fraction measured at the subsequent storage weeks, is graphically depicted in Figure 3. A visual inspection of this figure suggests that the RM samples exhibit stability throughout the three weeks storage period. To assess these results, regression analysis of data in Table 3 was conducted. The resulting regression slope (b_1) was determined to be 0.00014 with a standard error $s(b_1)$ of 0.010788. The calculated t-value, $t=|b_1|/s(b_1)$ (0.013) was found less than the critical t-value (2.306) for $\alpha = 0.05$ and $df = 8$ indicating no significant change in the patulin mass fraction over shipment time of the CRM. Therefore, these findings strongly support the conclusion that the patulin CRM can be safely shipped to users at a temperature not exceeding 40°C.

Table 3. The short-term stability results of the candidate RM obtained by the isochronous measurements

Storage time (w)	4 °C	23 °C	40 °C
0	26.75	26.58	26.47
1	26.76	26.45	26.43
2	26.46	26.50	26.69
3	26.45	26.50	26.65

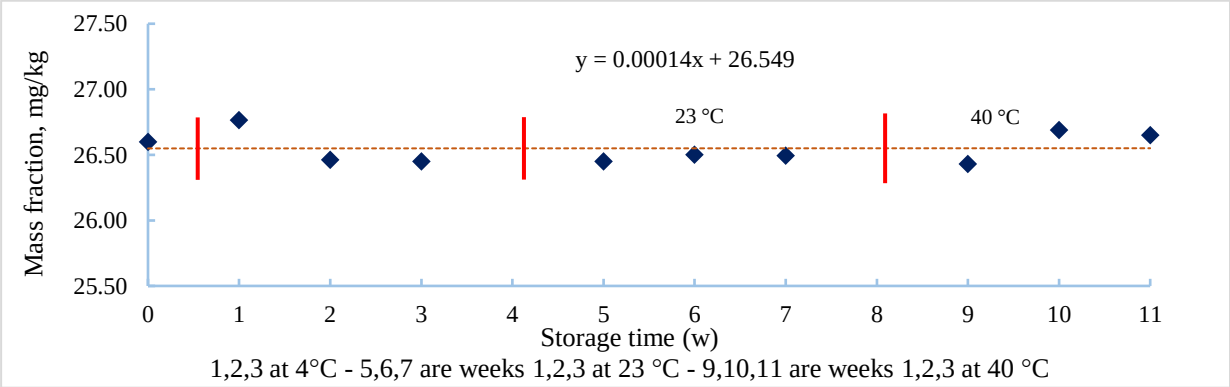

Figure 3. The regression line of the short-term stability data of the patulin RM samples

The uncertainty of the short-term stability measurements was calculated using equation 5 and was found to be 0.16 ppm [28].

$$u_{Sts} = \frac{SD}{\sqrt{\sum_{i=1}^{n}\left(t_i - \bar{t}\right)^2}}\, t \qquad (5)$$

where,

SD - standard deviation
t_i - the storage time (week)
$\bar{t}$ - average of the storage weeks
t - number of storage weeks (3)

3.4. The Characterization of Patulin RM

A stock solution of the CRM was prepared at a mass fraction of 176.53 mg/kg. The reference material was characterized over three different days and in each day, five fresh calibration solutions were prepared at mass fractions namely 10, 20, 30, 40, and 50 to calibrate the HPLC-UV instrument. Then, two ampoules from the six selected ampoules were measured five times each in each day. A typical calibration curve is shown in Figure 4 from which it can be seen that the response is highly linear giving rise to the good quality of the calibration process.

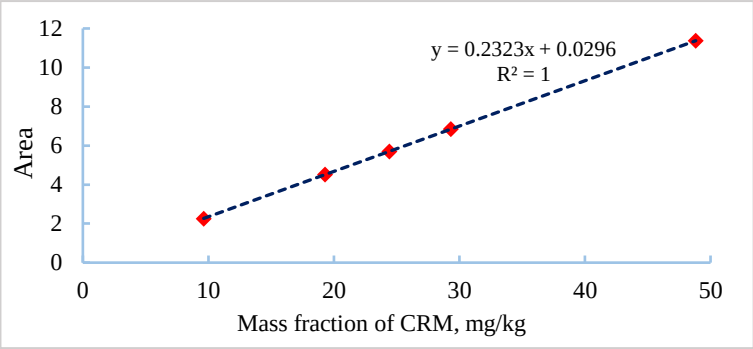

Figure 4. A typical calibration curve of HPLC-UV by patulin CRM

The good calibration response indicates a good efficiency of the chromatographic separation, which can be observed from the very well separated and symmetrical patulin peak shape in Figure 5. The absence of peak tailing and the apparent selectivity demonstrated by the absence of peaks related to any other compounds at the retention time of patulin (3.34 min) suggests that the mobile phase used for elusion and the column used for separation were optimally selected.

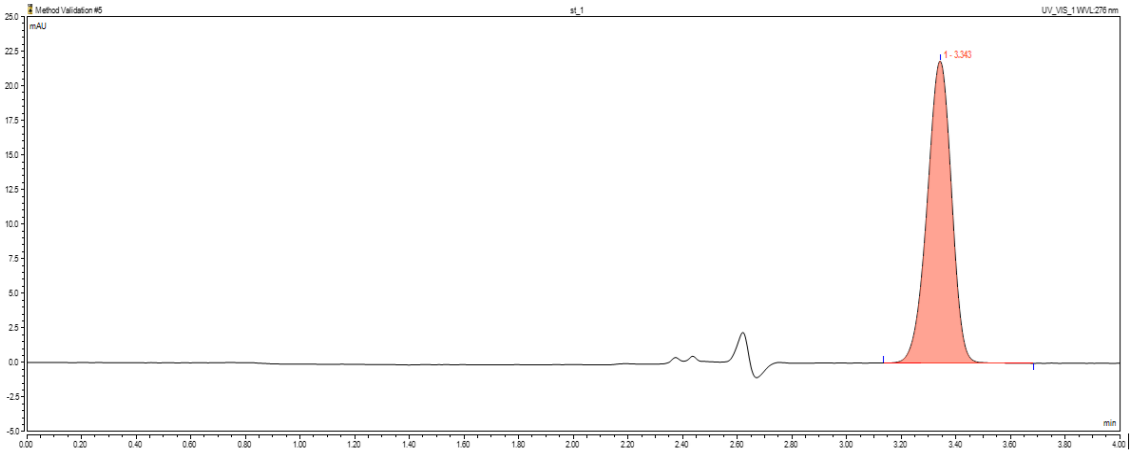

Figure 5. The HPLC-UV chromatogram containing the patulin peak at RT 3.34 min

The characterization results of patulin in the three days are presented in Table 4. They were tested for outliers by Grubbs test and no outlier values were found.

Table 4. The characterization results of patulin RM by HPLC-UV in D1-D3

Measurement days (D1-D3)	D1	D2	D3
Mass fraction (mg/kg)	26.77	26.68	26.84
	26.81	26.67	26.95
	26.79	26.67	26.91
	26.83	26.64	26.93
	26.89	26.72	26.80
	26.85	26.65	26.92
	26.88	26.67	26.86
	26.83	26.73	26.82
	26.83	26.65	26.89
	26.79	26.62	26.86
Ave	26.83	26.67	26.88
SD	0.039	0.035	0.049
Grand Mean		26.79	

Considering these results, one can find that they differ slightly in decimal places and that the difference between the highest and lowest value (0.33 mg/kg) is small indicating good intermediate precision of the results. On the other hand, one can note the close proximity of the grand mean (26.79 mg/kg) to the value produced from the gravimetric preparation (26.36 mg/kg), which means that the certified value can be derived from two closely related values of metrological traceability to the SI units.

3.4.1 Uncertainty Budget Estimation of Characterization Data

The uncertainty associated with the measurement of mass fraction of patulin RM by HPLC-UV was estimated based on ISO GUM and EURACHEM Guide [27,28]. The explicit sources of uncertainty were identified from the model equation 6 as: area A (repeatability), intercept, *b* and the slope, *a*.

$$x = \frac{A-b}{a} \tag{6}$$

There are more implicit sources of uncertainty which are the mass of patulin sample and the calibration solutions. Those two uncertainty sources were expressed as the term δC_{CRM} in condition that its mass fraction equals zero. This term was incorporated into the model as shown in equation 7.

$$x = \frac{A-b}{a} + \partial C_{CRM} \tag{7}$$

The combined standard uncertainty u_c, was calculated using equation 8 as described elsewhere [29].

$$u_c = \sqrt{\left(\frac{\partial x}{\partial A}.u_A\right)^2 + \left(\frac{\partial x}{\partial b}.u_b\right)^2 + \left(\frac{\partial x}{\partial a}.u_a\right)^2 + \left(\frac{\partial x}{\partial C_{CRM}}.u_{\partial C_{CRM}}\right)^2} \tag{8}$$

3.5. The Long-term Stability

The long-term stability of the candidate patulin RM is associated with its behavior when stored according to prescribed conditions by the user. It has been assessed experimentally by HPLC-UV at the real time storage intervals (0, 3, 6, 9 and 12 M) and the results obtained were presented in Table 5.

Table 5. The results of the RM long-term stability at real time storage intervals

Storage time (M)	0	3	6	9	12
Mass fraction (mg/kg)	26.18	24.74	24.71	25.81	24.45
	25.89	24.71	24.63	25.63	24.50
	25.74	24.72	24.69	25.87	24.32
	25.67	24.53	24.66	25.65	24.47
	25.30	24.62	24.38	25.67	24.53
	25.17	24.57	25.08	25.71	24.32
	25.07	24.41	24.44	25.71	24.45
	24.94	24.44	24.43	25.79	24.51
	24.82	24.39	24.51	25.70	24.29
	24.69	24.42	24.42	25.74	24.34

These results have been assessed to determine whether they suffer from any trend that could affect the shelf life of the RM. The regression line shown in Figure 6 was established between the storage time intervals and the measured mass fraction of the RM. The slope of the regression line, b_1 equals -0.0231 and the standard error of this slope, $s(b_1)$ was 0.01958. From these two values, the $t = |b_1|/s(b_1)$ was calculated and its value was found to be 1.2074. This value was found smaller than the tabulated value (3.182) for 3 degrees of freedom at a 95% confidence level. From this finding, it can be concluded that the results do not encounter any trend, which indicates a sufficient degree of stability during the specified shelf life of the patulin RM [24,30-31].

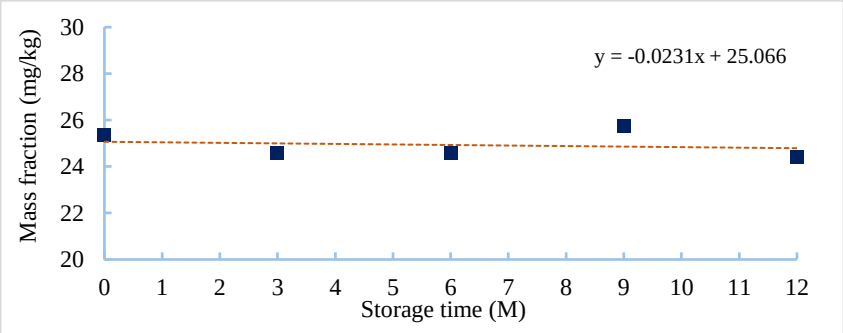

Figure 6. The regression line of the long-term stability results of the RM.

The uncertainty of measurements of the long-term stability was calculated from equation 9 and was found 0.69 mg/kg when t_{Cert} was 36 months [24].

$$u_{lts} = s(b_1) \times t_{Cert} \qquad (9)$$

3.6. The Assigned Value and Uncertainty Budget of CRM

According to ISO 33405, value assignment is the process of combining the results from the homogeneity and stability assessment with the results from the characterization studies to determine the assigned values and their uncertainties [24]. The weighted mean scheme was used since the reported uncertainties were shown to be reliable and the difference between the gravimetric value and the HPLC-UV value (0.33 mg/kg) can be wholly accounted for by the reported uncertainties [32-34]. The weighing scheme was started by calculating the weight, W_i of each method mean using equation 10, where u_i is the standard uncertainty.

$$W_i = \frac{1}{u_i^2} \qquad (10)$$

The weighted mean of each method was calculated by multiplying the method weight by the mean W_iX_i. Then the certified value (y_{char}) was calculated as the weighted mean by dividing the summation of the weighted means by the summation of the method weights according to equation 11.

$$y_{char} = \frac{\sum_{i=1}^{p} W_i X_i}{\sum_{i=1}^{p} W_i} \qquad (11)$$

To calculate the weighted uncertainty, the weighing factor w_i was calculated by equation 12 then squared and multiplied by the squared standard uncertainty, u^2 of each method.

$$w_i = \frac{W_i}{\sum_{i=1}^{p} W_i} \qquad (12)$$

The weighted uncertainty, u_{char} was calculated by taking the square root of Σw^2u^2 according to equation 13.

$$u_{char} = \sqrt{\sum w^2 u^2} \qquad (13)$$

The calculated results are given in Table 6 which shows that the weighted mean (certified value) is 26.78 mg/kg and the weighted uncertainty is 0.42 mg/kg.

Table 6. The weighted mean and weighted uncertainty, u_{char} results

	Gravimetry		HPLC-UV
Mean, X_i	26.36		26.79
Combined standard Uncertainty, u_c	0.413		0.072
Weight of the method mean, W_i	5.87		192.90
$\sum W_i$		198.77	
Weighing factor, w_i	0.03		0.97
$W_1 X_1$	154.83		
$W_2 X_2$			5167.82
Weighted mean, y_{char}		26.78	
Weighted uncertainty, u_{char}		0.42	

The certified uncertainty, U_{CRM} was calculated from the characterization (u_{char}), homogeneity, short- and long-term stability uncertainty contributions using Equation 14. A coverage factor, $k = 2$ was used which to provide a confidence level of 95% [24].

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{homo}^2 + u_{sts}^2 + u_{lts}^2} \qquad (14)$$

The values of the individual uncertainty components are summarized in Table 7 and the final certified uncertainty was determined to be ±1.66 mg/kg. It is worthy to mention that the gravimetric value, 26.36 mg/kg and the value produced by HPLC-UV, 26.79 mg/kg agree very well within the certified uncertainty, 1.66 mg/kg.

Table 7. Certified uncertainty components of the patulin CRM

Characterization uncertainty (u_{char})	0.42
σ homogeneity (σ_h)	0.089
Short-term stability (*sts*)	0.16
Long-term stability (*lts*)	0.69
k	2
Certified uncertainty	1.66 mg/kg

Thus, it can be concluded that the certified value and its associated uncertainty of the patulin CRM is 26.78 ±1.66 mg/kg.

3.7 The Traceability of Certified Value to SI Units

The traceability of the certified value to the SI units has been established through the purity value given in the certificate of the LCG reference material used in the preparation of the RM batch and the LCG CRM used in the calibration of the HPLC-UV. Moreover, the calibrated balance used in weighing the mass of patulin RM and the mass of the batch solution contributed to the traceability of measurements to the SI units.

3.8. Validation of the Gravimetric Preparation and Value Assignment

The key comparison, CCQM-K154.d on the preparation and value assignment of patulin (PAT) in acetonitrile (ACN) with 0.1 % formic acid (FA) was organized by the BIPM in the mass fraction range of 10 mg/kg to 100 mg/kg. SASO/NMCC, Saudi Arabia, participated in this comparison and the final report was issued on September 2023 [35]. The report pointed out that inspection of the degree of equivalence indicated that there was an excellent agreement of the results. This conclusion validates the capability of SASO/NMCC on the gravimetric preparation and value assignment of the patulin CRM.

4. Conclusion

The patulin reference material (RM) was gravimetrically prepared and its mass fraction was characterized by HPLC-UV. The material homogeneity was assessed and the ANOVA showed that F_{calc} (0.976) is smaller than $F_{critical}$ (2.073) indicating good homogeneity of the material. The short- and long-term stability were also assessed and the regression analysis showed no trend in the results giving rise to enough stability during shipment and shelf-life times. The characterization results of the patulin RM in three different days were found to be consistent, with a small variation in decimal places. The certified value was assigned as a weighted mean from the gravimetry and HPLC-UV results and was found to be 26.78±1.66 mg/kg with a well-established metrological traceability to the SI units. The produced patulin CRM is very useful for food testing laboratories in calibration of measuring equipment and in quality control purposes in food safety analysis.

ORCID
Adel B. Shehata: 0000-0002-6818-4825
Abdulrahman. R. Alaskar: 0000-0002-9846-5265
Mohammed. A. Alrasheed: 0000-0003-1392-9516
Abdulrahman. M. Alzahrany: 0000-0003-1077-4678
Fahd. A. Alkharraa: 0000-0002-9051-8131
Sowailem A. AlSowailem: 0009-0006-5799-0780

References

[1] R. N. Wu, Y. L. Dang, L. Niu and H. Hu (2008). Application of matrix solid-phase dispersion–HPLC method to determine patulin in apple and apple juice concentrate, *J. Food Composition Anal.* **21**, 582–586.

[2] A. Moukas, V. Panagiotopoulou and P. Markaki (2008). Determination of patulin in fruit juices using HPLC-DAD and GC-MSD techniques, *Food Chem.* **109**, 860–867.

[3] N. A. Al-Hazmi (2010). Determination of Patulin and Ochratoxin A using HPLC in apple juice samples in Saudi Arabia, *Saudi J. Biol. Sci.* **17**, 353-359.

[4] P. Majerus and Kapp, K. (2002). Reports on tasks for scientific cooperation, task 3.2.8. Assessment of dietary intake of Patulin by the population of EU Member States. Brussels: SCOOP Report.

[5] X. Zheng, W. Wei, W. Zhou, H. Li, S. Rao, L. Gao and Z. Yang (2021). Prevention and detoxification of patulin in apple and its products: a review, *Food Res. Int.* **140**, 110034.

[6] R. Fliege and M. Metzler (1999). The mycotoxin patulin induces intra- and intermolecular protein crosslinks in vitro involving cycteine, lysine, and histidine side chains, and alpha-amino groups, *Chem. Biol. Interact*. **123**, 85-103.

[7] R. Fliege and M. Metzler (2000). Electrophilic properties of patulin. N-acetylcysteine and glutathione adducts, *Chem. Res. Toxicol*. **13**, 373–381.

[8] D. K. Mahato , M. Kamle , B. Sharma, S. Pandhi, S. Devi, K. Dhawan, R. Selvakumar , D. Mishra , A. Kumar , S. Arora, N. A. Singh and P. Kumar (2021). Patulin in food: a mycotoxin concern for human health and its management strategies, *Toxicon* **198**, 12–23.

[9] M. M. Moake, O. I. Padilla-Zakour and R. W. Worobo (2005). Comprehensive review of patulin control methods in foods, *Compr. Rev. Food Sci. Food Saf.* **4**, 8-21.

[10] J. Harwig, Y. K. Chen, B. P. C. Kennedy and P. M. Scott (1973). Occurrence of patulin and patulin-producing strains of *Penicillium expansum* in natural rots of apple in Canada. *Canadian Inst, Food Sci. Technol. J.* **6**, 22–25.

[11] F. Sheu and Y. T. Shyu (1999). Analysis of patulin in apple juice by diphasic dialysis extraction with in situ acylation and mass spectrometric determination. *J. Agric.Food Chem.* **47**, 2711–2714.

[12] T. Subramanian (1982). Colorimetric determination of patulin produced by Penicillium patulum. *J. Assoc. Official Anal. Chemists* **65**, 57.

[13] H. Rupp and S. Turnipseed (2000). Confirmation of patulin and 5-hydroxymethylfurfural in apple juice by gas chromatography/mass spectrometry. *J. Assoc. Official Anal. Chemists* **83**, 612-620.

[14] K. Baert, B. Meulenaer, C. Kasase, A. Huyghebaert, W. B. Ooghe and F. Devlieghere (2007). Free and bound patulin in cloudy apple juice, *Food Chem.* **100**, 1278-1282.

[15] A. R. Brause, M. W. Trucksess, T. S. Frederick and S. W. Page (1996). Determination of patulin in apple juice by liquid chromatography. *J. Assoc. Official Anal. Chemists Int.* **9**, 451-455.

[16] S. MacDonald, M. Long, and J. Gilbert (2000). Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: Collaborative study, *J. Assoc. Official Anal. Chemists Int.* **83**, 1387–1394.

[17] Z. S. Iqbal, M. Waseem, A. F. Abdull Razis , I. A. Bhatti, A. M. Khaneghah, O. A. Mohammed, S. P. Lakshminarayanan and M. Iqbal (2024). Mycotoxin patulin contamination in various fruits and estimating its dietary impact on the consumers: From orchard to table, *Heliyon* **10**, e30252.

[18] G. Boonzaaijer, I. Bobeldijk and W. A. Van Osenbruggen (2005). Analysis of patulin in Dutch food, an evaluation of a SPE based method, *Food Control*, **16**, 587–59.

[19] D. R. Katerere, S. Stockenstro, G. S. Shephard (2008). HPLC-DAD method for the determination of patulin in dried apple rings, *Food Control* **19**, 389–392.

[20] ISO 17034: General Requirements for the Competence of Reference Material Producers (2016). ISO, Geneva.

[21] J. Bates, A. Bahadoor, Y. Cui, J. MeiJa, A. Y Windust, and J. e. Melanson (2019). Certification of Ochratoxin A Reference Materials: Calibration Solutions OTAN-1 and OTAL-1 and a Mycotoxin-Contaminated Rye Flour MYCO-1, *J. Assoc. Official Anal. Chemists Int.* **102**, 1756-1766.

[22] EA-4/14 INF: (2003). The Selection and Use of Reference Materials.

[23] S. Collin, E. Bodart, C. Badot, A. Bouseta and S. Nizet (2008). Identification of the main degradation products of patulin generated through heat detoxication treatments, *J. Inst. Brew*. **114**, 167-171.

[24] ISO 33405:2024(en). Reference materials -Approaches for characterization and assessment of homogeneity and stability, ISO, Geneva

[25] A. B. Shehata, A. R. Alaskar, M. A. Alrasheed, F. A. Alkharraa and A. M. Alzahrani (2020) Certification of sodium benzoate solution reference material by HPLC-UV, LC-MS/MS and UVVIS-NIR spectrophotometry for food and drug analysis, *J. Chem. Metrol.* **14**, 88-105.

[26] A. B. Shehata, A. R. Alaskar, R. A. Aldosari and F. R. AlMutairi (2023). Certification of buffer solutions reference materials using Baucke cell for supporting the quality of pH measurements, *J. Chem. Metrol*. **17**, 100-113.

[27] Eurachem (2013). Eurachem/CITAC Guide: Quantifying Uncertainty in Analytical Measurement.

[28] ISO (1993) GUM: Guide to the Expression of Uncertainty in Measurement. ISO, Geneva

[29] A. B. Shehata, A. R. AlAskar, M. A. AlRasheed, A. M. AlZahrany, F. A. AlKharraa and S. A. AlSowailem (2023). Development of a certified reference material from caffeine solution for assuring the quality of food and drug measurements, *Green Sustain. Chem.* **13,** 216-236.

[30] A. Lamberty, H. Schimmel and H. Schimmel (1998). The study of the stability of reference materials by isochronous measurements. *Fresenius J. Anal. Chem.* **360**, 359-361.

[31] T. P. J. Linsinger, J. Pauwels, A. M. H. Van der Veen, H. Schimmel and A. Lamberty (2001). Homogeneity and stability of reference materials. *Accred. Qual. Assur.* **6**, 20-25.

[32] A. B. Shehata, M. S. Rizk, A. M. Farag and I. F. Tahoun (2014). certification of three reference materials for α - and γ -tocopherol in edible oils. *MAPAN*, **29**, 183-194.

[33] A. B. Shehata, M. S. Rizk, A. M. Farag and I. F. Tahoun (2015). Development of two reference materials for all trans-retinol, retinyl palmitate, α - and γ -tocopherol in milk powder and infant formula. *Journal of Food Drug Anal.* **23**, 82-92.

[34] A. B. Shehata and I. F. Tahoun (2010). Preparation and certification of a fish oil natural matrix reference material for organochlorine pesticides, *Accred. Qual. Assur.* **15**, 563-568.

[35] Final Report CCQM-K154.d (2023). Key comparison study-organic solvent calibration solution gravimetric preparation and value assignment of patulin (PAT) in acetonitrile (ACN) with 0.1 % formic acid (FA).