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Validation of a Method for the Measurement of Caffeine in Water by HPLC-UV

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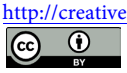

Abstract

For the production of a reference material from caffeine solution, one of the methods of characterization was HPLC-UV since caffeine is very sensitive to the UV. In this work, a batch solution of caffeine in water reference material of 1000 mg/kg has been gravimetrically prepared using a calibrated analytical balance. A sample of this solution was diluted to 25 mg/kg for measurement by HPLC-UV in the range 10 - 50 mg/kg. The chromatographic separation was carried out by C-18 column and a mobile phase assembled of 75% water and 25% methanol (*v*:*v*). The detection was made by the UV detector at 275 nm. The validation of this analytical method was carried out in accordance with requirements of the EURACHEM and ICH guidelines. The selectivity, linearity, accuracy, precision and trueness (recovery and bias) of the method were studied. The validation results proved that the method is fit-for-purpose of measuring the caffeine concentration in water in the range 10 - 50 mg/kg using HPLC-UV.

Keywords

HPLC-UV, Caffeine, Selectivity, Linearity, Precision, Accuracy

1. Introduction

Method validation is a procedure of performing numerous assessments designed to verify that an analytical method is suitable for its intended use and is capable of providing beneficial and legitimate analytical data [1]. The development of a method to analyse a specific analyte depends on the type of matrix, the range of concentration and the purpose of the analysis and when it is already developed, it is necessary to establish its validity [2] [3] [4] [5]. ISO/ICE 17025 requires that

the laboratory shall validate non-standard methods, laboratory-developed methods and standard methods used outside their intended scope or otherwise modified [6]. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory conducting method validation can obtain relevant basic information from published research, which will undoubtedly be useful in proposing the validation plan. The validation plan and the documented procedure for conducting it specify the performance characteristics that will be studied to determine the suitability of the method for its intended use [7] [8] [9]. The decision to choose the performance characteristics depends on the field of its application, the nature of the test samples, and the regulating regulations and legislation, if any [5]. Validation parameters vary depending on the field of application, whether industrial, regulatory, or laboratory, but evaluation of a common set of performance characteristics is usually included in any method validation [10]. In this paper, validation of a method developed for the analysis of caffeine in water to produce a reference material is illustrated [11]. The aim of this method was the characterization of a caffeine reference material of concentration of 1000 mg/kg using an HPLC-UV equipment. The linear range of analysis was 10 - 50 mg/kg and the measured caffeine RM sample was 25 mg/kg. This means that the reference material was diluted from 1000 mg/kg to 25 mg/kg and the result will be multiplied by the dilution factor. The validation plan included the limit of quantification (LOQ), selectivity, linearity, precision, accuracy, recovery and bias. Details of this work are described in this article.

2. Materials and Methods

2.1. Reagents and Solvents

Methanol (HPLC grade) was obtained from Merck, (Darmstadt, Germany). Pure caffeine (100%) was 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.

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 chromatographic separation was performed on a hypersll gold column (50 mm × 2.1 mm × 1.9 µm) and the HPLC-UV was run by the software 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.

3. Results and Discussion

3.1. Selectivity

Analytical selectivity relates to the extent to which the method can be used to

determine particular analytes in mixtures or matrices without interferences from other components of similar behavior [5] [12] [13] [14] [15]. The selectivity of the HPLC-UV method was evaluated by observing no peaks or distortions of the base line at the same retention time of caffeine when a blank sample was injected as it can be seen from **Figure 1(a)**. The selectivity of the method was then demonstrated by spiking a blank sample and observing the peak of caffeine at 1.875 min with no other peaks interfered with it as it can be seen from **Figure 1(b)**.

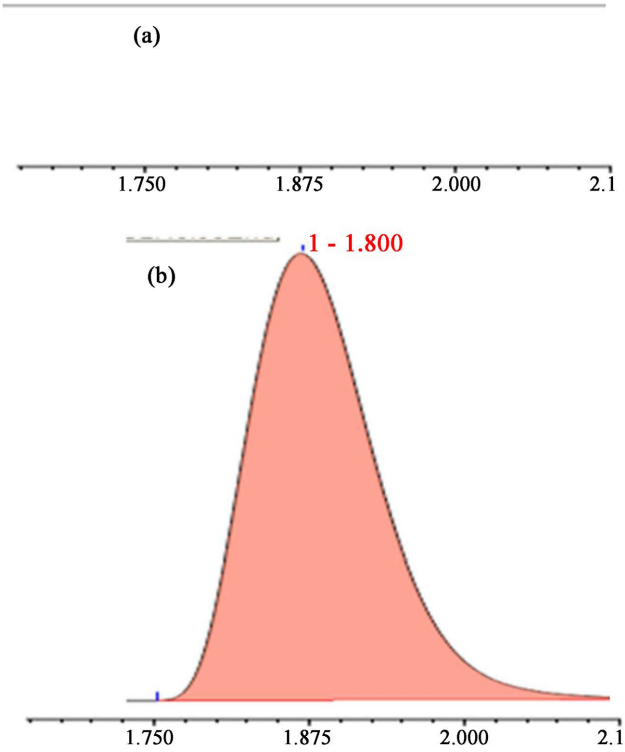

Figure 1. The HPLC-UV base line (a) and the caffeine peak at RT 1.875 min (b).

3.2. Linearity

Linearity of an analytical method can be defined as the ability to produce measurement results proportional to a defined number of calibration points of a calibrant [5] [16]. A linear multipoint calibration curve was obtained by five concentrations of caffeine CRM as shown in **Figure 2**.

The concentration levels were evenly distributed and the calibration mode was achieved by external standard (ESTD) methodology. This methodology was selected because the sample preparation and the HPLC-UV response were very good. The linear model was expressed by Equation (1) which shows a relationship between the concentration and the response of HPLC-UV and has a slope (a) and intercept (b). The symbol ε is the standard error of the residuals [17].

$$y = ax + b + \varepsilon \tag{1}$$

By visual inspection of the calibration data, it can be noticed that this data does not suffer outliers or nonlinear trends. The quality of the regression line was also evaluated by the coefficient R^2, which ideally equals one, but values higher than 0.990 are considered adequate [8]. In our method, R^2 approached 1 indicating a very good fitting of the linear model [5] [17]. Furthermore, linearity was evaluated by plotting the residuals produced by linear regression, which allow inspection of errors in the variance as it can be seen in **Figure 3**. From this figure, it was noticed that the residuals are randomly distributed around zero giving rise to a good linearity of the calibration line [18].

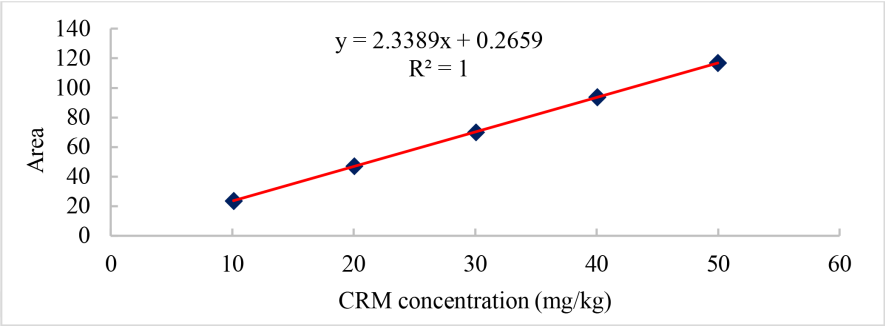

Figure 2. The calibration graph of HPLC-UV in range 10 - 50 mg/kg.

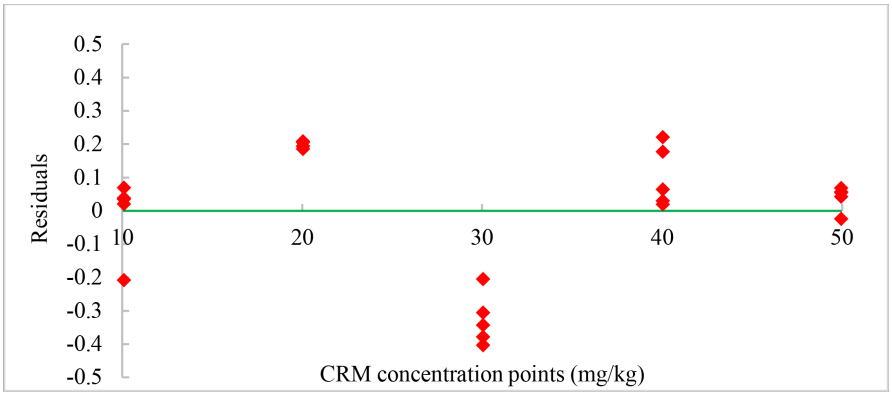

Figure 3. The residuals of the calibration line around 0-axis.

3.3. Limit of Quantification

The limit of quantitation (LOQ) is defined as the lowest amount of an analyte in a sample that can be quantitatively measured with suitable accuracy and precision under experimental conditions established for the analytical method [3]. When the LOQ corresponds to the first level of the analytical curve, it can be referred to as the lower limit of quantification (LLoQ). The LOQ is expressed as concentration and should be reported associated with its precision and accuracy [19] [20]. In our method, the blank sample did not give a response area when injected into the HPLC-UV, therefore, the study of LOQ was conducted by spiking the blank with different concentrations of caffeine (15, 20, 25 and 30 ppb) and measuring the area of each concentration 10 times. The obtained area results

were recorded in Table 1 and the average, SD and %RSD were calculated and recorded also in the same table. The LOQ is taken as the concentration value corresponding to an RSD value of approximately 5% at which quantification is considered to be with acceptable precision and accuracy. Looking to the %RSD values reported in Table 1, it can be noticed that the RSD value 4.069% resulting from the concentration 20 ppb can be taken as the LOQ of the method.

Table 1.The spiked concentrations (pbb) and the peak area for LOQ estimation.

Parameter	Spiked concentration (ppb)			
	15	20	25	30
Peak Area	0.0433	0.0566	0.0633	0.0738
	0.0408	0.0497	0.0599	0.0739
	0.0425	0.0512	0.0625	0.0790
	0.0414	0.0490	0.0641	0.0792
	0.0370	0.0521	0.0650	0.0803
	0.0341	0.0514	0.0679	0.0748
	0.0446	0.0532	0.0616	0.0790
	0.0374	0.0519	0.0621	0.0803
	0.0343	0.0510	0.0674	0.0790
	0.0356	0.0506	0.0667	0.0740
Average	0.0391	0.0517	0.0641	0.0773
SD	0.0039	0.0021	0.0027	0.0028
%RSD	9.913	4.069	4.162	3.638

3.4. Limit of Detection

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be reliably detected and identified but not necessarily quantified. Since the concentration of the target caffeine sample which, is to be measured as a reference material was 25 mg/kg, it has been measured in the range 10 - 50 mg/kg. This 25 mg/kg concentration was large enough and can be detected by HPLC-UV with very good precision and accuracy. Therefore, estimation of the limit of detection (LOD) parameter is not necessary. However, it has been calculated as an information value by dividing the LOQ by 3.3 and was found 6 ppb.

3.5. Accuracy

Accuracy is defined as the closeness of agreement between a measured quantity value and a true quantity value of a measurand [3] [5]. It can be assessed after confirmation of the method selectivity and determination of the linear range.

Accuracy should be verified using three levels (low, medium, high) along the linear range. For this purpose, three samples of a caffeine reference material were prepared as 10, 30 and 50 mg/kg and each one was measured 10 times by HPLC-UV calibrated by the CRM. The value of (x_i) was calculated from the linear equation of the calibration curve and the percentage accuracy was calculated by dividing each value (x_i) by the value (x_{CRM}) and multiplying this ratio by 100 as in Equation (2).

$$\%\ Acuracy = \frac{x_i}{x(CRM)} \times 100 \tag{2}$$

The results obtained are shown in Tables 2-4 for 10 mg/kg, 30 mg/kg and 50 mg/kg respectively. Looking to the accuracy values, it can be realized that in case of the three studied concentrations, the lowest value was 99.20% and the largest value was 100.24%.

Table 2. Results of the accuracy study at low level, 10 mg/kg.

y (Area)	a	b	y-b	x_i	x_{CRM}	% Accuracy
24.26790	2.3859	0.2642	24.0037	10.06	10.06	99.98
24.23590	2.3859	0.2642	23.9717	10.05	10.06	99.84
24.33190	2.3859	0.2642	24.0677	10.09	10.06	100.24
24.25230	2.3859	0.2642	23.9881	10.05	10.06	99.91
24.29360	2.3859	0.2642	24.0294	10.07	10.06	100.08
24.25580	2.3859	0.2642	23.9916	10.06	10.06	99.93
24.25790	2.3859	0.2642	23.9937	10.06	10.06	99.94
24.27790	2.3859	0.2642	24.0137	10.06	10.06	100.02
24.30110	2.3859	0.2642	24.0369	10.07	10.06	100.12
24.26300	2.3859	0.2642	23.9988	10.06	10.06	99.96

Table 3. Results of the accuracy study at medium level, 30 mg/kg.

y (Area)	a	b	y-b	x_i	x_{CRM}	% Accuracy
71.4842	2.3859	0.2642	71.22	29.85	30.00	99.51
71.5139	2.3859	0.2642	71.2497	29.86	30.00	99.56
71.4949	2.3859	0.2642	71.2307	29.85	30.00	99.53
71.5534	2.3859	0.2642	71.2892	29.88	30.00	99.61
71.6189	2.3859	0.2642	71.3547	29.91	30.00	99.70
71.5474	2.3859	0.2642	71.2832	29.88	30.00	99.60
71.5723	2.3859	0.2642	71.3081	29.89	30.00	99.64

Continued

71.5242	2.3859	0.2642	71.26	29.87	30.00	99.57
71.5091	2.3859	0.2642	71.2449	29.86	30.00	99.55
71.6346	2.3859	0.2642	71.3704	29.91	30.00	99.72

Table 4. Results of the accuracy study at high, 50 mg/kg.

y (Area)	a	b	y-b	x_i	x_{CRM}	% Accuracy
118.9517	2.3859	0.2642	118.6875	49.75	50.13	99.24
118.9687	2.3859	0.2642	118.7045	49.75	50.13	99.25
118.9593	2.3859	0.2642	118.6951	49.75	50.13	99.24
118.9683	2.3859	0.2642	118.7041	49.75	50.13	99.25
119.003	2.3859	0.2642	118.7388	49.77	50.13	99.28
118.9485	2.3859	0.2642	118.6843	49.74	50.13	99.23
119.0354	2.3859	0.2642	118.7712	49.78	50.13	99.31
118.935	2.3859	0.2642	118.6708	49.74	50.13	99.22
118.974	2.3859	0.2642	118.7098	49.75	50.13	99.26
118.9071	2.3859	0.2642	118.6429	49.73	50.13	99.20

3.6. Precision

The precision of the method is defined as the closeness of agreement between indications or measured quantity values obtained by replicate [3] [5]. It accounts for systematic and random errors. Systematic errors can result from instrumental, personal or procedural mistakes and they are repeatable in a set of measurements deviating the measured results from the reference value. Meanwhile, random errors occur by unknown variables that lead to dispersion of the measured values and they are unrepeatable. According to the EURACHEM guide, precision of the method can be estimated by repeatability and intermediate precision [21] [22]. Three analysts have prepared three samples of concentrations, 25.14, 25.01 and 25.00 mg/kg. Every analyst has measured a sample 10 times by a calibrated HPLC-UV. The results obtained were recorded in **Table 5** that shows the average and standard deviation by each analyst in addition to the grand mean.

In order to estimate the intermediate precision, the sets of results produced by the three analysts were analyzed by ANOVA one way and the results were shown in **Table 6**.

The standard deviation between groups, S_b was calculated by Equation (3) in which MS_b and MS_w are the mean square between and within groups respectively and n is the number of measurements [3]. The S_b was found 0.066 and was recorded in **Table 7**.

Table 5. Results obtained by 3 analysts for precision studies.

Anal 1	$\bar{x}$	SD	Anal 2	$\bar{x}$	SD	Anal 3	$\bar{x}$	SD
25.10			24.94			24.93		
25.09			24.93			24.93		
25.08			24.94			24.94		
24.90			24.92			24.94		
25.10	25.06	0.075	24.93	24.93	0.011	24.95	24.94	0.0094
25.08			24.92			24.95		
25.10			24.92			24.94		
25.07			24.91			24.95		
25.09			24.94			24.92		
24.93			24.93			24.93		
Grand mean					24.97 mg/kg			

Table 6. ANOVA one-way for studying the intermediate precision.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.100025	2	0.050012414	25.55295	5.92E−07	3.354130829
Within Groups	0.052845	27	0.001957207			
Total	0.152869	29				

Table 7. Repeatability, intermediate precision and precision results.

S_b	0.069
S_w	0.044
S_I	0.082
RSD%	0.33

$$S_{\text{between}} = \sqrt{\frac{MS_b - MS_w}{n}} \tag{3}$$

The standard deviation of the repeatability, S_r was calculated by Equation (4) as 0.044 and recorded in **Table 7** [3].

$$S_r = \sqrt{MS_w} \tag{4}$$

From the values of S_r and S_b, the intermediate precision S_I was calculated by Equation (5) as 0.082 and recorded in **Table 7** [3].

$$S_I = \sqrt{S_r^2 + S_{\text{between}}^2} \tag{5}$$

The precision expressed as %RSD was calculated by Equation (6) as 0.33 and recorded in **Table 7**.

$$\%\text{RSD} = \frac{S_I}{\bar{\bar{x}}} \times 100 \tag{6}$$

The %RSD value, 0.33 was small giving rise to a very good precision of the method.

3.7. Recovery and Bias

Recovery is defined as the proportion of the amount of analyte present in or added to the analytical portion of the test material, which is extracted and presented for measurement. Since the caffeine sample has been measured directly in water without extraction, the recovery of the method was studied by measuring a sample of concentration 25.14 mg/kg 10 times using a calibrated HPLC-UV and the results obtained were measured in Table 8. The average $\bar{x}$ was calculated and divided by the value x_{CRM} to obtain the % recovery as 99.78 % according to Equation (7).

Table 8. Results of the recovery study of the HPLC-UV method.

Area_{Un}	C_{Un} mg/kg	$\bar{x}$ (mg/kg)	x_{Ref} (mg/kg)	% Recovery	bias
58.9720	25.08	25.08	25.14	99.78	0.06 ppm or 0.22%
58.9629	25.08				
58.9785	25.08				
58.9503	25.07				
58.9424	25.07				
58.9764	25.08				
58.9512	25.07				
58.9737	25.08				
59.0215	25.10				
59.0095	25.10				

$$\%\,\text{Recovery} = \frac{\bar{x}}{x_{\text{CRM}}} \times 100 \tag{7}$$

The bias of the method was calculated using Equation (8) and was found 0.06 ppm and the %bias was calculated using Equation (9) and was found 0.22% [3].

$$\text{bias} = \bar{x} - x_{\text{Ref}} \tag{8}$$

$$\text{bias}(\%) = \frac{\bar{x} - x_{\text{Ref}}}{x_{\text{Ref}}} \times 100 \tag{9}$$

The acceptance criteria of the bias was expressed in Equation (10) in which σ is an uncertainty term, which was calculated by Equation (11) in which u_{CRM} is the uncertainty of the CRM used in the calibration of HPLC-UV and n is the number of measurements [23] [24] [25]. The u_{CRM} was calculated by dividing the expanded uncertainty 0.6% by 2.

$$-2\sigma \le b \le +2\sigma \tag{10}$$

$$\sigma = \sqrt{\left(S_b\right)^2 + \left(\frac{S_w}{n}\right)^2 + \left(u_{\mathrm{CRM}}\right)^2} \tag{11}$$

Since the uncertainty of the CRM purity is in % and that of the S_b and S_w are in mg/kg, the uncertainty ratios (u_x/x) of the three contributions in Equation (11) were combined under the square root and multiplied by the grand mean (24.97 mg/kg) to express σ in mg/kg. The values of σ, $+2\sigma$ and -2σ were recorded in Table 9. From these values, it can be noticed that no bias of the method was found since the observed bias (0.06 ppm) falls within $\pm 2\sigma$ at confidence level 95%.

Table 9. Calculation results of σ.

Parameter	Value (x)	Uncertainty (u_x)	unit	u_x/x
$\mathrm{Purity}_{\mathrm{CRM}}$	99.8	0.3	%	0.0030
σ	0.102		mg/kg	
$+2\sigma$	0.204		mg/kg	
-2σ	−0.204		mg/kg	

4. Conclusion

A method for the analysis of caffeine in water in the range 10 - 50 mg/kg by HPLC-UV was developed and validated. The validation results proved that the method is selective, precise and accurate enough for the purpose of measurements. The LOQ was found 20 ppb and the observed bias (0.06 ppm) came within the acceptance criteria $\pm 2\sigma$. The validated method can be used by the analytical laboratories measuring caffeine in water.

Conflicts of Interest

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

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