Hygeia.J.D.Med. May 2016 - Octoberer 2016 ISSN 2229 3590
Hygeia :: journal for drugs and medicines
May 2016
Open Accesswww.hygeiajournal.com
Research article section: Pharmaceutical Analysis
A Half Yearly Scientific, International, Open Access Journal for Drugs and Medicines
DOI:10.15254/H.J.D.Med.8.2016.152 Research Article

A COST EFFECTIVE HPLC METHOD FOR THE ANALYSIS OF CURCUMINOIDS

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Key words
Curcuminoids, HPLC validation, turmeric, Cost-effective method

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Received: 15 October 2015,
Revised: 20 November 2015
Accepted: 25January 2016,
Available online: 15 June 2016

ABSTRACT

***Plan**: Validation of a method for the HPLC estimation of curcuminoids*
***Preface**: Many methods are available for the assay of curcumin, the major pigment in turmeric rhizomes. These include direct fluorimetric, spectroscopic and HPLC methods. HPLC analysis of compounds is expensive, as HPLC grade water and solvents are used. Considering the expensive nature of solvents used in HPLC analysis, there is a need to develop cost-effective methods for the estimation of compounds using HPLC.*
***Methodology**: We modified a recently-reported HPLC method for the estimation of curcuminoids. The modified method was validated and found to be accurate, precise, specific, reproducible, and rugged.*
***Outcome**: This cost-effective method can be utilized for the speedy and routine HPLC estimation of curcuminoids.*

1. INTRODUCTION

Curcumin is the major pigment and biologically active constituent of turmeric rhizomes. All oriental medical traditions use this herb for the treatment of a variety of ailments. The scientific rationale behind these uses is well-known [1-4]. Many methods are available for the assay of curcumin. They include direct fluorimetric, spectroscopic (IR, NMR, MS) and HPLC methods [5]. However, HPLC methods are widely used considering ease and sensitivity.

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Hygeia journal for drugs and medicines, 2229 3590
Rid: D-2044-2014

HPLC analysis of curcumin was first attempted by Asakawa *et al* (1981), using Nucelosil C_{18} column as stationary phase, a mixture of Acetonitrile: H_2O: Acetic acid (51: 49: 5) as mobile phase and benzyl benzoate as an internal standard[6]. Many improved methods have been reported since then [7-10]. HPLC analysis of compounds is expensive, as HPLC grade water and solvents are used. Therefore, there is a need to develop cost-effective methods for the estimation of compounds using HPLC.

During literature survey we came across a HPLC method for the estimation of curcumin in rat plasma [11]. This method employed a mobile phase consisting of acetonitrile-5% acetic acid. While working with this method we noticed that this method could be modified to yield a better and less expensive analytical method. Development of this method is reported in this communication.

2. MATERIALS AND METHODS

2.1. Modification of the method

Li *et al* (2009) performed the chromatographic separation with a mobile phase consisting of acetonitrile-5% acetic acid (75:25 v/v) at a flow rate of 1.0 ml/ mi. the wavelength of detection was 420 nm. Injection volume was 50µl and a sample was analyzed in 3 minutes[11].

In the present study we performed chromatographic separation with a mobile phase composed of acetonitrile-2% acetic acid (55:45 v/v) at a flow rate of 0.5 ml /min. wavelength of 425 nm was used for detection. Injection volume was 10µl and running time was 10 minutes.

Transfer of method is best achieved by a systematic method validation process. This is carried out by challenging the method and determining the limits of allowed variability for the conditions required to run the method. The present method was validated with reference to parameters like accuracy, precision, specificity, linearity, limit of quantification (LOQ), limit of detection (LOD) and ruggedness [12, 13.]

2.2. Solvents

HPLC grade acetonitrile, methanol and water were procured from Merck India, Mumbai, HPLC grade acetic acid from Hi Media Laboratories Pvt Ltd., Mumbai and reference standard of curcuminoids from Merck KGaA, Darmstadt, Germany (Batch No. S6351554).

2.3. HPLC instrumentation and conditions

The HPLC system consisted of Agilent quaternary system with 1260 quat pump, injector, variable wavelength detector 1260 VWD VL and auto sampler 1260 ALS. A column (4.6 x 150 mm) packed with 5 µm particle size C_{18} material was used for the separations. Agilent Chem Station software was used for the control of the equipment and for data evaluation. Quantification of the compound was carried out using the peak areas method.

Chromatographic separation was achieved with a mobile phase composed of acetonitrile-2% acetic acid (55:45 v/v) at a flow rate of 0.5 ml/min. the mobile phase was filtered through a 0.45 µm membrane filter and ultrasonically degassed prior to use. Wavelength of 425 nm was used for detection. Injection volume was 10µl and running time was 10 minutes. All statistical analyses were carried out using *Graphpad Prism* Version 5.

3. RESULTS

3.1 Validation of the method

The method was validated according to the guidelines of International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [14].

3.2. Accuracy

A range of four concentrations of curcuminoids standard (0.5 ppm, 1 ppm, 2 ppm, 5 ppm) was prepared and analyzed four times by the same analyst under same conditions. Area was recorded and the mean, standard deviation and % relative standard deviation (% RSD) was calculated (Table 1). The % RSD was below 1% and therefore, this method is validated for accuracy. (Table.1)

3.3. Precision: System precision

To check system precision, the same concentration of curcuminoids standard was injected six times. The area recorded and concentration of the standard was calculated from regression equation. The standard deviation and % RSD are given in Table 2. The percentage RSD of < 1 confirms that the method has system precision.

3.4. Method repeatability

A sample of turmeric extract (CKL/SP/F/07-13/0080) was analyzed six times by the same analyst. The concentration and % purity were calculated from area and then the standard deviation and % RSD were calculated. Table 3: Repeatability of the method.

3.5. Reproducibility

Reproducibility of the methods was evaluated by analyzing the same sample on same day and different days by different analysts. The % purity values, standard deviation and % RSD were calculated from the data (Tables 4-6).

3.6. Linearity

A range of eight concentrations of curcuminoids standard was analyzed, regression equations calculated and correlated with calibration graph. A linear relationship was obtained between the peak areas and concentrations of DMC, BDMC and curcumin. The correlation coefficients for DMC, BDMC and curcumin were 0.99744, 0.99755 and 0.99729 respectively (Table 7).

Regression analysis demonstrated an excellent relationship between the peak areas and concentrations (Figures 1-3).

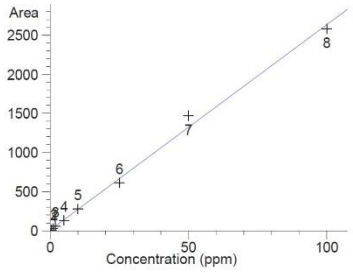

Figure1.Linearity plot of DMC

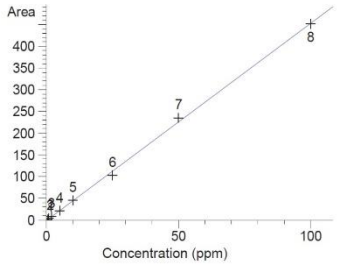

Figure 2. Linearity plot of BDMC

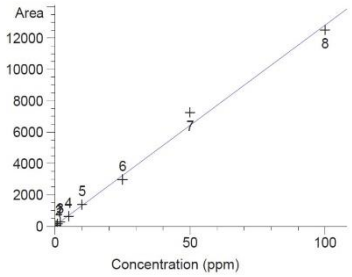

Figure 3. Linearity plot of curcumin

3.7. Recovery

Test sample was fortified by transferring 3 ml of sample solution to a 50 ml volumetric flask and spiking 0.85 ml of 296 ppm concentration of curcuminoids standard into it. The volume was made up to the mark with HPLC grade methanol. A concentration of 5.33 ppm was injected and analyzed seven times by the same analyst (Table 8).

3.8. LOD and LOQ

LOD and LOQ were determined from the specific calibration curve obtained using eight standard solutions (0.5, 1, 2, 5, 10, 25, 50 and 100 ppm). The following equations recommended in ICH (2005) were used for calculating LOD and LOQ:

$$LOD = \frac{3.3 \times \sigma}{S} \qquad LOQ = \frac{10 \times \sigma}{S}$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve. The data are provided in Table 9.

3.9. Specificity: Acid degradation

Specificity of the method was established by studying the degradation of the standard. After subjecting the standard to acid degradation by the addition of 1 M HCl and heating in a water bath for 2 hrs at 40^0C, the standard was analyzed six times by the same analyst. Percentage of degradation was calculated using curcuminoids standard (Table 10).

3.10. Base degradation

Base degradation was caused by the addition of 1M NaOH into the standard and keeping in a boiling water bath for 2 hrs at 40^0C. After that the solution was cooled and made up to the mark using HPLC grade methanol. This solution was analyzed by HPLC six times by the same analyst. Percentage of degradation was calculated using curcuminoids standard (Table 11).

3.11. Thermal degradation

Thermal degradation was caused by the addition of a small quantity of methanol into the standard and by keeping in a boiling water bath for 2 hrs at 80^0C. Thereafter, the solution was cooled and made up to the mark using HPLC grade methanol. This solution was analyzed six times by the same analyst. Percentage of degradation was calculated using curcuminoids standard (Table 12).

3.12. Ruggedness

Ruggedness was evaluated by small deliberate variations in experimental conditions, like changing mobile phase composition by ± 5 ml of acetonitrile, λ max by ± 5 nm and flow rate by ± 0.1 ml. The optimum conditions selected for the analysis are mobile phase composition of acetonitrile- 2% aqueous acetic acid 55:45, flow rate 0.5 ml/min and λ max 425 nm.

For alteration-I the conditions selected were mobile phase composition of acetonitrile- 2% aqueous acetic acid 60:40, flow rate 0.6 ml/min and λ max 430 nm.

For alteration-II the conditions selected were mobile phase composition of acetonitrile- 2% aqueous acetic acid 50:50, flow rate 0.4 ml/min and λ max 420 nm.

The alterations caused significant changes in resolution of peak area and retention time (Table 13) confirming the robustness of the method.

3.13. System suitability

3.14. Retention time

Single concentration of curcuminoids standard was injected six times and the % RSD calculated. % RSD was 0.176.

3.15. Tailing factor

Single concentration of curcuminoids standard was injected six times and tailing factor (TF) was calculated with the following formula: The data are presented in Table 14.

$$TF = \frac{\text{Peak width}}{\text{Half of peak width x 2}}$$

3.16. Retention factor

A single concentration of curcuminoids standard was injected six times and retention factor calculate using the formula $k = (t_r - t_o)/ t_o$ (Table 15).

3.17. Theoretical plates

Single concentration of curcuminoids standard was injected six times and the number of theoretical plates was calculated using the formula $N = 5.545 \times (t_r / W_{b1/2})^2$ (Table 16).

3.18. Resolution

Single concentration of curcuminoids standard was injected six times and resolution calculated by the following formula:

$$R_S = 2\,\frac{(t\text{R2} - t\text{R1})}{w\text{b1} + w\text{b2}}$$

Where tR2 = retention time of first peak, tR1 = retention time of second peak, wb1 = width of base peak 1 and wb2 is width of base peak 2 [15] (Table 17).

3.19. Measurement uncertainty

The sources of uncertainty for a HPLC method can be identified as repeatability, bias, measurement of the peak area, concentrations of standards and the mass and volume of the sample [16]. Uncertainty of these factors was calculated and a measurement uncertainty budget prepared (Table 18). Measurement uncertainty limit of curcuminoids was calculated to be 96.23 ± 7.93 at 95% confidence level.

4. DISCUSSION

Methanol, acetonitrile and tetrahydrofuran are the solvents commonly employed in reversed phase HPLC analysis. Among them methanol does not provide the required resolution/selectivity for the separation of curcuminoids [10]. The use of tetrahydrofuran instead of acetonitrile reverses the order elution of the curcuminoids [5,8,17]. Moreover, acetonitrile is the solvent of choice because of its low wavelength transparency, polarity, and intermediate position between methanol and tetrahydrofuran [18]. Therefore, we developed a method based on acetonitrile [10, 11].

The modified method consumes less HPLC solvents, and sample for injection. Li *et al* (2009) used a mobile phase of acetonitrile: 5% acetic acid [11]. However, we reduced the strength of acetic acid to 2%. The ratio of acetonitrile: acetic acid used in the original method was 75: 25. We could obtain good separation of curcuminoids with a ratio of 55:45. There was reduction in the injection volume as well. We injected 10µl of sample instead of 50µl used by Li *et al* (2009) [11]. Injection volumes ranging from 25µl – 50 µl have been used by others [19-22]. The analysis time was 3 minutes in the case of the original method. The peaks obtained were mixed in some cases. But we could resolve with the present method in 10 minutes, distinct peak at low concentration as 5 ppm (Figure 4).

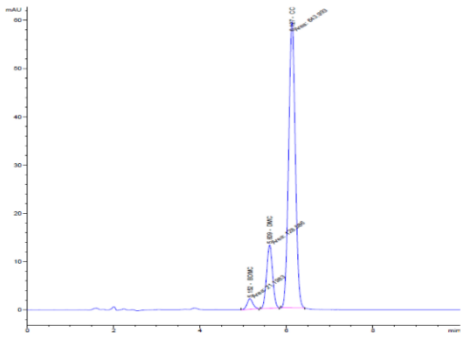

Figure 4. HPLC chromatogram of 5 ppm curcuminoids standard.

Though many methods are available for the estimation of curcumin using HPLC, several of them are not validated [19.21-24]. Among validated methods, many are validated for only a few parameters like linearity, precision, LOD and LOQ [11, 20, 25-27]. Therefore, we carried out full validation of the modified method based on Li *et al* (2009) [11], confirming its practical utility.

The % RSD of area of a range of four concentrations of curcuminoids was below 1%, indicating the accuracy of the method.

The % RSD related to system precision and method repeatability were also < 1 confirming the successful validation of these parameters. The measurement of the peak areas showed low values of % RSD (< 2) which suggested excellent accuracy and precision of the method.

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not quantified. It is expressed as a concentration at a specified signal: noise ratio, usually 3:1 [28]. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal: noise ratio 10:1 [14]. The LOD and LOQ were calculated in the present study, based on the standard deviation of the response (SD) and the slope of the calibration curve using eight standard solutions. The LOD for DMC, BDMC and curcumin were 0.3557, 1.796 and 0.0738 ppm respectively. Similarly, the LOQ for DMC, BDMC and curcumin were 1.0781, 5.445 and 0.2236 ppm respectively.

Analysis of the same sample on same day and different days by different analysts showed that the method is reproducible. The % RSD of these analyses were below1%, confirming the reproducibility of the method.

Small deliberate changes in mobile phase composition by ± 5 ml of acetonitrile, λ max by ± 5 nm and flow rate by ± 0.1 ml caused significant changes in resolution of peak area and retention time, indicating the ruggedness of the method. Thus it is evident that the modified HPLC method for estimation of curcuminoids reported in this communication is found to be economical, precise, specific, reproducible, and rugged for routine analysis.

Table 1 .Accuracy of the method

SI. No	*Concentration (ppm)*	*Detector Response*				*Mean*	*Standard Deviation*	*% RSD*
		I	II	III	IV			
1.	0.5	54.8	54.5	54.4	54.1	54.45	0.2886	0.5301
2.	1.0	135.6	134.7	134.0	134.1	134.60	0.7348	0.5459
3.	2.0	267.6	266.6	268.1	267.5	267.45	0.6245	0.2335
4.	5.0	629.9	629.9	631.2	631.5	630.63	0.8461	0.1341

Table 2 .System precision of the method

Replicate	*System precision*	
	*Area of curcumin **	*Concentration from graph (ppm)*
1	659.6	5.01
2	656.2	4.98
3	660.3	5.01
4	659.3	5.00
5	658.1	4.99
6	656.6	4.98
Mean		5.00
Standard deviation		0.014
% RSD		0.280

**Atomic units*

Table 3. Repeatability of the method

Replicate	*Repeatability of samples (area)**			*% Purity*			*% purity of curcuminoids*
	BDMC	*DMC*	*Curcumin*	*BDMC*	*DMC*	*Curcumin*	
1	95.4	741.5	4277.7	1.690	13.136	75.785	90.61
2	91.6	734.0	4273.0	1.622	13.003	75.702	90.32
3	92.3	765.9	4271.0	1.635	13.569	75.666	90.87
4	93.7	743.1	4287.8	1.660	13.165	75.964	90.78
5	89.3	739.5	4251.4	1.582	13.101	75.319	90.00
6	85.1	754.3	4249.3	1.507	13.363	75.282	90.15
Mean							90.46
Standard deviation							0.352
% RSD							0.389

**Atomic units*

Table 4. Inter day analysis-I

Replicate	Reproducibility of samples (area)*			% Purity			% purity of curcuminoids
	BDMC	DMC	Curcumin	BDMC	DMC	Curcumin	
1.	95.4	741.5	4277.7	1.690	13.136	75.785	90.61
2.	91.6	734.0	4273.0	1.622	13.003	75.702	90.32
3.	92.3	765.9	4271.0	1.635	13.569	75.666	90.87
4.	93.7	743.1	4287.8	1.660	13.165	75.964	90.78
5.	89.3	739.5	4251.4	1.582	13.101	75.319	90.00
6.	85.1	754.3	4249.3	1.507	13.363	75.282	90.15
Mean							90.46
Standard deviation							0.352
% RSD							0.389

**Atomic units*

Table 5.Inter day analysis-II

Replicate	Reproducibility of samples (area)*			% Purity			% purity of curcuminoids
	BDMC	DMC	Curcumin	BDMC	DMC	Curcumin	
1.	94.7	743.7	4316.1	1.67	13.17	76.46	91.39
2.	87.4	734.9	4282.2	1.54	13.01	75.86	90.43
3.	97.5	753.7	4307.0	1.72	13.35	76.30	91.38
4.	95.7	763.3	4300.1	1.69	13.52	76.18	91.40
5.	90.7	743.4	4279.9	1.61	13.17	75.82	90.60
6.	86.7	738.6	4261.2	1.53	13.08	75.49	90.11
Mean							90.89
Standard deviation							0.575
% RSD							0.630

**Atomic units*

Table 6.Intraday analysis

Replicate	Reproducibility of samples (area*)			% Purity			% purity of curcuminoids
	BDMC	DMC	Curcumin	BDMC	DMC	Curcumin	
1.	86.3	737.5	4273.3	1.52	13.06	75.70	90.30
2.	86.6	751.6	4272.6	1.53	13.31	75.69	90.54
3.	89.9	763.9	4280.6	1.59	13.53	75.83	90.96
4.	93	743.1	4287.8	1.64	13.16	75.96	90.77
5.	91.5	764.5	4299.6	1.62	13.54	76.17	91.33
6.	89	737.6	4275.4	1.57	13.06	75.74	90.38
Mean							90.71
Standard deviation							0.389
% RSD							0.429

*Atomic units

Table 7.Linearity of the method

Concentration (ppm)	Area*		
	DMC	BDMC	Curcumin
0.5	10.21	--**	54.98
1	26.07	4.37	135.66
2	51.71	7.55	270.73
5	124.87	23.37	643.17
10	271.05	50.75	1356.13
25	585.83	112.21	2953.37
50	1448.36	278.78	7231.31
100	2527.91	489.16	12529.90

*Atomic units, **Not detected

Table 8.Recovery analysis of fortified samples

SI. No	*Spiked Concentration (ppm)*	*Obtained Concentration (ppm)*		*Recovery %*
		*Area**	*Concentration*	
1	5.33	730.0	5.35	100.48
2	5.33	733.8	5.38	101.04
3	5.33	741.0	5.44	102.09
4	5.33	738.0	5.42	101.65
5	5.33	735.2	5.39	101.24
6	5.33	733.3	5.38	100.96
7	5.33	721.8	5.29	99.28
Mean				100.96
Standard deviation				0.903
% RSD				0.894

*Atomic units

Table 9.Derivation of LOD and LOQ

Sl. No.	*Concentration of standard (ppm)*	*Area of curcuminoids**		
		DMC	*BDMC*	*Curcumin*
1	0.5	10.21	1.54	54.98
2	1	26.06	4.36	135.66
3	2	51.71	7.55	270.73
4	5	124.87	23.37	643.17
5	10	271.05	50.75	1356.13
6	25	585.83	112.21	2953.37
7	50	1448.37	278.78	7231.31
8	100	2527.91	489.16	12529.90
Residual standard deviation		2.783	2.72	2.86
Slope (m)		25.820	5.00	128.05
LOD (ppm)		0.356	1.80	0.074
LOQ (ppm)		1.078	5.45	0.224

*Atomic units

Table 10. Effect of acid degradation on curcuminoids standard

Replicate	Before acid degradation		After acid degradation		
	Area of curcuminoids standard*	Purity of curcuminoids standard	Area of curcuminoids standard*	Purity of curcuminoids standard	% Degradation
1	1356.1	95.9	1284.8	90.41	5.48
2	1356.1	95.9	1286.3	90.51	5.38
3	1356.1	95.9	1285.8	90.48	5.41
4	1356.1	95.9	1289.1	90.71	5.18
5	1356.1	95.9	1286.4	90.52	5.37
6	1356.1	95.9	1282.5	90.24	5.65
Mean					5.41
Standard deviation					0.154
% RSD					2.84

**Atomic units*

Table 11. Effect of base degradation on curcuminoids standard

Replicate	Before base degradation		After base degradation		
	Area of curcuminoids standard*	Purity of curcuminoids standard	Area of curcuminoids standard*	Purity of curcuminoids standard	% Degradation
1	1356.1	95.9	858.1	60.38	35.52
2	1356.1	95.9	853.1	60.03	35.87
3	1356.1	95.9	851.2	59.89	36.00
4	1356.1	95.9	793.6	55.84	40.06
5	1356.1	95.9	789.0	55.52	40.38
6	1356.1	95.9	783.6	55.14	40.76
Mean					38.10
Standard deviation					2.54
% RSD					6.66

**Atomic units*

Table 12. Effect of thermal degradation on curcuminoids standard

Replicate	Before thermal degradation		After thermal degradation		
	Area of curcuminoids standard*	Purity of curcuminoids standard	Area of curcuminoids standard*	Purity of curcuminoids standard	% Degradation
1	1356.1	95.9	1158.4	81.515	14.68
2	1356.1	95.9	1157.3	81.438	14.46
3	1356.1	95.9	1156.0	81.346	14.55
4	1356.1	95.9	1155.3	81.297	14.60
5	1356.1	95.9	1152.5	81.100	14.80
6	1356.1	95.9	1155.9	81.339	14.56
Mean					14.61
Standard deviation					0.118
% RSD					0.808

**Atomic units*

Table 13.Confirmation of the ruggedness of the method

Experimental condition	Retention time & Response*	DMC*	BDMC*	Curcumin*
At optimum conditions	Retention time	5.638 ± 0.44	5.183 ± 0.50	6.15 ± 0.35
	Response**	131. 175 ± 0.67	20. 6 ± 0.76	629. 725 ± 0.20
Alteration-I	Retention time	3.53 ± 0.62	3.274 ± 0.69	3.83 ± 0.57
	Response	90. 85 ± 1.99	12.7 ± 1.80	3.70 ± 0.74
Alteration-II	Retention time	9.83 ± 0.24	8.94 ± 0.28	10.86 ± 0.18
	Response	161.875 ± 0.40	25.55 ± 1.56	749.6 ± 0.39

*Values are expressed as mean ± % RSD, **Atomic units

Table 14.Derivation of tailing factor

Replicate	Peak width	½ of peak width	Tailing factor
1	0.177	0.088	0.999
2	0.180	0.089	1.008
3	0.178	0.090	0.991
4	0.177	0.086	1.001
5	0.177	0.089	0.992
6	0.179	0.090	0.999
Mean			0.998
Standard deviation			0.006
% RSD			0.630

Table 15. Derivation of retention factor

Replicates	Curcumin			
	t_r	t_0	$t_r - t_0$	k
1	6.006	2.007	3.999	1.992
2	6.037	2.007	4.030	2.001
3	6.031	2.012	4.019	1.997
4	6.026	2.007	4.019	2.002
5	6.030	2.012	4.018	1.997
6	6.028	2.012	4.016	1.996
Mean				1.998
Standard deviation				0.004
% RSD				0.180

Table 16. Derivation of number of theoretical plates

Replicates	Peak RT	½ height of peak width	Number of theoretical plates
1	6.00	0.088	25388.02
2	6.04	0.089	25192.74
3	6.03	0.090	24863.17
4	6.03	0.088	26085.84
5	6.03	0.089	25078.09
6	6.03	0.090	24949.57
Mean			25259.57
Standard deviation			444.94
% RSD			1.76

Table 17. Derivation of resolution

Replicates	*t*R2	*t*R1	*w*b1	*w*b2	Resolution factor
1	6.01	5.49	0.177	0.164	3.01
2	6.04	5.51	0.180	0.167	3.02
3	6.03	5.51	0.177	0.164	3.03
4	6.03	5.51	0.177	0.166	3.03
5	6.03	5.51	0.177	0.165	3.05
6	6.03	5.51	0.179	0.166	3.02
Mean					3.03
Standard deviation					0.014
% RSD					0.462

Table 18.Uncertainty measurement budget of the method

Uncertainty – Budget Parameters	Value	Standard Uncertainty	RSU	RSU^2
Sample Weight	0.0148	0.00005	0.00337837	0.0000114134
Made up volume	50	0.0303169	0.00060633	0.0000003676
Calibration Standard (1 ppm)	1	0.025022	0.02502251	0.0006261261
Calibration Standard (2 ppm)	2	0.025022	0.01251107	0.0001565271
Calibration Standard (5 ppm)	5	0.024942	0.00498842	0.0000248844
Calibration Standard (10 ppm)	10	0.024942	0.00249421	0.0000062211
Calibration Standard (25 ppm)	25	0.024947	0.00099769	0.0000009954
Calibration Standard (50 ppm)	50	0.027568	0.00049894	0.0000002489
Calibration Standard (100 ppm)	100	0.02756	0.00027568	0.000000076
Standard deviation from graph	19.66	0.5779	0.02939471	0.000864049
Recovery (%)	100.78	0.00419	0.00004158	0.000000017
Repeatability (%)	96.2298	0.24	0.00249403	0.000006220
Combined uncertainty				0.041196238
Uncertainty in curcuminoids estimation				3.964305753
Effective degrees of freedom				372214.775
Coverage factor at 95% confidence level				2
Expanded uncertainty				7.9286

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