

## A Versatile High Performance Liquid Chromatography Method for Simultaneous Determination of Three Curcuminoids in Pharmaceutical Dosage forms

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### Abstract

A simple, precise, isocratic, reverse phase high performance liquid chromatography (HPLC) method was developed for the rapid determination of three curcuminoids viz. Curcumin (C), Desmethoxycurcumin (DMC) and Bisdesmethoxycurcumin (BDMC) using a Agilent RP C<sub>18</sub>, 4.6 mm × 150 mm, 5 μm XDB column. The run time was 7 min. The influence of mobile phase composition, injection volume, mobile phase pH, flow rate, temperature, and detector wavelength on resolution was investigated. The method was validated according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures with respect to precision, accuracy and linearity. The limit of detection and limit of quantitation were 0.015 and 0.050 μg/mL respectively. Linearity was from 0.05 to 15 μg/mL. Further, the proposed method was found to be reproducible and convenient for stability indicating analysis of curcumin in marketed formulation, polymeric nanoparticles and solubility studies.

**Keywords:** Curcuminoids; High performance liquid chromatography; Stability-indicating; Method validation; Nanoparticles; Solubility

### Introduction

Curcumin is a widely explored yellow colored phenolic pigment obtained from powdered rhizome of *Curcuma longa* Linn. (Family: Zingiberaceae) (Figure 1). It is found to be endowed with vast array of beneficial pharmacological effects including antioxidant, anti-inflammatory, anticarcinogenic, hypocholesterolemic, antibacterial, wound healing, antispasmodic, anticoagulant, antitumor, anti-angiogenic and hepatoprotective activities [1,2]. Marketed formulations of curcumin (C) generally contain curcuminoids; a mixture of related polyphenols which encompass the primary component C along with its co-purified derivatives DMC and BDMC [3,4]. Structurally, C is 1-(1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione, DMC is (1E,6E) 1,6-Heptadiene-3,5-dione,1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl) and BDMC is (1E,6E)-1,7-bis (4-hydroxyphenyl) hepta-1,6-diene-3,5-dione as shown in Figure 1 [5]. These three molecules show very small chemical modifications with respect to their number of methoxy groups (none for BDMC, one for DMC, and two for C) and the presence of methoxy groups in the ortho position on the aromatic ring [6]. However, these modifications influence the hydrophobic nature of three curcuminoids which is in the order of C > DMC > BDMC, with C being the relatively most hydrophobic among the three curcuminoids.

It is well documented that curcuminoids are unstable at basic pH and undergo alkaline hydrolysis in alkali/higher pH solution. In-vitro studies

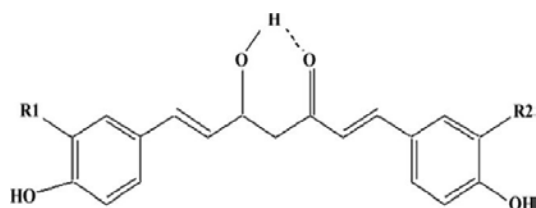
have also demonstrated hydrolytic decomposition in physiological condition (isotonic phosphate buffer, pH 7.2) [7-9]. Further, the curcuminoids are susceptible to photo degradation on exposure to light, both in solution and solid state [7]. In literature, various methods have been reported for the separation and analysis of curcuminoids per se. These methods include high performance thin layer chromatography (HPTLC) [10], high performance liquid chromatography (HPLC) [11-13], ultra performance liquid chromatography (UPLC) [14], capillary electrophoresis [15], microemulsion electrokinetic chromatography [16] and liquid chromatography mass spectrometry (LCMS) [17]. However, these methods have not been established for assessing the stability of curcuminoids during its shelf life. According to the ICH guideline, 'stability testing of new drug substances and products requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance'.

Thus, the aim of the present work was to develop an economic, accurate, specific, reproducible and stability-indicating HPLC method for the determination of C in the presence of its co-purified derivatives, degradation products and related impurities from a pharmaceutical dosage form in the form of hydrogel nanoparticles as well as capsules.

### Experimental

#### Materials

C (95% purity), C3 Caps were obtained as gift samples from Konark Herbals Ltd, India, Sanjivani Phytopharma Pvt. Ltd, Mumbai,



**Figure 1:** Chemical Structure of Curcuminoids. C: R<sub>1</sub> =R<sub>2</sub> =OCH<sub>3</sub>; DMC: R<sub>1</sub> =H, R<sub>2</sub> =OCH<sub>3</sub>; BDMC: R<sub>1</sub> =R<sub>2</sub> =H.

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India respectively. HPLC grade acetonitrile, methanol, sodium acetate, and orthophosphoric acid were purchased from s. d. Fine Chemicals (Mumbai, India). 0.45  $\mu$  membranes were purchased from Pall Life Sciences. All other chemicals used were of analytical grade unless otherwise indicated.

### Chromatographic conditions and equipment

The HPLC system consisted of Plus Intelligent LC pump PU-2080 from Jasco (Tokyo, Japan) equipped with a JascoUV-2075 Intelligent UV-Vis detector, a Rheodyne 7725 injector (Rheodyne, Cotati, CA, USA), along with Jasco ChromaPass Chromatography Data System Software (Version 1.8.6.1). Chromatographic separation was achieved on a 5  $\mu$  Agilent RP C<sub>18</sub> XDB column (4.6 mm  $\times$  150 mm). The mobile phase employed comprised of Solvent A: Aqueous sodium acetate buffer (0.04 M); adjusted to pH of 3 using dilute orthophosphoric acid and Solvent B: Acetonitrile (v/v). Prior to use, the mobile phase was filtered through 0.45  $\mu$  membrane. The flow rate was 1.0 mL/min; injection volume was 20  $\mu$ L and analysis was done at two wavelengths; 425 nm for C and 280 nm for both C and degradation products.

### Preparation of stock and standard solutions

A stock solution of C (100  $\mu$ g/mL) was prepared by dissolving accurately weighed 5 mg C in methanol using 50 mL volumetric flask. Standard solutions were prepared by dilution of the diluted stock solution with methanol to give solutions containing C in the concentration range of 0.05-15  $\mu$ g/mL.

### Forced degradation of curcumin

The working solution for the following studies was prepared by subjecting 1 mL of stock solution (previously described) to various forced degradation conditions to provide an indication of the stability indicating property and specificity of the proposed method. Prior to analysis, the resultant solution was diluted with methanol to give solutions containing C in the concentration of 10  $\mu$ g/mL [10,18].

To 1 mL of methanolic stock solution of C, 1 mL of HCl and NaOH of varying normality were added separately. These mixtures were heated separately for different time intervals at 80°C. The forced degradation in acidic and basic media was performed in 10 mL amber volumetric flasks in order to exclude the possible degradative effect of light. The neutralized solutions were injected in triplicate and chromatograms were run as previously described.

### Preparation of hydrogen peroxide induced degradation product

To 1 mL of methanolic stock solution of C, 1 mL of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (varying concentrations) was added. This solution was heated separately for different time intervals at 80°C. The forced degradation was performed in 10 mL amber volumetric flask in order to exclude the possible degradative effect of light. The final solutions were injected in triplicate and chromatogram was run as described previously.

### Photochemical and UV degradation product

1 mL of methanolic stock solution of C was diluted to 10 mL with methanol (10  $\mu$ g/mL of C) in transparent volumetric flask and the photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for different time intervals.

### Dry heat-induced degradation product

Powdered C was stored at 100°C for 1 h under dry heat conditions to study the inherent stability of C to dry heat-induced degradation which may be encountered during formulation methodology such as melt extrusion. The 10 mL methanolic solution (10  $\mu$ g/mL) was prepared from dry heat exposed drug as described above. The resultant solution was injected in triplicate and the chromatogram was run as described previously.

### Wet heat-induced degradation product

1 mL of methanolic stock solution of C was taken in 10 mL amber volumetric flask and heated for 2 h in water bath maintained at 80°C to study the wet heat degradation. The resultant cooled solution was injected in triplicate and the chromatogram was run as described previously.

### Method Validation

#### Accuracy and precision

Accuracy and Precision determination were carried out at 0.1, 2, 10 and 15  $\mu$ g/mL of C concentration. At each level of the amount, six determinations were performed and both, intra and inter day variation were expressed in terms of % Relative Standard Deviation (% R.S.D) and Standard Error (S.E). This was also done to check for the recovery of the drug at different levels in the formulations.

#### Robustness of the method

By introducing small changes in the mobile phase composition, injection volume, mobile phase pH, flow rate, temperature and detector wavelength at a concentration level of 10  $\mu$ g/mL in triplicate [19].

#### Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was injected six times following the same method as explained above. The LOD and LOQ for C were estimated at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

#### Linearity

Linearity test solutions for C were prepared by diluting the stock solution to the required concentrations. The solutions were prepared from LOQ to 15  $\mu$ g/mL (i.e. LOQ, 0.1, 0.25, 1, 2, 5, 10 and 15  $\mu$ g/mL). The calibration curves were drawn by plotting the peak areas of C against the corresponding concentration. The slope and Y intercept of the calibration curve was calculated.

#### Solution stability

The solution stability of C was carried out by leaving a spiked sample (10  $\mu$ g/mL) solution in a tightly capped volumetric flask at room temperature for 36 h. Content of C was determined at 12 h intervals by following the procedure as described previously.

### Applications of the Developed Method

#### Analysis of curcumin from hydrogel nanoparticles and marketed capsules

To determine the content of C in freeze dried hydrogel nanoparticles (label claim: 100 mg of C per 996 mg of freeze dried hydrogel nanoparticles), nanoparticles equivalent to 5 mg of C, i.e. 49.8 mg of

nanoparticles were accurately weighed and transferred to a volumetric flask containing 50 mL methanol. To ensure complete extraction of drug, it was sonicated for 10 min. The resulting solution was filtered through 0.45  $\mu$ m membrane and analyzed for drug content. 20  $\mu$ L (10  $\mu$ g/mL) of the filtered solution was injected and chromatogram was run. The analysis was repeated in six individual steps and the possibility of excipient interference in the analysis was studied.

Similar procedure was followed for determination of uniformity of content of 20 marketed capsules (C3 Caps) of C (label claim: 500 mg of C per capsule).

### Estimation of solubility of curcumin in different excipients

The solubility study of C in different excipients viz. surfactants and co-surfactants were determined by using shake flask method. Briefly, an excess amount of C was added individually to the surfactants and solubilizers (1 g each) in screw capped tubes. Subsequently, mixtures were shaken for 24 h in a water bath shaker (Remi, Mumbai, India) maintained at  $25 \pm 2^\circ\text{C}$ . After 24 h, each sample was centrifuged at 1200 x g for 10 min. The resultant supernatant (0.1 mL) was diluted suitably and analyzed using HPLC.

## Results and Discussion

### Development of the optimum mobile phase

The HPLC procedure was optimized with a view to develop a stability indicating assay method to quantify C and its degradation products (if arising on storage) from hydrogel nanoparticles. Further, it was aimed at separation of three curcuminoids in C sample. The pure drug was injected under both ambient and forced degradation conditions at different solvent systems. Various mobile phase compositions comprising of acetonitrile/tetrahydrofuran (THF)/methanol, pH 3 ammonium acetate/ pH 3 sodium acetate buffers in varying ratios were tried. The mobile phase acetonitrile: pH 3 ammonium acetate (70:30 v/v) gave symmetrical peak of C but failed to separate three the curcuminoids. In the same mobile phase composition, increase in concentration of buffer: acetonitrile (50:50 v/v) did show presence of three peaks but with poor resolution. On further increase in the concentration of buffer: acetonitrile (60:40 v/v), there was complete separation of peaks with relatively good resolution and prolonged elution time (more than 15 mins). Further increase in buffer did not show favorable effect on peak resolution and delayed the elution time for more than 25 mins. The replacement of pH 3 ammonium acetate buffer with pH 3 sodium acetate buffer did not improve the resolution of peak. Further, the inclusion of THF in varying concentrations of 5-10% v/v in the earlier composition failed to show desired results. Finally, the mobile phase consisting of acetonitrile: pH 3 sodium acetate buffer (40:60 v/v) was tried; it gave a well defined resolution of three curcuminoids. However, the run time exceeded 11 min, hence; the composition was altered to 50:50, which showed well-defined resolution and separation of three peaks within shortest run time of 7 min (Figure 2 and Figure 3). However, there was slight tailing in chromatograms; which could be very well ascribed to the mixture of two tautomeric forms: the keto- and enol-forms in the structure of curcuminoids as shown in Figure 1 [14]. The developed analytical procedure enabled complete analysis of C in shortest time of 7 min as well as provided clear demarcation between the three curcuminoids peaks. Moreover, the separation and order of elution of three curcuminoids were found

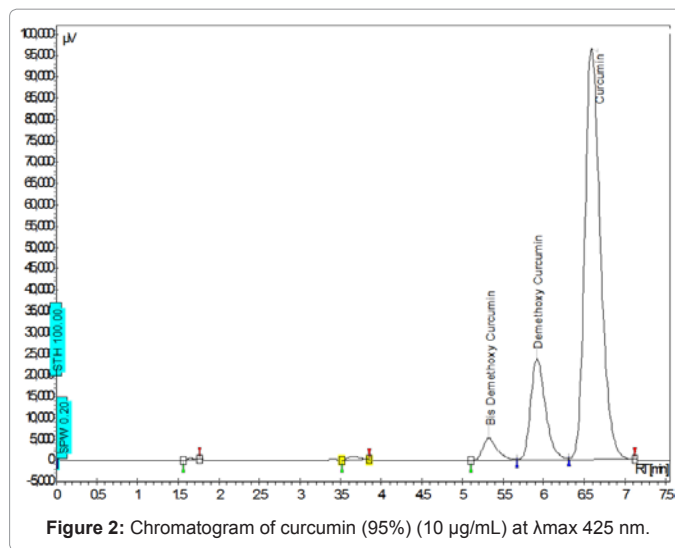


Figure 2: Chromatogram of curcumin (95%) (10  $\mu$ g/mL) at  $\lambda_{\text{max}}$  425 nm.

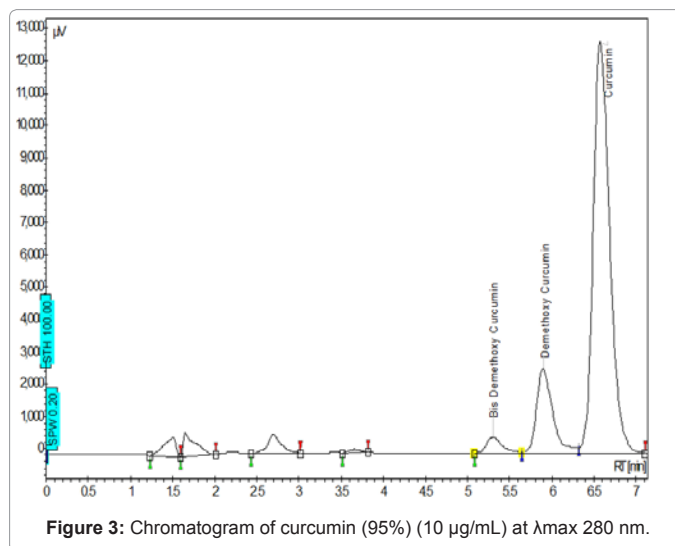


Figure 3: Chromatogram of curcumin (95%) (10  $\mu$ g/mL) at  $\lambda_{\text{max}}$  280 nm.

to be in agreement with the observations obtained using method employing acetonitrile as a component of mobile phase [20]. It has been noted that only THF shows reversal of order of elution of peaks of curcuminoids [21]. Additionally, the method proved its versatility in separation of three curcuminoids, the degradation peaks (arising due to the forced degradation conditions) and the related impurities.

The linear regression data for the calibration curves as shown in Table 1 showed a good linearity. No significant difference was observed in the slopes of standard curves (ANOVA,  $P < 0.0001$ ).

### Stability indicating property

The chromatograms of the samples treated with acid, base, hydrogen peroxide, dry and wet heat, and photochemical showed well separated chromatograms of pure BDMC, DMC and C as well as some degradation peaks at different retention times. The identification of degradants was based on comparison with the standard solution. The chromatograms of the degraded products were well resolved from the curcuminoids peaks as shown in Figures 4-7. The number of degradation products, content of curcuminoids remained, and percentage recovery was calculated and is listed in Table 2.

### Acid induced degradation product

The chromatogram of acid degraded sample of C, the chromatograms showed three additional peaks at 1.4, 1.7, 2.1 and 2.5 min, followed by curcuminoids peak as shown in Figure 4. Post 30 min heating on water bath, the areas of the acid-degraded product peaks were found to be higher than the area of standard C concentration (10 µg/mL) indicating that C undergoes significant degradation under mild acidic conditions (0.1 N HCl). Drug recovery of acid-degradation product peak was found at the level of 49.19%.

### Base induced degradation product

For base degradation (without heat), the chromatograms of the sample showed additional peaks at 1.7 and 2.8 min as shown in Figure 5. The presence of additional degradation peaks at mild conditions (0.1 N NaOH, 5 min exposure) reconfirmed the vulnerability of drug to degradation in basic conditions. Recovery at the level of 86.26% suggests susceptibility of C to degradation in basic conditions.

### Hydrogen peroxide induced degradation product

The chromatogram of the sample of C treated with H<sub>2</sub>O<sub>2</sub> (3% v/v) showed additional peaks at 1.5, 1.8 and 2.2 min as shown in Figure 6. Drug recovery at the level of 47.70% indicates the vulnerability of C to oxidation.

### Photochemical degradation product

The chromatogram of the sample of C exposed to photochemical degradation (sunlight for 30 min). C showed additional peaks at 1.7, 2.2 and 2.6 min as shown in Figure 7. This indicates that the drug is

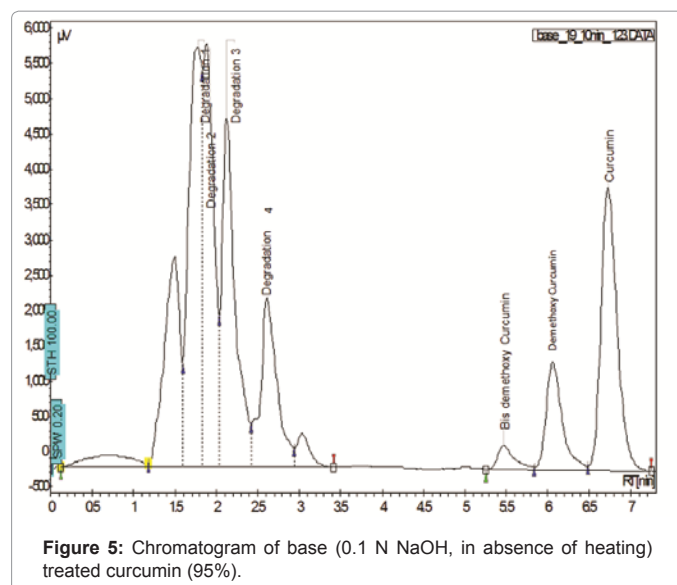


Figure 5: Chromatogram of base (0.1 N NaOH, in absence of heating) treated curcumin (95%).

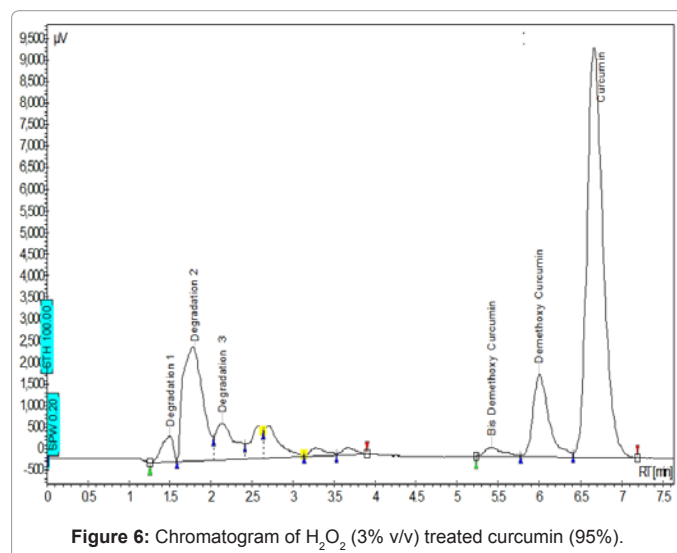


Figure 6: Chromatogram of H<sub>2</sub>O<sub>2</sub> (3% v/v) treated curcumin (95%).

Parameter	λ <sub>max</sub> -425 nm	λ <sub>max</sub> -280 nm
Linearity range (µg/mL)	0.05-15	0.05-15
Correlation coefficient (r <sup>2</sup> )	0.9999	0.9990
Slope	132200	17682
Confidence limit of slope <sup>a</sup>	131263 to 133137	17465 to 17900
Intercept	6731	156.3
Confidence limit of intercept <sup>a</sup>	488.4 to 12974	-1293 to 1606

<sup>a</sup>95% Confidence Intervals

Table 1: Linear regression data for calibration Curve (n=3).

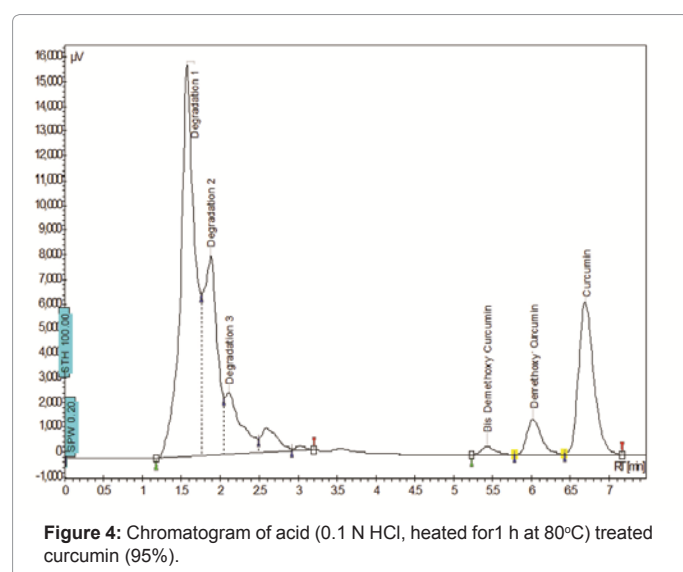


Figure 4: Chromatogram of acid (0.1 N HCl, heated for 1 h at 80°C) treated curcumin (95%).

unstable towards photochemical irradiations for the exposure period under study. However, on exposure to fluorescent light, C was found to be stable for 6 h duration.

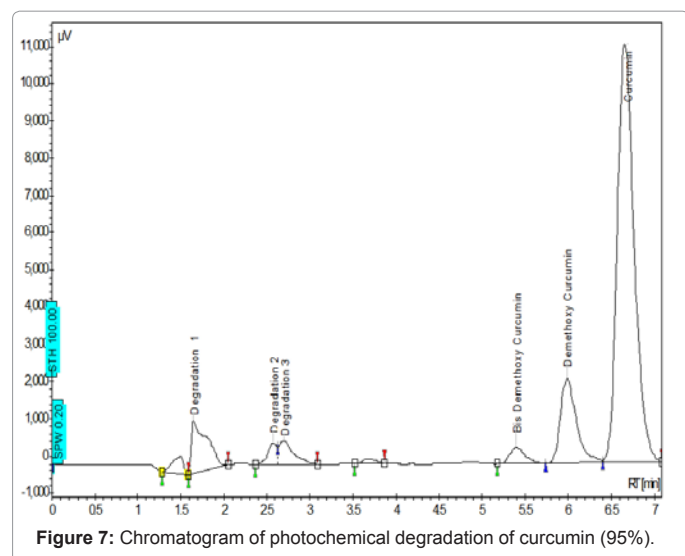
### Dry heat and wet heat degradation product

The samples subjected to dry heat (1 h at 100°C) and wet heat (1 h water bath at 80°C) conditions showed no additional peak. This study reflected the stability of drug under heating conditions encountered due to formulation methodologies such as hot melt extrusion technology.

### Validation of the Method

#### Accuracy and precision

Accuracy and precision of the method were determined by spiking known amount of C in triplicate at levels- very low, low, medium and high of the specified limit. The measurement of the peak area showed low values of % R.S.D. (<2) and very low values of the S.E. (<0.1) for inter and intra-day variation, which suggested an excellent accuracy and precision of the method (Table 3).



### Robustness of the method

The % R.S.D. and S.E. of the peak areas were calculated for change in mobile phase composition, injection volume, mobile phase pH, flow rate, composition of Solvent B was changed by 1%, temperature, and detector wavelength at a concentration level of 10 µg/mL in triplicate. The symmetry (<2) and asymmetry (>2) obtained after introducing small deliberate changes in the developed HPLC method indicated the robustness of the method (Table 4).

### Limit of detection and limit of quantification

Detection limit and quantification limit were calculated by the method as previously described. The signal-to-noise ratio of 3:1 and 10:1 were considered for LOD and LOQ respectively, and LOD and LOQ were found to be 0.015 and 0.050- µg/mL, respectively, which indicates adequate sensitivity of the method.

### Solution stability

The % of R.S.D for C concentration during solution stability

Sr. No.	Exposure condition	Time(h)	Drug remained (µg)	% of drug remained	% R.S.D	S.E.
1	Photo degradation	1.0	7.39	73.90	0.45	0.070
		0.5	8.08	80.08	0.61	0.056
2	Oxidation					
	3% H <sub>2</sub> O <sub>2</sub>	1.0	4.77	47.70	0.342	0.49
	30% H <sub>2</sub> O <sub>2</sub>	1.0	1.50	15.00	0.293	0.007
3	Base degradation					
	0.5 N NaOH	1.0	1.09	10.09	0.128	0.063
	0.1 N NaOH	1.0	2.04	20.40	1.30	0.28
	0.1 N NaOH	0.6	3.12	31.20	2.00	0.17
	0.1 N NaOH	No heat	8.62	86.26	0.483	0.18
4	Acid degradation					
	0.5 N HCl	1.0	3.068	30.68	0.12	0.04
	0.1 N HCl	1.0	4.919	49.19	0.806	0.13
5	Wet degradation	1.0	10.02	100.02	2.00	0.014
6	Dry heat degradation	1.0	10.04	100.40	0.85	0.037

**Table 2:** Forced degradation studies of curcumin (95%).

Intra-day accuracy and precision					Inter-day accuracy and precision			
Amount of drug added (µg)	Amount of drug remained (µg)	% of drug recovered	% R.S.D	S.E	Amount of drug remained (µg)	% of drug recovered (µg)	% R.S.D	S.E
0.1	0.1	100.00	1.46	0.001	0.107	107.60	0.81	0.006
2	1.989	99.45	1.47	0.020	2.05	102.67	1.58	0.023
10	9.992	99.92	1.32	0.01	9.991	99.91	0.10	0.079
15	15.02	99.34	0.17	0.016	15.13	100.90	0.184	0.0197

**Table 3:** Intra- and inter-day accuracy, precision and recovery studies of HPLC method (n=6).

experiments was within 1%. No significant changes were observed for the chromatograms of standard solution and the experimental solution. Further, absence of degradation peaks confirmed that sample is stable in solvent used during the assays for 36 h.

## Applications of the Developed Method

### Analysis of curcumin from hydrogel nanoparticles and marketed capsules

In the chromatograms of the C samples extracted from hydrogel nanoparticles and C capsules, separate peaks were observed corresponding to the three curcuminoids. There was no interference from the excipients commonly present in the both the formulations. The C content was found to be 101.80 and 97.65% with a % R.S.D of 0.88 and 1.0 and S.E. of 0.77 and 0.072 respectively for C hydrogel nanoparticles and C capsules. The low % R.S.D value indicated the suitability of this method for routine analysis of C in pharmaceutical dosage forms.

### Estimation of solubility of curcumin in different excipients

Considering the poor bioavailability of C in gastrointestinal milieu, nanoengineering of C has been sought. Although there are numerous literatures which state nano sized formulations of C, the accurate solubility of the drug in these excipients is still lacking. Thus, solubility studies were undertaken to estimate the solubilising potential of these excipients and further to investigate the effect of these components on the stability of C. As depicted in Table 5, the solubility of C in various excipients was not governed by any specific chemical composition. It was found that C exhibited maximum solubility in the following order Tween 20 > Tween 80 > Transcutol P > Softigen 767 > Gelucire 44/14 (Table 5). Further, though there was colour change from yellow to red, these components did not have deleterious effect on C, indicating the

Sr.No.	Parameter	Level	RT* (min)	As USP**	Res USP***
1	Flow rate (mL/min)	0.9	7.18	1.21	2.16
		1.0	6.41	1.18	2.18
		1.1	5.92	1.16	2.06
2	pH of the buffer	3.0	6.41	0.97	2.23
		3.3	6.5	1.16	2.15
3	Column temperature (°C)	25	6.41	1.18	2.18
		31	6.12	1.17	2.12
		37	6.1	1.2	2.02
4	Composition of solvent B (%)	49	5.97	1.2	2.01
		50	6.41	1.18	2.18
		51	6.3	1.17	2.08
5	Injection volume (µL)	20	6.41	1.18	2.18
		50	6.41	1.14	1.62
6	Wavelength λ max-(nm)	275	6.44	1.22	2.1
		280	6.41	1.18	2.18
		285	6.48	1.18	2.14

\*RT- Retention Time, \*\*As USP- Asymmetry USP, \*\*\* Res USP- Resolution USP

Table 4: Results of the robustness of the method.

Stabilisers, surfactants and co- surfactants	Solubility(mg/g)
<b>Stabilizers</b>	
Plasdone K 90 D (Ashland Speciality Ingredient): 1%	0.387 ± 0.0176
Plasdone K 90 D: 5%	0.383 ± 0.0381
Plasdone K 90 D 10%	0.415 ± 0.049
Poloxamer 407:1%	0.2665 ± 0.047
Lutrol F127 (BASF, India): 5%	0.731 ± 0.0721
Poloxamer 407:10%	8.785 ± 0.31
Poloxamer 188:1%	0.156 ± 0.0622
Poloxamer 188: 5%	0.1745 ± 0.02
Lutrol F68 (BASF, India): 10%	0.35 ± 0.070
<b>Surfactants and co -surfactants</b>	
Transcutol P (Gattefosse, India)	62.38 ± 1.84
Glycerine	0.094 ± 0.0014
Propylene glycol	3.5 ± 0.355
Softigen 767	16.3 ± 0.5
Gelucire 44/14 (Gattefosse, India)	13.245 ± 0.53
Tween 60	3.4 ± 0.42
Tween 80	72.94 ± 1.46
Tween 20	92.11 ± 6.25
Solutol HS 15 (India)	2.5 ± 0.35
Cremophor RH 40 (BASF, India)	3.01 ± 0.34
Cremophore EL (BASF, India)	6.2 ± 0.56
Labrasol (Gattefosse, India)	0.01515 ± 0.300

Table 5: Solubility of curcumin in different stabilizers, surfactants and co-surfactants.

colour change was representative of enhanced solubility of drug.

## Conclusion

The developed HPLC method is precise, specific, accurate and stability indicating for the determination of C (95%). Statistical analysis also proves that the method is reproducible and specific for the analysis of C along with its co-purified derivatives DMC and BDMC. The method is versatile in the separate analysis of curcuminoids in bulk drug as well as in formulations such as, hydrogel nanoparticles, marketed C capsules and stabilizers, surfactants and co-surfactants. Additionally, the solubility studies revealed that the colour change in various surfactants was ascribed to the high solubility as opposed to degradation.

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