

# Analytical Methods

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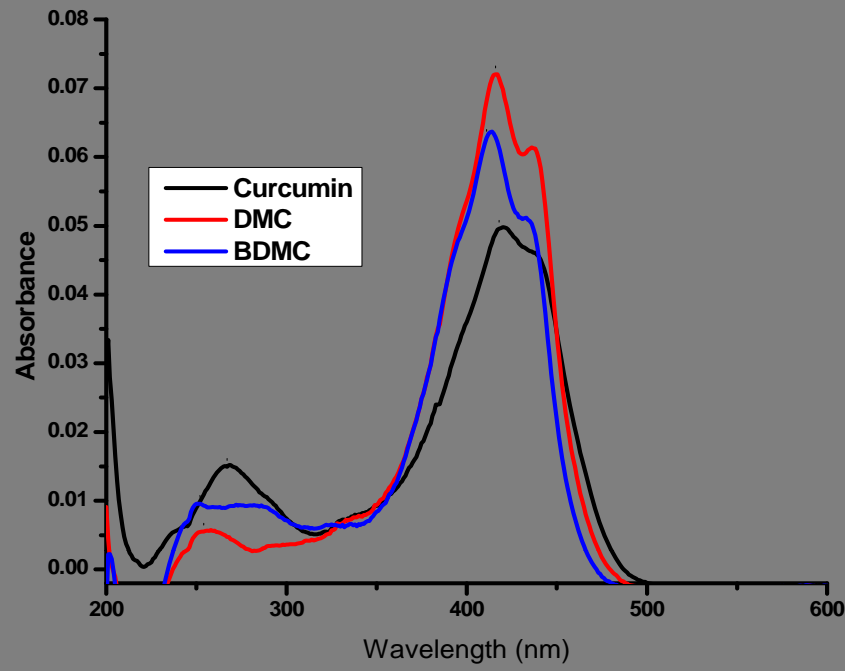
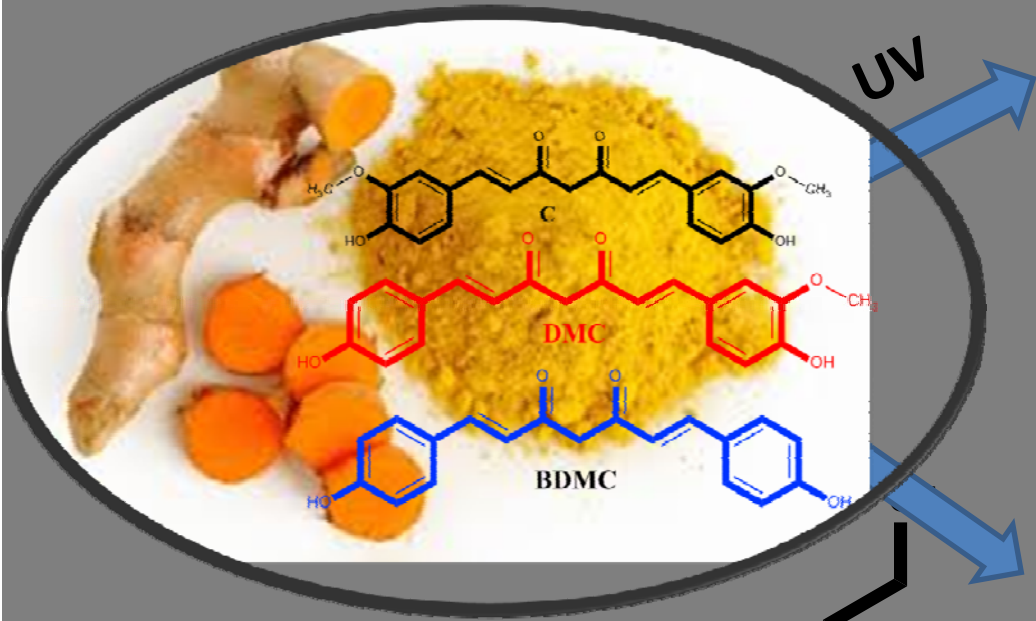
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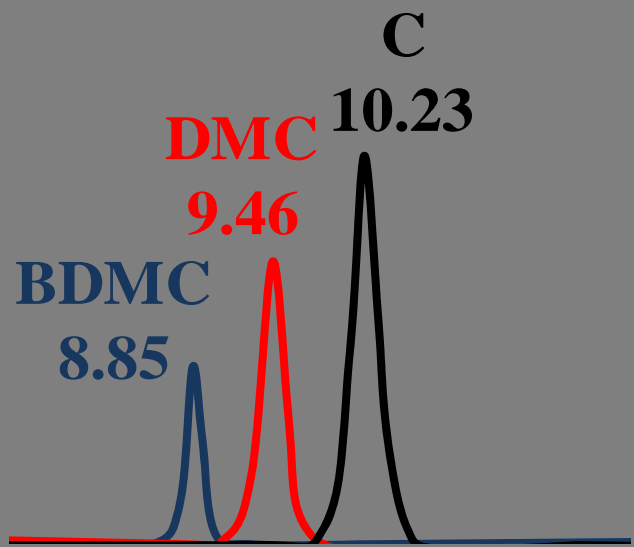
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Phenyl-propyl  
HPLC column



# Separation and Identification of Curcuminoids in Turmeric Powder by HPLC Using Phenyl Column

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**ABSTRACT**

The natural products including curcuminoids are globally used for treating several diseases. Curcumin, demethoxycurcumin and *bis*-demethoxycurcumin are the major constituents of *Curcuma longa L.* A rapid, selective, efficient and reproducible HPLC method for the separation and identification of curcuminoids was described on Sunniest PhE (phenyl) column (250 x 4.6 mm, 5.0  $\mu$ m). These constituents were separated within 10.5 min. using acetonitrile-methanol-water (40:20:40, v/v) as mobile phase with 1.0 mL/min. flow rate and 360 nm detection. The capacity factors ( $k$ , 4.2 to 4.9), separation factors ( $\alpha$ , 1.07 to 1.10) and resolution factors ( $R_s$ , 1.07 to 2.05) indicated a good separation of the compounds. The attempts have also been made to describe the separation mechanism of the reported method. The extraction of the curcuminoids from turmeric powder was 2.1, 0.46 and 0.1% of curcumin, demethoxycurcumin and *bis*-demethoxycurcumin, respectively. The reported method was considered as novel due to base lined separation of curcuminoids; with sharp peak and low LOD in comparison to the reported methods in the literature. Briefly, the described method may be used for the quality control of food stuffs and identification of curcuminoids in other natural products.

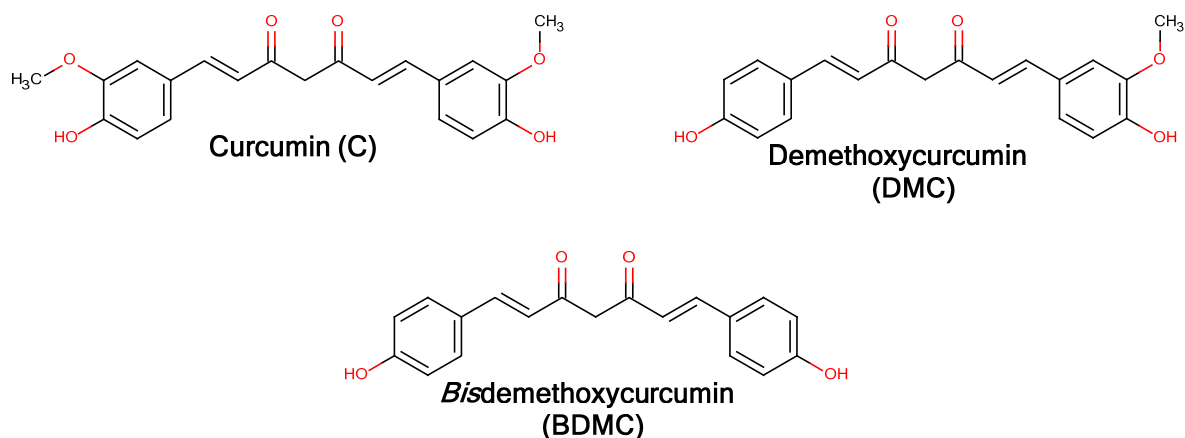
**KEYWORDS:** Curcuminoids, Turmeric powder, HPLC separation and identification, Phenyl column, Mechanism of separation.

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## INTRODUCTION

The rhizomes of turmeric (*Curcuma longa* L.); a plant of the *Zingiberaceae* family; provides yellow and flavourful powder. The yellow powder known as turmeric is mainly composed of curcuminoid pigments along with resin and turmerone. Turmeric is being used worldwide from the ancient time as food ingredient due to its bright colour and promising health properties.<sup>1</sup> For long time turmeric (mixture of curcuminoids) is known for its several medicinal values. It is being used for curing sprains, swellings, biliary disorders, rheumatism, sinusitis, abdominal pains, icterus etc. Besides, turmeric has several other important pharmaceutical properties such as anti-HIV, anti-microbial, anti-oxidant, anti-parasitic and anti-cancer.<sup>2-10</sup> Mainly these activities of curcuminoids are due to the presence of three structurally correlated curcuminoids. *viz.* 1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Curcumin, C), 1-(4-hydroxy phenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (demethoxy curcumin, DMC) and 1,7-*bis*(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione(*bis*-demethoxycurcumin, BDMC).<sup>10</sup> The anticancer activities order of these curcuminoids is BDMC > DMC > C.<sup>11</sup> The structures of these curcuminoids are given in Figure 1, indicating two substituted phenyl rings separated by heptadienone spacer.



**Figure 1:** Structures of curcuminoids.

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2  
3 The commercially available turmeric powder is a mixture of naturally occurring  
4 curcuminoids; with curcumin (C) as the major constituent with other two (DMC and BDMC) as  
5 minors (C: DMC: BDMC: 77: 18: 5%).<sup>2,12</sup> Due to the different pharmaceutical properties of  
6 three curcuminoids, their separation and identification are important issues in medicinal  
7 chemistry. A thorough search of literature was carried out and some methods for analyses of  
8 curcuminoids are available. The reported methods include TLC,<sup>12-17</sup> column chromatography,<sup>12,18</sup>  
9 HPTLC,<sup>19</sup> and HPLC.<sup>12,20-40</sup> A comparison of the reported methods was carried out with  
10 observation that TLC and HPTLC are time consuming methods with poor limits of detection. In  
11 all cases C<sub>18</sub>, amine and core shell (accucore) columns were used in HPLC, which were able to  
12 separate three curcuminoids, but with some limitations. The major limitations includes extreme  
13 low pH of mobile phases, high separation time and detection limits. In some papers peaks were  
14 very close to one another while peaks were broad in some other papers. It may be due to poor  
15 interactions of these molecules with reverse phase materials because of the absence of  $\pi$ - $\pi$   
16 interactions; required for such kind of molecules due the presence of phenyl moieties. Therefore,  
17 the reported methods showed poor separation, broad peaks with high limit of detection and  
18 quantification. In view of these facts, the attempts have been made to develop, optimize and  
19 validate a fast, selective and reproducible method for the separation and identification of a  
20 mixture of curcuminoids using phenyl HPLC column. The developed, optimized and validated  
21 method was applied for the analyses of these curcuminoids in commercial turmeric powder. The  
22 results of these findings are reported herein.

## 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 **EXPERIMENTAL**

### 48 49 50 **CHEMICALS AND REAGENTS**

51  
52 Turmeric powder was purchased from local market, New Delhi, India. The standard  
53 samples of the curcuminoids were kindly supplied by SAMI Labs, Bangalore. Acetonitrile and  
54 methanol of HPLC grade were purchased from Fisher Scientific, Fairlawn, New Jersey, USA.  
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Purified water was prepared by using a Millipore Milli-Q, Bedford, USA water purification system. UV spectrometer of PG instruments (model T80) was used.

### PREPARATION OF STANDARD SOLUTIONS

The standard solutions ( $1.0 \text{ mgmL}^{-1}$ ) of each curcuminoid and their mixture in commercial sample were prepared in acetonitrile. The stock solutions were protected from light by covering with aluminium foil and stored at  $4 \text{ }^{\circ}\text{C}$ . A grade bulb pipettes and  $10.0 \text{ mL}$  volumetric flask were used for serial dilutions of these curcuminoids with acetonitrile to obtain the required concentration ranges ( $0.01\text{-}0.10 \text{ mgmL}^{-1}$ ).

### EXTRACTION OF CURCUMINOIDS FROM TURMERIC POWDER

Curcuminoids are soluble in various organic solvents including ethanol, dichloromethane and ethyl acetate. Some research papers describe the application of various solvents for extraction of curcuminoids from the turmeric powder.<sup>41-43</sup> Besides, sequential extraction with number of solvents has also been used for this purpose.<sup>44,45</sup> It was observed that DCM is the best solvent for the extraction of curcuminoids from turmeric powder. Therefore, DCM was used as extracting solvent as per the procedure describe by Anderson et al.<sup>42</sup>  $50.0 \text{ g}$  of turmeric powder was taken in  $250 \text{ mL}$  round bottom flask and  $125.0 \text{ mL}$  dichloromethane was added followed by constant stirring on magnetic stirrer. The mixture was refluxed for  $1.0 \text{ hr}$  at  $50 \text{ }^{\circ}\text{C}$ . It was filtered by Buchner's funnel followed by the separation of mother liquor. The mother liquor was concentrated on rotary evaporator resulting into dark orange oily liquid, which was precipitated with the addition of  $50.0 \text{ mL}$  hexane. The precipitate was further filtered through Buchner's funnel, which gave a mixture of curcuminoids. The purity of the extracted curcuminoids was confirmed by UV-vis. spectrophotometer. The resulted product was analysed by HPLC for curcuminoids.



## HPLC INSTRUMENT

HPLC system used was of ECOM (Prague, Czech Republic) consisting of solvent delivery pump (model Alpha 10), manual injector, absorbance detector (Sapphire 600 UV-Vis.), chromatography I/F module data integrator (Indtech. Instrument, Mumbai, India) and Winchrome software. The column used was Sunniest PhE (phenyl) column (250 x 4.6 mm, 5.0  $\mu\text{m}$ ) of Chromanik Japan.

## HPLC CONDITIONS

All the experiments were carried out by HPLC system as described above. The aliquots of 5.0  $\mu\text{L}$  of standard solutions of each curcuminoid and their mixture in commercial sample (1.0  $\text{mgmL}^{-1}$  in acetonitrile) were loaded onto HPLC instrument, separately and respectively. The mobile phase used was acetonitrile-methanol-water (40:20:40, v/v) in isocratic mode (1.0  $\text{mL}/\text{min}$ ). The mobile phase was prepared, filtered and degassed daily before use. All the experiments were carried out at  $27\pm 1$   $^{\circ}\text{C}$  temperature with detection at 360 nm. The chromatographic parameters such as retention ( $k$ ), separation ( $\alpha$ ) and resolution ( $R_s$ ) factors were calculated. The order of elution was ascertained by running individual curcuminoid. The qualitative and quantitative analyses were carried out by using retention times and peak areas, respectively. The chromatographic method was optimized and validated by carrying out an extensive experimentation followed by applied analyses of curcuminoids in extracted turmeric powder.

## VALIDATION

The validation of HPLC method was carried out by calculating various HPLC parameters. The linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, selectivity, robustness and ruggedness were determined for the purpose. The limits of detection (LOD) and quantitation (LOQ) were determined by injecting more diluted samples of curcuminoids. The results of the statistical analyses of the experimental data such as relative

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2  
3 standard deviation, correlation coefficients and confidence limit were calculated by Microsoft  
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5 Excel software program. Good linearity of the calibration graphs and the negligible scatter of  
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7 experimental points were considered for calculations of correlation coefficients and relative  
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9 standard deviations.<sup>46</sup> The robustness of the method was demonstrated by the versatility of the  
10  
11 experimental factors that affected the peak areas.  
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### 13 14 15 **LINEARITY**

16  
17 The linearity was tested by least squares linear regression analysis of the calibration  
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19 curve.<sup>46</sup> The linearity of calibration curves (peak area vs. concentration) for curcuminoids  
20  
21 standards as well as in turmeric powder was checked over the concentration ranges of 0.01.0-  
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23 0.10 mgmL<sup>-1</sup> Equal volumes (5.0 µL) of the standards as described above were loaded onto  
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25 HPLC instrument. The chromatograms were recorded, separately and respectively. The  
26  
27 calibration curves of all curcuminoids were constructed, separately and respectively, using the  
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29 observed peak areas versus nominal concentrations of the analytes.  
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### 32 33 **DETECTION AND QUANTITATION LIMITS**

34  
35 The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as  
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37 three and five times to the baseline noise, respectively, following the United States  
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39 Pharmacopoeia.<sup>46</sup>  
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### 42 43 **SPECIFICITY**

44  
45 The specificity of the method was investigated by observing any interference in  
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47 chromatographic parameters due to the present of some impurities in standard samples. The  
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49 standard samples were mixed with little amount of turmeric powder to make them impure.  
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### 51 52 **PRECISION**

53  
54 To calculate precision data, three different concentrations (0.01, 0.05 and 0.10 mgmL<sup>-1</sup>)  
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56 of each curcuminoids were used. Five sets of the chromatographic runs were carried out for all  
57  
58 the three concentrations.  
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## ACCURACY

The different concentrations of curcuminoids were used to determine the accuracy of HPLC method. Three concentrations i.e. 0.01, 0.05 and 0.10 mgmL<sup>-1</sup> were used. The chromatographic runs were carried out five times (n = 5). The accuracy was determined by interpolation of five replicates peak areas of these molecules.

## ROBUSTNESS

The method robustness was determined by a slight variation in the chromatographic parameters such as flow rate, temperature, mobile phase composition and wavelength. The retention time, peak area and shape were analyzed under the established and slightly varied experimental conditions.

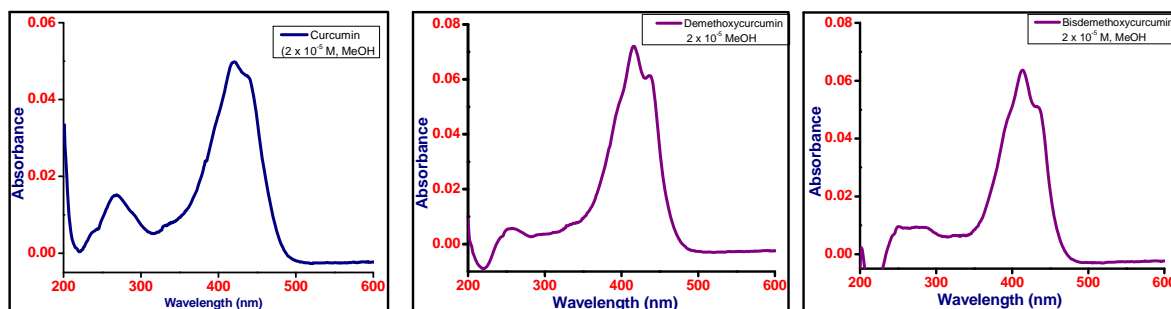
## RUGGEDNESS

The ruggedness of the method was determined by the change of the experimental environment such as different operators, different days.

## RESULTS AND DISCUSSION

### EXTRACTION OF CURCUMINOIDS FROM TURMERIC POWDER

As discussed above 1.40 g of curcuminoids were obtained from 50.0 g turmeric powder. The amounts of curcumin, demethoxycurcumin and *bis*-demethoxycurcumin were 1.06, 0.23 and 0.05 g, respectively (Table 1). Figure 2 depicts the UV-vis. spectra of the curcuminoids in methanol ( $2 \times 10^{-5}$  M) (Figure 2).  $\lambda_{\text{max}}$  values of C, DMC and BDMC were 420, 416 and 412 nm, respectively.



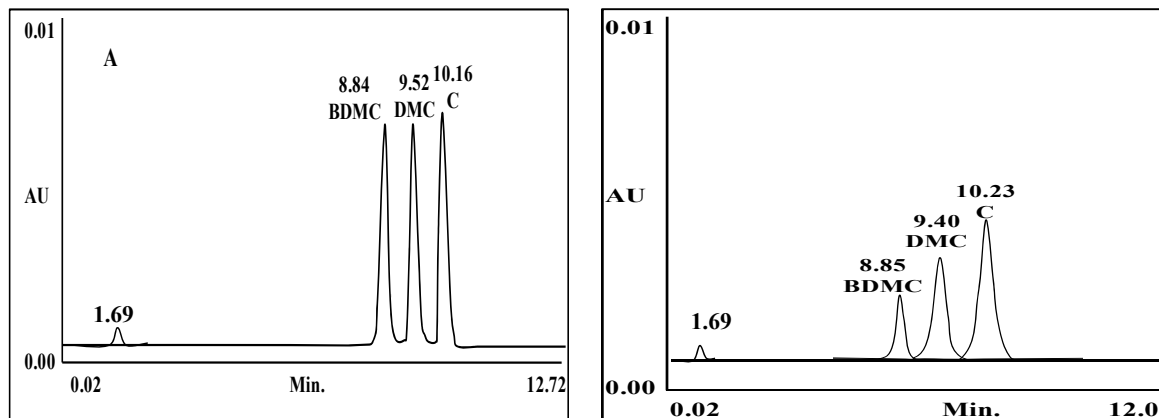
**Figure 2:** UV-vis. Spectra of curcuminoids in methanol ( $2 \times 10^{-5}$  M).

The percentage recoveries of these were 2.1, 0.46 and 0.1, respectively. The values of RSD, correlation coefficients and confidence levels were 2.0-2.5, 0.9998-0.9999 and 98.80-99.00, respectively (Table 1). The attempts have been made to extract these curcuminoids from turmeric powder by using methanol, acetonitrile, ethyl acetate and ethanol as pure or their mixtures but maximum recoveries could be achieved with pure dichloromethane only. Therefore, dichloromethane was used as an extracting solvent for the reported curcuminoids. The attempts have been made to compare the extraction recoveries with those reported in the literature<sup>41-45</sup>. It was observed that the extraction recoveries of these curcuminoids were comparable. However, the extracted curcuminoids were pure as there was no extra peak in HPLC studies.

## HPLC METHOD DEVELOPMENT

The separation and identification of curcuminoids were carried out on column and mobile phase as described in HPLC instrumentation section. The separated curcuminoids in commercial samples were identified by running and comparing the retention times of the individual curcuminoid. The calibration curves were plotted for all curcuminoids and used to determine their concentrations in turmeric powder. The quantitative analyses of curcuminoids in turmeric powder were carried out by comparing their peak areas with those of standards. The capacity ( $k$ ), separation ( $\alpha$ ) and resolution ( $R_s$ ) factors for these compounds in standard solutions and turmeric powder were calculated and given in Table 2, respectively. The chromatograms of curcuminoids mixtures in standard and extracted sample are given in Figure 3. It is clear from this figure that

all three curcuminoids are base lined separated with sharp peak within 10.5 min. The order of elution observed was BDMC > DMC > C. The capacity, separation and resolution factors of curcumin were 4.2 to 4.9, 1.07 to 1.10 and 1.07 to 2.05, respectively (Table 2).



**Figure 3:** HPLC chromatograms of (A): standard mixture and (B): extracted curcuminoids. Experimental conditions as given in text.

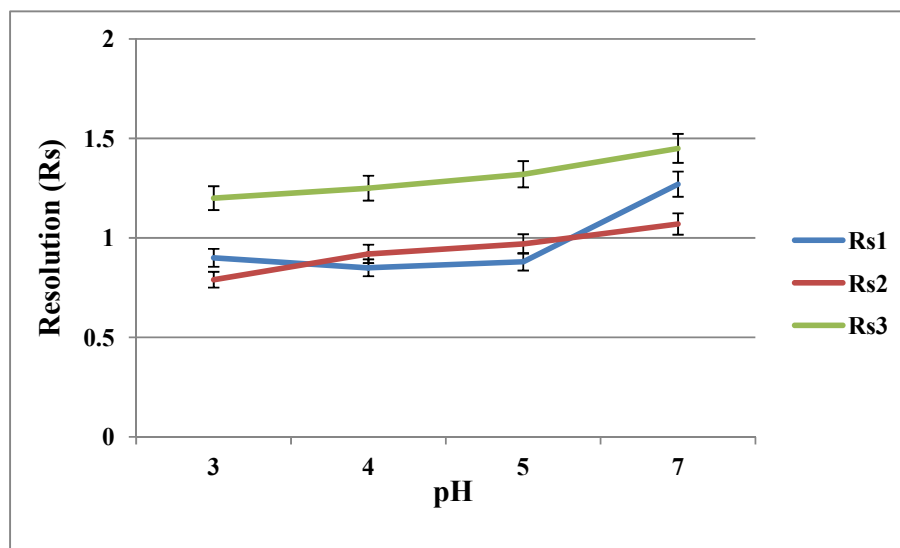
### HPLC METHOD OPTIMIZATION

To optimize the chromatographic conditions, various combinations of acetonitrile-methanol-water (40:20:40, v/v) were tried. The influence of flow, pH, detection wave length and amount injected were studied. Besides, other solvents such as phosphate buffer, acetate buffer and ratio of different organic solvents were also tested. As a result of exhaustive experimentation, the best chromatographic conditions were optimized and reported herein. The optimizations of chromatographic parameters are discussed in the following paragraphs.

#### Effect of pH

pH of the mobile phase is one of the important factors for controlling HPLC separation. pH of the mobile phase was varied from 3.0-7.0. Above pH 7.0, the chances of curcuminoids decomposition are high,<sup>32</sup> that's why the experiments were carried out maximum up to pH 7.0. The resolution factors ranged from 0.88 to 1.32 for these molecules at pH 3.0-6.0. Contrarily, these values at pH 7.0 were 1.07 to 1.45 (Figure 4). Good separation of the reported molecules at pH 7.0 might be due to the presence of curcuminoid phenoxide ions (having higher  $\pi$  electron

density), leading to  $\pi$ - $\pi$  interactions with phenyl column. Contrarily, this situation is not available at low pH values (in acidic media) and, hence, poor separation.

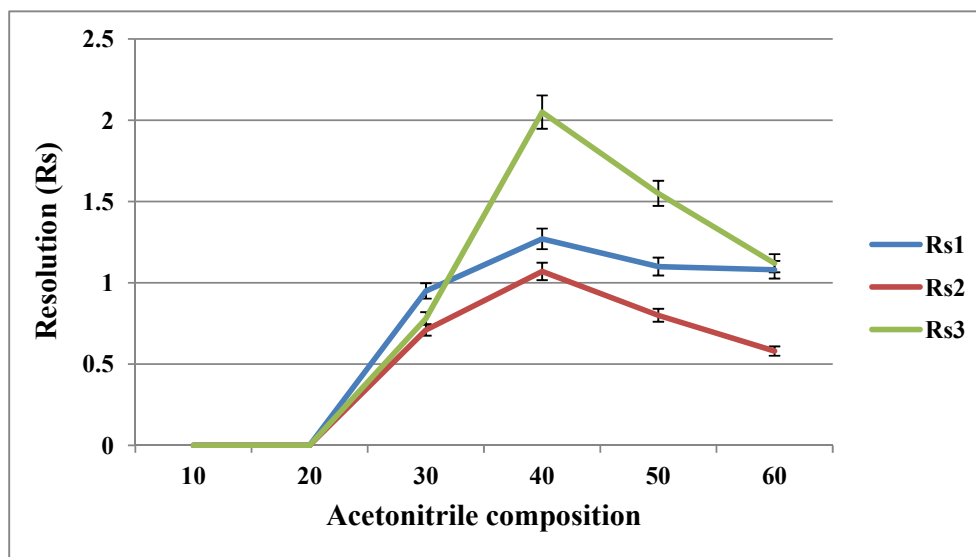


**Figure 4:** Effect of pH on the resolution of curcuminoids. ( $Rs_1$ : resolution between peak 1-2,  $Rs_2$ : resolution between peak 2-3, (Peak 1: BDMC, Peak 2: DMC and Peak 3: C).

#### Effect of Acetonitrile

Amount of acetonitrile was varied from 10 to 60 parts, keeping methanol and water constant. Interestingly, it was observed that there were no peaks within 20 mins. at 10 mL acetonitrile (low amount). Further increasing volume of acetonitrile from 10  $\rightarrow$  20, two peaks (10.5 and 11.1 min.) were detected. Furthermore, at 30 parts of acetonitrile, three clear peaks (14.6, 15.5 and 16.6 min.) were observed; with  $R_s$  values 0.95, 0.71 and 0.78 of C, DMC and BDMC, respectively. However, this system was laced with drawback of low detection of DMC and BDMC. On further increment to 40 mL, better peak shape with clear separation was observed; with  $R_s$  values 1.27, 1.07 and 2.05 of C, DMC and BDMC, respectively. These values indicated satisfactory separation. Further increase in acetonitrile concentration to 50 and 60 mL resulted into poor resolution. It might be due to weak bonding among molecules and column stationary phase due to acetonitrile effect ( $\pi$ -electron of nitrile functionality involved in bonding

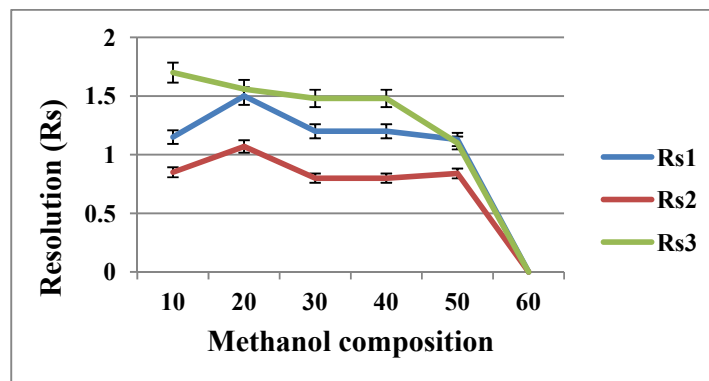
with phenyl group by replacing analyte molecules).<sup>47</sup> As a result, the best volume of acetonitrile was selected as 40 mL (Figure 5).



**Figure 5:** Effect of acetonitrile on the resolution of peaks. ( $Rs_1$ : resolution between peak 1-2,  $Rs_2$ : resolution between peak 2-3), (Peak 1: BDMC, Peak 2: DMC and Peak 3: C).

#### Effect of Methanol

Amount of methanol was varied from 10 to 60 mL. The resolution was poor ( $R_s = 1.15$ , 0.85 and 1.7) at low amount of methanol (10 mL). However, at 20 mL of methanol  $R_s$  values were slightly higher i.e. 1.5, 1.07 and 1.56. Further increase of methanol  $R_s$  values decrease with broad peaks. It might be attributed to decreasing concentration of acetonitrile; responsible for sharp peaks. The results of this optimization are shown in Figure 6.



**Figure 6:** Effect of methanol on the resolution of peaks. ( $Rs_1$ : resolution between peak 1-2,  $Rs_2$ : resolution between peak 2-3, (Peak 1: BDMC, Peak 2: DMC and Peak 3: C).

### Effect of Water

Amount of water was varied from 10 to 60 mL. Only two peaks were (3.73 and 4.32 min) observed with 10 mL water in mobile phase. It might be attributed to high polarity of solvent. Further increasing of water contents (20→40) three peaks were observed within 10.5 min. with poor resolution at 30 mL. However, peaks were well resolved and sharp at 40 mL of water. Further increase in water content resulted into partial separation with broad peaks. Therefore, 40 mL water gave the best results.

### Effect of Flow Rate

Flow rate of the solvent system acetonitrile-methanol-water (40:20:40, v/v) was varied from 0.5 to 2.0 mL/min. and the chromatograms are shown in Figure 7. It was observed that at low flow rate (0.5 mL/min), the peaks were poorly resolved with high retention time (Fig. 7). Further increase in flow rate to 1.0 mL/min resulted into sharp and base lined separation. On the other hand, high flow rates (1.5 and 2.0 mL/min.) decreased retention times with partial separation. Figure 8 depicts a typical trend of  $R_s$  change with respect to flow rate. At 0.5 mL/min., the values of  $R_s$  were 0.79, 0.95 and 1.25 of C, DMC and BDMC, respectively. On increasing the flow rate to 1.0 mL/min., there was an increase in these values ( $R_s = 1.06, 1.26$  and 1.6). At high flow rates (1.5 and 2.0 mL/min.), there was decrease in resolution among the peaks ( $R_s = 0.92, 1.18$  and 1.3). Briefly, the peaks were well resolved at 1.0 mL/ min. flow rate, which was considered as the best flow rate.



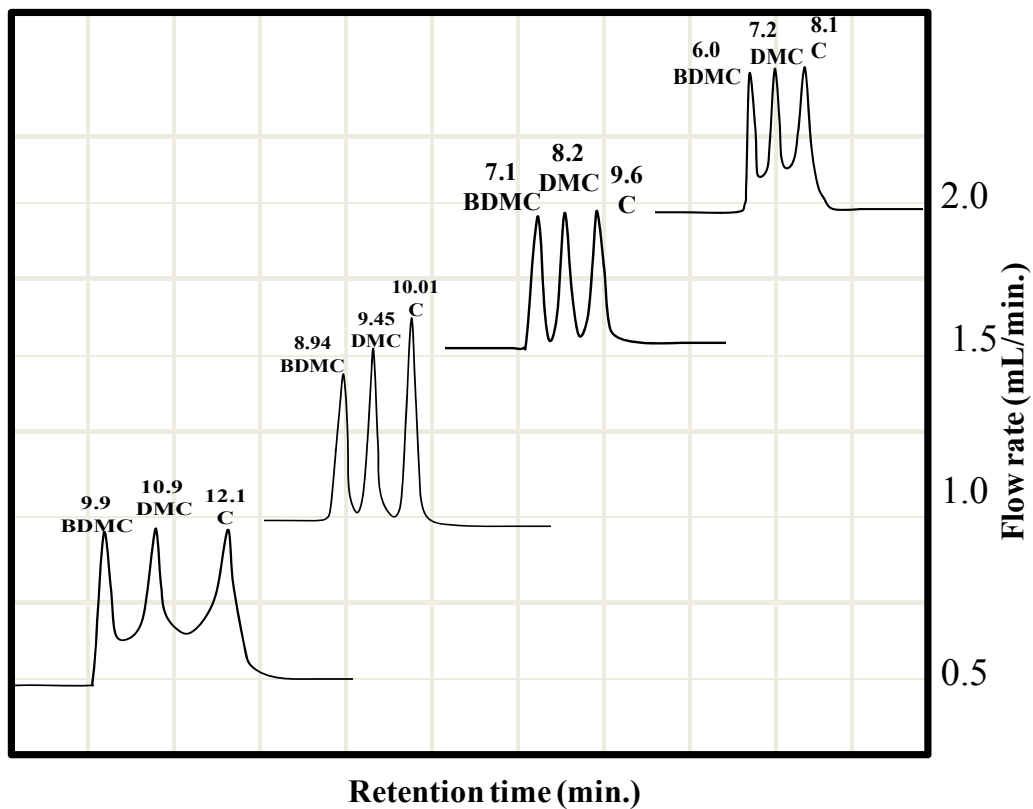


Figure 7: Effect of flow rates on the retention times of curcuminoids.

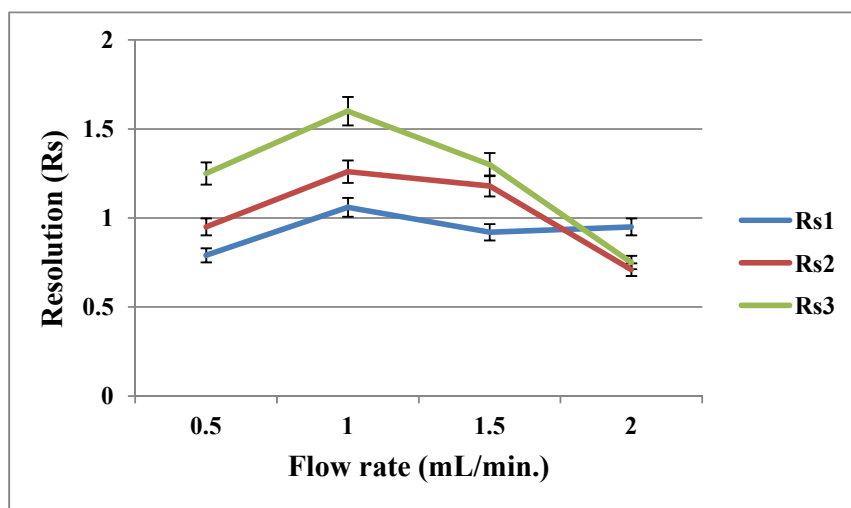


Figure 8: Effect of flow rate on the resolution of peaks. ( $Rs_1$ : resolution between peak 1-2,  $Rs_2$ : resolution between peak 2-3, (Peak 1: BDMC, Peak 2: DMC and Peak 3: C).

## MECHANISM OF SEPARATION

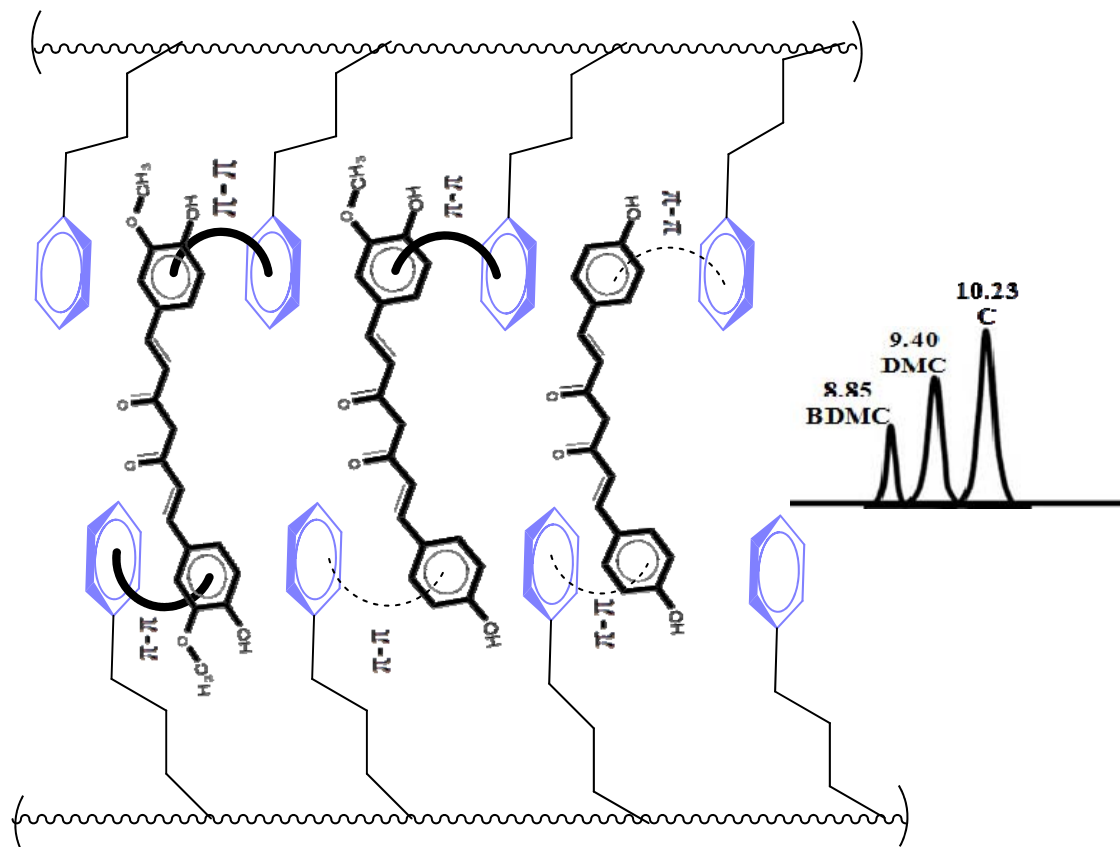
As mentioned in the introduction part that curcuminoids were poorly resolved on C<sub>18</sub> and amine columns but the present method was more advantageous such as working at normal temperature, acid free conditions and good efficiency. It is well known that van der Waals forces, hydrogen bondings, dipole-induced dipole interactions, ionic interactions and coordination bondings are the separation controlling forces in HPLC. It is interesting to note that the reported curcuminoids have little difference in their structures and such types of forces are almost of equal magnitudes. Hence, C<sub>18</sub> and amine phases involving above cited interactions are not capable to separate them successfully. Contrarily,  $\pi$ - $\pi$ -interactions, cation- $\pi$  or anion- $\pi$  interactions are of quite good magnitude and are ideal for separation of such closely related molecules. Figure 1 clearly indicates the presence of two phenyl moieties in these molecules on each side. Therefore, phenyl column was used to overcome the problem of their poor separation. For these molecules,  $\pi$ - $\pi$ -interactions played major role for their separation. Additionally, the above cited forces also contribute in separation mechanism. Phenyl column comprises several Si-O-(CH<sub>2</sub>)<sub>3</sub>-Ph groups (Figure 9). The phenyl group on stationary phase retained the reported molecules at different extents which might be due to dissimilar magnitudes of  $\pi$ - $\pi$ -interactions among curcuminoids and stationary phase; resulting into good resolution. Besides, due to the comparatively stronger  $\pi$ - $\pi$ -interactions the diffusion of curcuminoids gets reduced; resulting into big and sharp peak (low detection limits). Briefly, the separation of the reported curcuminoids is controlled by  $\pi$ - $\pi$  interactions along with other forces.

Phenyl-propyl column (alkyl chain stationary phase (C<sub>8</sub>/C<sub>18</sub>) is replaced by aromatic ring) has good potential for the analyses of natural products and other pharmaceuticals by exploiting the  $\pi$ - $\pi$  type interactions with analytes.<sup>48</sup> On the reported column  $\pi$ - $\pi$ -reversed-phase ( $\pi$ -RP) retention mechanism is found to be prominent,<sup>49</sup> with some other additional interactions.<sup>50</sup> Even stereoisomers of identical properties can be separated by exploiting  $\pi$ - $\pi$  type interactions on

1  
2  
3 chiral columns.<sup>47, 51-53</sup> It has already been established that solutes with  $\pi$ -electron systems  
4  
5 displayed different retention behaviors on  $\pi$ -RP-phases than on ordinary RP-columns. Contrarily,  
6  
7 the solutes without  $\pi$ -electrons or with sterically hindered  $\pi$ -electron systems behaved almost in  
8  
9 similar fashion on C<sub>8</sub>/C<sub>18</sub> and  $\pi$ -RP-phases.<sup>54</sup> Stronger  $\pi$ - $\pi$  interactions are responsible for higher  
10  
11 aromatic solute retention on  $\pi$ -RP-phases.<sup>55,56</sup> As per Euerby et al.<sup>57</sup> selectivity of phenyl ring  
12  
13 bonded stationary phase varied with the length of spacer between phenyl group and silica  
14  
15 surface. The authors reported low selectivity on column having phenyl ring directly attached to  
16  
17 silica. However, selectivity of the stationary phase augments as the number of linker atoms  
18  
19 between phenyl and silica increases. The authors observed poor  $\pi$ - $\pi$  interactions on column  
20  
21 having phenyl ring close to silica surface due to  $\pi$ - $\pi$  stacking among analytes and stationary  
22  
23 phases. On introduction of linker atoms,  $\pi$ - $\pi$  interactions among analytes and stationary phase  
24  
25 were quite enough, resulting into good separation. These results indicated phenyl propyl column  
26  
27 as the best choice for the separation of analyses having  $\pi$  electrons.  
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32 The attempts have been made to develop the mechanism of separation at supra-molecular  
33  
34 level (Figure 9). Curcuminoid structures can be differentiated from one another by the presence  
35  
36 or absence of methoxy group (an electron donating group). Greater the number of methoxy  
37  
38 group, greater will be electron density on aromatic ring and, hence, more will be  $\pi$ - $\pi$  interactions.  
39  
40 The experiments were carried out at pH 7.0 and all curcuminoids occur in phenoxide ionic form  
41  
42 at this pH, increasing  $\pi$  electron density on phenyl rings. Therefore, there are stronger  $\pi$ - $\pi$   
43  
44 interactions at pH 7.0 in comparison to acidic pHs. On the other hand, methoxy group effect  $\pi$   
45  
46 electron density on phenyl rings of these molecules. C and DMC have two methoxy groups while  
47  
48 BDMC is devoiding it. It means  $\pi$  electron density on these molecules is in the order of C >  
49  
50 DMC > BDMC. Similarly, the binding capacity of these molecules on stationary phase will be in  
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52 the same order. Consequently, the elution order of these compounds will be reversed, which was  
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observed experimentally. Therefore, it may be assumed that the separation of curcuminoids on phenyl phase is being controlled by  $\pi$ - $\pi$  interactions along with other forces.



**Figure 9:** Possible  $\pi$ - $\pi$  interactions between stationary phase (propyl phenyl phase) and curcuminoids (C, DMC and BDMC). Solid and dotted lines represent strong and weak interactions, respectively.

## VALIDATION OF HPLC METHOD

HPLC method was validated with respect to various parameters including linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, selectivity, robustness and ruggedness.<sup>58</sup>

## LINEARITY

The linearity of calibration curves (peak area vs. concentration) for curcuminoids standards as well as in turmeric powder were checked over the concentration ranges of 0.01.0-

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3 0.10 mgmL<sup>-1</sup>. The plotted curves were linear over these concentration ranges (n = 5) for all  
4  
5 curcuminoids. The peak areas of curcuminoids were plotted versus their respective  
6  
7 concentrations. The linear regression analysis was performed on the resultant curves. The  
8  
9 correlation coefficient (R<sup>2</sup>) (n = 5) were found to be 0.9999 for all curcuminoids. The values of  
10  
11 RSD and confidence levels were in the range of 2.00-2.10 and 98.80-99.00, respectively, across  
12  
13 the concentration ranges studied.  
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15

### 16 17 **DETECTION AND QUANTITATION LIMITS**

18  
19 The values for LOD and LOQ for curcuminoids ranged from 0.30-0.50 ng and 1.00-2.00  
20  
21 ng, respectively. The resultant RSDs for these studies were in the range of 2.00-2.50%.  
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23

### 24 25 **SPECIFICITY**

26  
27 The method is a quite good specific as can be seen from Fig. 3. The retention times of all  
28  
29 curcuminoids are almost similar in both standard solutions and turmeric powder. There was no  
30  
31 effect of the added impurities in standard on the retention times and peak shape of these  
32  
33 curcuminoids. These findings indicate a good specificity of the reported method.  
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35

### 36 37 **PRECISION**

38  
39 The precision data was calculated by taking three concentrations of all curcuminoids *i.e.*  
40  
41 0.01, 0.05 and 0.10 mgmL<sup>-1</sup>. Five chromatographic runs were carried out for all the three  
42  
43 concentrations. RSDs were calculated and ranged from 2.00 to 2.80%; indicating HPLC method  
44  
45 precise.  
46

### 47 48 **ACCURACY**

49  
50 The accuracy of the method was tested by analyzing different samples of curcuminoids as  
51  
52 described in experimental section. The accuracy was determined by interpolation of replicate (n  
53  
54 = 5) peak areas of three accuracy standards. In each case, the percent error was calculated and  
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3 found in the range of 0.60 to 1.20%. This range indicated a good accuracy of the developed  
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5 method.  
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### 7 8 **ROBUSTNESS**

9  
10 The small changes made include in mobile phase compositions, flow rates, amounts  
11  
12 loaded and detection wavelengths. It was observed that there were no remarkable variations in  
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14 HPLC results. No change in HPLC results by varying above experimental conditions indicated  
15  
16 the reported method as robust.  
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### 18 19 **RUGGEDNESS**

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21 The ruggedness assessment was performed during the development of HPLC method.  
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23 RSD values for intra- and inter-days of curcuminoids were in the range of 2.00-2.51; indicating  
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25 the robustness of the method. Besides, the results obtained with different operators were  
26  
27 unaffected, which also indicated ruggedness of the method.  
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### 30 31 **COMPARISON WITH OTHER METHODS**

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33 The reported results of curcuminoids separation were compared with those of reported in  
34  
35 the literature.<sup>12,20,23,24,27,29-40,59</sup> The comparison was carried out in terms of columns, mobile  
36  
37 phase, flow rates, detection limits, peak shapes and economy. The data is given in Table 3. It is  
38  
39 clear from this Table that all the methods used reversed phase C<sub>18</sub>, accucore and amine columns  
40  
41 with acid in mobile phases. pHs of these mobile phase are expected between 1-2, which damage  
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43 HPLC column. Only one column is based on amine group, which has a catalytic effect upon  
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45 curcuminoids degradation and compositional variations; leading to variable results. Mostly  
46  
47 methods used 1.5-2.0 mL/min as flow rates using moderate amounts of costly HPLC grade  
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49 solvent. Besides, the retention times of these methods ranged 11-28 mins. In this ways these  
50  
51 methods are costly and time consuming. Besides, in most of the cases the detection limits are not  
52  
53 given as the separation is poor on C<sub>18</sub> columns. In some cases the detection limits are given but  
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55 these are high and not acceptable. In some papers the peaks are not well resolved and sharp. Of  
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3 course, the separation time is 3.0 min on accucore column but this column is not capable to  
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5 separate curcuminoids under natural laboratory conditions. HPLC instrument needs a heating  
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7 device to achieve 40 °C temperature for good separation. Besides, low pH damaging column was  
8  
9 another major drawback on Accucore column. On the other hand, the reported method on phenyl  
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11 based reversed phase column showed sharp peaks with good resolution factors. The separation  
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13 time is only 10.5 with low limits of detection i.e. 0.30-0.50 ng. pH of the mobile phase is 7.0,  
14  
15 which did not affect column life. Low experimental time and use of water made this method  
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17 economic and eco-friendly. By considering all these factors, it was concluded that the presented  
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19 HPLC method was superior to the reported ones in terms of economy and eco-friendship, low  
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21 limits of detection and sharp peaks.  
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#### 24 25 **APPLICATION OF DEVELOPED AND VALIDATED HPLC METHOD**

26  
27 The validity of the developed method was applied to analyze curcuminoids in turmeric  
28  
29 powder extract. The qualitative and quantitative analyses of curcuminoids were carried out by  
30  
31 using the above mentioned HPLC conditions. The chromatograms of curcuminoids in turmeric  
32  
33 powder are shown in Figure 3. The quantitative analyses of curcuminoids in turmeric powder  
34  
35 were carried out by comparing their peak areas with those of standards. For calculation of  
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37 concentrations of curcuminoids in turmeric powder extract, five sets of HPLC experimentations  
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39 were carried out under identical experimental conditions. The amounts of curcumin,  
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41 demethoxycurcumin and *bis*-demethoxycurcumin in turmeric powder were 21.2, 4.60 and 1.0  
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43 g/kg, respectively.  
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#### 47 48 **CONCLUSION**

49  
50 A fast, reproducible, selective and effective HPLC method on phenyl reversed phase  
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52 column was described for the analyses of curcuminoids within 10.5 minutes at a 360 nm. The  
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54 values for LOD and LOQ for curcuminoids ranged from 0.30-0.50 ng and 1.00-2.00 ng,  
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56 respectively. The linearity was observed in the concentrations ranges of 0.01 to 0.10 mgmL<sup>-1</sup> for  
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3 all curcuminoids. The method was used for analyses of curcuminoids in turmeric powder with  
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5 concentrations of curcumin, demethoxycurcumin and *bis*-demethoxycurcumin as 21.2, 4.60 and  
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7 1.0 g/kg, respectively. The results of the proposed method were compared (Table 3) and  
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9 observed that the present method was more advantageous such as working at normal  
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11 temperature, acid free conditions and good efficiency. Besides, it can work under, the reported  
12  
13 method can be used for the quality control of any food stuff having turmeric. Besides, the  
14  
15 reported method can be used to identify these curcuminoids in food stuffs and some other natural  
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17 products. The base lined separation; with good values of separation and resolution factors; of  
18  
19 these molecules on phenyl column dictated its application at preparative scale for the separation  
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21 of individual curcuminoids.  
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**Table 1:** Regression analyses data for the extraction of curcuminoids from turmeric powder.

Curcuminoids	Extraction			
	Recoveries (%)	% RSD	Correlation coefficients (R <sup>2</sup> )	Confidence levels (%)
Curcumin	2.1	2.50	0.9998	98.78
Demethoxycurcumin	0.46	2.40	0.9999	98.80
<i>Bis</i> -demethoxycurcumin	0.1	2.00	0.9999	99.00

n = 5

**Table 2:** Chromatographic and precision data of curcuminoids.

Curcuminoids	k	$\alpha$	R <sub>s</sub>	%RSD	Correlation Coefficients	Confidence Levels (%)
<b>Standard Solutions</b>						
Curcumin	4.28	1.07	1.27	2.00	0.9999	99.00
Demethoxycurcumin	4.59					
<i>Bis</i> -demethoxycurcumin	4.92	1.10	1.07	2.05	0.9999	99.00
<b>Turmeric Powder Extract</b>						
Curcumin	4.23	1.05	1.06	2.00	0.9999	98.80
Demethoxycurcumin	4.50					
<i>Bis</i> -demethoxycurcumin	4.86	1.08	1.06	2.10	0.9999	99.00

n = 5

Note: k: Capacity factor,  $\alpha$ : Separation factor and R<sub>s</sub>: Resolution factor.

Column: Phenyl column (250 x 4.6 mm, 5.0  $\mu$ m).

Isocratic HPLC Conditions: Acetonitrile-Methanol-Water (40:20:40, v/v), Flow Rate: 1.0 mL/min, UV detection: 360 nm, Temperature: 27 $\pm$ 1  $^{\circ}$ C.

**Table 3:** A comparison of curcuminoids separation by HPLC.

S. No	Columns	Mobile Phases	Sep. Time (min.)	Detection Limits & Flow Rates	Drawbacks	Refs.
1.	C <sub>18</sub>	CH <sub>3</sub> OH-2% CH <sub>3</sub> COOH- CH <sub>3</sub> CN	6.75	- 1 mL/min.	Very low difference in retention time, low pH leading to column damage; detection limits not given	12
2.	C <sub>18</sub>	(A) H <sub>2</sub> O (0.25% CH <sub>3</sub> COOH and(B) CH <sub>3</sub> CN, 0–17 min, 40– 60% B; 17–32 min, 60–100% B; 32–38 min, 100% B; 38–40 min, 100–40% B	14.0	- 0.2 ml/min.	Carried out at 48 °C, low pH leading to column damage; detection limits not given	20
3.	RP-5- NH <sub>2</sub>	C <sub>2</sub> H <sub>5</sub> OH-H <sub>2</sub> O (96:04, v/v)	20.73	- 1 mL/min.	High separation time and, hence costly; amino-bonded stationary phase has a catalytic effect upon curcumin degradation; composite-onal variations leads to variable results (due to the use of azeotropic mixture of ethanol, detection limits not given	23
4.	C <sub>18</sub>	0.1 M of acetate buffer (pH 4.0) - CH <sub>3</sub> CN (57:43, v/v)	~ 28	1.5, 0.9 and 0.09 ngmL <sup>-1</sup> for C, DMC & BDMC, 1 mL/min.	High retention time and, hence, costly; low pH leading to damage of column	24
5.	C <sub>18</sub>	Gradient Elution Mobile	~ 11	- 1 mL/min.	Low pH leading to column	27

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		phase solvents (A) 2% CH <sub>3</sub> COOH in H <sub>2</sub> O and (B) CH <sub>3</sub> CN			damage, detection limits not given	
6.	C <sub>18</sub>	CH <sub>3</sub> CN-CH <sub>3</sub> OH-H <sub>2</sub> O (40:23:37, v/v) pH 3.0	~ 12	100-5000 ngmL <sup>-1</sup> , 1 mL/min.	Low pH leading to damage of column; high detection limits	29
7.	C <sub>18</sub>	Gradient Elution Mobile phase solvents (A) 3 mM H <sub>3</sub> PO <sub>4</sub> in H <sub>2</sub> O and (B) CH <sub>3</sub> CN	13.2	- 0.7 mL/min	Retention times too close; low pH leading to column damage; detection limits not given	30
8.	C <sub>18</sub>	0.05% CH <sub>3</sub> COOH-CH <sub>3</sub> OH (15:85, v/v)	~ 26	- 1 mL/min.	High retention time and, hence, costly; low pH leading to column damage; detection limits not given	31
9.	C <sub>18</sub>	Gradient Elution Mobile phase solvents (A) 0.25% CH <sub>3</sub> COOH in H <sub>2</sub> O and (B) CH <sub>3</sub> CN	17.0	- 1 mL/min.	High retention time and, hence, costly; low pH leading to column damage; detection limits not given	32
10.	C <sub>18</sub>	CH <sub>3</sub> CN - 2% CH <sub>3</sub> COOH (40:60, v/v)	13.6	0.90, 0.84 and 0.08 µg/ for C, DMC & BDMC 2 mL/min.	Low pH leading to damage of column; high detection limits	33
11.	C <sub>18</sub>	CH <sub>3</sub> CN-0.1% CF <sub>3</sub> COOH- (50:50, v/v), (pH adjusted to 3.0 with NH <sub>3</sub> )	9.0	27.99, 31.91 and 21.81 ng mL <sup>-1</sup> for C, DMC & BDMC 1.5 mL/min.	Low pH leading to damage of column; moderate detection limits	34
12.	TSK-GEL ODS 80 Ts	0.1% HCOOH-CH <sub>3</sub> CN (50:50, v/v)	10.0	- 1.0 mL/min.	Low pH leading to damage of column; detection limits not given	35
13.	C <sub>18</sub>	40% THF - 60% H <sub>2</sub> O with 1% citric acid,	9.27	- 0.7 mL/min.	Low pH leading to damage of column; broad	36



		pH 3.0			peaks, detection limits not given	
14.	C <sub>18</sub>	CH <sub>3</sub> CN-5% CH <sub>3</sub> COOH (75:25, v/v)	2.0	- 1.0 mL/min.	Extremely low pH leading to damage of column; detection limits not given	37
15.	C <sub>18</sub>	1% H <sub>3</sub> PO <sub>4</sub> - CH <sub>3</sub> CN	6.36	2.5 µg/mL 1.0 mL/min.	Low pH leading to damage of column; detection limit high	38
16.	Chromolith column (Monolithic C <sub>18</sub> )	H <sub>2</sub> O-CH <sub>3</sub> CN-CH <sub>3</sub> COOH (60:40:1, v/v)	20.5	50 ng/mL 1.0 mL/min.	High retention time and, hence, costly; retention times too close	39
17.	C <sub>18</sub>	5.0 mM CH <sub>3</sub> CN - H <sub>3</sub> PO <sub>4</sub> (45:55, v/v)	18.6	0.207, 0.202 and 0.514 ngmL <sup>-1</sup> for CUR, DMC and BDMC 1.0 mL/min.	High retention time and, hence, costly; detection limit high	40
18.	C <sub>18</sub> and Accucore	MeOH-10 mM H <sub>3</sub> PO <sub>4</sub> (80:20, v/v)	3.0	- 0.8 mL/min.	Not capable to separate under normal conditions (room temperature) and Low pH leading to damage of column	59
19.	<b>RP-Phenyl Column</b>	<b>ACN-MeOH-H<sub>2</sub>O (40:20:40, v/v)</b>	<b>10.5</b>	<b>0.30-0.50 ng</b> 1.0 mL/min.	<b>Fast, reproducible, at a new wave length</b>	<b>Present work</b>

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