

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR QUALITATIVE AND QUANTITATIVE ESTIMATION OF CURCUMIN AND SIMILAR COMPOUNDS IN BULK MIXTURE

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ABSTRACT:

A simple, rapid and efficient method for the determination of Curcumin and other curcuminoids contents in turmeric samples was here developed. The method relied on sample extraction with methanol and extract analysis by liquid chromatography. The separation of components was carried out in reversed phase mode using a methanol-acetonitrile (2:3, v/v) as the components of the mobile phase. Chromatograms were recorded at 425nm for specific monitoring of Curcumin and related compounds. Extraction and separation conditions were optimized by experimental design and multi criteria response functions. Figures of merit were established under the selected experimental conditions. In general, repeatability of peak areas was better than 0.69, detection limits were below 1.94µg/ml and quantitative recoveries expressed as about below 6.64µg/ml. The method was applied to quantify curcuminoids in commercial samples. It was found that apart from Curcumin, demethoxycurcumin and bis-demethoxycurcumin, other related molecules also occurred in the samples. Differences in the compositional profiles among samples were encountered to be relevant, so that the resulting HPLC data was exploited for characterization of turmeric samples. Samples were successfully discriminated according to matrix types, geographic varieties and origins.

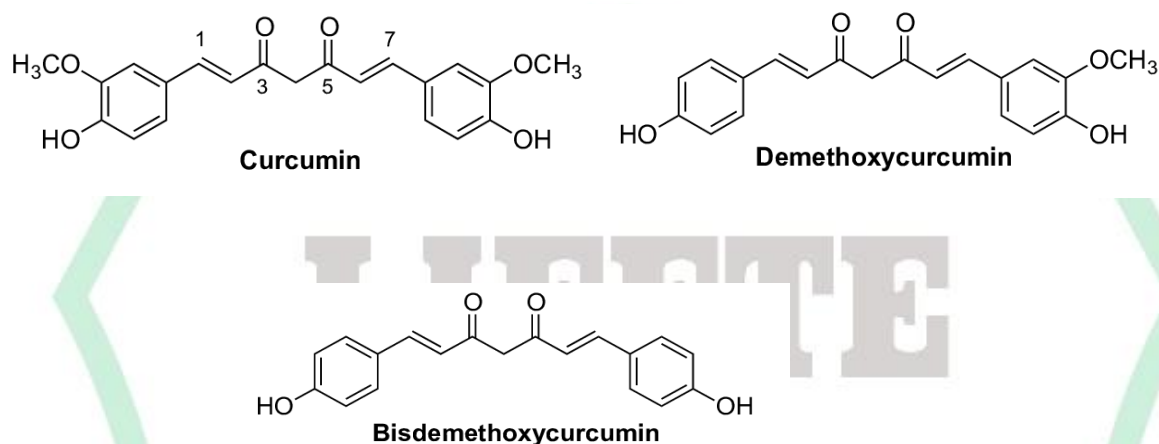
Keywords: Curcumin, HPLC, method development

INTRODUCTION:

Curcumin is a polyphenolic compound obtained from turmeric (*Curcuma longa*, family Zingiberaceae) and has long been known for its medicinal effects. It has attracted medical and scientific attention because it deals with the management of oxidative and inflammatory disorders like cancer, metabolic syndrome and arthritis. Its antioxidant and anti-inflammatory qualities are responsible for the majority of these benefits. Curcumin consumption on its own does not result in allied health advantages due to its low blood levels, because of inadequate absorption, rapid metabolism, and rapid excretion. Bio-availability can be boosted by a variety of ingredients. Piperine, for example, is the main active ingredient in black pepper and has been known to improve bioavailability by 2000% when coupled with Curcumin. Curcumin, in combination with boosting mediators, has a number of health effects.¹⁻²

Quercetin is most significant bio-flavonoids existing in about twenty plants and which is recognized due to its anti-hypertensive, anti-inflammatory, vasodilation properties, anti-obesity and anti-cholestermic actions. The word "quercetin" comes from the Latin word "quercetum," which meaning "oak forest, belongs to the flavanol class of compounds that cannot be synthesized in the human body.³ It is yellow in color and is poorly in hot water soluble, alcohol soluble in a moderate amount and lipids and insoluble in cold water.

The quantification of curcumin and related molecules in food samples entails an efficient separation of components in order to avoid mutual interferences. High performance liquid chromatography (HPLC) with UV-Vis spectroscopic detection is the current technique of choice for a rapid, feasible and accurate determination of curcumin in food samples.⁴ Curcuminoids are typically monitored at 425 nm on the basis of their intense yellow color. Reversed-phase HPLC with C18 column is commonly used, although anion exchange and micellar chromatography also been introduced.⁵⁻⁶ Some recent modifications to enhance the separation performance are based on phenyl, monolithic and fused-core columns. An alternative separation technique to resolve curcuminoid mixture, in the last years, capillary electrophoresis (CE) has also been proposed. Various CE modes, such as capillary zone electrophoresis, non-aqueous capillary electrophoresis, micellar electrokinetic chromatography and microemulsion electrokinetic chromatography, have been applied to determine Curcumin related molecules, including degradation products such as vanillic and ferulic acids and 4-hydroxybenzaldehyde, in food samples.⁷ apart from Curcumin, demethoxycurcumin and bis-demethoxycurcumin, other related molecules also occurred in the samples.



MATERIALS AND METHODS:

Chemical and Reagents:

Curcumin (99%) was obtained as a gift sample from Sun Pure Extract in New Delhi was obtained from Otto Chemicals in Marine Lines, Mumbai, and HPLC grade methanol, water, and acetonitrile were obtained from Avi Chemicals in Mumbai.

Instruments:

Shimadzu High Performance Liquid Chromatography (HPLC) equipped with SPD-10A UV-visible detector. Column- Zodiac 100 C-18 of dimension (150 x 4.5 mm, 5 μ m particle). Mobile phase methanol-acetonitrile (2:3, v/v), Detection Wavelength 425nm, Flow rate 1.0ml/min, Temperature- Ambient, Sample size-5.0 μ l were selected to develop an accurate method.

Selection of Wavelength:

The standard solution of Curcumin standard was scanned over the range of 200-500nm wavelength. The wavelength of absorption was found to be 425nm. So, this wavelength was selected for determination of Curcumin standard.

Chromatographic Parameter:

The mobile phase consisting of methanol-acetonitrile (2:3, v/v), was filtered through 0.45 μ nylon filter, sonicated and was pumped from the solvent reservoir. The flow rate of mobile phase was maintained at 1ml/min and the response was monitored at 425nm with a run time of 60min. The volume of injection loop was 10 μ l. The column and the HPLC systems were kept at ambient temperature.

Preparation of Standard Stock Solutions:

Curcumin standard standard stock solution: (100 μ g/ml) A 100 mg of Curcumin standard was weighed and transferred to a 100 ml volumetric flask. 50 ml of methanol-acetonitrile (2:3, v/v) was transferred to this volumetric flask and sonicated for 10 min. The flask was shaken and volume was made up to the mark with methanol-acetonitrile (2:3, v/v) to give a solution containing 1000 μ g/ml Curcumin standard. From this solution 10 ml was transfer to 100 ml volumetric flask. The volume was adjusted to the mark with the methanol-acetonitrile (2:3, v/v) to give a solution containing 100 μ g/ml Curcumin standards.

Sample Preparation:

Specific aliquots from stock solutions were used to make the sample solutions. Turmeric powder extract stock solution (10 $\mu\text{g}/\text{ml}$): A 100 mg of Turmeric powder extract was weighed and transferred to a 100 ml volumetric flask. 50ml methanol-acetonitrile (2:3, v/v) was transferred to this volumetric flask and sonicated for 10 min. The flask was shaken and volume was made up to the mark with methanol-acetonitrile (2:3, v/v) to give a solution containing 1000 $\mu\text{g}/\text{ml}$ Turmeric powder extract. From this solution 10 ml was transfer to 100 ml volumetric flask. The volume was adjusted to the mark with the methanol-acetonitrile (2:3, v/v) to give a solution containing 100 $\mu\text{g}/\text{ml}$ turmeric powders extract.

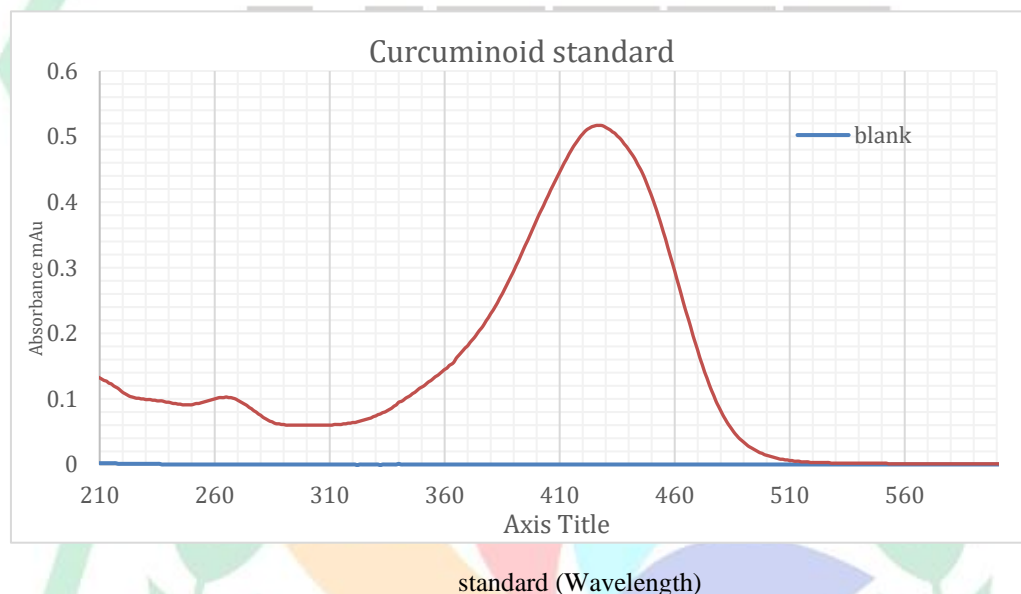


Figure 1: UV spectra of Curcumin

Analytical Method Validations:

The developed RP-HPLC method of Curcumin, BDMC and DMC was validated as per the ICH guidelines. The method was tested with linearity, precision, specificity, sensitivity, accuracy, ruggedness, robustness, limit of quantification (LOQ), and limit of detection (LOD).

RESULTS AND DISCUSSION:

RP-HPLC method was designed with the goal of estimating Curcumin, BDMC and DMC in bulk, and system suitability criteria such as peak resolution factor, tailing factor, number of theoretical plates, runtime, and cost effectiveness were considered. Curcumin elution took 12.125 min, while BDMC took 10.018 min and DMC took 11.014 using the developed improved procedure. The produced chromatogram of Curcumin, BDMC and DMC is shown in Figure. Curcumin was discovered to have 7908.036, 8344.274 and 6350.359 theoretical plates, respectively. Each trial took 15 min to complete.

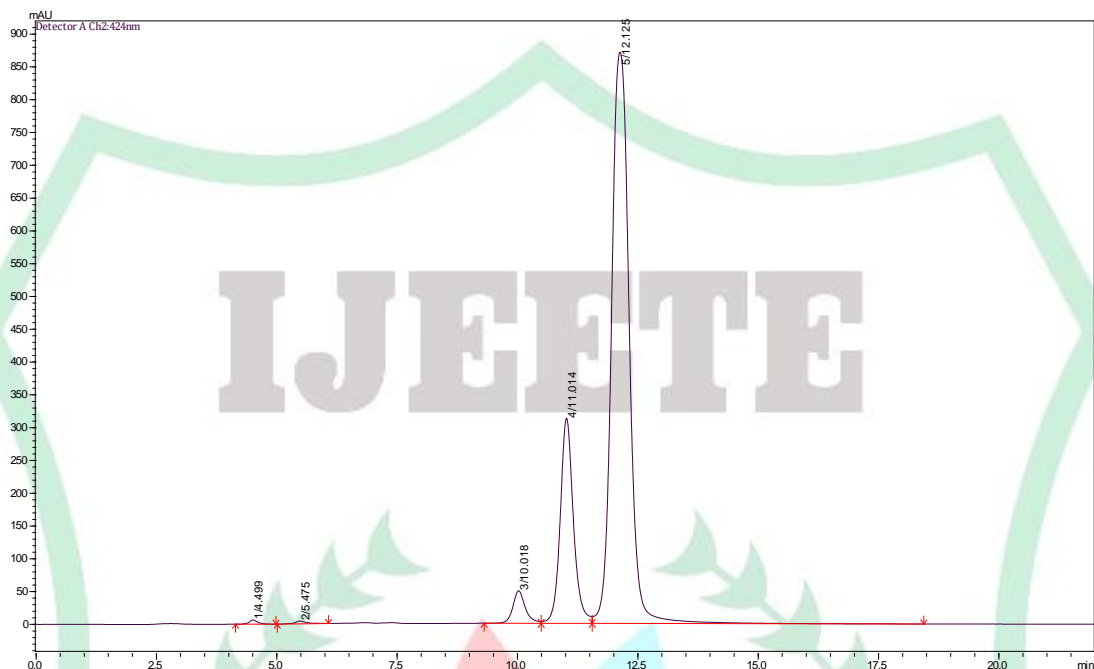


Figure 2: The developed chromatogram of the optimized method for Curcumin, BDMC and DMC

Table 1: The system suitability parameters 5

Peak#	Ret. Time	Area%	T.Plate#	Resolution	Tailing F.
1	4.499	0.2761	3375.37	--	1.158
2	5.475	0.2487	3172.746	2.796	1.301
BDMC	10.018	3.0547	7908.036	10.823	--
DMC	11.014	20.4422	8344.274	2.134	1.155
CURCUMIN	12.125	75.9782	6350.359	2.038	1.092

Method Validation:

Specificity:

The developed chromatogram of the optimized method for Curcumin, BDMC and DMC for standard drug solutions, shown in Figure 3, reveals that the peaks obtained in the standard solutions at working concentrations are only due to the drugs, as the blank has no peak at the retention time of Curcumin, BDMC and DMC. As a result, it is possible to conclude that the established approach is specific.⁸

Linearity:

The linearity of HPLC method represents its ability to explicit the results that should be proportional to the concentration of studied analytes within a selected range.⁹ Therefore, as observed from figures () and tables () over the selected concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ppm) of curcuminoids (BDMC, DMC and curcumin) their relative peak areas were highly proportional since in all studies their regression coefficients (R²) were almost 0.999 which is closest to 1. It represents that the developed method has a high degree of linearity. Furthermore, based on the standard deviation of the response and the slope of the regression equation their limits of detection (LOD) and limits of quantification (LOQ) were calculated. As observed, the LOD and LOQ for all selected compounds were well below the 5 µg/ml which signifies the selected wavelength is more sensitive enough to detect the lowest amount of drugs either from pharmaceutical drugs or biological fluids.

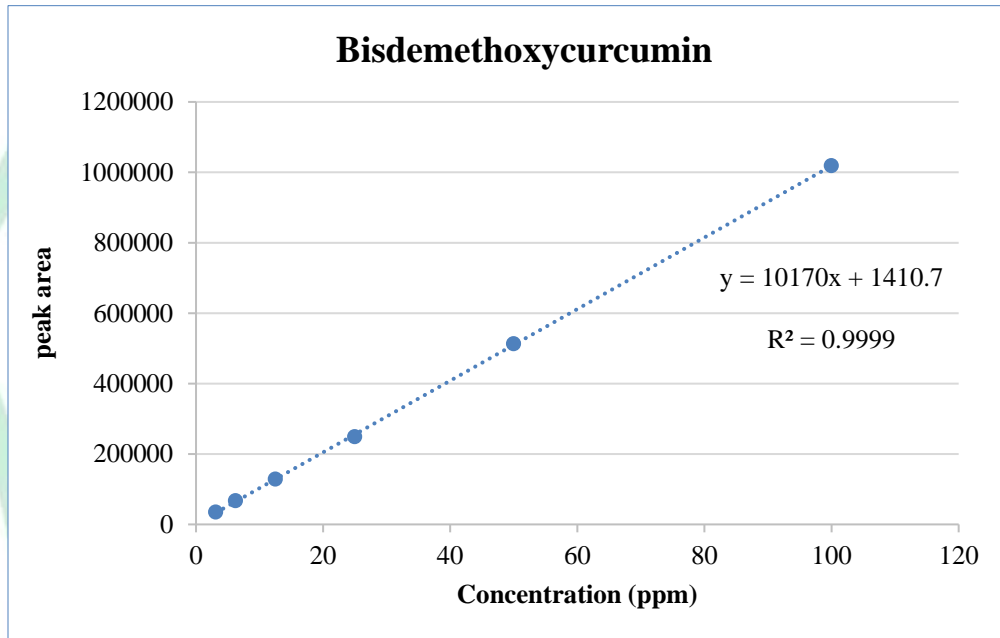


Figure 3: Linearity curve of BDMC

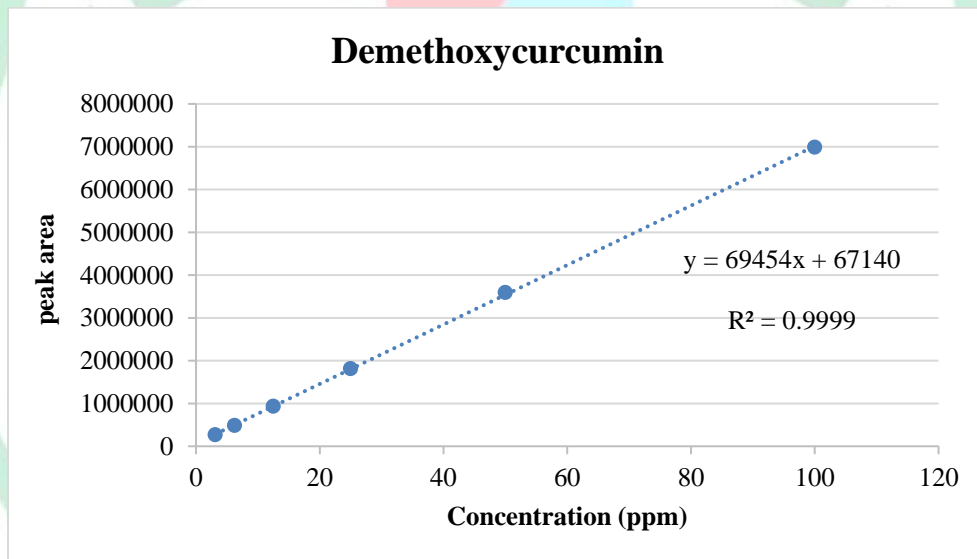


Figure 4: Linearity curve of DMC

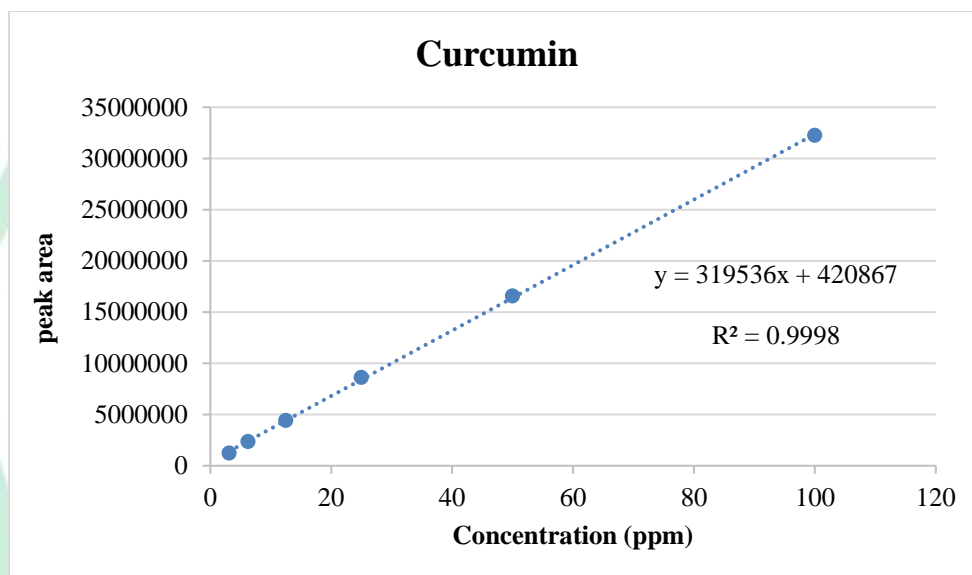


Figure 5: Linearity curve of Curcumin

Repeatability study of curcuminoids:

Implementing the procedure mentioned under experimental section, the curcuminoid standard was tested for six injections within the same day.¹⁰ Furthermore, their RSD in percentage was calculated and as displayed in table they are for BDMC, DMC and curcumin, respectively. Thus, calculated RSDs for all studied curcuminoids were less than 2% which are quite significant in accordance with the ICH guidelines.

Table 2: Repeatability data of BDMC, DMC and Curcumin

S. No.	BDMC	DMC	Curcumin
	Peak Area; Conc. 100 ppm	Peak Area; Conc. 100 ppm	Peak Area; Conc. 100 ppm
1	894451	5985636	32246983
2	907420	5991863	31855075
3	897463	5848211	31989302
4	893506	5871173	32295637
5	906753	5874998	31602758
6	904023	5852606	32358821
Mean	900603	5904081	32058096
Std. Dev.	6230.000021	66419.0509	295236.3075
RSD (%)	0.69	1.12	1.34

Precision studies for curcuminoids:

The precision (intraday and interday/intermediate) of HPLC method reflects its closeness to the agreement among the series of repetitive results, those derived after multiple sampling of the same homogenous mixture of selected drugs under the given conditions.¹¹ As displayed in Table 2; after calculating their relative standard deviation (RSD) in percentage, this developed method found to be significantly precise for curcuminoids.¹² Moreover, the peak

area of the studied drugs was also correlated with their selected concentration; where the % RSDs for curcuminoids were <2%. The RSDs were observed well below 2% that reflects an acceptable precision with minimum variations of the proposed method.

Intraday precision:

Implementing the procedure mentioned under ICH guidelines, the homologous mixture of BDMC, DMC and curcumin of three replicates of same concentrations were tested and evaluated.¹³ Their RSD values in percentage have been determined and they were found less than 2% for all selected analytes.

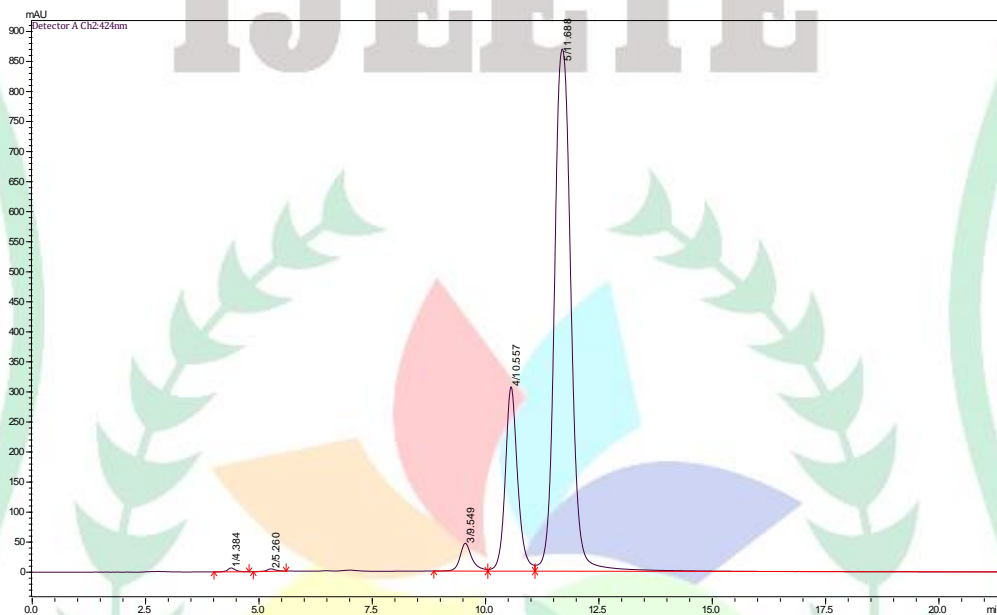


Figure 6: intraday precision; HPLC Analysis of curcuminoids standard

Interday (Intermediate) Precision studies of Curcuminoids:

Implementing the procedure mentioned under ICH guidelines, the homologous mixture of BDMC, DMC and curcumin of three replicates of selected similar concentrations; were tested and evaluated in three successive days (interday/intermediate precision).¹⁴ The RSDs in percentage were calculated and they are found to be less than 2% for all selected analytes.

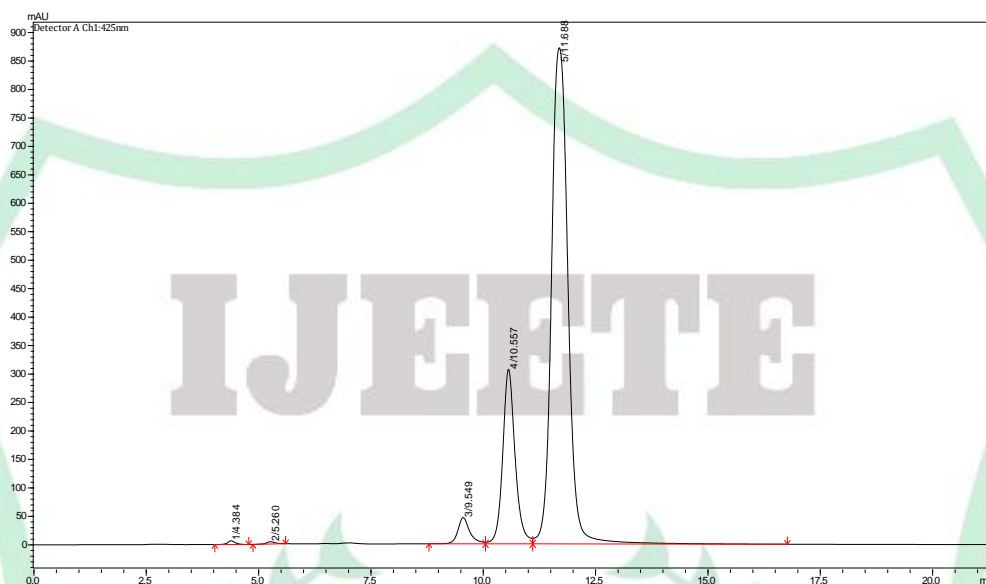


Figure 7: Interday/intermediate precision data of BDMC

Table 3: The precision results of optimized method

Parameters	BDMC	DMC	CURCUMIN (CM)
Repeatability (% RSD)	0.69	1.12	1.34
Intra-Day Precision (% RSD)	0.42 – 1.45	0.37 -0.64	0.33 -1.08
Inter-Day Precision (% RSD)	0.59 – 1.14	0.48 -0.76	0.69 -0.87
Linearity range	3.12 – 100 $\mu\text{g.ml}^{-1}$	3.12 – 100 $\mu\text{g.ml}^{-1}$	3.12 – 100 $\mu\text{g.ml}^{-1}$

Sensitivity:

It is limit for reliable method to detect minimum level of analyte. Lower level of BDMC, DMC and curcumin were evaluated by signal to noise ratio. The smallest concentration of analyte that yields an accurate response but cannot be quantified is known as the limit of detection (LOD).¹⁵ the smallest quantity of analyte that yields a correct response is known as the limit of quantitation (LOQ). The signal-to-noise ratio was used to calculate LOD and LOQ. The LOD value found for BDMC, DMC and curcumin were 5.44, 6.46 and 1.32 $\mu\text{g/ml}$ respectively, while LOQ values were found to be 1.63, 1.94 and 0.39 $\mu\text{g/ml}$ respectively.

Table No. 4: Validation Parameters of Curcuminoids

System suitability parameters	BDMC	DMC	CURCUMIN (CM)
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Theoretical plates (N)	7908	8344	6350
Retention time (t_R)	10.01 min.	11.01 min.	12.12 min.
Wavelength of Detection (nm)	424 nm	424 nm	424 nm
Repeatability (% RSD)	0.69	1.12	1.34
Intra-Day Precision (% RSD)	0.42 – 1.45	0.37 -0.64	0.33 -1.08
Inter-Day Precision (% RSD)	0.59 – 1.14	0.48 -0.76	0.69 -0.87
Linearity range	3.12 – 100 $\mu\text{g.ml}^{-1}$	3.12 – 100 $\mu\text{g.ml}^{-1}$	3.12 – 100 $\mu\text{g.ml}^{-1}$
Regression equation	$y=10170x + 1410.7$	$y = 69454x + 67140$	$y = 319536x + 420867$
Correlation Coefficient (r^2)	0.9999	0.9994	0.9997
LOQ ($\mu\text{g.mL}^{-1}$)	5.44 $\mu\text{g/mL}$	6.46 $\mu\text{g/mL}$	1.32 $\mu\text{g/mL}$
LOD ($\mu\text{g.mL}^{-1}$)	1.63 $\mu\text{g/mL}$	1.94 $\mu\text{g/mL}$	0.39 $\mu\text{g/mL}$

CONCLUSION:

For the simultaneous quantification of BDMC, DMC and curcumin, the RP-HPLC method reported here is accurate, sensitive, precise, and repeatable. It is cost effective method. It can be utilized for quality control analysis on a regular basis of BDMC, DMC and curcumin in combination as per the regulatory guidelines. With the use of a C18 column, the established approach allows for good separation of BDMC, DMC and curcumin in bulk mixtures. Using a solvent system of methanol-acetonitrile (2:3, v/v) at ambient temperature and a detection wavelength of 424 nm, the flow rate was 1ml/min with a run time of 15 min. Elution took 10.01 min, 11.01 min and 12.12 min respectively using the developed improved procedure. The developed method's system suitability parameters were investigated and determined to be within acceptable ranges. LOD and LOQ were reported to be 5.44, 6.46 and 1.32 $\mu\text{g/ml}$ respectively, while LOQ values were found to be 1.63, 1.94 and 0.39 $\mu\text{g/ml}$ respectively. The linearity precision, specificity, sensitivity, accuracy, ruggedness, robustness, LOD, and LOQ of the devised method were all validated according to ICH recommendations. The results of the preceding observations show that the procedure is effective in both qualitative and quantitative analysis of the in the mixture.

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

ABBREVIATIONS:

BDMC: Bis-demethoxycurcumin

DMC: Demethoxycurcumin

RP-HPLC: Reverse Phase High Performance Liquid Chromatography;

LOQ: Limit of Quantitation

LOD: Limit of Detection

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