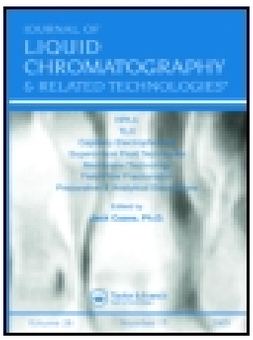

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Journal of Liquid Chromatography & Related Technologies

ISSN: 1082-6076 (Print) 1520-572X (Online) Journal homepage: https://www.tandfonline.com/loi/ljlc20

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To cite this article: Prafulla Kumar Sahu, Jagadeesh Panda, Y. V. V. Jogendra Kumar & S. Karunya Ranjitha (2020): A robust RP-HPLC method for determination of turmeric adulteration, Journal of Liquid Chromatography & Related Technologies, DOI: 10.1080/10826076.2020.1722162

To link to this article: https://doi.org/10.1080/10826076.2020.1722162

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
https://doi.org/10.1080/10826076.2020.1722162

A robust RP-HPLC method for determination of turmeric adulteration

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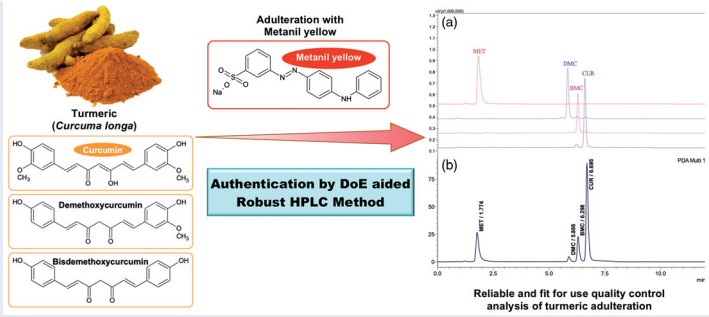
ABSTRACT

A gradient reverse phase high pressure liquid chromatographic method has been proposed for simultaneous determination and separation of curcumin (CUR), bisdemethoxycurcumin (BMC), demethoxycurcumin (DMC), and metanil yellow (MET). The separation was achieved on an Agilent Eclipse XDB-C18 (4.6 mm × 150 mm, 3.5 µm) analytical column using photodiode array (PDA) detection at 425 nm. Gradient elution of solvent A (mixture of 0.1% trifluoro acetic acid and 0.1% formic acid 50:50 v/v) and solvent B (acetonitrile) at a flow rate of 0.8 mL/min was used to accomplish adequate separation of the analytes. The developed method was extensively validated with respect to linearity, detection and quantitation limits, precision, recovery, and robustness testing. Method variables, viz., mobile phase flow rate (0.90 ± 0.05 mL/min), percentage of B at start of gradient (40 ± 2%) and detection wavelength (425 ± 5 nm) were studied by a Box–Behnken Design (BBD) for testing the robustness of the proposed method. The method was found robust, with no significant variations in the method performance, linear in the range of 10–80 µg/mL ($r^2 = 0.999$) and precise (RSD < 2%) satisfying regulatory criteria. The method is selective for rapid determination of turmeric adulteration with a detection limit of 0.37–2.48 µg/mL.

KEYWORDS

Adulteration; curcuminoids; food fraud; metanil yellow; turmeric; RP-HPLC; robustness

GRAPHICAL ABSTRACT



Introduction

The use of food colors has a long history as part of the human diet is now a commercial reality. Consumption of spices containing natural pigments has been an Indian tradition not just to color the food but for their flavor and notably the positive health attributes they impart. Among them, turmeric (*Curcuma longa*) rhizome is widely consumed as a fresh root, powder, decoction, dry extract or oleoresin, or alcoholic tincture for multiple uses. Due to the characteristic deep orange-yellow color and its therapeutic benefits, it became accustomed to every household as a cosmetic, antiseptic, condiment, spice (a common ingredient to curry and ethnic dishes), dye (coloring cotton, wool and silk), etc. Turmeric is highly vulnerable to adulteration due to the fact that it is typically sold in powdered form rendering the visual authentication impossible.[1–3] The overwhelming world demand is another important reason what appears to be responsible for fraud trade of turmeric that frequently admixed or substituted in part or whole with pre-extracted rhizomes, inferior species (containing lower content of CUR), chalk powder, starch, low-cost natural ingredients and artificial dyes.[4,5] Added to this, the desirability of artificial coloring has been earmarked today to meet the demand of food industries and for technological advancements. However, stringent regulatory policies were implicated to restrict the uses of synthetic dyes to the minimal owing to their possible ill effects. Consequently, most of the synthetic dyes were delisted from the national legislation permitted colors due to their severe toxicity. Contrary to many cases, the uses of banned dyes are still

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evidenced by the increased prevalence of associated food-borne diseases.

Metanil yellow (MET), a synthetic compound with bright yellow color is very often added to turmeric powder that poses potential health hazards such as cancer, damages to the gastrointestinal tract, liver and many more.[6] Such an adulteration practiced is economically motivated or for reasons due to the unmet market demand. The term "economically motivated adulteration," generally called as "food fraud" has been defined by the USFDA as "fraudulent, intentional substitution or addition of a substance for the purpose of increasing the apparent value of the product or reducing the cost of its production."[7] The term is more appropriate only when the additions (adulterants) are unwanted and could deprive nutrients essentials.[8] The fraud trade practice is not only noncompliance with food laws but also involve serious public health risk. Hence, authentication of food quality and safety by analytical methods has become the primary means of consumer protection and compliance with regulatory standards.

A thorough literature survey evidenced that many analytical techniques have been used for detection of turmeric alone or in combination with other analytes. UV-Vis spectroscopy,[9] HPLC,[9–20] TLC,[21] HPTLC,[22] UPLC,[23] LC-MS/MS,[24–27] UHPLC-MS/MS,[28–33] FT-IR,[13,34,35] FT-NIR,[36,37] ^{1}H-NMR,[38,39] supercritical chromatography,[40,41] and fluorescence spectroscopy[42] were the current analytical trend for turmeric determination. A study applied UV, FT-IR, ^{1}H NMR, and HPLC to construct a metabolic fingerprint while assessing the turmeric quality. When the results were analyzed, by chemometrics, UV was found to be a simple and rapid alternative to HPLC; while FTIR failed to differentiate the same species; and ^{1}H NMR was more reliable for metabolic variability between samples in fatty acid or essential oil region.[43] However, very few of the methods are dealt with the quality authentication and detection of turmeric adulteration. In addition, strategic approaches for determining adulteration of turmeric with MET are limited to spectroscopy (chemometrics assisted NIR,[44] FT-Raman and FTIR,[45] UV, and FT-MIR[46]) and electrochemical detection[47] only. Availability of rapid, accurate, and efficient analytical tools is imperative to prevent food adulteration and fraud before it occurs.

The present study aimed at developing a validated quality control tool for the simultaneous determination of BMC, DMC, CUR, and MET (Figure 1) using RP-HPLC coupled with PDA detection that would provide a rapid and sensitive estimation of turmeric adulteration. The method involves a gradient elution and a multivariate domain for robustness testing that makes it selective not only for the determination of the curcuminoids but also to detect MET contamination.

Materials and methods

Chemicals and reagents

All the materials were of analytical-reagent grade unless stated otherwise. HPLC-grade water (Qualigens, Mumbai, India), HPLC grade acetonitrile and methanol (FINAR, Ahmedabad), CUR (Molychem, Mumbai, India), MET (Molychem, Mumbai, India), trifluoroacetic acid (FINAR, Mumbai, India), formic acid (EMPARTA, ACS, Mumbai, India) were procured from the local vendor, Visakhapatnam.

Apparatus

A binary gradient HPLC system (Shimadzu, Kyoto, Japan) consisting two pumps LC 20AD, a photo diode array detector SPD-M20A, with a manual sample injector all from Shimadzu was used for the entire study. The output signal was monitored and integrated using LC solutions software (Shimadzu, Kyoto, Japan). Other apparatus used were Electronic balance (Shimadzu), Ultra sonicator (SONICA, Soltec), pH meter (Elico), microfilter paper (0.45 µ, Millipore), filtration apparatus (Borosil), etc.

Chromatographic condition

The chromatographic analysis was performed on an Agilent eclipse XDB-C18 (4.6 mm × 150 mm, 3.5 µm) analytical column using photo diode array (PDA) detection at 425 nm. A gradient elution (Table 1) of solvent A and solvent B at a flow rate of 0.8 mL/min was used to accomplish better

Table 1. Gradient elution program.

Time (min)	0.01	6.00	8.00	10.00
Acetonitrile content in mobile phase (%)	40	60	40	40

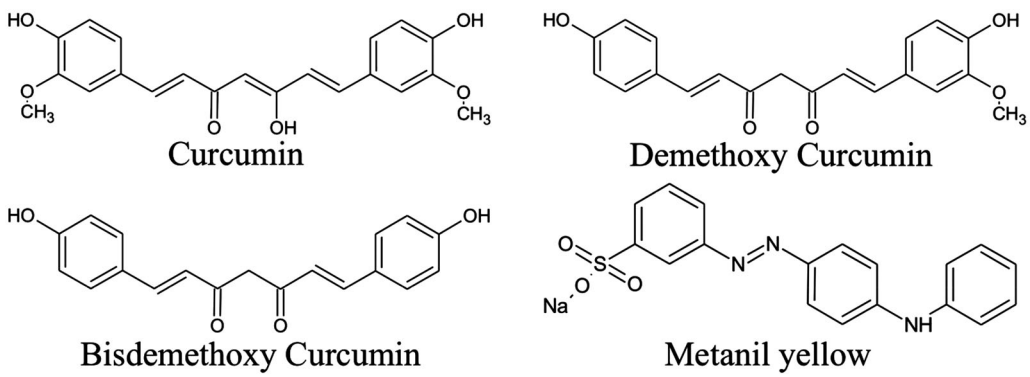

Figure 1. Chemical structure of curcumin, bisdemethoxycurcumin, demethoxycurcumin and metanil yellow.

separation of the analytes. Where, solvent A is a mixture of 0.1% trifluoro acetic acid and 0.1% formic acid (50:50 v/v) and solvent B used is acetonitrile (HPLC grade).

Standards and quality control solutions

All the solutions and samples were prepared using the diluent comprising mixture of acetonitrile and water (50:50) unless stated otherwise. For the preparation of primary stock solutions (1 mg/mL), accurately weighed CUR, BMC, DMC and MET were dissolved in small amount of methanol prior to volume adjustment with the diluent. Working standards (100 µg/mL) were prepared from suitable dilution of the primary stock solutions individually. All the standard solutions were refrigerated until use. In a similar fashion, a mixture of all analytes was also prepared freshly before analysis, which was further diluted to get a series of calibration standards of appropriate concentrations. Calibration curves (five points) were constructed between the concentration range of 10 to 80 µg/mL. Quality control (QC) samples for precision and robustness testing were prepared by diluting working (a mixture of all analytes) or calibration standards.

Commercial sample solutions

For application to real sample analysis, the developed method was used to determine the percentage of CUR and added artificial color (MET) if any in the marketed turmeric powders. Commercially available branded and loose turmeric powders were procured from the local markets of cities in Andhra Pradesh. About fifteen branded samples were collected, out of them four were locally manufactured popular brands. Loose turmeric powders were procured from local suppliers and vendors of twelve major cities in the state. For the preparation of stock solutions, 15 mg each of the commercial powder samples were transferred to a 15 mL glass centrifuge tubes (Borosil) and centrifuged in acetonitrile at 1500 rpm for 30 min. The supernatant layer (10 µL) was used for direct injection or further dilution as essential for the recovery study. To demonstrate the accuracy of the sample preparation step, a sample recovery study was performed. Sample recovery was assessed in duplicate by analyzing a known concentration of the commercial sample (30 µg/mL) spiked with different amounts of standard CUR (50, 100, and 150%). The three fortified QC samples were then injected for HPLC analysis and percentage CUR recovery was calculated by Equation (1) as follows:

$$\%\text{Curcumin} = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \frac{\text{Concentration of standard}}{\text{Concentration of sample}} \times 100 \quad (1)$$

Robustness testing

It is an important regulatory criterion to establish the robustness of the optimized method. It has been stated that "*Robustness of an analytical method is the property that indicates insensitivity against changes of known operational parameters on the results of the method and hence its suitability for its defined purpose.*"[48] Contrary to traditional trial and error strategy, the use of the design of experiments (DoE) was evidenced to furnish more valid results to establish how robust the method is.[49] In the present study, we have considered determining the robust domain (tolerable variations) via response surfaces using a Box–Behnken Design (BBD). The study was limited to investigating the influence of the basic HPLC parameters only. Three key parameters viz. flow of mobile phase (mL/min), percentage of B at the start of gradient and wavelength (nm) were studied (Table 2) at 3 levels for 17 experimental trials. Chromatographic factors such as critical resolutions (RS2, RS3) and tailing factor of first eluate (T1) were adapted as the responses to be investigated. The responses obtained for all the 17 experimental runs were then ensured for reproducibility and to be within the acceptable criteria. Analysis of variance (ANOVA) with non-significant results demonstrates that small but deliberate variations in method parameters did not lead to drastic changes of responses (beyond the acceptable criteria).

Table 2. Operational parameters selected along with their levels for robustness testing of the method by BBD.

Operational parameters	Low	Optimal	High
Flow rate (mL/min)	0.85	0.90	0.95
Percentage of B at start of gradient	38	40	42
Wave length (nm)	420	425	430

Results and discussion

Method development and optimization

The development of a simple RP-HPLC method for efficient separation of the three curcuminoids was critical and challenging. As a starting point, initial trials were done using methanol/(or) acetonitrile with water as the mobile phase at various compositions and flow rates. The separation was inadequate and dictated an acidic buffer (ortho-phosphoric acid, trifluoroacetic acid, formic acid) and gradient elution of organic phase from 0 to 100% for 30 min. Trials were also carried out with triethylamine, ammonium acetate, ammonium bicarbonate buffers, but good peak shape and adequate separation were never achieved. Sharp peaks were obtained when a mixture of 0.1% trifluoroacetic acid and 0.1% formic acid (50:50 v/v) is used as the buffer (pH 2.5 ± 0.2) with acetonitrile as the organic modifier. Nevertheless, inadequate separation of the curcuminoids has resulted that could be improved by adopting a gradient elution of the mobile phase. Optimum separation of all the analytes were achieved on an Agilent Eclipse XDB-C18 (4.6 mm × 150 mm, 3.5 µm) analytical column using elution of mobile phase consisting of equivalent mixture of 0.1% trifluoroacetic acid and 0.1% formic acid as buffer and acetonitrile in a gradient mode (Table 1) at a flow rate of 0.8 mL/min. The injection volume was 10 µL and detection was done using photodiode array (PDA) detector set at 425 nm.

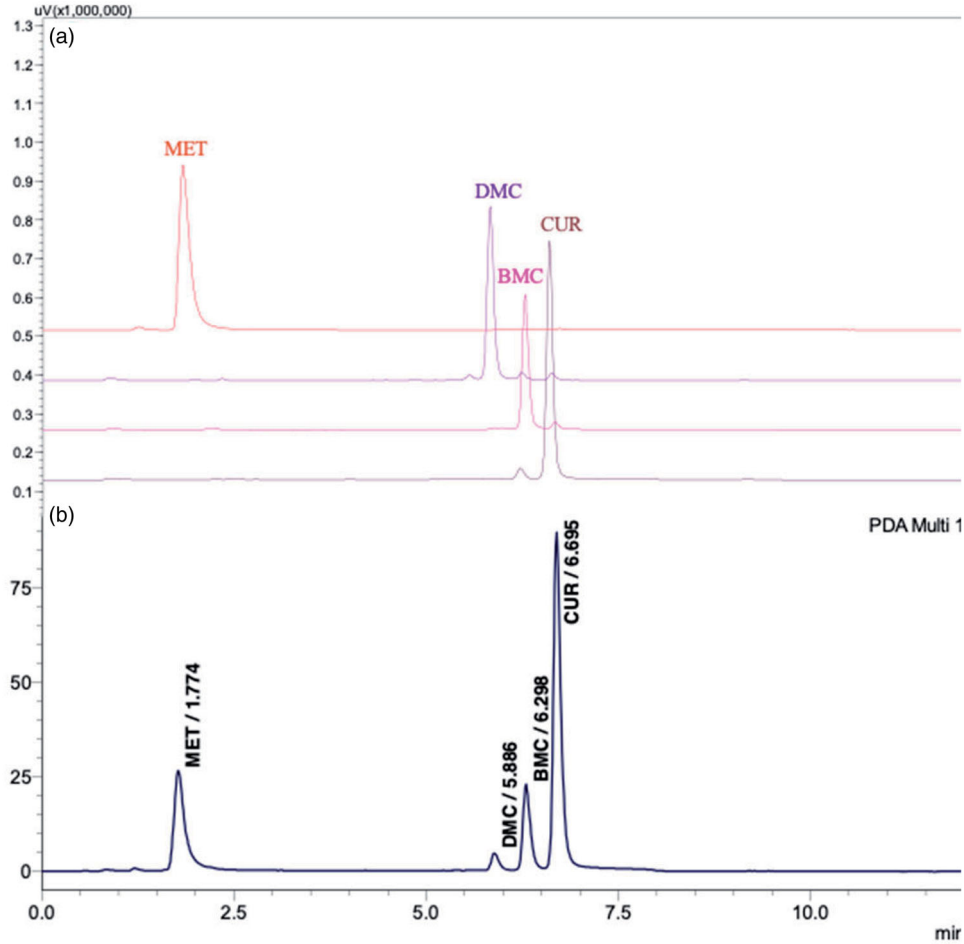

Figure 2. Chromatograms obtained at optimal condition: (a) overlaid chromatograms of individual analyte standards (20 µg/mL); (b) real commercial sample fortified with MET (20 µg/mL).

Chromatograms of analytes at the optimal condition are portrayed in Figure 2.

Robustness

The robustness study was designed for assessing the ability of the RP-HPLC method to remain unbiased by small variations in the three key operational parameters (mobile phase flow, percent organic modifier at the start of the gradient and wavelength). A BBD was adapted to module the influence of the basic parameters on the method performance mathematically. Critical resolutions between the curcuminoids and tailing of MET were considered as method performance index. BBD experimental designs are based on three-level factorial designs and classified as rotatable or nearly rotatable second-order response surface designs.[50] Unlike full-factorial or other optimization designs, BBD is augmented with convenient, less expensive and more efficient for achieving the desired separation. The factor settings with the resulted HPLC responses for 17 experimental runs are shown in the following design matrix (Table 3). The resulted chromatograms from the experimental trials are shown in Figure 3. Analyzing the variance (Table 4) for RS_2, RS3, and T_1 revealed that regression models were insignificant violating the respective 95% confidence intervals for each of the method variables (p-value > 0.05). It was hence demonstrated that deliberate variations in operational parameters did not observe any drastic alteration in the separation performance so that the developed method was considered to be robust.

Table 3. Box–Behnken Design and responses obtained for testing robustness of the RP-HPLC method.

	Factors			Responses		
Run	Flow (mL/min)	Acetonitrile (%)	Wave length (nm)	RS2	RS3	T1
1	0.9	40	425	1.969	1.942	1.882
2	0.95	40	430	1.953	1.941	1.814
3	0.9	40	425	1.969	1.921	1.913
4	0.85	40	420	1.989	1.953	1.748
5	0.85	40	430	2.058	2.046	1.783
6	0.95	38	425	2.167	2.134	2.414
7	0.95	40	420	1.981	1.964	1.647
8	0.85	42	425	2.027	1.979	1.79
9	0.9	40	425	1.969	1.942	1.882
10	0.9	38	420	1.994	1.989	1.637
11	0.95	42	425	2.173	2.113	2.34
12	0.9	42	420	2.172	2.097	2.398
13	0.9	38	430	1.947	1.955	1.748
14	0.9	40	425	1.969	1.921	1.913
15	0.85	38	425	2.202	2.013	1.683
16	0.9	40	425	1.969	1.942	1.882
17	0.9	42	430	2.108	2.093	2.309

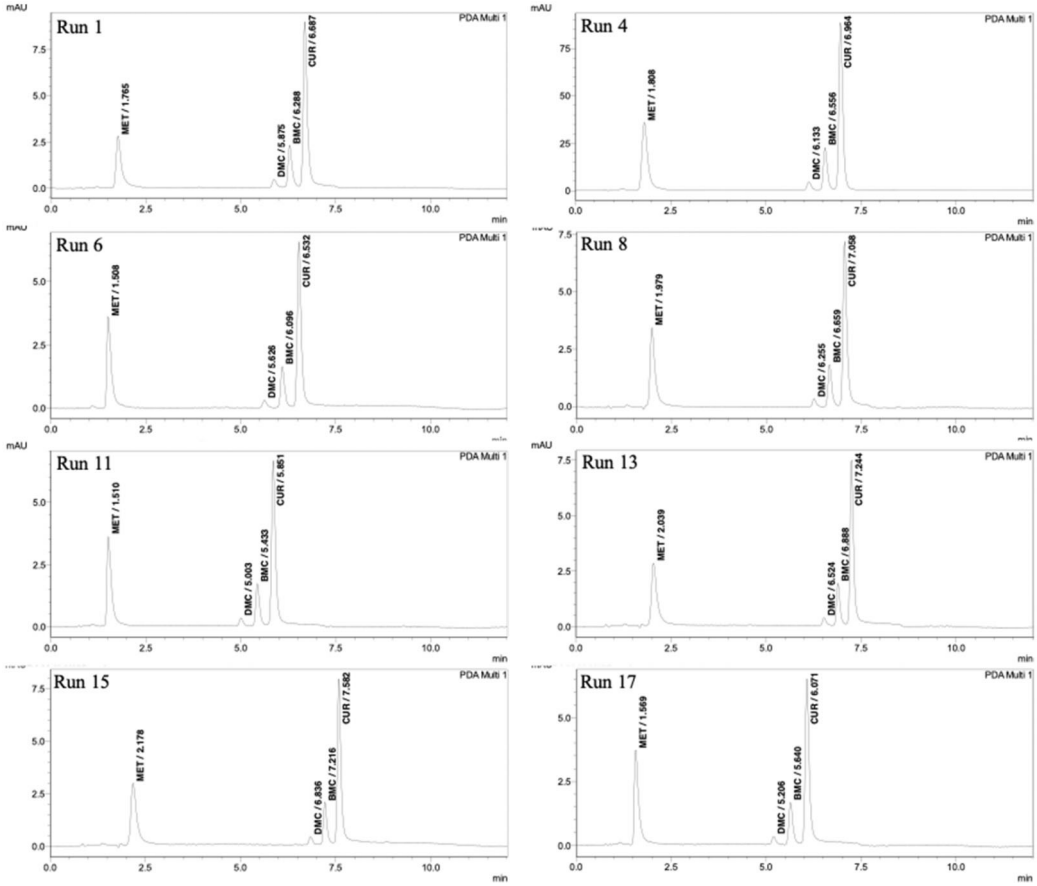

Figure 3. Represented chromatograms from BBD experimental trials.

Table 4. ANOVA results for the three critical responses.

	RS2		RS3		T1*	
Variables	*F*	*p*	*F*	*p*	*F*	*p*
Model	1.70	0.2481	1.49	0.3079	2.59	0.0975
A: flow	0.0001	0.9929	0.7948	0.4023	3.40	0.0882
B: B concentration (%)	0.6105	0.4602	1.12	0.3254	4.25	0.0597
C: wave length	0.1035	0.7570	0.0314	0.8644	0.1163	0.7386
AB	1.38	0.2778	0.0104	0.9218		
AC	0.3976	0.5484	0.8252	0.3939		
BC	0.0122	0.9151	0.0552	0.8210		
A^2	2.28	0.1747	1.22	0.3065		
B^2	9.68	0.0171	8.71	0.0214		
C^2	0.6566	0.4444	0.0673	0.8027		

*The response (T1) follows linear model.

Table 5. Inter and intra-day precision data when performed in triplicate.

	Actual concentration (μg/mL) found (mean ± SD; CV[%])					
	Inter-day			Intra-day		
Theoretical concentration (μg/mL)	30	50	70	30	50	70
MET	32.64 ± 0.39; 1.21	49.83 ± 0.5; 1.01	71.75 ± 0.5; 0.69	29.30 ± 0.39; 1.35	49.5 ± 0.53; 1.08	69.53 ± 2.37; 1.41
DMC	31.85 ± 0.37; 1.17	49.95 ± 0.5; 1.07	72.98 ± 1.1; 1.62	29.86 ± 0.31; 1.04	49.09 ± 0.6; 1.22	70.55 ± 1.05; 1.49
BMC	31.67 ± 0.35; 1.13	49.19 ± 0.49; 0.99	69.19 ± 0.48; 0.7	30.32 ± 0.31; 1.02	48.9 ± 0.82; 1.68	68.66 ± 0.4; 0.61
CUR	32.49 ± 0.37; 1.14	50.45 ± 0.5; 0.99	70.43 ± 0.63; 0.90	29.95 ± 0.18; 0.62	50.32 ± 0.30; 0.61	69.92 ± 0.14; 0.2

Table 6. Overall recovery data ($n = 2$) for spiked samples.

	Overall recovery (%)			
Analytes	MET	DMC	BMC	CUR
QC1	92.8	98.87	96.82	96.41
QC2	93.3	96.64	98.3	94.11
QC3	96.98	97.8	92.73	93.74

Linearity and sensitivity

The calibration curve was obtained by plotting respective peak areas against five drug concentrations. The method demonstrated good linearity ($r^2 > 0.999$) in the concentration range of 10–80 μg/mL for all the analytes. Sensitivity of the

Table 7. Determination of CUR content (%) in commercial turmeric powders.

Samples	Total no of samples	Samples contain CUR (content [%])	Samples contain MET (content [%])	Samples with CUR > 3% (BIS standard [%])	Overall adulteration (%)
Loose	12	12 (2.54–33.4)	3 (3.73–7.5)	8	33.33
Branded	15	15 (10.11–54.37)	0	15	0

method was studied by establishing the limit of detection (LOD) = 3.3 (σ/S) and limit of quantitation (LOQ) = 10 (σ/S); where, σ is the standard deviation of the responses and S is the slope of the calibration curve. The measured LODs were 0.371814, 2.489206, 1.599097, and 2.430587 µg/mL respectively and LOQs were 1.23938, 8.297355, 5.330322 and 8.101957 µg/mL for MET, DMC, BMC, and CUR, respectively.

Precision and recovery studies

All the experiments for the precision study were conducted in triplicates and obtained data were summarized in Table 5. Studies for intra- and inter-day precision of the developed RP-HPLC method were performed for QC samples at three different concentrations 30, 50, and 70 µg/mL. The method was considered to be precise, where the estimated coefficient of variation (CV) was < 1.68%. Recovery results for three fortified QC samples are summarized in Table 6. The overall recovery of CUR was ranged from 93.74 to 96.41%, whereas those of MET, DMC, and BMC ranged from 92.8 to 96.98%, 96.64 to 98.87%, and 92.73 to 98.3% respectively.

Application to marketed sample analysis

The developed RP-HPLC method was applied to analyze the commercial samples collected from the market place. Analysis of commercial turmeric samples was performed in a view to check their quality and authentication (admixture with MET). Total CUR content is considered as the quality index and can partially serve as a tool to check the authenticity of turmeric samples. It has been well evidenced that substituted turmeric samples are frequently reported with lower CUR content. The Bureau of Indian Standards recommended a minimum CUR content of 3% for powdered turmeric products.[51] The developed RP-HPLC method is simple and efficient than any spectrophotometric methods in the literature. Unlike spectrophotometric methods, the present method distinctly measures the content of CUR, BMC, DMC and MET in turmeric samples. Observed data in Table 7 demonstrated that most of the loose samples are adulterated with MET and exhibit lower CUR content when compared to the branded. All of the branded samples analyzed were devoid of any adulteration and reported a high CUR percentage.

Conclusions

Turmeric is an essential commodity to every Indian household and widely consumed for multiple uses. However, the frequent substitution of turmeric with MET has been allegedly reported from time to time. MET is a synthetic extraneous color, which upon consumption imparts potential health hazards. Such a fraudulent practice is illegal and dictates continuous monitoring to ensure consumer protection and compliance with the rules and standards. Moreover, analytical methods for quality authentication and detection of turmeric adulteration is occasionally reported and mostly limited to spectrophotometric determinations. The proposed analytical method employing gradient HPLC and PDA detection demonstrates adequate separation and quantification of CUR, BMC, DMC, and MET. A multivariate approach for its robustness testing rendered the method more reliable for routine quality control of turmeric.

Disclosure statement

All authors declare that they have no conflict of interest.

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