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Determination of Bioactive Markers in *Curcuma longa* Rhizome

Kedar Kumar Rout* • **Satyaranjan Mishra** • **Sagar Kumar Mishra** • **Sabuj Sahoo**

University Department of Pharmaceutical Sciences, Utkal University, Vani Vihar Bhubaneswar-751004, Orissa, India

Corresponding author: * **kd_rout@yahoo.co.in**

ABSTRACT

A simple, precise and rapid HPTLC method has been developed and validated for quantitative determination of biologically active curcuminoids in four *Curcuma longa* samples collected from Kandhamal, Sambalpur, Bhubaneswar and local market of Orissa (India). The HPTLC method was optimized with the mobile phase consisting of chloroform: methanol, 9.6: 0.4 (v/v). The compounds were quantified at their maximum absorbance, at 430 nm. Limit of detection was found to be 15 ng for each curcuminoids and limit of quantification was found to be 50, 40, 50 ng for curcumin (**1**), demethoxycurcumin (**2**), and bisdemethoxycurcumin (**3**), respectively. The response was a linear function in the ranges 50-300, 40-200, and 50-250 ng with correlation coefficients of 0.9999, 0.9991, and 0.9986, respectively for **1**, **2** and **3**. Instrumental precision was found to be 0.46, 0.79, and 0.92% and repeatability of the method was 1.75, 1.80, and 2.25% for **1**, **2** and **3** respectively. The mean recovery values were found to be 99.71% for **1**, 98.74% for **2**, and 99.23% for **3**. Among the samples investigated the highest content of bioactive markers were accumulated in the Kandhamal sample.

Keywords: bisdemethoxycurcumin, *Curcuma longa*, curcumin, demethoxycurcumin, haridra, HPTLC
Abbreviations: CV, coefficient of variation; **HPTLC**, high performance thin layer chromatography; **ICH**, International Conference on Harmonization; **LOD**, limit of detection; **LOQ**, limit of quantification; **NMR**, nuclear magnetic resonance; **UV**, ultraviolet

INTRODUCTION

'Haridra' is a single Ayurvedic drug that consists of the dried and cured rhizomes of *Curcuma longa* Linn. Belonging to the family Zingiberaceae. It is used as a spice by the Indian population for its colour and flavour. The rhizome of turmeric is a valuable cash crop, which is widely cultivated in Asia, particularly in India, China, and other tropical countries (Govindarajan 1980). It has a special importance that its powder, when added to various food preparations, preserves their freshness and imparts a characteristic flavour (Jayaprakasha *et al*. 2005). In the Indian traditional system of medicine it has been prescribed for several human diseases (Mukhopadhyay *et al*. 1998) and is well known for its cosmetic properties (Wealth of India 1950). It is also used as a nontoxic Ayurvedic drug for treatment of skin diseases, intestinal worms, rheumatism, intermittent, hepatic disorders, biliousness, urinary discharges, dyspepsia, inflammations, constipation, leukoderma, amenorrhea, and colic (Jain and DeFilipps 1991). However, its extracts have been reported to possess anti-oxidant, antimicrobial, anti-inflammatory, and anticancer effects (Kelloff *et al*. 1996). Curcuminoids are the major bioactive principle derived from the rhizomes of *C. longa* comprising of curcumin (diferuloylmethane), demethoxycurcumin (*p*-hydroxycinnamoyl, feruloylmethane), and bisdemethoxycurcumin (di-*p*-hydroxycinnamoylmethane). These phenolic phytochemicals are yellow crystalline powder and practically insoluble in water and ether but soluble in methanol, ethanol, dimethylsulfoxide, and acetone (Sharma *et al.* 2005). Among the curcuminoids, curcumin is generally considered a major bio-active constituent of turmeric (Srinivasan 1953) and attributed with various pharmacological effects such as hepatoprotective (Despande *et al*. 1998; Subramanian and Selvam 1999; Park *et al*. 2000), hypocholesterolemic (Rao *et al*. 1970; Patil and Srinivasan 1971), antioxidant, anti-inflammatory, anticarcinogenic (Ruby *et al*. 1995; Bush *et al*. 2001; Shao *et al*. 2002), antibacterial (Ramaprasad and Sirsi 1956), wound healing, antispasmodic, anticoagulant, and antitumor activities (Ammon and Wahl 1991). Similarly demethoxycurcumin and bisdemethoxycurcumin are also attributed with several activities which include antioxidant (Jayaprakasha *et al*. 2006), antimutagenic, anticarcinogenic (Anto *et al*. 1996) and effect on *WT1* gene expression in leukemia cell lines (Anuchapreeda *et al*. 2006). Besides the above mentioned activities, the curcuminoids have gained popularity for medicinal purposes, such as anticoagulative (Tokuo *et al.* 1985), potent anticancer agent (Ramadasan *et al.* 1985) and as modest inhibitors of HIV-1 and HIV-2 proteases (Sui *et al.* 1993).

In view of excellent therauptic uses of turmeric in traditional system of medicine and varieties of beneficial biological and pharmacological activities of curcuminoids, it was planned to determine the content of curcuminoids in various samples of *C. longa* from different districts of Orissa, India. Different samples of *C. longa* were investigated by HPTLC and a potent sample was identified for its commercial utilization. A few reports are available for the estimation of curcuminoids by HPTLC (Rasmussen *et al*. 2000; Ansari *et al*. 2005; Pathania *et al*. 2006; Pozharitskaya *et al*. 2008; Paramasivam *et al*. 2008), however the proposed method is very simple, rapid and selective for the accurate quantification of curcuminoids.

MATERIALS AND METHODS

Instrumentation

A Camag (Muttenz, Switzerland) HPTLC system equipped with a Linomat 5 applicator fitted with 100–µl syringe, TLC scanner 3 linked with winCATS software, twin-through plate development glass chamber (20 × 10 × 4 cm). E Merck grade, aluminium foil backed HPTLC plates pre-coated with silica gel 60 F_{254} (20 × 10 cm, 0.2 mm thickness) used during the analysis.

Materials and chemicals

Rhizomes of C. *longa* were collected from different districts of

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Orissa: Kandhamal (s_1), Sambalpur (s_2), Bhubaneswar (s_3) and from a local market (s_4). Analytical grade solvents were used during the analysis. Standard curcuminoids were isolated from a methanolic extract of *C. longa* by preparative TLC method followed by column chromatography, and finally crystallized from methanol and characterized by UV, infrared, NMR and mass spectral studies (Sastry 1970; Gupta *et al*. 1999).

Sample and standard solution preparation

Air dried (30-35°C) powdered sample (1 g each) was placed in a thimble and extracted exhaustively with hot methanol in a Soxhlet extractor for 36 hrs. The soluble portions were concentrated under vacuum and transferred to a 25 ml volumetric flask. Then 5 ml of each sample solution was further diluted to 100 ml with methanol.

Working standard solutions of pure isolated curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) were prepared by dissolving each 2.5 mg in 25 ml (100 ng/µl) of methanol in a volumetric flask (stock solution). For the determination of limit of detection (LOD) and limit of quantification (LOQ), 5 ml of the stock solution was diluted to 50 ml (10 ng/µl) and 5 ml of the same solution diluted to 25 ml (20 ng/µl) for linearity study.

Chromatography

Chromatography was performed on prewashed (methanol) and pre-activated (at 50°C for 30 min) silica gel 60 F_{254} HPTLC plates (20 × 10 cm, 0.2 mm layer thickness). Different concentrations of standard and test sample solutions were applied on the plate as 6 mm band with 8 mm band gap by Linomat 5 applicator (fitted with a 100-µl syringe) under the flow of inert gas, positioned 10 mm from the bottom. The application volume for each sample was 2 µl and a constant application rate of 150 nl/s was used. For the determination of LOD and LOQ of curcuminoids, the standard solution (10 ng/µl) was applied on separate plates. Linear ascending development with chloroform: methanol (9.6: 0.4, v/v) as the mobile phase was performed in a filter-paper-lined Camag twin-trough glass chamber previously saturated with mobile phase vapor (optimum saturation conditions) for 3 min at 21 ± 3°C with 32 ± 4% relative humidity. The solvent migration distance was approximately 55 mm. The developed HPTLC plates were dried with an air-dryer, and scanning was performed at 430 nm (λ_{max} for curcuminoids) by using a Camag TLC scanner 3 with winCATS software (version 1.4.2) in absorbance-reflectance scan mode. The slit dimension of the scanner was 5.0 × 0.45 mm (micro) with 20 mm/s scanning speed and 100 µm per step data resolution was used. Concentration of the chromatographed compound was determined from the intensity of diffusely reflected light. Evaluation was done on the basis of peak area versus concentration in linear regression mode. The robustness of the method was studied by slightly changing the composition of the binary mobile phase. To confirm the specificity of the method, the sample solution was spotted in the TLC plate which was then developed and scanned.

RESULTS AND DISCUSSION

Several methods are available for the analysis of curcumanoids in *C. longa*. Among these, the paper and thin-layer chromatographic method are having lack of precession and describes a poor separation of curcuminoids. Spectrophotometric method used for expressing the total curcuminoid content and analysis of individual compound are not possible. In GC-MS analysis the column efficiency was found unsatisfactory. The liquid chromatographic methods like HPLC, LC-MS are solvent consuming and required a longer period for analysis.

The major advantage of HPTLC is its simplicity, accuracy, cost-effectiveness and rapidity. Several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. It facilitates automated application and scanning *in situ*. Simultaneous assay of several components in a multicomponent system is also possible.

The developed HPTLC method describes the detection of curcuminods in low concentration rages and enables rapid qualitative and quantitative analysis because the chromatogram development distance is short – up to 55 mm. The optimized mobile phase, chloroform: methanol (9.6: 0.4, v/v) enables satisfactory separation of the components in the extracts with symmetrical and well resolved peaks (**Figs. 1A-1D**). The R_F values for curcuminoids were found to be 0.40, 0.23, and 0.15, respectively for **1**, **2** and **3**. The identities of curcuminoids in the sample extract were confirmed by overlaying their absorption spectra with those of the standard compounds using the TLC Scanner 3. The peak purity of the compounds was tested by recording the absorption spectra at the start-to-middle and middle-to-end of the peak [for **1**, r(s, m) = 0.9994, r (m, e) = 0.9992; for **2**, r(s, m) = 0.9995, r (m, e) = 0.9987; and for **3**, r(s, m) = 0.9999, r (m, e) = 0.9997]. The LOD was 15 ng for each curcuminoids and the LOQ were found to be 50, 40, 50 ng for compounds **1**, **2** and **3** respectively. The linear calibration plots for the compounds were found in the range of 50-300, 40-200, and 50-250 ng/spot, respectively for **1**, **2** and **3**, which were reported by the equations y=11.53x−124.709 for compound **1**, y=16.380x−62.165 for compound **2**, and y=8.270x−73.954 for compound **3**, where y is the peak area response and x is the amount of standard curcuminoids (ng); the corresponding correlation coefficients (r) were 0.9999, 0.9991, and 0.9986.

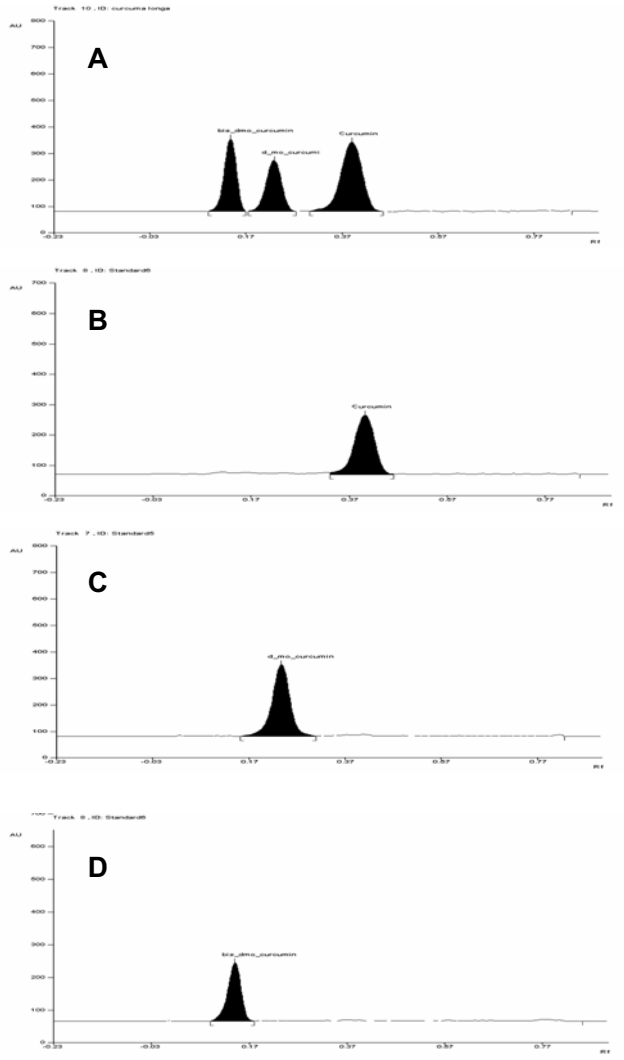

Fig. 1 HPTLC chromatograms obtained for (**A**) *Curcuma longa*, (**B**) standard curcumin, (**C**) standard demethoxycurcumin and (**D**) standard bisdemethoxycurcumin at 430 nm.

Method validation

The ICH guideline was followed for the validation of the analytical procedure (Rout *et al*. 2008). The HPTLC me-

Table 1 Method validation parameters for curcuminoids.

Parameters	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
Instrument precision (%CV, n=7)[a]	0.46	0.79	0.92
Repeatability of method (%CV, n=6)	1.75	1.80	2.25
Intermediate precision (%CV, n=6)	1.89	2.12	2.38
Limit of detection (ng/spot)	15	15	15
Limit of quantification (ng/spot)	50	40	50
Correlation coefficient	0.9999	0.9991	0.9986
Linearity range (ng/spot)	50-300	40-200	50-250
Robustness[b]	Robust	Robust	Robust
Specificity	Specific	Specific	Specific

[a] n = Number of determinations
[b] Robustness is the ability of the procedure to give the same results with small deliberate changes in experimental parameters

Table 2 Intra-day and inter-day precision of curcuminoids.

Marker compound	Concentration	Intra-day precision		Inter-day precision	
		Mean area	% R.S.D[a]	Mean area	% R.S.D[a]
Curcumin	80	888.5	1.52	835.2	2.13
	130	1392.7	1.75	1381.6	1.87
	180	1982.6	1.98	1925.3	1.66
Demethoxycurcumin	80	1194.6	1.67	1187.4	1.98
	120	1969.0	1.42	1950.7	2.13
	160	3195.5	2.11	3163.8	2.24
Bisdemethoxycurcumin	100	770.0	2.13	757.3	2.60
	150	1216.5	2.63	1208.1	2.02
	200	1528.2	1.98	1496.2	2.53

[a] Relative Standard Deviation

Table 3 Results from the recovery study of curcuminoids by HPTLC.

Compound	Amount present in the sample (ng)	Amount spiked (ng)	Amount found[a] (ng)	Recovery (%)	Average Recovery (%)
Curcumin	80.96	50	128.94	98.46	99.71
	80.96	100	180.13	99.54	
	80.96	150	233.55	101.12	
Demethoxycurcumin	50.37	50	98.18	97.82	98.74
	50.37	100	148.46	98.73	
	50.37	150	199.70	99.67	
Bisdemethoxycurcumin	53.29	50	101.40	98.17	99.23
	53.29	100	152.09	99.22	
	53.29	150	203.90	100.30	

[a] Each value is the mean of three determinations.

thod was validated for precision, repeatability (**Tables 1**, **2**), and accuracy (**Table 3**). The instrument precision was studied by repeat scanning of curcumin (150 ng), demethoxycurcumin (200 ng), and bisdemethoxycurcumin (250 ng) six times, and CV was calculated. Intra-day precision was determined by analyzing aliquots of a standard solution over the entire calibration range of curcumin (80, 130, and 180 ng/spot), demethoxycurcumin (80, 120, and 160 ng/spot), and bisdemethoxycurcumin (100, 150, 200 ng/spot), six times on the same day (intraday precision) and inter-day precision was determined by analyzing same standard solution concentration for six days, the results were expressed as CV.

The method's accuracy was tested from the recovery experiment, which was performed by spiking a known amount of standard at three different levels (50, 100, 150 ng for each compound). The percentage recovery for curcumin was found to be 98.46, 99.54, and 101.12% with an average of 99.71%; for demethoxycurcumin 97.82, 98.73, and 99.67% with an average of 98.74%; and for bisdemethoxycurcumin 98.17, 99.22, and 100.3% with an average of 99.23% (**Table 3**). No significant change in the R_F of curcuminoids was observed when the composition of the mobile phase was varied slightly and it was also found that the analytes were stable in solution as well as in adsorbent layer, which confirmed the robustness of the method. It was found that the other components present in the extract were not interfering with the analysis of curcuminoids indicating the specificity of the method for the analysis of marker compounds.

The content of curcumin, demethoxycurcumin and bisdemethoxycurcumin in four varieties of *C. longa* samples were determined in triplicate by the above method and presented in **Table 4**.

Table 4 Percentage content of curcuminoid in different Haridra sample.

Samples	Curcumin[a]	Demethoxycurcumin[a]	Bisdemethoxycurcumin[a]
s_1	8.516	2.730	4.942
s_2	4.892	2.621	4.231
s_3	4.328	2.548	3.329
s_4	2.025	1.511	2.252

s_1- Kandhamal Sample , s_2- Sambalpur Sample, s_3- Bhubaneswar Sample, s_4- Market Sample
[a]Each value is the mean of three determinations.

CONCLUSION

The established method for quantitative analysis of bioactive markers in the rhizomes of *C. longa* was found to be very simple and rapid since the chromatogram development distance is very short. So, it can be used for routine quality evaluation of the raw materials in a short time. Among the samples investigated, the Kandhamal sample (s_1) contained the highest, where as the market sample (s_4) has the lowest percentage of curcuminoids. Therefore, s_1 may be treated as the most potent variety available in the state of Orissa (India) for its commercial utility.

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