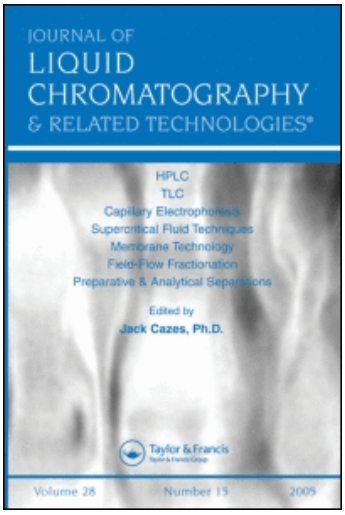

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Improved HPTLC Method for Determination of Curcuminoids from *Curcuma longa*

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Improved HPTLC Method for Determination of Curcuminoids from *Curcuma longa*

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Abstract: A rapid, improved, simple, and precise high performance thin layer chromatography method has been developed for the quantification of curcuminoids in *Curcuma longa*. Separation of curcuminoids was achieved by employing a mobile phase consisting of chloroform-methanol (98 : 2 v/v) on precoated HPTLC LiChrosphere aluminium plates Si $60F_{254}$. Densitometry analysis was carried out at 366 nm in adsorption reflection mode. The method was validated for precision and recovery. Use of LiChrosphere HPTLC plates gave better resolution, reproducibility, good selectivity and compact band as compared to other plates. Plant material collected from mid-hills showed higher contents of curcuminoids.

Keywords: *Curcuma longa*, HPTLC, Curcumin, Demethoxycurcumin, Bis-demethoxycurumin

INTRODUCTION

The genus *Curcuma*, a perennial herb, is a member of the Zingiberaceae (ginger) family and cultivated in Asia, India, China, and other tropical countries. *Curcuma longa* grows to a height of 0.9–1.5 m, has oblong, pointed leaves and bears funnel-shaped yellow flowers.[1] The rhizomes of *C. longa* are used in foods for both its flavour and color. In Chinese and Ayurvedic systems of medicine, it is used as an anti-inflammatory agent and in the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic.[2]

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Present research is focused on antioxidant, hepatoprotective, anti-carcinogenic, and anti-microbial properties of turmeric, in addition to its use in cardiovascular diseases and gastrointestinal disorders. Curcumin is the main bioactive compound, possessing anti-inflammatory, hepatoprotective, anti-microbial, wound healing, anti-cancer, anti-tumor, and anti-viral properties.[3]

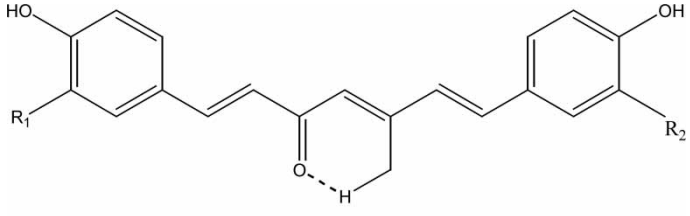
The main yellow coloured substances in the rhizomes of *Curcuma* species are curcumin, demethoxycurcumin, and bis-demethoxycurumin (Figure 1).[4] Separation of the curcuminoids has been achieved by thin layer chromatography and paper chromatography.[5,6] Spectrophotometric methods lack precision due to interference by other pigments present in the plant.[7,8] A few HPLC methods have been reported for quantification of curcuminoids.[3,9–11] Due to its simplicity, accuracy, and lower cost than HPLC, a TLC scanning procedure[12,13] for curcumin, HPTLC, and a fingerprint preparation method for curcuminoids have been reported.[14,15]

As a continuation to our work on development of rapid and simple methods for the determination of bioactive substances from various medicinal plants of commercial utility,[16–18] herein we report an improved method for rapid determination of curcumoids from *Curcuma longa* from different locations of the western Himalayas. This is the first report on LiChrospere HPTLC plates for the quantification of curcuminoids in *Curcuma longa.*

EXPERIMENTAL

Chemicals

All the solvents were of analytical grade from Merck (India). The HPTLC Aluminum sheet, LiChrosphere $60F_{254}$ (20×20 cm) (Cat. No. 1.05586.0001) was purchased from E. Merck (Darmstadt, Germany).



$R_1 = R_2 =$ OCH_3, CURCUMIN

$R_1 =$ OCH_3, $R_2 =$ H, DEMETHOXYCURCUMIN

$R_1 = R_2 =$ H, BIS-DEMETHOXYCURCUMIN

Figure 1. Structures of curcuminoids.

Curcuminoids, namely curcumin, demethoxycurcumin, and bis-demethoxycurcumin were isolated by column chromatography and preparative TLC from the acetone extract of *Curcuma longa*, and identity of the compounds was confirmed by spectral analysis.[15]

Plant Material

Samples of *C. longa* were collected from different altitudes ranging from 610–3165 m amsl of the western Himalayan region. The collected rhizomes were dried at room temperature (25°C–30°C).

Stock Solution

The stock solutions containing (1 mg/20 mL) of curcumin, demethoxycurcumin, and the bis-demethoxycurcumin were prepared in methanol. Appropriate quantities of these stock solutions were spotted to obtain curcumin, demethoxycurcumin, and bis-demethoxycurcumin in the range of 100–1000 ng.

Preparation of Crude Extract

Air dried (25–30°C) 0.1 gm of *C. longa* rhizomes were extracted with acetone (3 × 10 mL), filtered and concentrated under vacuum, dissolved in methanol, and made up in a 5 mL volumetric flask for HPTLC analysis.

Apparatus

A Camag HPTLC system equipped with an automatic TLC sampler ATS4, Scanner 3, and integrated software Win CATS version 1.2.3 was used for the analysis. HPTLC was performed on a precoated HPTLC LiChrosphere Si $60F_{254}$ (20 × 20 cm) plate. Samples were applied on TLC plates using a spray on technique.

Chromatographic Conditions

Chromatographic studies were performed using the following conditions. Stationary phase: HPTLC LiChrosphere aluminium sheet silica $60F_{254}$ precoated (20 × 20 cm); Mobile phase: Chloroform: Methanol (98: 02 v/v); Volume of mobile phase: 20 mL; Chamber saturation time: 30 min; Temperature: 25 ± 1°C; Relative humidity: 35–40%; Migration distance: 80 mm; Migration time: 25 min; Wavelength of detection: 366 nm; Scanning speed: 20 mm/s; Data resolution: 100 μm/step; Band width: 6 mm; Space between two bands: 10 mm.

A Camag Video Documentation system in conjunction with the Reprostar 3 was used for imaging and archiving the thin layer chromatograms. The object was captured by means of a high sensitive digital camera with 4.0 M pixel CCD sensor and 3× optical zoom, model Power shot G2 (Canon, Singapore) Figure 2. A special digitizing board (frame grabber) assisted in rapid processing via the personal computer system. Image acquisition processing and archiving were controlled via Win CATS software.

Chromatographic Separation

Each extract of 5 μL *C. longa* solution was spotted on the HPTLC LiChrosphere plate, 10 mm from the bottom and 10 mm from the side, using a Camag ATS4 automatic TLC sampler spotting device. The TLC plate was developed in ascending mode in a twin trough chamber pre-saturated for 30 min with mobile phase, chloroform-methanol (49 : 1 v/v; 20 mL). The plate was removed from the chamber, dried in air, and scanned in absorbance/reflectance mode of a Camag TLC scanner 3 at 366 nm (Figure 3). Peak area data were recorded using Camag Win CATS software.

Calibration Curve

Standard solution volumes of 2–10 μL of curcumin, demethoxycurcumin, and bis-demethoxycurcumin were applied to the plate corresponding to a concentration of 100 ng–1,000 ng for the preparation of a 5-point calibration curve.

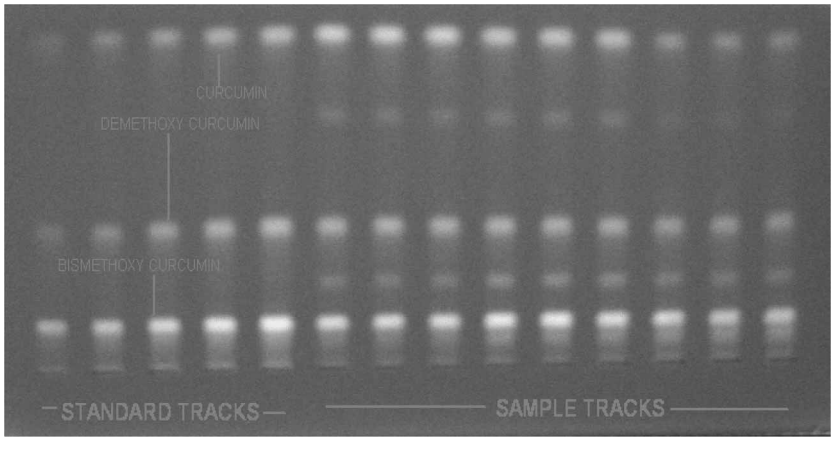


Figure 2. CCD image of TLC plate of *Curcuma longa* at 366 nm. Lane: 1–5 = standards of curcumin, demethoxycurcumin, bis-demethoxycurcumin, 6–14 = samples from different altitude.

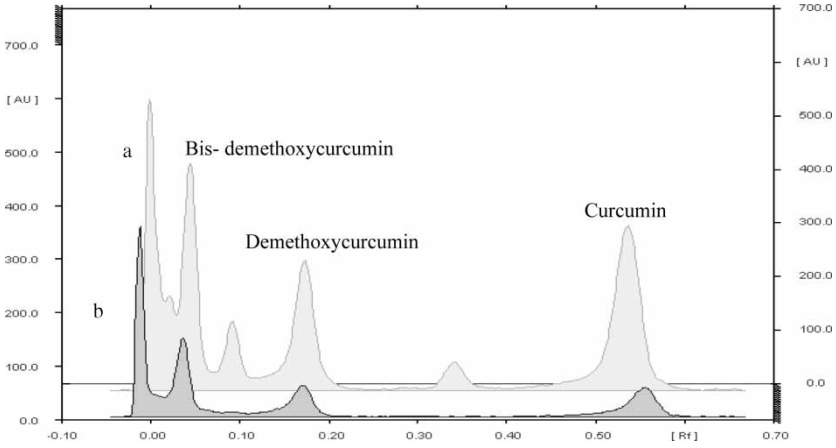


Figure 3. 3D overlay chromatogram of (a) samples of rhizomes of *Curcuma longa* and (b) standard track.

Validation of HPTLC Densitometry Method Specificity

Specificity

The specificity of the method was ascertained by analyzing standard compounds and samples. The spots for curcumin, demethoxycurcumin, and bis-demethoxycurcumin in samples were confirmed by comparing the R_f and spectra of the spots with that of the standards. The peak purity of curcuminoids was assessed by comparing the spectra at three different levels, i.e, peak start, peak apex, and peak end positions of the spot.

Accuracy

The results summarized in Table 1 show the accuracy of the method according to mean values and the %CV value calculated from the three analyses for curcuminoids.

Precision

Six bands of 10 μL of each curcumin, demethoxycurcumin, and bis-demethoxycurcumin were applied from a single stock solution (1000 ng each) on LiChrosphere Si $60F_{254}$ plates and analyzed by the proposed method for system precision studies (Table 2). To determine variations due to the instrument, six different samples of the same concentration (1000 ng each) were spotted on HPTLC LiChrosphere plates and analyzed by the proposed method to determine variations arising due to method itself (Table 3).

Table 1. Curcuminoids content in *C. longa* from different locations of western Himalaya by HPTLC method

S. No.	Altitude (m)	Longitude	Latitude	HPTLC values in triplicate (ng) per spot				Mean per spot (ng)	CV (%)
1	350	77°45′ 9.98E	31°19′ 49.47 N	C[a]	304.1	306.7	306.2	305.7	0.45
				D[b]	127.3	127.5	124.8	126.5	1.17
				B[c]	114.2	116.3	235.4	115.7	1.11
2	754	76°55′ 6.86E	31°43′ 0.21 N	C	614.1	618.6	594.4	609.0	2.11
				D	511.5	507.4	503.0	507.0	0.83
				B	121.7	117.2	117.8	118.9	2.01
3	800	76°30′ 47.81E	31°40′ 49.97 N	C	389.6	397.5	430.2	405.8	5.30
				D	318.6	320.3	310.1	316.3	1.73
				B	111.8	111.1	104.0	109.0	3.94
4	1000	76°16′ 3.54E	32°06′ 14.94 N	C	732.1	727.0	734.2	731.1	0.50
				D	714.4	724.6	738.8	725.9	1.69
				B	132.8	126.1	128.8	129.2	2.57
5	1290	77°5′ 47.96E	31°57′ 46.48 N	C	755.2	749.8	756.7	753.9	0.48
				D	533.1	501.7	496.9	510.6	3.85
				B	116.8	113.4	111.6	113.9	2.32

6	1350	76°47′ 0.51E	31°59′ 14.91 N	C	479.0	487.4	488.4	484.9	1.07
				D	486.2	464.6	473.2	474.7	2.28
				B	125.2	123.0	129.4	125.9	2.58
7	1400	76°07′ 35.04E	32°06′ 36.84 N	C	235.4	237.4	238.4	237.1	0.64
				D	105.2	105.5	103.4	104.7	1.09
				B	103.0	103.5	102.0	102.8	0.75
8	2769	76°07′ 47.96E	32°33′ 52.94 N	C	514.0	497.3	497.4	502.9	1.91
				D	205.4	201.9	190.0	199.1	4.05
				B	129.5	124.3	126.9	126.9	2.02
9	3165	77°02′ 29.00E	32°34′ 34.37 N	C	250.4	242.0	245.5	246.0	1.71
				D	148.5	149.5	147.5	148.5	0.65
				B	117.8	117.6	115.8	117.0	0.92

C^a = Curcumin.
D^b = Demethoxycurcumin.
B^c = Bis-demethoxycurcumin.

Table 2. System precision study of the method

S. No.	Area of curcumin	Area of demethoxycurcumin	Area of bis-demethoxycurcumin
1	7286	7020	17883
2	7321	6631	17683
3	7559	7030	17844
4	7272	7152	17873
5	7689	6963	17850
6	7116	6650	17829
Mean average	7425.9	6959	17827
CV (%)	2.53	2.81	0.46

Recovery

The recovery of the method was determined at two levels (50% and 100% addition) by adding a known amount of curcumin, demethoxycurcumin, and bis-demethoxycurcumin to the powder of the *C. longa* rhizomes of pre-analysed samples, and the mixtures were analysed according to the proposed method. The average recoveries for curcumin, demethoxycurcumin, and bis-demethoxycurcumin were 99.79, 96.97, and 99.48 at two levels, respectively, (Table 4).

Ruggedness

The ruggedness of the proposed method was studied using reagents from different lots and different manufacturers.

Table 3. Method precision study of the method

S. No.	Area of curcumin	Area of demethoxycurcumin	Area of bis-demethoxycurcumin
1	6980	6880	18230
2	6992	6902	18140
3	7062	6709	18235
4	7107	6808	18143
5	7034	6878	18175
6	7036	6872	18124
Mean average	7035.16	6841.50	18174.5
CV (%)	0.6	1.0	0.26

Table 4. Recovery studies of curcumin, demethoxycurcumin and bis-demethoxycurcumin by the HPTLC method (n = 3)

S. No.	Compound	Amount present (ng)	Amount of std. added (ng)	Average amount found in mix (ng)	Average recovery (%)
1	Curcumin	104.7	100	206.2	100.73
	Demethoxycurcumin	237.1	200	416.9	95.37
	Bis-demethoxycurcumin	102.8	100	198.3	97.78
2	Curcumin	104.7	200	301.2	98.85
	Demethoxycurcumin	237.1	400	628.0	98.57
	Bis-demethoxycurcumin	102.8	200	306.4	101.18

Limit of Detection and Limit of Quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained in the experimental section. The signal to noise ratio was determined. The limit of detection was calculated to be three times of the SD (3 : 1), and ten times of the SD value (10 : 1) gave the limit of quantitation (Table 5).

RESULTS AND DISCUSSION

Various compositions and combinations of the mobile phase were tried for the desired resolution of curcumin, demethoxycurcumin, and bis-demethoxycurcumin. A solvent combination of chloroform and methanol (98 : 02, v/v) gave a dense and compact spot. The identification of curcuminoids was carried out by matching the R_f values of curcuminoids in samples with the standard track, and further, they were confirmed by *in situ* UV-VIS

Table 5. Linear regression equations and R_f values for curcuminoids

Compounds	R_f	Regression equation	r^a	Sdv.	LOD (ng)	LOQ (ng)
Curcumin	0.79	$Y = 297.883 + 5884.330 \times X$	0.99776	3.77	40	100
Demethoxycurcumin	0.37	$Y = 34.663 + 2881.132 \times X$	0.99941	2.07	40	100
Bis-demethoxycurcumin	0.16	$Y = 1551.557 + 10325.040 \times X$	0.99616	4.62	20	100

r^a = Correlation coefficient.

spectra of the above bands of the densitogram. The sample bands at R_f 0.79, 0.36, and 0.16 corresponded to curcumin, demethoxycurcumin, and bis-demethoxycurcumin, respectively (Figure 2). The calibration curves of the curcuminoids were linear in the range of 100–1000 ng. Linear regression equations, R_f values, and standard deviations are given in (Table 5). For the examination of recovery rates, known amounts (50 and 100%) of known stock solutions of pure curcuminoids were added to one of the samples; the analysis was repeated three times, and average values for curcumin, demethoxycurcumin, and bis-demethoxycurcumin were 99.79%, 96.67%, and 99.48%, respectively.

The present HPTLC method (use of LiChrosphere plate) overcomes the problem of broadness of the spots. The chromatographic separation before quantitation permits determination of low levels of curcuminoids without any interference. The minimum solvent consumption, offline technique, zero waiting time for the instrument set up, makes the method more suitable for the routine analysis in a crop improvement plan. Plate to plate variation and limit of quantitation (as compared to LC) can be a limiting factor as far as the use of HPTLC as a method of analysis is concerned. However, this limitation can be overcome by spotting known concentrations along with the sample in each chromatographic run. Higher contents of curcuminiods (755.26 ng, 533.16 ng, 116.8 ng, and 732.17 ng, 714.40 ng, and 132.8 ng) of curcumin, demethoxycurcumin, and bis-demethoxycurcumin, respectively, in the midhills at 1,290 m and 1,000 m amsl of Himalaya were recorded, whereas, at higher (3,165 m and 2,769 m) altitudes and lower altitudes (350 m and 800 m), low contents of these curcuminiods were observed. Further studies are needed to acertain the role of the age and stage of harvest on curcuminoids contents in *Curcuma longa*. Curcuminoids contents of all samples collected from different altitudes are given in Table 1.

CONCLUSION

The proposed HPTLC method of analysis with the use of HPTLC LiChrosphere aluminium plates, Si $60F_{254}$, is rapid, selective, and gives good compact bands and reproducible peaks. The method is found to be economical and can be employed for routine and large-scale analysis with reproducible results.

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