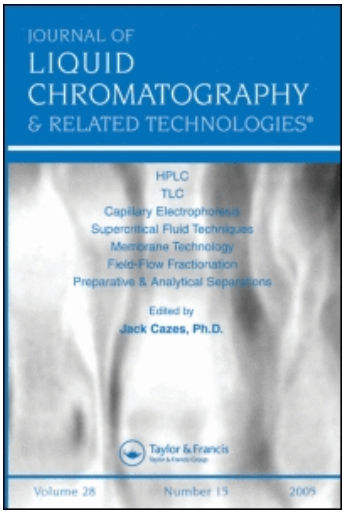

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SIMULTANEOUS DETERMINATION OF CURCUMINOIDS IN *CURCUMA* SAMPLES USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A rapid and simple high performance thin layer chromatographic method has been developed for the simultaneous quantitation of pharmacologically important diaryl heptanoids curcumin, demethoxy curcumin, and bis-demethoxy curcumin in *Curcuma longa* and *C. amada*. The assay combines the isolation and separation of curcuminoids on silica gel $60F_{254}$ high performance thin layer chromatographic plates, followed by scanning of the spots at 366 nm using a UV detection mode.

INTRODUCTION

The rhizomes of plants in the genus Curcuma have a traditionally important role as a coloring agent in food, cosmetics, and textiles. *Curcuma amada* Roxb. (Zingiberaceae), commonly known as mango-ginger is one of the species with rhizomes having the characteristic odor of raw mangoes. Its root is considered to be stomachic, bitter, aromatic, cooling, astringent, and carminative.[1] Rhizomes are used externally in the form of paste over sprains and skin diseases.

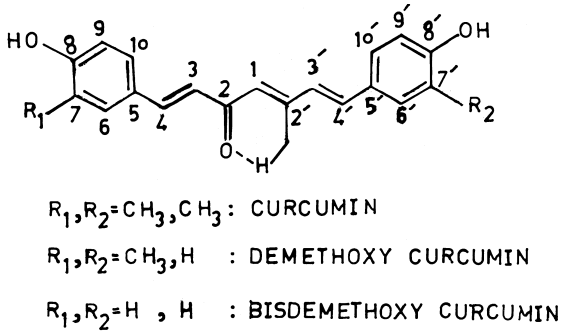

Figure 1. Chemical structures of curcuminoids.

Combined with other medicines, they are also used for improving the quality of blood.[2] The main colored substances in the rhizomes of *Curcuma* species are curcumin and two related demethoxy compounds, demethoxy curcumin and bis-demethoxy curcumin (Fig. 1). Curcumin is the main active compound, possessing anti-inflammatory, hepatoprotective, anti-microbial, wound healing, anticancer, anti-tumor and anti-viral[3] properties. Usually, separation of the curcuminoids has been achieved by thin layer chromatography/ paper chromatography.[4,5]

Spectrophotometric methods[6,7] lack precision due to interference by other pigments present in the plant.

A few HPLC methods are reported for quantification of curcuminoids.[3,8-11] Due to its simplicity, accuracy, and lower cost than HPLC, HPTLC is now utilized frequently in the crop improvement programs involving rapid plant screening.

Although a TLC-scanning procedure[12] for curcumin and an HPTLC fingerprint preparation method[13] for curcuminoids have been reported, HPTLC methods for simultaneous analysis of major curcuminoids in *Curcuma* species have not yet been reported.

Here, we have developed a simple high performance thin layer chromatographic method for the rapid analysis of major curcuminoids in *C. longa* and *C. amada*. The method was found suitable for rapid screening of plant materials for their genotypic assessment and can be performed without any special sample pretreatment.

EXPERIMENTAL

Plant Material

The plant materials of *C. longa* and *C. amada* were obtained from the experimental farm of this Institute at Lucknow. The sample materials of the genotypes used are available in the Gene Bank of this Institute.

Curcuminoids

Curcumin, demethoxy curcumin, and bis-demethoxy curcumin have been isolated by column chromatography ($CHCl_3$-MeOH; 98:2, 95:5) of the acetone extract of *C. amada*. Compounds were identified by spectral analysis.

Reagents

Reagents used were from E. Merck, India and the HPTLC plates used were from E. Merck, Germany.

Isolation of Curcuminoid Standards

Curcumin, demethoxy curcumin, and bis-demethoxy curcumin were isolated from the acetone extract of the *C. amada* rhizomes as shown below. About 100 g of rhizomes were extracted using acetone and a 7 g extract sample was column chromatographed over silica gel for the isolation of curcuminoids.

Curcumin (A) was obtained from fractions 12-20 of chloroform-methanol (98:2) and crystallized in MeOH, melting point, 183-185 ^{0}C, ^{1}H NMR $(CD_3)_2CO$ δ: 3.95 (6 H, s, 2 x OCH_3), 5.82 (1 H, s,1-H), 6.48 (2 H, d, J=16 Hz, 3, 3' -H_2), 6.84 (2 H, d, J = 8 Hz, 9, 9' - H_2), 7.22 (2 H, brd, J = 8 Hz , 10, 10' - H_2), 7.28 (2 H, brs, 6, 6' - H_2), 7.62 (2 H, d, J = 16 Hz , 4, 4' - H_2).

Demethoxy curcumin (B), isolated from fractions 64-77 of chloroform-methanol (95:5) and crystallized in MeOH, melting point, 170-173 ^{0}C, ^{1}H NMR $(CD_3)_2CO$ δ: 3.95 (3 H, s, OCH_3), 5.96 (1 H, s, 1-H), 6.62 (2 H, d, J = 16 Hz, 3, 3' - H_2), 6.85 (2H, d, J = 8Hz, 9, 9' - H_2), 6.88 (1 H, d, J = 8 Hz, 7-H), 7.15 (1 H, brd, J = 8 Hz, 10 H), 7.30 (1 H, brs, 6 H), 7.52 (2 H, d, J = 8 Hz, 6'-H, 10'-H), 7.60 (2 H, d, J = 16 Hz, 4, 4' - H_2).

Bis-demethoxy curcumin (C) was isolated by preparative thin layer chromatography of fractions 90-110) from chloroform-methanol (95:5). Preparative thin layer chromatography was performed in chloroform-methanol (95:5) on a preparative Si gel plate. Compound C was crystallized in MeOH, melting point, 222-225 ^{0}C, ^{1}H NMR $(CD_3)_2$ CO δ: 5.90 (1 H, s, 1-H), 6.58 (2 H, d, J = 16 Hz , 3, 3' - H_2), 6.82 (4 H, d, J = 8 Hz, 7, 7', 9, 9' - H_4), 7.50 (4 H, d, J = 8 Hz, 6, 6', 10, 10' - H_4), 7.60 (2 H, d, J = 16 Hz, 4, 4' - H_2). All structures were confirmed by comparison with spectral analysis data reported in literature.[14]

Analytical Procedures

Chromatographic conditions

Chromatography was performed on a pre-activated (110^{0}C) silica gel HPTLC plate 60$F_{254,}$ 10x10 cm. Samples and standards were applied to the plate as 6 mm wide bands with an automatic TLC applicator, Linomate IV, with N_2 flow (Camag, Muttenz, Switzerland), 10 mm from the bottom of the plate at a delivery speed of the syringe 10 s/µL. The application parameters were identical for all the analyses performed.

Detection of curcumin and related compounds

The TLC plates were developed using a Camag twin-trough glass tank which was pre-saturated with the mobile phase chloroform-methanol (95:5) for 1 hour and each plate was developed to a height of about 8 cm. The composition of mobile phase was optimized by using different mobile solvents of varying polarity. The TLC runs were made under laboratory conditions of 25 ± 5^{0}C and 50 % relative humidity. After development, the plate was removed and dried and spots were visualized in UV light (UV cabinet, Camag, Switzerland).

Quantification

Curcuminoids were quantified with a Camag TLC Scanner 3, equipped with CATS4 software and computer under the following conditions: slit width 6 x 0.4 mm, wave length 366 nm, absorption/reflection detection mode.

Extraction procedure

Air dried (35-50^{0}C) rhizomes of *C.. amada* and *C. longa* (0.2 g each) were extracted separately in 10 mL acetone for 12 hours, filtered, and evaporated. The individual extracts of *C. amada* and C. *longa* were re-dissolved in 0.5 and 2.0 mL of acetone, respectively, for quantification.

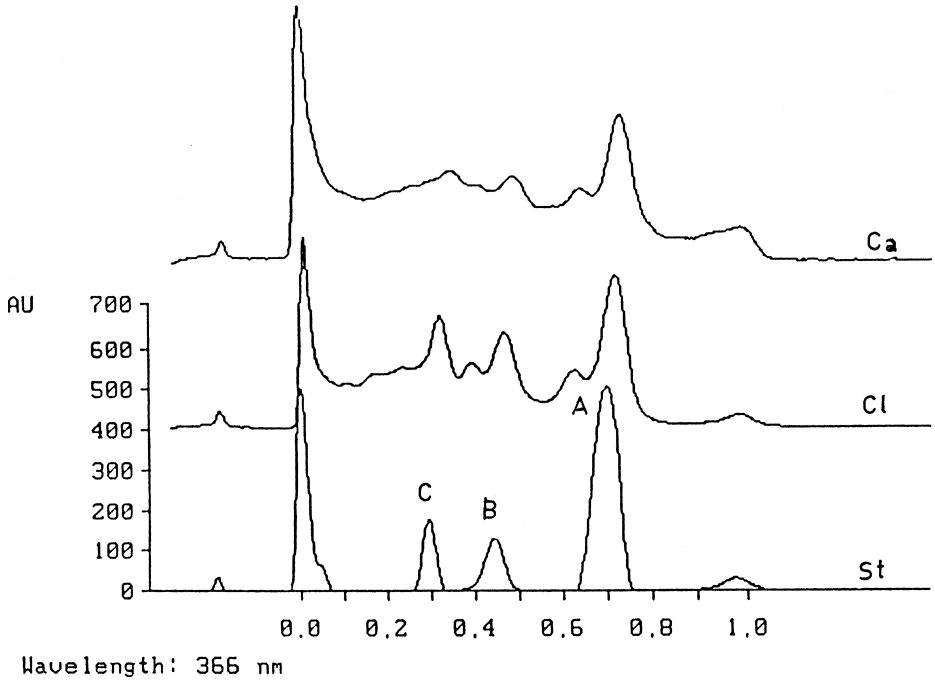

Figure 2. HPTLC separation of curcumin (A), demethoxy curcumin (B), and bis-demethoxy curcumin (C) in standard (St), *Curcuma longa* (Cl) and *Curcuma amada* (Ca) tracks at 366 nm UV absorption/reflection mode.

Calibration graphs

Stock solutions of curcumin, demethoxy curcumin (1mg/mL) and bis-demethoxy curcumin (0.5 mg/mL) were prepared in acetone and different amounts (1-20 µL) of these were loaded on a TLC plate, using Linomate IV for preparing calibration graphs.

RESULTS AND DISCUSSION

Different compositions of the mobile phase were tested and the desired resolution of curcuminoids with symmetrical and reproducible peaks was achieved by using chloroform-methanol (95:5) as mobile phase (Fig. 2). Peaks corresponding to curcumin (A), demethoxy curcumin (B), and bis-demethoxy curcumin (C) were at R_f. 0.69, 0.44, and 0.29, respectively.

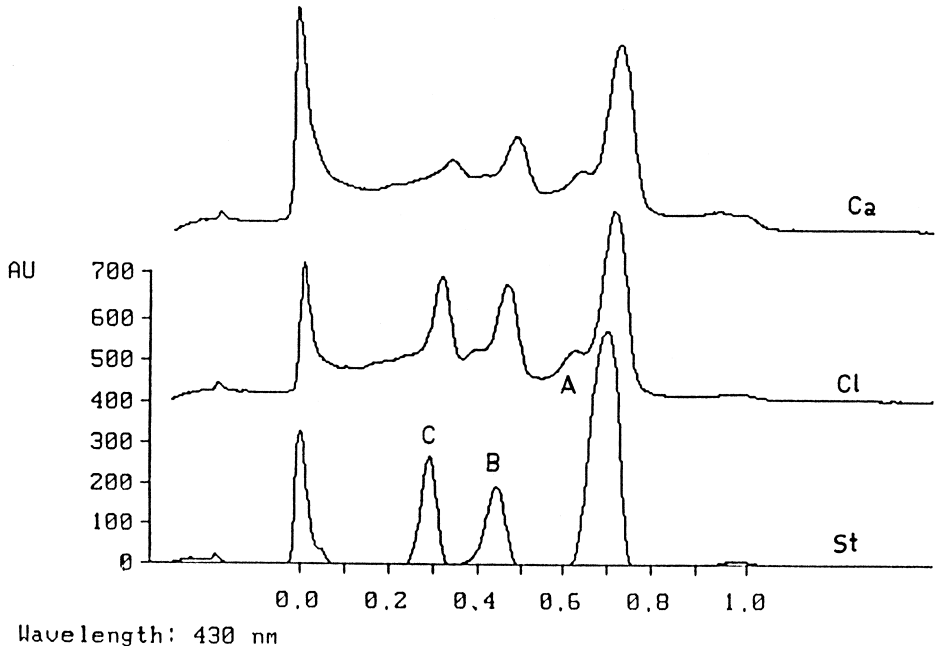

Figure 3. HPTLC separation of curcumin (A), demethoxy curcumin (B), and bis-demethoxy curcumin (C) in standard (St), *Curcuma longa* (Cl) and *Curcuma amada* (Ca) tracks at 430 nm visible absorption/reflection mode.

Table 1

Linear Regressions and R_f for Curcuminoids

Curcuminoids	R_f	Equation	r
Curcumin	0.69	Y = 567.69X - 92.526	0.999
Demethoxy Curcumin	0.44	Y = 162.97X - 22.451	0.998
Bis-Demethoxy Curcumin	0.29	Y = 105.09X + 3.0301	0.998

The calibration curves of A, B, and C were linear in the range of 1 μg to 20 μg. Linear regression and R_f for these curcuminoids are given in Table 1. For the examination of recovery rates, known amounts of stock solutions of pure A, B and C were added in *C. longa* rhizome extract and quantitative

Table 2

Effect of Solvent in the Extraction of Different Curcuminoids from the Rhizomes of the Accession No. 2 of *Curcuma longa*

Solvent Used For Extraction	- - - - - - - % Content - - - - - - - Curcumin (A)	Demethoxy Curcumin (B)	Bis-Demethoxy Curcumin (C)
$CHCl_3$	1.04	0.80	0.68
MeOH	1.62	1.00	0.68
$(CH_3)_2CO$	1.96	1.19	1.02
EtOH	1.80	1.05	0.94

Table 3

Percent Content of Curcuminoids in the Rhizomes of *Curcuma longa* and *C. amada* Accessions

S. No.	Accession No. and Species	A	B	C
1	2 (*C. longa*)	1.96	1.19	1.02
2	5 (*C. longa*)	1.45	1.21	1.08
3	27 (*C. longa*)	0.66	0.46	0.48
4	1 (*C. amada*)	0.16	0.02	0.01

analyses were repeated three times. Values were 95, 95, and 96 % for A, B, and C, respectively. Peak purity tests were done on all the three curcuminoids by comparing the spectra of all these in standard and sample tracks. Fig. 2 and Fig. 3 are the chromatograms, in UV (366 nm) and visible (430 nm) absorption/reflection modes, respectively. Resolution by UV scanning was better than that observed by visible light, as some of the compounds were not fully responding in the sample track when the visible mode was applied.

Acetone was suitable for extraction, as the recoveries of curcuminoids were optimum in this solvent (Table 2). The results are the average of three readings.

In the method applied here, curcuminoids were well separated from each other, and peaks were symmetrical and suitable for rapid screening purpose of plant samples for the improvement of crops under the plant breeding program. The method has been applied for the analysis of a large number of accessions for crop improvement of *Curcuma* species for curcuminoids. Results of a few are given in Table 3.

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