JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Improved HPLC Method for the Determination of Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin

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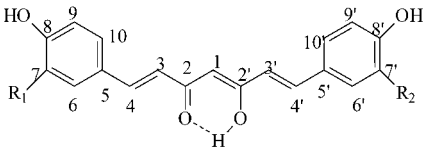
Commercially available curcumin, a bright orange-yellow color pigment of turmeric, consists of a mixture of three curcuminoids, namely, curcumin, demethoxycurcumin, and bisdemethoxycurcumin. These were isolated by column chromatography and identified by spectroscopic studies. The purity of the curcuminoids was analyzed by an improved HPLC method. HPLC separation was performed on a C_{18} column using three solvents, methanol, 2% AcOH, and acetonitrile, with detection at 425 nm. Four different commercially available varieties of turmeric, namely, Salem, Erode, Balasore, and local market samples, were analyzed to detect the percentage of these three curcuminoids. The percentages of curcumin, demethoxycurcumin, and bisdemethoxycurcumin as estimated using their calibration curves were found to be 1.06 ± 0.061 to 5.65 ± 0.040, 0.83 ± 0.047 to 3.36 ± 0.040, and 0.42 ± 0.036 to 2.16 ± 0.06, respectively, in four different samples. The total percentages of curcuminoids are 2.34 ± 0.171 to 9.18 ± 0.232%.

KEYWORDS: ***Curcuma longa*; curcuminoids; curcumin; demethoxycurcumin; bisdemethoxycurcumin; HPLC analysis**

INTRODUCTION

Turmeric (*Curcuma longa* L.) is a coloring agent, and it has been found to be a rich source of phenolic compounds, namely, curcuminoids (*1*). *C. longa* extracts contain three different diarylheptanoids, curcumin (diferuloylmethane), demethoxycurcumin (*p*-hydroxycinnamoyl, feruloylmethane), and bisdemethoxycurcumin (di-*p*-hydroxycinnamoylmethane) (**Figure 1**). Commercially available curcumin consists of a mixture of three naturally occurring curcuminoids with curcumin as the main (≈77%) constituent (*2*). Curcuminoids are responsible for the biological activity of *C. longa* and present to the extent of 2−5% (*1*). Curcuminoids are recognized for their broad spectrum of biological activities and safety in foods or pharmaceuticals. Curcumin, the principal natural yellow pigment, is widely used for the coloring of foods, for example, pickles and snacks. (*3*). Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is known for its antioxidant (*4*), anti-inflammatory, antimicrobial, antiparasitic, antimutagenic, and anticancer properties (*5*). The potential use of curcumin in the prevention of cancer and in the treatment of infection with human immunodeficiency virus (HIV) is the subject of intensive laboratory and clinical research (*5*). Recently, the effect of curcuminoids was examined on the proliferation of MCF-7 human breast tumor cells. It was reported that demethoxycurcumin was the best inhibition of MCF-7 cells followed by the curcumin and bisdemethoxycurcumin (*6*). Kim et al. (*7*) reported the strong antioxidant activity of demethoxycurcumin and bisdemethoxycurcumin to be as efficient as the well-known strong antioxidant curcumin. Ahsan et al. (*2*) reported the antioxidant and pro-oxidant activities of curcumin and the structure relationship between curcumin, demethoxycurcumin, and bisdemethoxycurcumin.



Compound	R1	R2
Curcumin (**1**)	OMe	OMe
Demethoxycurcumin (**2**)	H	OMe
Bisdemethoxycurcumin (**3**)	H	H

Figure 1. Chemical structures of curcuminoids.

A variety of methods for the quantification of the curcuminoids have been reported. Most of these are spectrophotometric methods, expressing the total color content of the sample (*8*). Commercial curcumin/turmeric products contain mixtures of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (*2*). However, it is not possible to quantify the individual curcuminoids with spectrophotometric method. Recently, Gupta et al. (*9*) reported the determination of curcuminoids in turmeric using high-performance thin-layer chromatography. However, linearity was found in the concentration range between 1 and 20 μg. Rasmussen et al. (*10*) reported the simple and efficient separation of curcuminoids using dihydrogen phosphate impregnated silica gel TLC plates. Although this method can be used for the separation and identification of curcuminoids, it may not

10.1021/jf025506a CCC: $22.00
Published on Web 05/18/2002

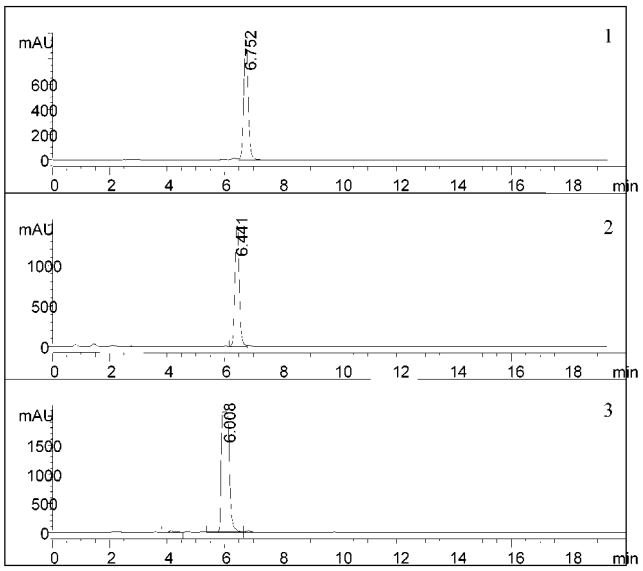


Figure 2. HPLC chromatograms of curcumin (**1**), demethoxycurcumin (**2**), and bisdemethoxycurcumin (**3**).

be possible to quantify individual curcuminoids. Karasz et al. (*11*) and Tonnesen and Karlsen (*12*) reported direct fluorometric methods for the analysis of curcumin in food materials. The analysis of individual curcuminoids is possible by HPLC on normal phase or on reversed phase C_{18} columns (*13*−*15*). Due to the very labile characteristics of curcuminoids C_{18} columns are preferred for HPLC analysis (*16*). Of the three commonly used reversed phase solvents, methanol, acetonitrile, and tetrahydrofuran (THF), methanol does not provide the necessary resolution/selectivity for the separation of curcuminoids. Using THF instead of acetonitrile as the organic modifier reverses the elution order of the curcuminoids (*14*, *15*). He et al. (*17*) reported the on-line HPLC-UV diode array and electrospray mass spectrometry methods used to analyze curcuminoids and sesquiterpenoids in a fresh turmeric extract. The curcuminoids and sesquiterpenoids were identified at a column temperature set at 48 °C. Taylor and McDowell (*18*) reported separation of three curcuminoids, using a non-silica polymeric column and aqueous 55% acetonitrile as the mobile phase. Hiserodt et al. (*19*) reported LC-MS and GC-MS methods for the separation of curcuminoids. These involve an octadecyl stationary phase using a mobile phase consisting of ammonium acetate with 5% AcOH and acetonitrile. The presence of inorganic salt may contaminate the mass spectrometer ion source.

The pigment curcumin is industrially produced using turmeric oleoresin as the starting material. The mother liquor (∼70−80%), after isolation of curcuminoids from oleoresin, has a composition of oil, resin, and leftover curcuminoids enriched with demethoxycurcumin and bisdemethoxycurcumin. This fraction has no commercial value at present (*20*−*22*), but the demand for demethoxycurcumin and bisdemethoxycurcumin is increasing due to the discovery of their new biological activities (*2*, *6*, *7*). Hence, a method has been developed for the isolation of curcuminoids from curcumin-removed turmeric oleoresin (CRTO) (*23*). The present paper describes the isolation and identification of curcuminoids from mother liquor by reporting the HPLC separation conditions for curcumin, demethoxycurcumin, and bisdemethoxycurcumin, and the results on the content of the individual curcuminoids are presented.

MATERIALS AND METHODS

Materials. Commercial samples of different turmeric varieties of *C. longa* were obtained: Salem (A), Erode (C) (Tamil Nadu State), Balasore (D) (Orissa State), and local market samples (B) from Mysore (Karanataka State). Spent oleoresin (CRTO) was collected from a local oleoresin industry. All solvents/chemicals used were of AR/HPLC grade and obtained from E-Merck, Mumbai, India.

Equipment. ^{1}H and ^{13}C NMR spectra (DMSO-d_6) were recorded at 400 and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). TMS was used as the internal standard. The HPLC system consisted of a Hewlett-Packard quaternary HPLC model HP 1100 series (Hewlett-Packard, Palo Alto, CA), fitted with a Waters μ-Bondapack (Waters Corp., Milford, MA) C_{18} column (300 × 4.6 mm i.d.). The injection system (Rheodyne) used was a 20 μL sample loop. An HP 1100 series variable-wavelength detector used at a wavelength of 425 nm was used for detection. A Millipore Swinnex type filter (pore size = 0.45 μm) was obtained from Millipore (Bangalore, India).

Isolation of Curcuminoids. Five grams of CRTO was impregnated with 8 g of silica gel, loaded onto a column of silica gel (100 g), and eluted with 500 mL of hexane. Then the column was eluted with benzene and ethyl acetate with increasing polarity to get three 400 mL fractions. Compound **1** was eluted with benzene/EtOAc (82:18 v/v), whereas compounds **2** and **3** were eluted with benzene/EtOAc (70:30 v/v) and benzene/EtOAc (58:42 v/v), respectively. The solvents from elutes were concentrated under vacuum (Büchi, Switzerland) and recrystallized to obtain compounds **1** (110 mg), **2** (223 mg), and **3** (170 mg).

Identification of Compounds. The melting points of compounds **1**, **2**, and **3** were recorded as 186−187, 175−176, and 231−232 °C, respectively. The purified compounds were subjected to TLC on silica gel (10 × 20 cm) plates (E-Merck, Darmstadt, Germany), with chloroform/methanol (98:2) as the developing solvent system and visualized as yellow spots. The R_f values of compounds **1**, **2**, and **3** were found to be 0.55, 0.50, and 0.43, respectively. Furthermore, the isolated compounds were subjected to ^{1}H and ^{13}C NMR spectral analysis.

Preparation of Stock Solution of Curcuminoids for HPLC. Methanolic stock solutions of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were prepared separately at a concentration of 0.5 mg/mL.

Sample Preparation. Turmeric powder samples (1.0 g each) were extracted with hexane (50 mL) by using a Soxhlet extractor for 30 min separately. The hexane extract was discarded, and the powder was re-extracted with 50 mL of methanol for 2 h. One milliliter of this solution was transferred to a 10 mL volumetric flask, and the volume was adjusted to 10 mL with methanol.

Chromatographic Conditions. The elution was carried out with gradient solvent systems with a flow rate of 1.0 mL/min at ambient temperature. The mobile phase consisted of methanol (A), 2% acetic acid (B), and acetonitrile (C). Quantitative levels of curcuminoids were determined using the above solvents programmed linearly from 45 to 65% acetonitrile in B for 0−15 min. The gradient then went from 65 to 45% acetonitrile in B for 15−20 min, with a constant of 5% A. The compounds were quantified using HP ChemStation software.

Validation of HPLC Method. *Calibration and Linearity.* The linearity of the method was evaluated by analyzing a series of standard curcuminoids. Ten microliters of each of the five working standard solutions containing 0.0625−2.0 μg of standard curcumin, demethoxycurcumin, and bisdemethoxycurcumin was injected into the HPLC. The elution was carried out as described above, and standard calibration curves were obtained by plotting the concentration of standard curcuminoids versus peak area (average of three runs).

Range. The calibration range was chosen to reflect normal curcuminoid concentrations in turmeric samples. This range included concentrations from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ).

Determination of the Limit of Quantification (LOQ). The LOQ was defined as the lowest standard curcuminoid concentration, which can be determined with an accuracy and precision of <20%.

Table 1. ^{1}H NMR ($CDCl_3$) Spectral Data of Compounds **1**–**3**[a]

H	1	2	3
1	6.06 (s)	5.97 (s)	6.03 (s)
2-OH	16.41 (bs)		16.4 (bs)
3,3′	7.57 (d, $J = 16$ Hz)	7.60 (d, 16 Hz)	7.56 (d, $J = 15.9$ Hz)
4,4′	6.75 (d, $J = 16$ Hz)	6.69 (d, $J = 16$ Hz), 6.64 (d, $J = 16$ Hz)	7.56 (d, $J = 15.9$ Hz)
6,6′	7.32 (d, $J = 2$ Hz)	7.34 (d, $J = 1.7$ Hz), 6.9 (d, $J = 8$ Hz)	6.84 (d, $J = 8.2$ Hz)
7,7′		7.56 (d, $J = 8$ Hz)	7.56 (d, $J = 8.2$ Hz)
8,8′-OH	9.64 (s)		10.03 (s)
9,9′	6.85 (d, $J = 8.1$ Hz)	7.56 (d, $J = 8$ Hz); 6.88 (d, $J = 8$ Hz)	7.56 (d, $J = 8.2$ Hz)
10,10′	7.16 (dd, $J = 2$, 8.1 Hz)	7.27 (dd, $J = 8$, 1.7 Hz), 6.9 (d, $J = 8$ Hz)	6.84 (d, $J = 8.2$ Hz)
OMe	3.85 (3H, s)	3.92 (3H, s)	

[a] Chemical shifts are followed by coupling constant J values in parentheses. s, singlets; d, doublets.

Table 2. ^{13}C NMR ($CDCl_3$) Spectral Data of Compounds **1**–**3**

C	1	2	3
1	100.8	101.6	100.9
2,2′	183.2	184.4, 184.5	183.2
3,3′	121.1	122.1, 122.3	120.8
4,4′	140.7	141.4, 141.0	140.3
5,5′	126.4	128.2, 127.7	125.8
6,6′	111.5	111.5, 130.9	130.3
7,7′	148.0	148.8, 116.8	115.9
8,8′	149.4	150.0, 160.5	159.8
9,9′	115.8	116.2, 116.8	115.9
10,10′	123.0	123.8, 130.9	130.3
OMe	55.7	56.3	

Determination of Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin in Samples. The sample volume was 20 μL. Curcuminoid concentrations were calculated on the basis of linear calibration functions and with regard to the dilution factor. The content of curcumin, demethoxycurcumin, and bisdemethoxycurcumin was expressed as grams per 100 g of plant material.

RESULTS AND DISCUSSION

Pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin are not commercially available. Hence, curcuminoids **1**−**3** were isolated from the spent turmeric oleoresin by column chromatography on silica gel after initial elution with hexane for the removal of volatile oil. Separation of compounds **1**−**3** was achieved on elution with benzene and ethyl acetate mixtures with increasing polarity. Compounds **1**−**3** showed single spots on TLC. Also, HPLC analysis of compounds **1**, **2**, and **3** showed single peaks at retention times of 6.93 ± 0.092, 6.51 ± 0.065, and 6.11 ± 0.079 min, respectively (**Figure 2**). Compounds **1**−**3** were characterized and identified as curcumin, demethoxycurcumin, and bisdemethoxycurcumin, respectively, using ^{1}H and ^{13}C NMR spectra. Chemical shifts of the curcuminoids were in accordance with reported values (**Tables 1** and **2**) (*24*, *25*). ^{13}C NMR spectral assignments were confirmed with the help of SEFT spectra.

CRTO contains more demethoxycurcumin and bisdemethoxycurcumin as compared to commercially available turmeric samples. Hence, CRTO may be a good source for the isolation of these two curcuminoids. The major curcuminoid in different commercial varieties of turmeric is curcumin, as detected by HPLC (**Figure 3**). Two minor peaks were identified as demethoxycurcumin and bisdemethoxycurcumin by co-injection of standards. The percentage compositions of curcuminoids in four different varieties of *C. longa* by HPLC are summarized in **Table 3**. Curcumin was found to be the major compound in all of the tested varieties. It was found that Erode and Salem varieties have greater amounts of total curcuminoids content. Hence, these two varieties may be good sources for the isolation of curcuminoids. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin were resolved as individual peaks in all of the samples analyzed with no interference from other compounds. The identity of each peak was confirmed by determination of retention times and by spiking with standards.

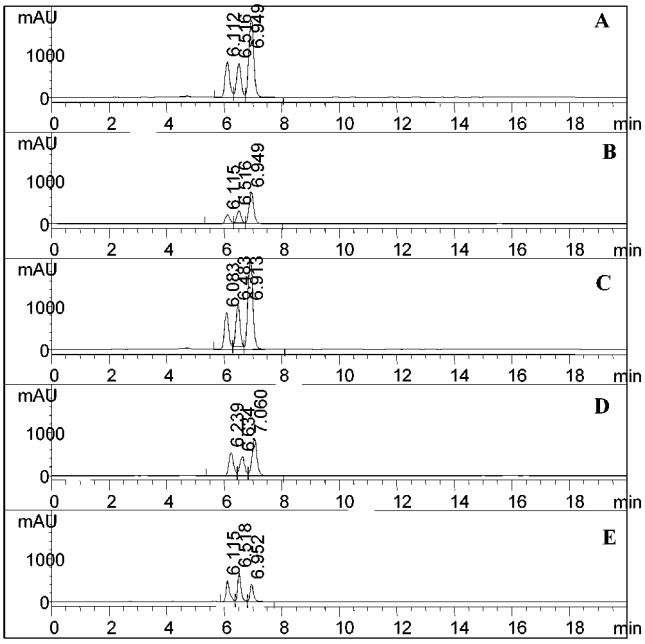


Figure 3. HPLC chromatograms of different varieties of *C. longa*: Salem (A), local (B); Erode (C); Balasore (D); standard bisdemethoxycurcumin, demethoxycurcumin, and curcumin (E).

Table 3. Percentage (w/w) of Composition of Curcuminoids in Four Different Varieties of *C. longa* by HPLC[a]

sample	curcumin	demethoxy-curcumin	bisdemethoxy-curcumin	total
Salem (A)	4.14 ± 0.150	2.88 ± 0.025	2.16 ± 0.060	9.18 ± 0.232
Mysore (B)	1.06 ± 0.061	0.86 ± 0.074	0.42 ± 0.036	2.34 ± 0.171
Erode (C)	4.00 ± 0.061	3.36 ± 0.040	1.75 ± 0.050	9.11 ± 0.150
Balasore (D)	5.65 ± 0.040	0.83 ± 0.047	0.62 ± 0.031	7.10 ± 0.119

[a] Average of three replications.

The purpose of this study was to develop an improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Asakawa et al. (*26*) and Amakawa et al. (*27*) reported that the HPLC system based on C_{18} stationary phases does not completely resolve these three curcuminoids. The separation of colored compounds can be achieved by the use of an amino-bonded stationary phase using the mobile phase with <10% water content. Tonnesen and Karlsen (*12*) and

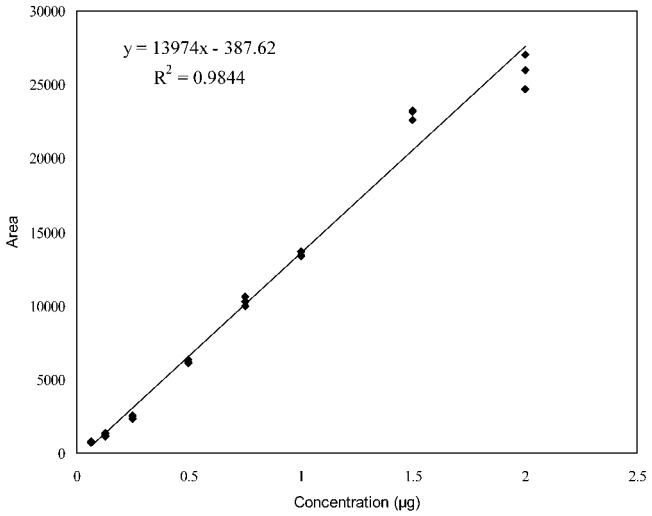


Figure 4. Linear relationship between peak area response and concentration of curcumin.

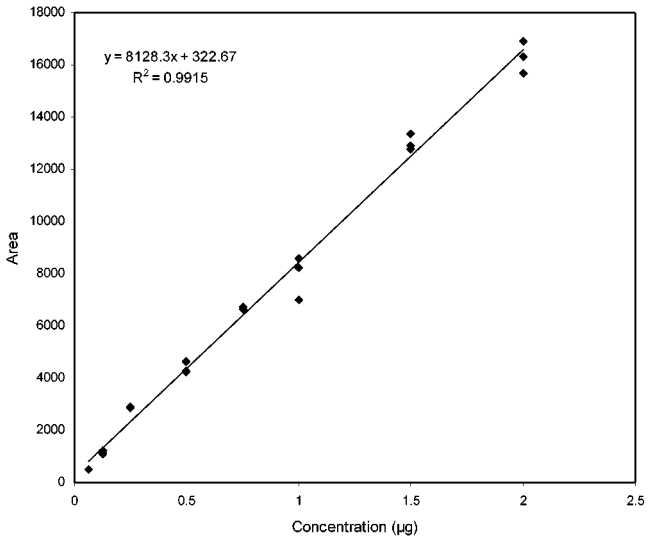


Figure 5. Linear relationship between peak area response and concentration of demethoxycurcumin.

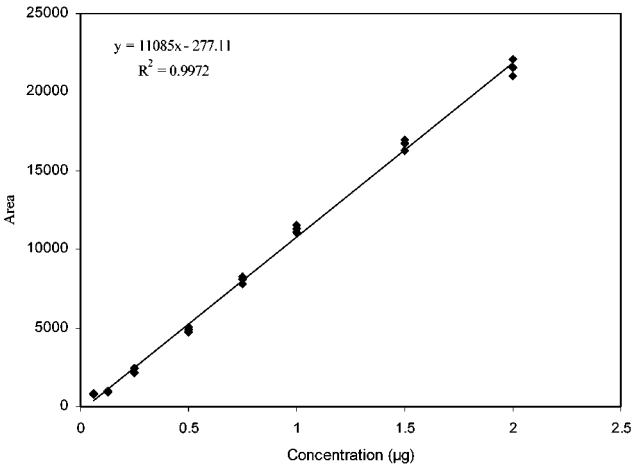


Figure 6. Linear relationship between peak area response and concentration of bisdemethoxycurcumin.

Karasz (*11*) reported the HPLC method for the separation of curcuminoids by using a fluorescence detector. In general, these methods are not stable enough to get reproducible results, and problems such as tailing peaks and poor resolution occur. He et al. (*17*) reported the analysis of curcuminoids and sesquiterpenes using HPLC-UV-ES-MS. The column used was C_{18}, and it was maintained at 48 °C. Marsin et al. (*28*) reported the HPLC analysis of curcuminoids extracted with supercritical CO_2 using acetonitrile/acetate buffer as the mobile phase. Another method (*17*) involves the separation and identification of curcumin using a Supelcosil LC-18 column using gradient elution with ammonium acetate/acetic acid and acetonitrile. The presence of inorganic salt in the mobile phases may contaminate the mass spectrometer ion source. There are some reports on the use of citric acid and sodium hydroxide in the HPLC mobile phase (*29*), but these mobile phases cannot be used when the HPLC is interfaced with a mass spectrometer because of the requirement for volatile mobile phases. With regard to the selection of the mobile phase, a mixture of methanol/2% acetic acid/acetonitrile gave optimum chromatographic separation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (**Figure 2**). In the present study methanol/2% acetic acid/acetonitrile was used as mobile phase, and the same method can be extended to LC-MS. It was found that the resolution was very good in the present study. This is an alternative HPLC analysis method to fluorometric detection, HPTLC, and amino-bonded silica column for the determination of individual curcuminoids and total content in turmeric samples.

Calibration graphs were prepared to determine the curcuminoids content of different turmeric samples. Calibration curves were derived from three independent injections of seven concentrations of curcumin, demethoxycurcumin, and bisdemethoxycurcumin versus the peak area. Linearity was found in the concentration range between 0.0625 and 2.0 μg, with high reproducibility and accuracy (**Figures 4**−**6**). Regression analysis of the experimental data points showed a linear relationship with excellent correlation coefficients (r^2) of curcumin, demethoxycurcumin, and bisdemethoxycurcumin of 0.9844, 0.9915, and 0.9972, respectively. The linear regression equations for the curves for curcumin, demethoxycurcumin, and bisdemethoxycurcumin are $y = 13974x - 387.62$, $y = 8128.3x - 322.67$, and $y = 11085x - 277.11$, respectively. The estimated LOQ in this study was found to be 0.05 μg.

Recently, demethoxycurcumin and bisdemethoxycurcumin were found to possess strong antioxidant activities, and inhibition of the proliferation of MCF-7 human breast tumor cells has been reported (*2*, *6*, *7*). The concentration of these two components is increased abundantly in spent oleoresin compared to normal oleoresin. These compounds can be isolated by silica gel column chromatography on a large scale for various application purposes. The results of the present study are indicative of the utilization of spent oleoresin having no commercial application at present, as a natural bioactive component. In the present study, curcuminoid levels in rhizomes of *C. longa* were determined by a simple analytical procedure requiring minimal sample preparation. The method described is suitable for the routine analysis of a large number of commercial samples of *C. longa*.

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Received for review February 25, 2002. Revised manuscript received April 25, 2002. Accepted April 29, 2002.

JF025506A