

Quantitative Determination of Curcuminoids from the Roots of *Curcuma longa*, *Curcuma* species and Dietary Supplements Using an UPLC-UV-MS Method

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Abstract

An UPLC-UV-MS method was developed for the determination of curcuminoids and ar-turmerone from roots of *Curcuma longa* L., different species of *Curcuma* (*C. zedoaria*, *C. phaecaulis*, *C. wenyujin* and *C. kwangsiensis*) and dietary supplements that claimed to contain *C. longa*. The separation was achieved within 3.5 minutes using a C-18 column, a water/acetonitrile mobile phase, containing formic acid at a temperature of 35°C. The method was validated for linearity, repeatability, limits of detection and limits of quantification. The limits of detection and limits of quantification of curcuminoids were found to be 0.01µg/mL and 0.03µg/mL, respectively. The total content of curcuminoids (curcumin, demethoxycurcumin, bisdesmethoxycurcumin) was found to be in the range from 1.16-4.92% and 0.83-35.37% in samples of *C. longa* and dietary supplements, respectively. The curcuminoid content was 0.004% for *C. zedoaria* and 0.0006% for *C. phaecaulis*. The curcuminoids were not detected in root samples of *C. wenyujin* and *C. kwangsiensis*. The developed method is simple, economic, rapid and especially suitable for quality control analysis of curcuminoids. LC-mass spectrometry with electrospray ionization (ESI) was used for the identification and confirmation of compounds in various plant samples and dietary supplements.

Keywords: Curcuminoids; *Curcuma* species; Dietary supplements

Introduction

The rhizomes of turmeric (*Curcuma longa* L., family Zingiberaceae) play an important role as a coloring agent in foods, cosmetics and textiles [1]. The main yellow bioactive substances in the rhizomes are curcumin and two closely related demethoxy compounds, viz., demethoxycurcumin and bisdemethoxycurcumin. The rhizomes have long been used in traditional medicine (mainly in India and China) for multiple pharmacological activities including anti-inflammatory, hepatoprotective, antitumour, antiviral, anticancer remedies. They are also used to treat gastrointestinal and respiratory disorders [2-4]. *Curcuma longa* and other *Curcuma* species are also widely used in the treatment of snakebite poisoning [5]. The anti-venom activity was due to a different component, viz., ar-turmerone. The antivenin effect of *Curcuma longa*, reported by Ferreira et al. [6] against the haemorrhagic activity of a venomous pit viper (*Bothrops jararaca*) was also due to ar-turmerone.

A number of analytical methods have been reported for the analysis of curcumin or mixtures of curcuminoids [7-23]. Spectrophotometric methods were used to determine the total content of curcuminoids [7]. Commercial curcumin/turmeric products contain mixtures of curcumin, demethoxycurcumin, and bisdesmethoxycurcumin. However, it is not possible to quantify the individual curcuminoids with spectrophotometric methods. Estimation of curcuminoids has been reported by thin-layer chromatography method (TLC) [8-9], high performance thin-layer chromatography (HPTLC) [10-12], near-infrared spectroscopic analysis [17], microemulsion electrokinetic chromatography [18], capillary electrophoresis [15,16], supercritical fluid chromatography [17] and LC-ESI-MS/MS [18-20]. The analysis of individual curcuminoids is possible using HPLC with modified stationary phases [21-22]. Among the methods mentioned above, the HPLC methods are probably the most convenient. However, there are

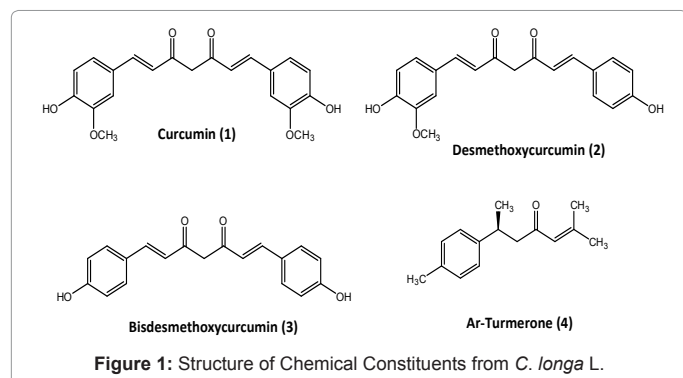
many problems in the application of HPLC method: it is difficult to produce complete separation of three curcuminoids and the analysis is time consuming. It is, therefore, desirable to develop a rapid and reliable method for the simultaneous quantification of three curcuminoids in *C. longa* and its related preparations that can produce complete resolution of the curcuminoids and ar-turmerone using isocratic elution. A UPLC method was developed for the analysis of curcuminoids [curcumin (1), demethoxycurcumin (2), bisdesmethoxycurcumin (3) and ar-turmerone (4)] from the roots/rhizomes of *C. longa* using UPLC with PDA and MS detection. The developed method is applied to the identification of curcumins and ar-turmerone in four different species of *Curcuma* (*C. zedoaria*, *C. phaecaulis*, *C. wenyujin* and *C. kwangsiensis*), and dietary supplements that claimed to contain *C. longa* (Figure 1). The three curcuminoids could form the basis for quality control of *C. longa* and dietary supplements claiming to contain rhizomes of *C. longa*. A mass spectrometer coupled with quadrupole MS and an ESI source was used for the confirmation of curcumins in various *Curcuma* species, and in dietary supplements. The compounds were numbered by the order of elution using LC-UV method. The method described is suitable for the routine analysis of a large number of commercial and biological samples of *C. longa*.

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Experimental

Instrumentation and chromatographic conditions

UPLC-UV-MS analysis: All analyses were performed on a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) including binary solvent manager, sampler manager, column compartment and PDA (Waters Acquity model code UPD) connected to Waters Empower 2 data station. An Acquity UPLC™ BEH Shield RP18 column (50mm×2.1mm I.D., 1.7µm) also from Waters was used. The column and sample temperature were maintained at 40°C and 25°C, respectively. The column was equipped with a LC-18 guard column (Vanguard 2.1 x 5 mm, Waters Corp., Milford, MA, USA). The mobile phase consisted of water (0.05 % formic acid) (A), Acetonitrile (B) (0.05 % formic acid) at a flow rate of 0.25 mL/min, which were applied in the following linear gradient elution: 0 min, 55% A : 45% B in next 5 min to 20% A : 80% B. Separation was followed by a 1 min washing procedure with 100 % B and re-equilibration period of 2.5 min. Strong needle wash solution (95/5; acetonitrile/water) and weak needle wash solution (10/90; acetonitrile/water) were used. All solutions were filtered via 0.20 µm membrane filters and degassed before their usage. The total run time for analysis was 5 minutes. The injection volume was 2 µL. The detection wavelength was 420 nm for three curcuminoids and 240 nm for ar-turmerone compounds. Peaks were assigned by spiking the samples with standard compounds and comparison of retention times.

The effluent from the LC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The source temperature and the desolvation temperature were maintained at 150 and 350°C, respectively. The probe voltage (capillary voltage), cone voltage and extractor voltage were fixed at 3.0kV, 23V and 3V, respectively. Nitrogen was used as the source of desolvation gas (650 L/hr) and drying gas (25 L/hr). Compounds were confirmed in selected ion recording (SIR) mode. Curcumins were confirmed in selected ion recording (SIR) mode. [M-H]⁻ = 367, 337 and 307 ion for curcumin, desmethoxycurcumin, bisdesmethoxycurcumin, were selected as detecting ions. Mass spectra were obtained at a dwell time of 0.1 s in SIR and 500 Da/sec of scan rate.

Chemicals and plant materials: The standard compounds (1-4) were purchased from Chromadex (Santa Ana, CA, USA). Acetonitrile, water and formic acid were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA).

Rhizomes of *Curcuma longa* L. (MPG# A104) (CL-1) and *Curcuma zedoaria* Roscoe (MPG # A018) (CZ) were obtained from the cultivated living collection of the NCNPR Medicinal Plant Garden, University of Mississippi. These two samples were identified by Dr. Aruna

Weerasooriya of Medicinal Plant Garden, Coy Waller Complex, The University of Mississippi. Rhizomes of *Curcuma longa* L. (# 835, CL-2), *Curcuma kwangsiensis* S. K. Lee & C. F. Liang (# 640, CK), *Curcuma phaeocaulis* Valetton (# 823, CP) and *Curcuma wenyujin* Y. H. Chen & C. Ling (# 659, CW) were obtained from Beijing Yuke Botanical Development Co. Ltd, China. Rhizomes of *Curcuma longa* L. (# 5213, CL-3) was obtained from CRISM, New Delhi, India. All dietary supplements (CLP-1 to CLP-6) were purchased online. Specimens of sample were deposited at the National Center for Natural Products Research (NCNPR), University of Mississippi, University, Mississippi, USA.

Preparation of standard solutions: An individual stock solution of standard compounds was prepared at a concentration of 100µg/mL in methanol. The calibration curves were prepared at seven different concentration levels. The range of the calibration curves was 0.03-25µg/mL for compounds 1-3 and 0.1-25µg/mL for compound 4 using UPLC-UV method. Table 1, shows the calibration data and the calculated limits of detection using UPLC-UV method.

Preparation of sample solutions: Dry ground rhizomes (25-50 mg) of *C. longa* or solid dosage forms or about 500 mg of *Curcuma* species were sonicated in 2.5 mL of methanol for 30 min followed by centrifugation for 15 min at 3300 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated thrice and respective supernatants combined. The final volume was adjusted to 10.0 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.2µm nylon membrane filter. The first 1.0mL was discarded and the remaining volume was collected in an LC sample vial. Each sample solution was injected in triplicate.

Validation procedure: The UPLC method was validated in terms of precision, accuracy, and linearity according to ICH guidelines [24]. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio equal to 2 or 3 and 10, respectively. The accuracy of the assay method was evaluated in triplicate using two concentration levels of 1 and 10µg/mL. Intra- and inter-day variation of the assay was determined on

Analyte	Regression Equation	r ²	LOD (µg/mL)	LOQ (µg/mL)
1	Y = 6.80e+004x - 4.89e+003	0.9997	0.01	0.03
2	Y = 8.63e+004x - 1.42e+004	0.9991	0.01	0.03
3	Y = 8.09e+003x - 1.18e+004	0.9991	0.01	0.03
4	Y = 3.23e+004x + 1.21e+003	0.9996	0.05	0.1

Table 1: Regression Equation, Correlation Coefficient (r²), Limit of Detection (LOD) and Limit of Quantitation (LOQ) for chemical constituents from rhizomes of *C. longa* L. using UPLC-UV method.

Compounds	Intra-Day (n=3)			Inter-Day (n=9)
	Day 1	Day 2	Day 3	
1	2.74±0.96	2.74±0.20	2.74±0.46	2.74±0.08
2	1.17±2.49	1.15±2.54	1.15±0.52	1.15±0.23
3	1.02±1.99	1.03±0.69	1.03±0.14	1.03±0.22
4	0.73±2.55	0.72±4.21	0.73±4.15	0.73±0.97

Values in mg/100 mg of plant sample; relative standard deviation (% CV) are given in parentheses.

Table 2: Intra- and inter-day precision of plant sample CL-1 assayed under optimized conditions for compounds 1-4 by using LC-UV method.

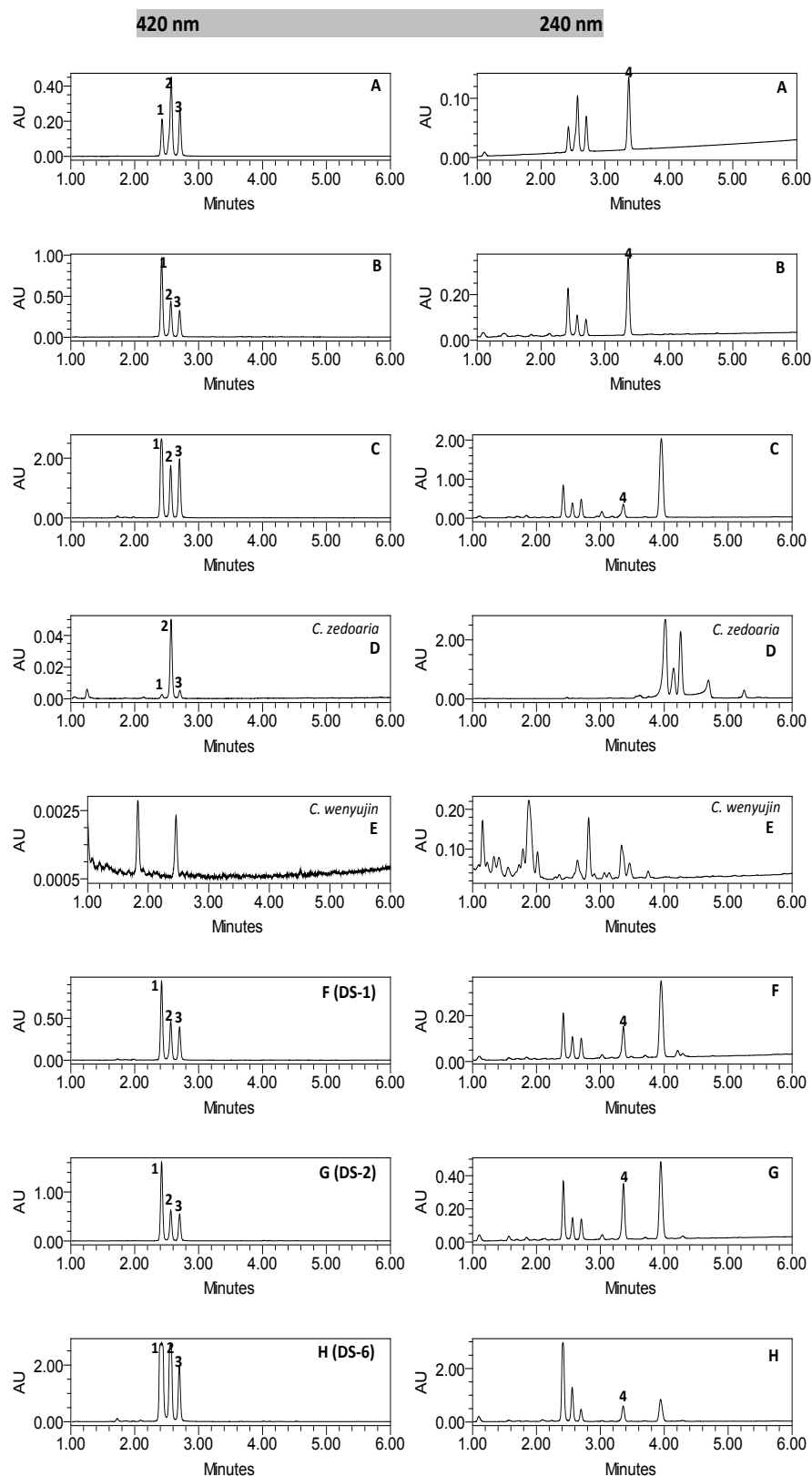


Figure 2: UPLC Chromatograms of a mixture of standard (A), roots of *C. longa* (B, C), *Curcuma* species (D, E) and dietary supplements (F-H) at 420 nm (compounds 1-3) and 240 nm (Compound 4).

3 consecutive days with 3 repetitions each.

Results and Discussion

Chromatographic conditions

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed phase C18 column. The different columns tried for UPLC were Acquity UPLC BEH C18 (100 mm × 2.1 mm I.D., 1.7µm), Acquity UPLC BEH C18 (50 mm × 2.1 mm I.D., 1.7µm) and Acquity UPLC BEH Shield RP18. The best results were observed with BEH shield RP18 column (50 mm × 2.1 mm I.D., 1.7µm) water and acetonitrile, both containing 0.05% formic acid as the mobile phase. Acetonitrile was preferred over methanol as the mobile phase as its use resulted in improved separation as well as a significantly reduced column back pressure.

Accuracy, precision and linearity

The seven point calibration curves for all four compounds showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity ($r^2 > 0.999$) of the detector response for all standard compounds was 0.03-25 µg/mL for compounds 1-3 and 0.1-25µg/mL for compounds 4. The limits of detection and limits of quantification for compounds 1-4 were found to be in the range from

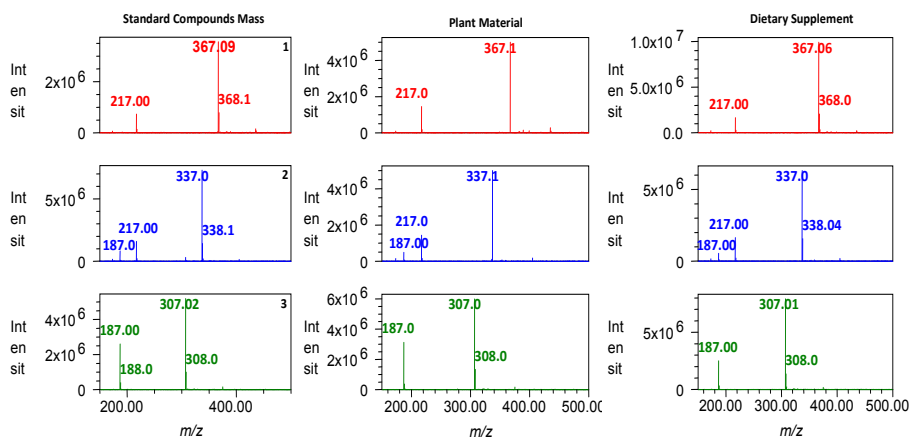
0.01-0.05µg/mL and 0.03-0.1µg/mL, respectively. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible with low standard error. Accuracy of the method was confirmed by performing the following recovery experiment; *C. longa* (CL-1) was spiked with known amounts of the standard compound then extracted and analyzed. Intra- and inter-day variation of the assay was determined and showed to be lower than 5%, with a maximum RSD of 4.21%. It was performed three times on three different days and was injected in triplicate (Table 2).

Analysis of plant samples

The identification of the compounds in *Curcuma* samples was based on the retention times and the comparison of UV spectra and MS with those of authentic standards. The developed method was used for analysis of four compounds in *Curcuma* species and commercial products of *Curcuma* (Figure 2, 3).

In this work, a reverse phase chromatographic method was developed using UPLC for chemical fingerprint analysis of curcumins and ar-turmerone. The method can also be used for determination of these compounds in *Curcuma* species and dietary supplements that claim to contain *Curcuma longa*. The calibration curve showed good linearity ($r^2 > 0.999$) within the range. The LOD and LOQ were

(a) MS of compounds 1-3



(b) UV spectra of compounds 1-4

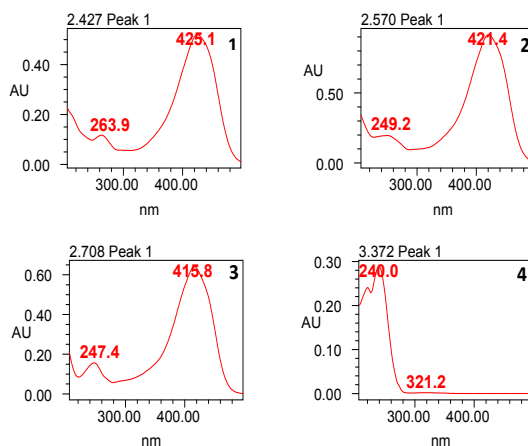


Figure 3: (a) UPLC-MS of a mixture of curcuminoids, roots of *C. longa* L. and dietary supplement, (b) UV spectra of compounds 1-4.

Plant Sample/ Commercial Products	Analytes				Serving size	Servings/day	Ave. wt of each capsule content/tablet (mg)	<i>C. longa</i> L. (Amount, mg)/ serving
	1	2	3	4				
CL-1	2.74 (0.16)	1.15 (0.19)	1.03 (0.24)	0.73 (0.12)	-	-	-	-
CL-2	0.75 (0.09)	0.25 (0.83)	0.16 (0.65)	1.32 (0.12)	-	-	-	-
CL-3	0.91 (0.29)	0.32 (0.79)	0.23 (0.41)	0.51 (0.71)	-	-	-	-
CZ	0.00031 (0.91)	0.003 (0.42)	0.00055 (0.26)	ND	-	-	-	-
CP	0.0002 (2.13)	0.0004 (1.45)	DUL	ND	-	-	-	-
CW	ND	ND	ND	ND	-	-	-	-
CK	ND	ND	ND	ND	-	-	-	-
CLP-1	0.699 (0.23)	0.322 (0.09)	0.220 (0.13)	0.271 (0.16)	2 capsules	1	827.5	1440
CLP-2	1.186 (0.12)	0.405 (0.07)	0.305 (0.56)	0.632 (0.10)	1 capsule	1-3	798.2	250 mg extract +250 mg root
CLP-3	1.138 (0.04)	0.373 (0.21)	0.279 (0.08)	0.575 (0.03)	1 capsule	3-6	510.9	400
CLP-4	0.467 (0.12)	0.203 (0.097)	0.155 (0.04)	0.187 (0.06)	2 capsules	1	832.9	1440
CLP-5	10.848 (1.54)	2.845 (2.60)	0.913 (0.85)	1.100 (0.31)	2 capsules	2	528.9	350 mg rhizome + 100 mg extract
CLP-6	27.669 (0.17)	5.818 (2.15)	1.884 (1.78)	0.291 (0.53)	1 tablet	3	911.9	450

DUL = Detected under limits of quantification

ND = Not Detected

Mean (n=3) ± relative standard deviation (% CV) are given in parentheses.

Table 3: Content (% w/w) of curcumins in *Curcuma* samples (mg/100 mg dried material) and dietary supplements (mg/average wt of capsule) using UPLC-UV method: curcumin (1), desmethoxycurcumin (2), bisdesmethoxycurcumin (3) and ar-turmerone (4).

found to be 0.01 and 0.035 µg/mL for curcuminoids and 0.05 and 0.1 µg/mL for ar-turmerone by UPLC-UV method. The curcuminoids were detected in species of *C. longa*, *C. zedoaria*, *C. phaeocaulis*. The total content of curcuminoids (curcumin, desmethoxycurcumin, bisdesmethoxycurcumin) was found to be in the range from 1.16-4.92% and 0.83-35.37% in *C. longa* and dietary supplements, respectively. The curcuminoid content was 0.004% for *C. zedoaria* and 0.0006% for *C. phaeocaulis* samples. The curcuminoids were not detected from roots of *C. wenyujin* and *C. kwangsiensis*. Ar-turmerone was detected only in *C. longa* samples which was in the range from 0.19-1.10% and was not detected in other species of *Curcuma* analyzed. The lowest concentration of curcuminoids was present in sample CLP-4 (0.83%) and highest amounts in sample CLP-6 (35.57%) (Table 3).

UPLC-Mass spectrometry coupled with electrospray ionization (ESI) method is described for the identification of curcuminoids in plant samples and dietary supplements claiming to contain *C. longa*. LC-MS is a powerful qualitative and quantitative technique for the determination of molecular masses of analytes, because analyte identification on the basis of molecular mass is extremely selective. This method involved the use of [M+H]⁺ ions of compounds 1-4 which were observed for standard compounds in positive ion mode with extractive ion monitoring (EIM) at *m/z* 369.1 [M+H]⁺, 339.1 [M+H]⁺, 309.1 [M+H]⁺ and 217.1 [M+H]⁺, respectively. The method also involved the use negative ion mode (deprotonated ions) with extractive ion monitoring (EIM) at *m/z* 367.1 (C₂₁H₁₉O₆, [M-H]⁻) to the product ion *m/z* 217 [M-C₉H₁₀O₂]⁻ for curcumin, *m/z* 337.1 (C₂₀H₁₇O₅, [M-H]⁻) to the product ions at *m/z* 217 [M-C₈H₈O]⁻ and 187 [M-C₉H₁₀O₂]⁻, respectively, for demethoxycurcumin analysis, and *m/z* 307.1 (C₁₉H₁₅O₄, [M-H]⁻) to product ion at *m/z* 187 [M-C₈H₈O]⁻ for bisdesmethoxycurcumin analysis. Curcumin (1) was found to be the major compound among the analyzed curcuminoid. Compounds 1-4 in *C. longa* L. and in the dietary supplement that claim to contain *C. longa* L. were identified

by comparison of the retention time and mass spectral data with those of the standards. In general, mass spectrometric methods do not require any chemical modifications on the analyte compounds. In conclusions, the newly developed UPLC-UV-MS method for the determination of curcuminoids (curcumin, desmethoxycurcumin, bisdesmethoxycurcumin) and ar-turmerone was found to be capable of giving fast retention times while maintaining better resolution than that achieved with conventional HPLC. This method exhibited excellent performance in terms of sensitivity and is a suitable method for rapid analysis of curcuminoids & ar-turmerone and for chemical fingerprint analysis. The developed method was validated for all the parameters tested and successfully applied to the identification of five different species of *Curcuma* (*C. zedoaria*, *C. phaeocaulis*, *C. wenyujin*, *C. longa* and *C. kwangsiensis*), and dietary supplements that claimed to contain *C. longa*. LC-mass spectrometry with SQD is described for the identification of four compounds in plant samples and dietary supplements. This method involved the use of [M+H]⁺ ions in the positive ion mode with selective ion recording (SIR). Furthermore, the short run time analysis allows increased sample throughput for routine purposes and pharmacokinetic application.

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