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ARTICLES

Purification of Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin by High-Speed Countercurrent Chromatography

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Curcuminoids are substances of great interest because of their important pharmacological activities, particularly anti-inflammatory, anticarcinogenic, and anti-Alzheimer's activities. In this study, we report the first procedure and effect of processing for the high, efficient, and useful purification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin from turmeric powder. Purification involves high-speed countercurrent chromatographic (HSCCC) separation of these curcuminoids using a simple two-phase solvent system composed of *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5, v/v). The HSCCC-fractionated effluent peaks indicated that the peak resolutions were 1.7 between curcumin and demethoxycurcumin and 2.1 between demethoxycurcumin and bisdemethoxycurcumin for 25 mg of loaded turmeric powder. These purified substances were analyzed by liquid chromatography−tandem mass spectrometry with scan and daughter scan negative modes, and the wide absorbance from 200 to 500 nm was monitored by photodiode array detection. The separation yielded 1.1 mg of curcumin, 0.6 mg of demethoxycurcumin, and 0.9 mg of bisdemethoxycurcumin (>98% purity). Moreover, the antioxidant effect of curcuminoids was measured by a 1,1-diphenyl-2-picrylhydrazil assay. The order of antioxidant activity was purified curcumin > purified demethoxycurcumin > purified bisdemethoxycurcumin > turmeric powder. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin can be used for various evaluations of their pharmacological activities.

KEYWORDS: Turmeric powder; curcumin; demethoxycurcumin; bisdemethoxycurcumin; high-speed countercurrent chromatography; liquid chromatography−tandem mass spectrometry

INTRODUCTION

Turmeric, *Curcuma longa* L., is a well-known herb of the *Zingiberacea* family and a natural colorant. Three main curcuminoids have been identified in turmeric (see **Figure 1**): curcumin [molecular weight (M.W.), 368], demethoxycurcumin (M.W., 338), and bisdemethoxycurcumin (M.W., 308). All three compounds are responsible for the hallmark yellow pigmentation of the turmeric plant and its rhizome. Recently, many studies have provided evidence suggesting that curcuminoids are responsible for turmeric's broad spectrum of biological activities, such as anti-inflammatory, antioxidant, and anticarcinogenic effects (*1*−*3*). Moreover, a study has suggested that a low dose of curcumin effectively disaggregates amyloid-β peptides, as well as prevents fibril

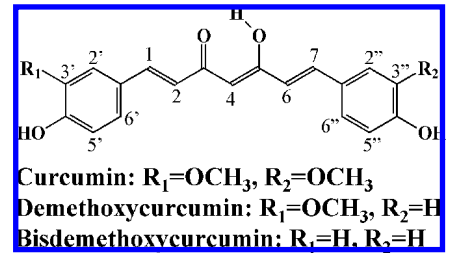

Figure 1. Structural formulas of the main curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

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and oligomer formation, supporting the rationale for the use of curcumin in clinical trials for preventing Alzheimer's disease (*4*). Many reports of clinical and animal studies have provided several molecular mechanisms that elucidate multiple biological effects of curcuminoids.

Many methods for the quantification of the curcuminoids have been reported by using high-performance thin-layer chromatography (HPTLC) (*5*), high-performance liquid chromatography (HPLC) (*6*−*8*), and mass spectrometry (MS) (*9*−*11*). Recently, Yang et al. performed herbal analysis of the roots of *Curcuma longa* L. by liquid chromatogramphy with tandem mass spectrometry (LC-MS/MS) (*12*). LC-MS/MS is very useful to quantify individual curcuminoids from biological, food, and plant samples for controlling the quality of the processed product and its pharmacokinetic and metabolic fate (*9*−*12*). However, many studies used curcuminoids of low and/or unknown purity as reference standards in animal studies and analytical evaluations. It is not available to purchase high-quality standards of curcuminoids commercially.

On the other hand, individual curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin are substances of interest because of their different biological activities. On the basis of HPTLC separation with 1,1-diphenyl-2-picrylhydrazil (DPPH) antioxidant assay, the order of activity was curcumin > demethoxycurcumin > bisdemethoxycurcumin (*13*). Recent studies on basic cancer chemotherapy suggested that curcumin, demethoxycurcumin, and bisdemethoxycurcumin inhibit the function of multidrug resistance-related protein and P-glycoprotein, and they have multiple beneficial effects (*14*−*16*). In an in vitro assay for studying anticarcinogenic effects, the observations of inhibited proliferation and increased apoptosis in colon cancer cells appeared to be associated with the cellular uptake of individual curcuminoids (*17*). Moreover, a recent study suggested that bisdemethoxycurcumin may improve the innate immune system and increase amyloid-β clearance from the brain of patients with sporadic Alzheimer's disease (*18*). There is an increasing demand for individual curcuminoids due to the discovery of their biological effects. On the other hands, high-purity individual curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin are not available from commercial sources. Jayaprakasha et al. used the specific resin [(named curcumin removed turmeric oleoresin (CRTO)] for the isolation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin from turmeric powder (*6*). This specific resin polymer is not a common material. This purification using CRTO is a novel technique but is not useful for routine purifications of curcuminoids. Thus, in spite of intensive research, no simple, efficient, and low-cost technique for specific purification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin has yet been achieved for use in practical methods.

It was reported that the preparative separation of individual curcuminoids from turmeric powder was attempted via pH zone-refining countercurrent chromatography, but the actual separation and HPLC evaluation of the purified curcuminoids were not completed (*19*). In addition, this technique is very confusing, and thus, various parameters of the mobile phase such as solvents, pH, and fraction ranges had to be adjusted. A number of studies using high-speed countercurrent chromatography (HSCCC) have suggested that very useful, simple, efficient, and low-cost purification of natural products can be achieved (*20, 21*). We have performed simple and efficient purification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin by HSCCC using a two-phase solvent system. The purified curcuminoids were analyzed by liquid chromatography−electrospray ionization tandem mass spectrometry (ESI-LC-MS/MS) and nuclear magnetic resonance (NMR) and then were subjected to a simple antioxidant assay using a DPPH reagent to determine the antioxidants of curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

MATERIALS AND METHODS

Reagents. Turmeric powder and curcumin standard were obtained from San-Ei Gen FFI Co., Inc. (Osaka, Japan). HPLC-grade water, *n*-hexane, ethyl acetate, acetonitrile, *n*-butanol, methanol, acetic acid (AA; HPLC-grade), formic acid (FA; 99%, LC/MS-grade), and trifluoroacetic acid (TFA; 98%) were obtained from Wako Chemical Co., Inc. HPLC-grade *tert*-butyl methyl ether was obtained from Sigma-Aldrich (St. Louis, MO). Butylated hydroxytoluene (BHT) was obtained from Wako Chemical Co., Inc. for an antioxidant assay. Purified water was obtained using a Milli-Q Simplicity UV system (Millipore, Bedford, MA).

Standard Solution of Turmeric. Concentrated solutions (1.0 mg/mL) of turmeric powder were prepared in methanol. The obtained standard solutions were treated in a centrifuge (8000 rpm, 10 min) and were then diluted to 50 μg/mL as required by the addition of methanol/water (50/50, v/v) for HPLC analysis.

HPLC Analysis of Curcuminoids. HPLC was performed using a LC-20AD pump, SPD-20AV detector, CTO-20AC column oven with injector, and C-R8A recorder system (Shimadzu Co., Kyoto, Japan). HPLC columns were used (TSK-GEL ODS 100 V, 100 Z, and 80 Ts; 4.6 mm × 150 mm, 5.0 μm, Tosoh Co., Tokyo, Japan) for the separation of curcuminoids, and the temperature was 40 °C. Separation was carried out using an isocratic mobile phase of 0.1% FA in water/acetonitrile (50/50, v/v) at a flow rate of 1.0 mL/min for 15 min. A sample volume of 10 μL was injected. The elution of curcuminoids was monitored by visible absorbance at 405 nm.

HSCCC Isolation of Curcuminoids from Turmeric Powder. HSCCC was performed using an HSCCC-1A prototype model (multilayer coil planet centrifuge, Shimadzu Co.) with a 10 cm orbital radius that produces a synchronous type-J planetary motion with a maximum speed of 800 rpm. A multilayer coil was prepared using ca. 160 m long polytetrafluoroethylene (PTFE) tubing with a 10 cm hub diameter and a 15 cm hub length wound onto a column holder, making six coiled layers with a total capacity of 270 mL.

Ten milligrams of turmeric powder was added to two mutually equilibrated solvent phases (1 mL each; see **Table 1**) in a test tube, mixed to equilibrate, and then centrifuged (8000 rpm, 10 min). After the mixture settled, equal volumes of the upper and lower phases were transferred into separate test tubes, which were then diluted using equal volumes of methanol. Each phase was assessed by HPLC, and the area of each peak was used to determine the partition coefficient (K) values for each component. The K value was calculated as follows:

$$K = \frac{\text{HPLC peak area of solute in upper phase}}{\text{HPLC peak area of solute in lower phase}}$$

The two phases were mutually saturated by shaking in a separatory funnel and were then separated immediately before use. Either phase can serve as the mobile or stationary phase, depending on the direction of the column rotation.

The two-phase solvent system composed of hexane/chloroform/methanol/water solution (5/10/7.5/2.5, v/v, total 2 L) at room temperature was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking three separate times, followed by inverting the vessel and manipulating its stopcock. First, the column was entirely filled with the upper aqueous stationary phase. Second, 25 mg of turmeric powder was dissolved in 2 mL of each phase and was centrifuged (2000 rpm, 10 min). Finally, the supernatant was then loaded into the column. The column was rotated at 780 rpm, while the lower organic mobile phase was pumped into the head of the column at a flow rate of 1.0 mL/min using an HPLC pump (LC-6A, Shimadzu Co.) (the head−tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects with different densities, either lighter or

heavier than the surrounding medium, are driven toward the head of the coil). The effluent from the outlet of the column was fractionated into test tubes at 1 min/tube using a fraction collector (model 2128 Fraction Collector, Bio-Rad Laboratories, Inc., NY). After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column using pressurized nitrogen gas under slow coil rotation in the tail-to-head elution mode.

The HSCCC fractions of the curcuminoids were analyzed by flow injection analysis (FIA). This analysis was performed using a LC-20AD pump, SPD-20AV detector, SIL-20AC autosampler, and C-R8A recorder system (Shimadzu Co.). The solvent for FIA was 0.1% FA in water/0.1% FA in acetonitrile (50/50, v/v) with a flow rate of 1.0 mL/min. These HSCCC fractions were monitored at 405 nm.

Peak Resolutions (Rs) of Curcuminoids for HPLC and HSCCC Methods. On the basis of HPLC chromatograms and HSCCC fraction curves of curcuminoids, we calculated the Rs as follows:

$$\mathrm{Rs} = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}}$$

The retention time and peak width of the midpoint for each curcuminoids were t_R and $W_{0.5\,h}$, respectively.

LC/MS/MS Analysis of Curcuminoids. LC analyses were performed using a Waters Ailiance 2695 system (Waters, Milford, MA). LC separation was performed using a TSK-GEL ODS 80Ts (4.6 mm × 150 mm, 5 μm: Tosoh Co., Tokyo, Japan) maintained at 40 °C. The mobile phase consisted of 0.1% FA in water (solvent A) and 0.1% FA in acetonitrile (solvent B). The LC stepwise gradient was as follows: 50% solvent B at 0 min, 50% solvent B at 15 min, 98% solvent B at 15.1 min, 98% solvent B at 20 min, and 50% solvent B at 20.1 min with a flow rate of 1.0 mL/min. The injection volume was 10 μL. For MS with ESI detection, a microsplitter valve (GL Science Co., Tokyo, Japan) was used to obtain a flow rate of 0.2 mL/min after a photodiode array detector (PDA, Waters 2996) monitored from 200 to 500 nm. The mass spectrometer (a Waters Micromass Quattro Premier triple quadrupole mass spectrometer) was operated with an ESI source in the negative ionization mode. The ionization source conditions were as follows: capillary voltage of 3.0 kV, extractor voltage of 4 V, RF lens voltage of 0 V, source temperature of 110 °C, and desolvation temperature of 400 °C. The cone and desolvation gas flows were 50 and 850 L/h, respectively, and were obtained using a nitrogen source (N_2 Supplier model 24S, Anest Iwata Co., Yokohama, Japan). We used argon as the collision gas and regulated it at 0.35 mL/h, setting the multipliers to 650 V.

NMR Analysis of Curcuminoids. ^{1}H NMR spectra were recorded on a JEOL ECA-500 spectrometer in acetone-d_6 using tetramethylsilane as an internal standard. Used are the following abbreviations: s, singlet; d, doublet; and dd, doublet-of-doublets. These positions of the structure are shown in **Figure 1**. Deuterated NMR solvent of pyridine (C_5D_5N: Isotec, Miamisburg, OH) was used and adjusted to 0.6 mL for one sample.

DPPH Antioxidant Assay of Curcuminoids. The free radical scavenging activity was measured by the DPPH method. Curcumin standard, BHT, and HSCCC fraction A−C solutions at concentrations of 25, 50, and 100 μg/mL were obtained by the addition of methanol in different test tubes. Four milliliters of 0.1 mM DPPH in methanol was added to these test tubes, which were then shaken vigorously. The tubes were then incubated in a dark at room temperature for 20 min. A control sample was prepared without curcuminoids or BHT and was used for baseline corrections. The absorbance of sample solutions was measured at 517 nm (V-530 UV/vis Spectrophotometer, JASCO Co., Tokyo, Japan). All analyses were performed three times, and their results were averaged. The radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: radical scavenging activity (RSA %) = [(control OD − analyte OD)/control OD] × 100, where OD is the opitical densitiy, which the absorbance values measured in the test sample and control, respectively.

RESULTS AND DISCUSSION

HPLC Analysis of Curcuminoids. Turmeric powder usually contains three dye components having different structures. Curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin possess similar chemical structures. For the chromatographic separation of these three curcuminoids, a C_{18}-based column was commonly used. Recently, Bos et al. reported that they developed an HPLC/PDA method for the determination of these three curcuminoids, wherein the mobile phase consisted of a mixture of methanol/0.1% aqueous TFA/acetonitrile (39.5/350/468, w/w/w) (*22*). In addition, Jayaprakasha et al. reported that the chromatographic condition for the separation of these curcuminoids was the gradient mode using methanol/2% aqueous AA/acetonitrile (*6*). If a mass spectrometric detector is used for monitoring the curcuminoids, analysis of substances with different M.W.s is possible by applying an HPLC isocratic elution with simple and favorable solvents, buffers, and/or other additives (*9, 11*). On the other hand, a mass spectrometric detector is rather costly for developing a routine and useful method for separating the three curcuminoids from various samples. However, because of the extremely similar structures of curcuminoids, very limited chromatographic retention and/or resolution could be achieved. Therefore, in our study, simple HPLC separation based on three C_{18} columns [TSK-GEL ODS 100 V, 100 Z, and 80 Ts (4.6 mm × 150 mm, 5.0 μm, Tosoh Co., Tokyo, Japan)] was investigated using a mobile phase consisting of 0.1% FA in water/acetonitrile (50/50, v/v) at a flow rate of 1.0 mL/min. **Figure 2** shows the chromatograms of the three curcuminoids. The Rs of HPLC separation based on TSK-GEL columns are 0.6 (peak 1 vs peak 2, peak 1/2) and 0.5 (peak 2 vs peak 3, peak 2/3) for 100 V, 0.7 (peak 1/2) and 0.6 (peak 2/3) for 100 Z, and 1.9 (peak 1/2) and 1.8 (peak 2/3) for 80 Ts. When TSK-GEL 80Ts and a simple mobile phase of 0.1% FA in water/acetonitrile (50/50, v/v) were used for analysis of the three curcuminoids, the Rs values showed that a good separation of analytes (Rs > 1.5) was achieved (see **Figure 2**). Therefore, we decided to monitor three

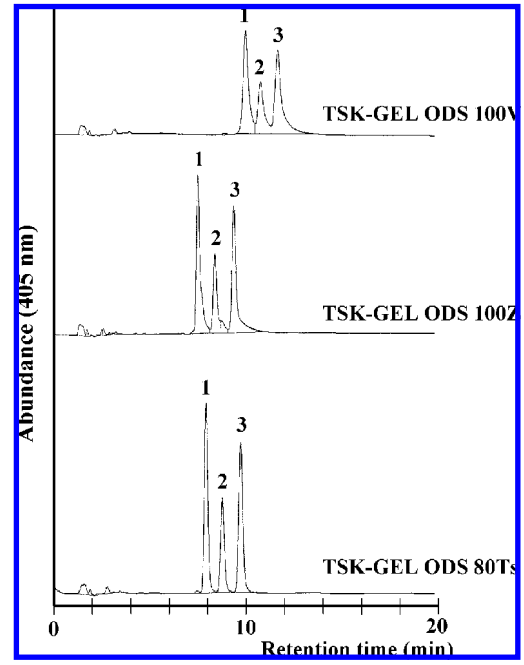

Figure 2. HPLC chromatograms of turmeric powder. Peaks: 1, bisdemethoxycurcumin; 2, demethoxycurcumin; and 3, curcumin. Columns are TSK-GEL ODS 100V, 100Z, and 80Ts (4.6 mm × 150 mm, 5.0 μm, Tosoh Co., Tokyo, Japan). The mobile phase consisted of 0.1% FA in water/0.1% FA in acetonitrile (50/50, v/v). The monitoring absorbance is visible at 405 nm.

Table 1. Partition Coefficient Ratios (*K*) of Curcuminoids for Different Two-Phase Solvent Systems

two-phase solvents	concentration of acid in water (%)	ratio (v/v)	partition coefficient (*K*) [a]		
			bisdemethoxycurcumin	demethoxycurcumin	curcumin
n-hexane/ethyl acetate/*n*-butanol/methanol/water	NA[b]	10/0/0/5/5	0	0	0.02
	NA	9/1/0/5/5	0.04	0.06	0.08
	NA	8/2/0/5/5	0.19	0.21	0.22
	NA	7/3/0/5/5	0.48	0.48	0.46
	NA	6/4/0/5/5	1.07	1.02	0.94
	NA	5/5/0/5/5	2.09	1.94	1.75
	NA	4/5/0/4/5	9.27	8.19	7.15
	NA	3/5/0/3/5	57.02	47.87	40.00
	NA	2/5/0/2/5	209.38	194.89	175.45
	NA	1/5/0/1/5	366.66	413.91	388.97
	NA	0/5/0/0/5	73.09	74.39	73.38
	NA	0/4/1/0/5	84.41	85.83	84.54
	NA	0/3/2/0/5	112.91	114.41	113.95
	NA	0/2/3/0/5	92.67	94.44	92.89
	NA	0/1/4/0/5	95.29	100.35	102.32
	NA	0/0/5/0/5	120.46	131.64	132.45
	0.1% AA[c]	5/5/0/5/5	2.18	2.00	1.81
	0.3% AA	5/5/0/5/5	2.22	2.05	1.86
	0.5% AA	5/5/0/5/5	3.85	3.57	3.22
	1.0% AA	5/5/0/5/5	3.83	3.52	3.17
	0.1% FA[d]	5/5/0/5/5	2.65	2.43	2.19
	0.3% FA	5/5/0/5/5	2.95	2.71	2.42
	0.5% FA	5/5/0/5/5	2.44	2.26	2.03
	1.0% FA	5/5/0/5/5	3.03	2.79	2.51
	0.1% TFA[e]	5/5/0/5/5	2.79	2.58	2.31
	0.3% TFA	5/5/0/5/5	1.76	1.65	1.49
	0.5% TFA	5/5/0/5/5	2.77	2.57	2.32
	1.0% TFA	5/5/0/5/5	2.51	2.34	2.11
tert-butyl methyl ether/*n*-butanol/acetonitrile/water	NA	1/0/0/1	102.24	110.47	116.34
	NA	4/0/1/5	165.24	191.90	200.84
	NA	6/0/3/8	140.34	144.51	144.35
	NA	2/0/2/3	113.56	108.02	99.47
	NA	4/2/3/8	116.56	111.73	102.97
	NA	2/2/1/5	93.89	94.44	93.32
chloroform/methanol/water	NA	10/0/10	0.01	0	0
	NA	10/1/9	0.01	0	0
	NA	10/2/8	0.01	0	0
	NA	10/3/7	0.02	0	0
	NA	10/4/6	0.02	0.01	0
	NA	10/5/5	0.04	0.01	0
	NA	10/6/4	0.07	0.03	0.01
	NA	10/7/3	0.16	0.10	0.07
	NA	10/7.5/3	0.18	0.12	0.08
	NA	10/8/3	0.28	0.20	0.14
	NA	10/7.5/2.5	0.31	0.23	0.17
	NA	10/7/2.5	0.24	0.16	0.11
	NA	10/9/4	0.20	0.13	0.09

[a] The partition coefficient (*K*) was a value of "area of solute in upper phase/area of solute in lower phase". [b] NA, no addition. [c] AA, acetic acid. [d] FA, formic acid. [e] TFA, trifluoroacetic acid.

curcuminoids by HPLC using a simple mobile phase and TSK-GEL 80Ts and to measure the partition coefficient (*K*) of the three peaks using HPLC chromatograms to select a two-phase solvent system for the HSCCC.

Partition Coefficients of Curcuminoids. For achieving successful HSCCC separation to obtain pure standards of the curcuminoids, the two-phase solvent system should satisfy the following requirements: (i) the settling time of the two-phase solvent system with samples should be less than 30 s, (ii) the partition coefficient (*K*) value of the curcuminoids should be close to 1.0, (iii) the separation factor ($\alpha = K_m/K_n$, $K_m > K_n$) should be greater than 1.5, (iv) the two-phase solvents should be nearly equal volumes for each phase, and (v) the two-phase solvents should be a volatile solvent system. In this study, the *K* values of three curcuminoids were determined in the following three solvent systems: *n*-hexane/ethyl acetate/*n*-butanol/methanol/water (added AA, FA, or TFA), *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water, and chloroform/methanol/water each at various volumes (see **Table 1**). We calculated the separation factors (α) of the curcuminoids based on the values in **Table 1**. These α values of the curcuminoids for the solvent systems of *n*-hexane/ethyl acetate/*n*-butanol/methanol/water (added AA, FA, or TFA) and *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water ranged from 1.0 to 1.4. In

Table 2. Partition Coefficient Ratios (*K*) of Curcuminoids for New Two-Phase Solvent Systems

two-phase solvent	ratio	partition coefficient (*K*)			separation factor (α)	
		bisdemethoxycurcumin	demethoxycurcumin	curcumin	α1	α2
n-hexane/chloroform/methanol/water	1/10/7.5/3	0.33	0.22	0.14	1.51	1.50
	2/10/7.5/3	0.41	0.27	0.17	1.55	1.52
	3/10/7.5/3	0.67	0.41	0.26	1.63	1.57
	1/10/8/3	0.36	0.25	0.18	1.43	1.41
	2/10/8/3	0.52	0.35	0.24	1.49	1.46
	3/10/8/3	0.72	0.46	0.30	1.55	1.52
	1/10/7.5/2.5	0.37 ± 0.03[f]	0.27 ± 0.03	0.19 ± 0.03	1.37	1.42
	2/10/7.5/2.5	0.48 ± 0.01	0.33 ± 0.02	0.24 ± 0.03	1.45	1.38
	3/10/7.5/2.5	0.80 ± 0.06	0.53 ± 0.06	0.37 ± 0.06	1.51	1.43
	4/10/7.5/2.5	1.25 ± 0.14	0.81 ± 0.12	0.54 ± 0.10	1.54	1.50
	5/10/7.5/2.5	1.64 ± 0.15	1.03 ± 0.13	0.68 ± 0.11	1.59	1.51
	6/10/7.5/2.5	2.29 ± 0.18	1.42 ± 0.19	0.91 ± 0.16	1.61	1.56
	1/10/7/2.5	0.37	0.25	0.17	1.50	1.46
	2/10/7/2.5	0.41	0.26	0.17	1.57	1.50
	3/10/7/2.5	0.63	0.40	0.26	1.58	1.52
	5/5/5/5	0.86	0.22	0.07	3.97	3.24

[f] Values are means ± standard deviations (SDs, $n = 3$).

addition, the *K* values of the three curcuminoids for the solvent system of chloroform/methanol/water were lower than 0.5. These solvent compositions did not prove satisfactory as an HSCCC solvent system for the separation of curcuminoids. Therefore, we found a new two-solvent system of *n*-hexane/chloroform/methanol/water for measuring *K* and α values of the three curcuminoids for HSCCC separation. By evaluating *K* and α values shown in **Table 2**, it was determined that the new solvent system of *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5 and 6/10/7.5/2.5, v/v) could be used to separate these curcuminoids. This solvent composition provides satisfactory reproducible *K* values. In addition, **Table 2** shows that the α values of the three curcuminoids are greater than 1.5. Next, we determined that the settling time was less than 30 s using *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5 and 6/10/7.5/2.5, v/v), which ensures a satisfactory retention level of the stationary phase in HSCCC (*23*). This result proved that the ratio of 5/10/7.5/2.5 was a better time (<30 s) than that of 6/10/7.5/2.5. On the basis of these results, we decided to use the new solvent system of *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5, v/v) for the separation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin standards by HSCCC.

HSCCC Separation of Curcuminoids. HSCCC has been used for the separation and isolation of various natural products. This advanced method can be useful for obtaining pure standards on the industrial scale (*24*). Therefore, preparative isolation and purification by HSCCC were more useful, simple, and automatic than using silica gel column chromatography. Patel et al. reported that the preparative separation of individual curcuminoids from turmeric powder was attempted by pH zone-refining countercurrent chromatography (*19*). However, this method is very confusing due to various parameters of the mobile phase, such as solvents, pH, and fraction ranges. Therefore, this method cannot be used for simple and routine purification of these three curcuminoids. On the other hand, completely validated purification and isolation of curcuminoids by the HSCCC methods have not been reported thus far. Therefore, we have developed an efficient and effective HSCCC method for the purification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin from turmeric powder using the new solvent system of *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5, v/v). We used 25 mg of turmeric powder in the above-mentioned HSCCC system; however, insoluble components in this powder should not be injected into an HSCCC system. Therefore, to facilitate the separation of the curcuminoids from other insoluble components, we centrifuged (2000 rpm, 10 min) the sample solution before loading. The amount of insoluble components was 9.0 mg in 25 mg of turmeric powder. Therefore, the actual injected sample for isolation of curcuminoids by HSCCC was 16.0 mg. **Figure 3** shows the HSCCC elution curve of the three curcuminoids, monitored at 405 nm by FIA. The retention of the stationary phase was 74.3%. The total separation time was 5 h, and the total elution volume was 300 mL. The HSCCC fractionated effluent peaks of A, B, and C indicated that the Rs values were 1.7 and 2.1. Moreover, the repeatability tests of elution time were that the RSDs ($n = 3$, interday) of the three effluent peaks were 6.6 (peak A), 7.3 (peak B), and 12.0% (peak C),

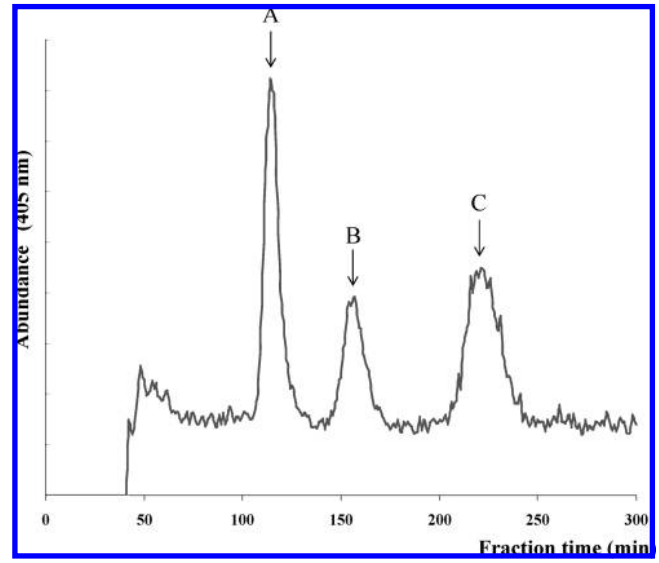

Figure 3. Separation, elution curve, and fraction peaks (fractions A−C) of turmeric powder by HSCCC. Fraction A, from 105 to 130 min; fraction B, from 145 to 175 min; and fraction C, from 205 to 245 min. The HSCCC solvent system is *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5, v/v). These fractions are analyzed by FIA with monitoring at 405 nm. The solvent is 0.1% FA in water/0.1% FA in acetonitrile (50/50, v/v). Peaks: A, curcumin; B, demethoxycurcumin; and C, bisdemethoxycurcumin.

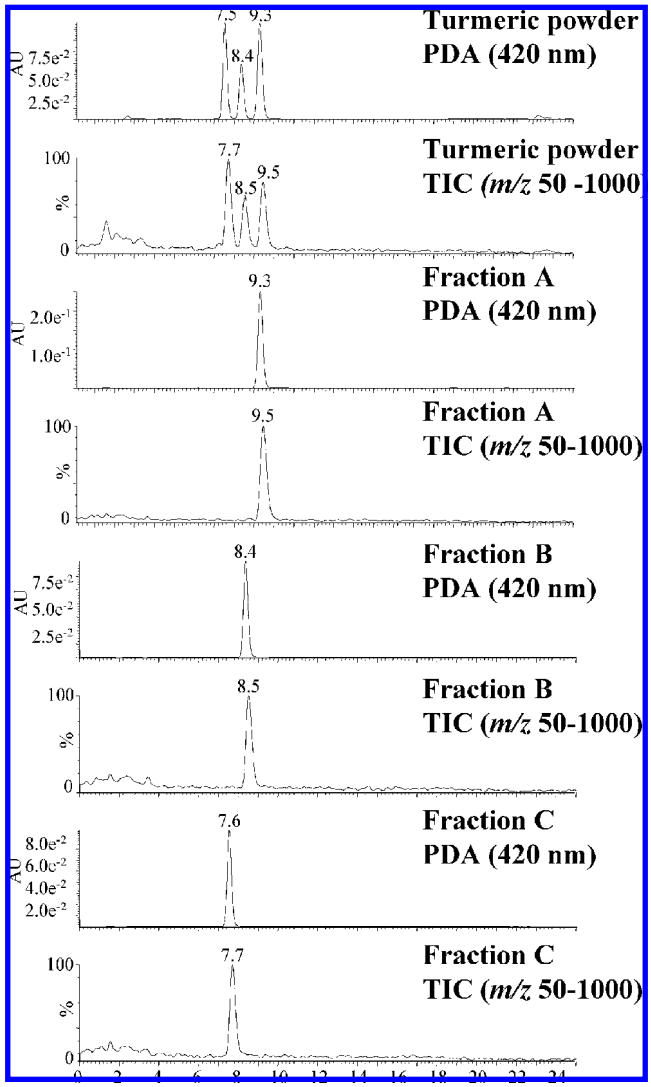

Figure 4. LC/PDA and MS chromatograms of turmeric powder and purified curcuminoids (fractions A−C) by HSCCC. PDA detection is monitored from 200 to 500 nm (higher absorbance, 420 nm). TICs from *m/z* 50 to 1000 are obtained by a Waters Micromass Quattro Premier triple quadrupole mass spectrometer based on ESI-negative mode. A, fraction A was identified as curcumin; B, fraction B was identified as demethoxycurcumin; and C, fraction C was identified as bisdemethoxycurcumin.

respectively. On the basis of this elution curve, the collected fractions were combined into three pooled fractions (A−C, see **Figure 3**). The amounts of the peak fractions were 0.9 (fraction A), 0.6 (fraction B), and 1.1 mg (fraction C). These results showed that a very useful and efficient purification method for three curcuminoids was developed by HSCCC using a new solvent system.

Identification of Curcuminoids by LC-MS/MS and NMR. Many diarylheptanoids are present in turmeric rhizomes and powder and are identified by MS (*25, 26*). LC-MS/MS can provide sufficient information to tentatively identify diarylheptanoids (curcuminoids) from HSCCC fractions of turmeric powder, because curcumin, demethoxycurcumin, and bisdemethoxycurcumin have been shown to exhibit similar fragmentation patterns in ESI-MS/MS (*11*). On the basis of the developed HPLC method for measuring curcumin, demethoxycurcumin, and bisdemethoxycurcumin, we applied LC-MS/MS analysis using the microsplitter valve adjusted to a flow rate of 0.2 mL/min for ESI. **Figure 4** shows the LC-MS/MS and PDA chromatograms of the turmeric standard (the three curcuminoids), that is, fractions A−C. The negative total ion chromatogram (TIC) and photodiode array (200−500 nm) chromatograms showed that the purity of the purified curcuminoids was greater than 98%. In addition, **Figure 5** shows the negative-MS scan and MS/MS daughter spectra of fractions A−C. From the TIC of these purified curcuminoids, we detected the $[M - H]^-$ ions (*m/z* 367 for fraction A, 337 for B, and 307 for C) of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (*9, 11, 26*). As shown in MS/MS daughter scan mode (see **Figure 5**), the ion of *m/z* 217 or 187 is the base peak in the MS/MS spectra of deprotonated $[M - H]^-$ curcuminoids. Other product ions of *m/z* 173 and 149 for fraction A (curcumin), *m/z* 187, 173, 149, 143, and 119 for B (demethoxycurcumin), and *m/z* 143 and 119 for C (bisdemethoxycurcumin) were reported by HPLC with ion-trap MS/MS (*11*). We also recorded very similar fragmentation behavior of three curcuminoids by quadrupole MS/MS with negative ESI. Moreover, we were able to identify HSCCC purified references of curcumin (A), demethoxycurcumin (B), and bisdemethoxycurcumin (C) by LC-MS/MS analysis as well as the NMR technique. The ^{1}H NMR spectrum of fraction A showed a signal due to the methoxy group at 3.91 ppm, a singlet signal (5.96 ppm), a set of the trans olefin, and the trisubstituted benzene ring. In comparison with the integration of the signal at 5.96 ppm with the other signals, the molecule should be symmetrical and can be assigned as curcumin (see **Table 3**). In the spectrum of fraction B, the signals due to one methoxy group, two sets of olefins, one trisubstituted benzene, and one a para-substituted benzene ring appeared. Fraction C gave very simple spectrum with a singlet signal, one set of the trans olefin, and the para-substituted benzene ring. This indicated that the compound in C was a symmetrical molecule without methoxy groups, which could be assigned as bisdemethoxycurcumin. In this study, a pyridine (C_5D_5N) for NMR solvent was used and performed by the comparison with the authentic specimens (*27, 28*). On the basis of these LC-MS/MS and NMR data for the three curcuminoids, HSCCC-purified curcuminoids were identified as curcumin (fraction A), demethoxycurcumin (fraction B), and bisdemethoxycurcumin (fraction C).

DPPH Antioxidant Assay of HSCCC Purified Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin. A number of reports have been presented on free radical scavenging and antioxidant activities of curcuminoids (*13, 29*). It was suggested that curcumin had the strongest activity among the three tested curcuminoids, and the 8,8 (*para*)-hydroxyl groups are important for the free radical scavenging activity of the curcuminoids (*30*). However, a great number of studies used curcuminoids of low and/or unclear purity for reference standards for studying free radical scavenging and antioxidant activities. In this study, high-purity curcumin, demethoxycurcumin, and bisdemethoxycurcumin purified by HSCCC were applied to a DPPH radical scavenging assay. These reference compounds were curcumin standard (purity >95%) and BHT. From the DPPH assay data (see **Figure 6**), it was established that three purified curcuminoids were capable of scavenging DPPH radicals. The order of antioxidant activity is curcumin standard = purified curcumin (A) > purified demethoxycurcumin (B) > purified bisdemethoxycurcumin (C) > BHT (see **Figure 6**). This assay and other studies yield similar antioxidant profiles for the curcuminoids (*13, 29*). These purified curcuminoids can be used in other biological assays and food assessments.

In this paper, we described the development of a very easy and useful HPLC separation method using 0.1% FA in water/acetonitrile (50/50, v/v) and the high-quality purification of three curcuminoids (purity >98%) by HSCCC using the new

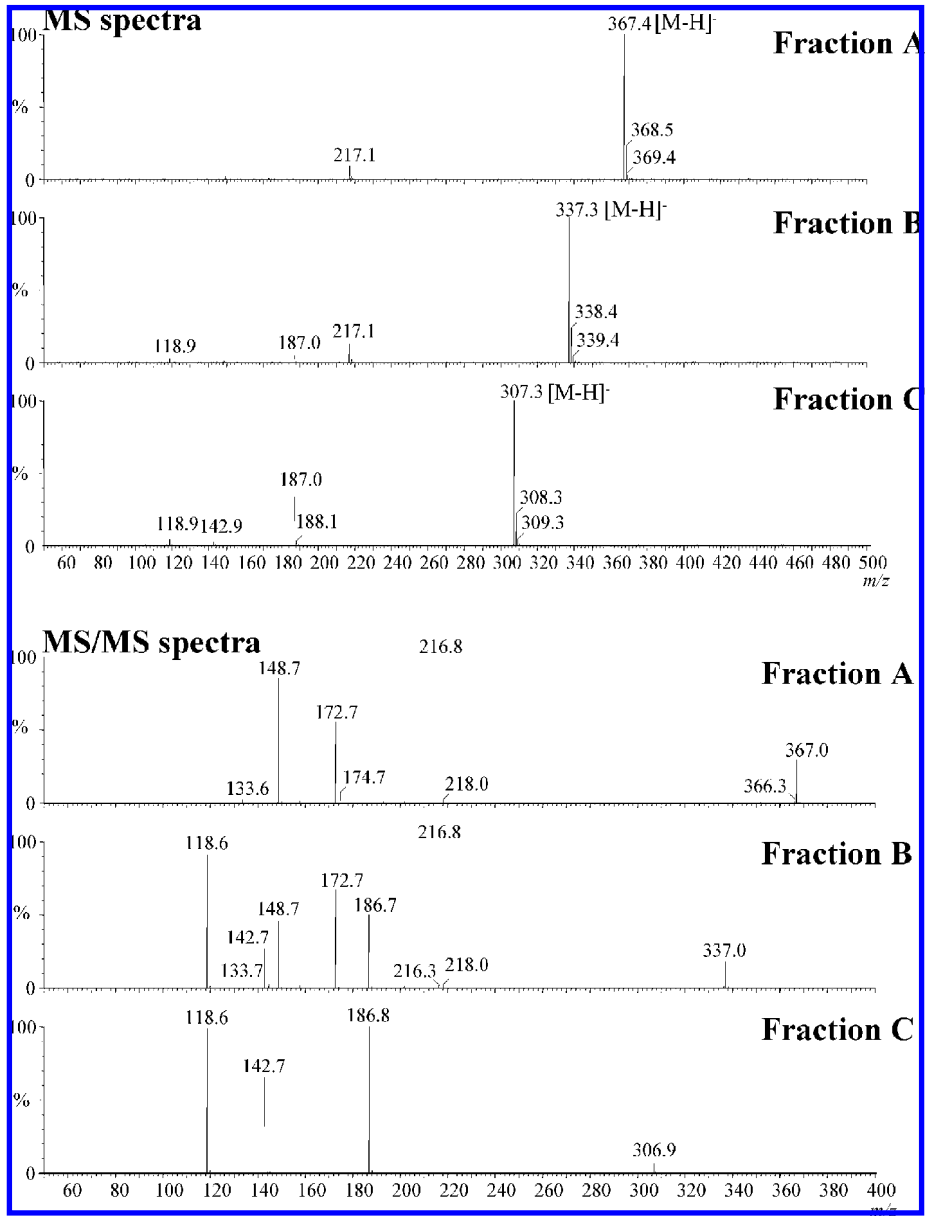

Figure 5. MS/MS spectra of purified curcuminoids (fractions A−C) by HSCCC. Spectra on MS scan and MS/MS daughter scan are obtained by a Waters Micromass Quattro Premier triple quadrupole mass spectrometer based on ESI-negative mode. A, fraction A was identified as curcumin; B, fraction B was identified as demethoxycurcumin; and C, fraction C was identified as bisdemethoxycurcumin.

Table 3. NMR Data of Purified Curcuminoids by HSCCC[a]

positions	fraction A = curcumin δ (ppm)	positions	fraction B = demethoxycurcumin δ (ppm)	positions	fraction C = bisdemethoxycurcumin δ (ppm)
1, 7	7.58 (2H, d, J = 16 Hz)	1	7.58 a (1H, d, J = 16 Hz)	1, 7	7.59 (2H, d, J = 16 Hz)
2, 6	6.69 (2H, d, J = 16 Hz)	2	6.69 b (1H, d, J = 16 Hz)	2, 6	6.65 (2H, d, J = 16 Hz)
4	5.96 (1H, s)	4	5.96 (1H, s)	4	5.95 (1H, s)
		6	6.65 b (1H, d, 16 Hz)		
		7	7.59 a (1H, d, 16 Hz)		
2′, 2″	7.33 (2H, d, J = 1 Hz)	2′	7.33 (1H, d, J = 1 Hz)	2′, 2″	7.56 (4H, d, J = 8 Hz)
5′, 5″	6.87 (2H, d, J = 8 Hz)	5′	6.87 (1H, d, J = 8 Hz)	6′, 6″	
6′, 6″	7.17 (2H, dd, J = 1, 8 Hz)	6′	7.17(1H, dd, J = 1, 8 Hz)	3′, 3″	6.89 (4H, d, J = 8 Hz)
		2″, 6″	7.56 (2H, d, J = 8 Hz)	5′, 5″	
		3″, 5″	6.89 (2H, d, J = 8 Hz)		
OMe	3.91 (6H, s)	OMe	3.91 (3H, s)		

[a] Used are the following abbreviations: s, singlet; d, doublet; and dd, doublet-of-doublets. For letters a and b, signal assignments may be interchangeable in the same column.

solvent system of *n*-hexane/chloroform/methanol/water (5/10/7.5/2.5, v/v). We evaluated the behaviors of the subsequent fragments of curcuminoids by quadrupole LC-MS/MS with negative ESI and established the antioxidant activity of the

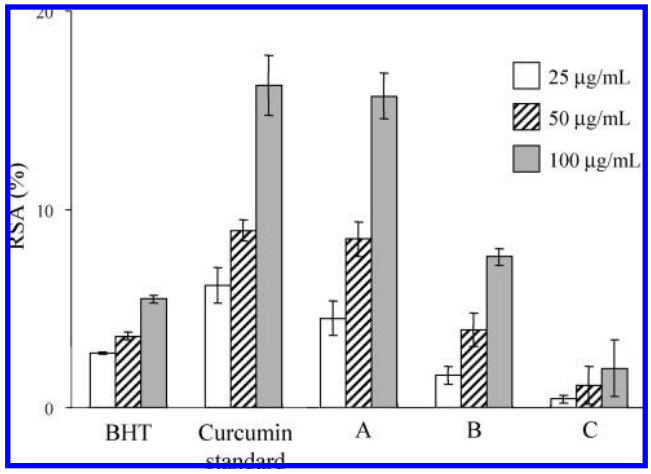

Figure 6. DPPH radical scavenging activity (RSA) of curcumin standard, BHT, and fractions A and B. A, fraction A was identified as curcumin; B, fraction B was identified as demethoxycurcumin; C, fraction C was identified as bisdemethoxycurcumin; and BHT, butylated hydroxytoluene. Curcumin standard was obtained from San-Ei Gen FFI Co., Inc. (Osaka, Japan).

purified compounds using a scavenging DPPH radical assay. It is suggested that this high purification of three curcuminoids is a very useful, simple, and efficient technique because this advanced HSCCC approach is easy to apply on an industrial scale and routine purification. Recent papers have reported that isolation and identification of individual curcuminoids and their radical scavenging activity have been investigated by specific methods (*6, 13, 29*). Jayaprakasha et al. used the CRTO for isolation of three curcuminoids from turmeric (*6, 29*). This oleoresin is a less common material and method for routine purifications. Pozharitskaya et al. used the HPTLC with two different mobile phases for separation and DPPH assay of three curcuminoids from turmeric (*13*). This aim of study was not to develop the efficient isolation but to determine simultaneously the qualitative and quantitative composition of curcuminoids from turmeric (*13*). On the other hand, our reported HSCCC method and solvent system will highlight the advantages of high purification and predictable scale-up for the development process that will cut their costs and enable them to reduce the time because HSCCC is suggested to emerge as a major enabling technology for industry (*24*). This study is shown to the effect of processing on safety food additives for three curcuminoids and/or turmeric by useful and efficient HSCCC purification and basic analytical data with HPLC, LC-MS/MS, and NMR.

ABBREVIATIONS USED

M.W., molecular weight; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC-MS/MS, liquid chromatogramphy with tandem mass spectrometry; HSCCC, high-speed countercurrent chromatography; ESI, electrospray ionization; NMR, nuclear magnetic resonance; DPPH, 1,1-diphenyl-2-picrylhydrazil; AA, acetic acid; FA, formic acid; TFA, trifluoroacetic acid; BHT, butylated hydroxytoluene; PTFE, polytetrafluoroethylene; K, partition coefficient; FIA, flow injection analysis; Rs, peak resolutions; t, retention time; $W_{0.5\ h}$, peak width of the midpoint; PDA, photodiode array detector; TIC, total ion chromatogram.

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