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Optimization and Validation of High-Performance Liquid Chromatography Method for Individual Curcuminoids in Turmeric by Heat-Refluxed Extraction

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ABSTRACT: Turmeric is a plant in the *Zingiberaceae* family which contains curcuminoids as anticancer agents and has been widely used as a main ingredient in curry powder. However, there is a lack of suitable high performance liquid chromatography-diode array detection (HPLC-DAD) methods for reducing sample preparation time and peak resolution improvement of curcuminoids (bisdemethoxycurcumin, demethoxycurcumin, and curcumin). No significant differences in yield concentrations were observed after 60 min of heat-refluxed extraction ($p < 0.05$). Simultaneous chromatographic separation of all three curcuminoids achieved satisfactory results with a separation factor of 1.08 and a resolution factor of 3.39 with validation results in compliance with FDA guidelines. The expanded relative measurement uncertainty results 5.71−6.60 complied with CODEX draft. The method was successfully applied to the turmeric samples ($n = 107$, range 2.70−4.41 g/100 g, total curcuminoids 3.58 g/100 g). These results show that heat-refluxed extraction can be carried out easily with excellent precision and accuracy of total curcuminoids in turmeric samples.

KEYWORDS: *turmeric, curcumin, demethoxycurcumin, bisdemethoxycurcumin, method validation, high-performance liquid chromatography (HPLC)*

INTRODUCTION

Turmeric (from the rhizome of *Curcuma longa* L) is a plant in the *Zingiberaceae* family and the main ingredient of curry, which has been widely used as a coloring and flavoring agent.[1,2] Turmeric was originally consumed as a food additive in curries and used to enhance storage, preservation, and palatability of food.[3] Turmeric is cultivated mostly in India and surrounding regions such as Bangladesh, China, Cambodia, Malaysia, Thailand, Philippines, and Indonesia.[4] It is particularly a staple in India, where 94% of the turmeric in the world originates.[5] Furthermore, it has long been used traditionally as an herbal medicine for treating a variety of inflammatory conditions such as coryza, cough, diabetic wounds, hepatitis, and other diseases.[6]

The major bioactive compounds in turmeric are three different curcuminoids consisting of curcumin as the dominant constituent, demethoxycurcumin, and bisdemethoxycurcumin. Pharmacological studies conducted so far on turmeric have shown that curcuminoids have several biological activities, such as an antioxidant, antiprotozoal, antimicrobial, antivenom, anti-HIV, antitumor, anti-inflammatory, anticancer, and anticarcinogenic.[3,7−12] Thus, turmeric has a wide range of health benefits and antioxidant activity in food and biological systems.

Curcuminoids make up 3−5% of turmeric depending on the variety.[13] However, there is a lack of information about individual curcuminoids according to the diverse varieties and origins. Various methods have been established to determine individual curcuminoids in turmeric powder and extracts, such as spectrophotometric measurements, capillary electrophoresis,[14] high performance liquid chromatography (HPLC),[2,15−18] liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ES-MS/MS),[1,19] near-infrared spectroscopy[20] and nuclear magnetic resonance.[21] Refluxing for 1−2.5 h,[1,22,23] Soxhlet extraction for 2−5 h,[15,16] shaking for 20 min[19,20] sonication[14] for 2 min, and magnetic stirring for 20 h[24] have been reported as methods used to extract curcuminoids. However, none of these studies compared different refluxing times during sample extraction or conducted measurement uncertainty of individual curcuminoids analysis using HPLC. The use of a conventional HPLC method is preferred for analysis of curcuminoid concentrations in a large number of turmeric samples because of its low-cost and ease of application.

The objectives of this study were (1) to improve a routine analytical method to determine curcuminoids in turmeric samples, including optimization of sample preparation (heat-refluxed extraction) and chromatographic conditions to enhance peak resolution; (2) to validate linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, recovery, stability and measurement uncertainty for reliability of analysis; and (3) to further elucidate differences in the curcuminoid content in turmeric consumed in Korea.

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MATERIALS AND METHODS

Reagents and Chemicals. The chemicals used were all analytical grade. Pure reference standards of curcumin (96.4% purity, CAS no. 458−37−7), demethoxycurcumin (95.1% purity, CAS no. 22608−11−3), and bisdemethoxycurcumin (91.8% purity, CAS no. 22608−12−4) were supplied by Chromadex Co. (Irvine, CA). The chemical structures of these compounds are shown in Figure 1. Methanol and acetonitrile were obtained from J.T. Baker Chemical Co. (Deventer, The Netherlands) and Fisher Scientific (Pittsburgh, PA), respectively. High purity water (18.2 MΩ) as the HPLC eluent was purified using a Milli-Q Water system (Millipore, Bedford, MA). Liquid chromatography solvents were filtered with a 47 mm, 0.45 μm HVLP (Millipore, Billerica, MA).

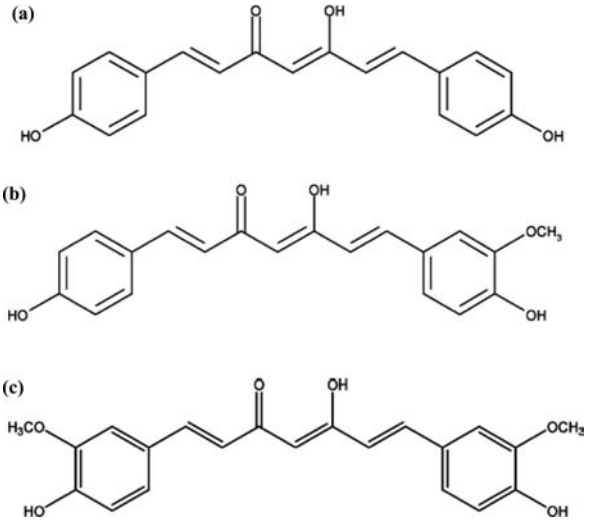

Figure 1. Chemical structures of bioactive constituents detected in turmeric; bisdemethoxycurcumin (a), demethoxycurcumin (b), and curcumin (c).

Preparation of Standard Solutions. Individual standard solutions were prepared by dissolving 5 mg of each compound in 10 mL of methanol to obtain a final concentration of 500 mg/L. The working standard solutions were prepared at concentrations of 0.2, 1.0, 5, 10, 25, 50, and 100 mg/L by serially diluting the curcuminoid solutions in methanol to fit the calibration curves and determine the linearity of the responses.

Turmeric Sample Preparation. Dried turmeric samples were powdered using a Knifetec 1095 sample mill (Foss Tecator, Höganäs, Sweden). They were then passed through a 250 μm stainless steel sieve before extraction. One hundred mg of turmeric was weighed and dissolved in about 30 mL of methanol in a 100 mL round-bottomed flask and refluxed in a cooled condenser, according to the modified procedures described in the ASTA method.[22] We observed refluxing time at 30 min intervals for up to 180 min to determine optimum extraction efficiency. After cooling, the extract was transferred to a 100 mL volumetric flask with a methanol rinse and penetrated through glass wool. Extracted sample solutions were filtered through 15 mm, 0.45 μm RC membrane filters (Sartorius, Goettingen, Germany) for HPLC analysis. The extract was placed in a light resistant amber colored glass vial, and 5 μL of the sample solution was injected into the HPLC system.

Apparatus. All chromatographic analyses were carried out using an Agilent Technologies HPLC (1200 series, Palo Alto, CA) with an automatic degasser, quaternary pump, autosampler, column thermostat, and DAD for analysis. A Capcell Pak C18 UG120 column (4.6 mm i.d. × 250 mm; 5 μm; Shiseido, Inc., Tokyo, Japan) was employed for analytical separation and quantification with the column temperature set to 25 °C. HPLC analysis was performed using mobile phase A (1% acetic acid in distilled water) and B (acetonitrile) in a gradient program at a flow rate of 1.0 mL/min. The mobile phase started with an initial gradient of 45% A and then increased from 45% solvent A to 50% in 10 min. The system was then kept at the initial mobile phase condition for another 5 min for the next run. The DAD was positioned at a wavelength of 424 nm (at 4 nm bandwidth) with a 550 nm reference wavelength (at 50 nm bandwidth). Full spectral scanning was also performed from 200 to 600 nm, with a range step of 2 nm. Agilent Chemstation software was used to control all analytical conditions and the chromatographic data processing. Sample quantification was calculated by comparing peak area with the external calibration curve from neat standard solution. The content of each curcuminoid was estimated as follows: $C_{spl} = (A_j - B_o)/B_1 \times F$, where C_{spl} is the curcuminoid concentration; A_j, is the jth measurement of the area of the ith calibration standard; B_1 is the slope of the calibration curve; B_o is the intercept of the calibration curve, and F is a dilution factor.

Column Performance Test. Peak asymmetry (A_s) was estimated at 10% of peak height from the ratio of the widths of the rear and front sides of the peak.[25] Retention factors (k') were calculated as $k' = (t_r - t_0)/t_0$, where t_0 is retention time of unretained solvent and t_r is retention time of the analyte. The k' values were within the optimum range ($1 \leq k' \leq 10$) for satisfactory chromatographic elution of curcuminoids.[26] Separation factors (α) were >1, which indicated acceptable separation.[26] The resolution factor (R_s), which is a measurement of how well two peaks are separated, was calculated as $R_s = 2 \times (t_1 - t_2)/(w_1 + w_2)$, where t_1 and t_2 are the retention time for two adjacent peaks; and w_1, w_2 are peak widths at the bases. In addition, the number of theoretical plates (N), which is a measurement of peak dispersion, was calculated from peak width at half height ($w_{0.5}$) using the formula $N = 5.54(t_r/w_{0.5})^2$.[25]

HPLC Method Validation. The HPLC-DAD method was fully validated for selectivity, LOD, LOQ, linearity, precision, accuracy, recovery, and stability according to the FDA guideline.[27]

a. Selectivity. Selectivity is the ability of an analytical method to differentiate and quantify the individual curcuminoids that are expected to be present. Blank matrix samples were tested for interference, and selectivity was evaluated by comparing the chromatograms of blank and turmeric sample solutions by confirming DAD. Retention times were determined to verify peaks which were compared to a ratio of sample peaks from DAD to a ratio of reference standard peaks.

b. LOD, LOQ, and Linearity. The LOD and LOQ were defined according to Knoll's research[28] as the lowest concentration of analyte in the standard solution that triggers a significantly different instrumental signal from the blank or background noise, which is equal to signal-to-noise ratios of 3 and 10, respectively. Linearity of the detector response was verified with bisdemethoxycurcumin, demethoxycurcumin, and curcumin standard solutions over the range of 0.2−100 mg/L. Calibration curves were prepared each experiment day, and the concentration of the analytes in the samples was calculated.

c. Precision and Accuracy. The intraday precision of the HPLC−DAD method was tested six times/day with a quality control sample (fortified turmeric solution), which was a standard solution of bisdemethoxycurcumin, demethoxycurcumin, and curcumin. For interday precision, three measurements/day on three different days were conducted. The precision of the method was expressed as the relative standard deviation (RSD) for the repeated measurements. The accuracy of the method was calculated as the relative difference between the determined and nominal concentrations of the analyzed samples.

d. Recovery. Turmeric samples were spiked with curcuminoid standards. Turmeric samples (bisdemethoxycurcumin for Indian turmeric, demethoxycurcumin for Chinese turmeric, and curcumin for Korean turmeric sample 100 mg used) were analyzed in triplicate to quantify each curcuminoid concentration as a blank. Standard-added samples were prepared containing individual curcuminoids (0.05 mL, 0.5 mL, and 2.5 mL of 1000 mg/L stock solution) to give final results at three concentrations (for 0.05 g/100 g, 0.5 g/100 g, and 2.5 g/100 g, dilution factor = final volume;100 mL volumetric flask/sample weight; 100 mg, that is 1,000). The recovery (R) was calculated by the method of Rodriguez et al.[29] as $R = (C_{found} - C_{sample})/C_{added}$,

where C_{found} is the concentration in the standard added sample, C_{sample} is the concentration as a blank sample, and C_{added} is the added concentration.

e. Stability. The stability of curcuminoids in turmeric extracted solution was conducted under various conditions; freeze−thaw stability (at −20 °C and room temperature for three cycle), short-term stability (at room temperature for 12 h), long-term stability (−20 °C for 6 weeks), which were analyzed through triplicate determinations in low, mid and high concentration (1 mg/L, 5 mg/L and 25 mg/L in matrix solution), respectively. Furthermore, postpreparative stability was evaluated in the sample solution by tests in HPLC autosampler (sample solution, at room temperature, for 12).

f. Measurement Uncertainty Assessment. Uncertainty of a measurement is defined as "a parameter, which is associated with the result of a measurement and characterizes the dispersion of the values that could reasonably be attributed to the measurand".[30] The measurement uncertainty budget for the methods was evaluated based on the modified of EURACHEM/CITAC Guide and Guide to the Expression of Uncertainty in Measurement (GUM) method.[31] The sources of measurement uncertainty (i.e., standard stock solution (u_{SSS}), sample preparation (u_{SP}), calibration curve (u_{Cal}), and repeatability for the determination of curcuminoids in turmeric sample (u_{RP})) in associated with the analysis of curcuminoids were evaluated. There error components were estimated and calculated as an expanded uncertainty (U_c) using a coverage factor (k) of 2 at the confidential level of 95%.

Statistical Analysis. Samples were prepared and analyzed in triplicate. The results are reported as mean values and standard deviations. Differences were detected by one-way analysis of variance (ANOVA) using the SPSS 12.0.1 version 4 software package (SPSS, Inc., Chicago, IL) with a significance level of 0.05.

RESULTS AND DISCUSSION

Analytical Characteristics. *a. Optimizing Sample Preparation Efficiency.* The sample preparation procedure for HPLC is one of the most important and time-consuming steps, which may also affect errors. Various extraction methods for turmeric have been described in the literature. Turmeric powder samples have been extracted with hexane for 30 min using a Soxhlet extractor and re-extracted with methanol for 2 h.[15] Bos et al.[16] extracted turmeric powder samples with pentane and methanol for 2 and 3 h using a Soxhlet extractor, respectively, and the methanol solution was concentrated using a rotary evaporator. These studies may not be suitable for routine analysis of a large number of turmeric samples because sample extraction takes a long time. The ASTA method, which is a common method for measuring turmeric color, uses a reflux time of 2.5 h.[22] However, information related to turmeric reflux extraction time for determining curcuminoids by HPLC is scarce. To determine the reflux time (0, 30, 60, 90, 120, 150, and 180 min, at 65 °C) for optimum extraction efficiency, turmeric powder was extracted at each reflux time with three replicate extractions. Each compound concentration was analyzed by HPLC to determine the effectiveness of sample preparation. As shown in Figure 2, significant differences were observed between unrefluxed (0 min) and refluxed samples (30−180 min). In case of demethoxycurcumin and curcumin, no significant differences in yield concentrations with good precision and no thermal decomposition were observed by comparing 30−180 min heat-refluxed samples. However, bisdemethoxycurcumin showed significant differences between 30 min and 60−180 min refluxing time period ($p < 0.05$). Based on these results, we chose 60 min as optimum refluxing time. This indicated that refluxing sample extraction resulted in outstanding thermal stability and repeatability (RSD%: < 1.4% of total curcuminoids). In addition, the standard reflux time of 60 min showed good recovery rates (bisdemethoxycurcumin 101.09%, demethoxycurcumin 99.41%, and curcumin 100.45%, Table 4).

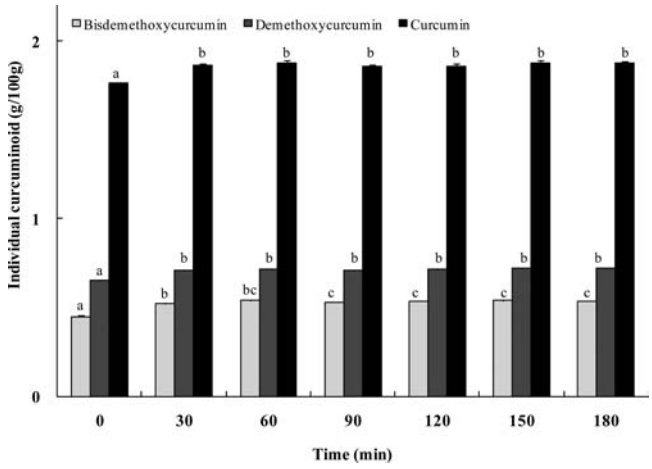

Figure 2. Individual curcuminoids of turmeric powder for refluxing different time periods (0−180 min). * Different letters in individual curcuminoids are significant differences ($p < 0.05$), based on the ANOVA test. Analysis was conducted three times in each refluxing time. Bar indicate ± standard deviation.

b. Optimization of Chromatographic Analysis. Several preliminary studies have been conducted to develop an HPLC or LC-MS/MS method for separating individual curcuminoids in turmeric using conventional C18 columns.[15,16,18−20] It is difficult to separate the individual curcuminoids due to their similar chemical characteristic.[19] To overcome the problems associated with the chromatographic separation, attempts were made to use short and long columns, respectively, HPLC columns including Zorbax Eclipse Plus C18 column (4.6 mm i.d. × 150 mm; 5 μm) and Capcell Pak C18 UG120 column (4.6 mm i.d. × 250 mm; 5 μm) with isocratic and gradient elution were used to achieve efficient resolution. Several mobile phases, composed of mixtures of acetonitrile, methanol, acetic acid, trifluoroacetic acid (TFA), and water in different ratios, were used to optimally separate the curcuminoids. However, Williams reported that a considerable ghost peak appeared originating from the TFA in the mobile phase.[32] Therefore, our current study did not use TFA as a mobile phase for curcuminoid analysis. In addition, the more complicated the mobile phase, the more possibility of impurities and ghost peaks are possible.[32]

Optimum separation was achieved with the Capcell Pak C18 UG120 column, which showed better resolution between individual curcuminoid compounds. A reverse-phase HPLC system was applied to optimally separate individual curcuminoids within 10 min. A typical chromatogram for the curcuminoids in turmeric powder is shown in Figure 3. Table 1 shows the column performance values obtained for the curcuminoids analysis. A_s over 0.92 in our result presented a good indicator of column deterioration or errors in the mobile phase preparation.[25] The k' value over 2.80 indicated a better separation compared to a previous study by Bos et al.[16] All α factors were 1.08, which indicated acceptable separation.[26] In addition, R_s values (3.39) and N were obtained by applying our method.

For confirming the robustness of slight variations in method, the proposed analytical method has been performed with three different manufacturer's 250 mm length columns (Shiseido

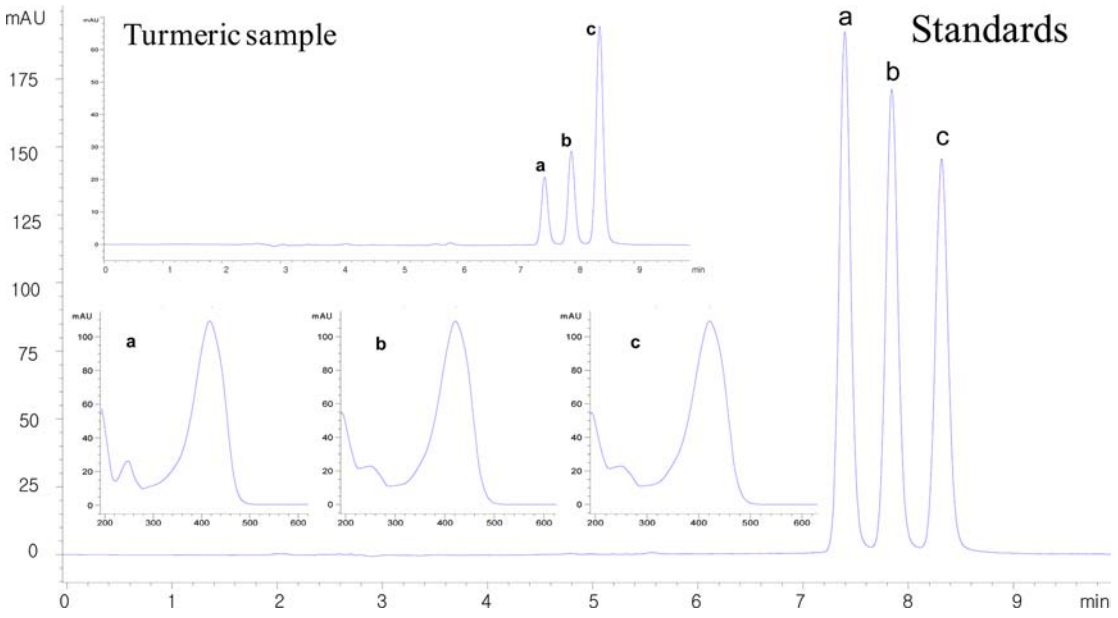

Figure 3. HPLC-DAD Chromatogram and UV-vis spectra of a standard mixture at 25 mg/L level and a turmeric sample solution showing curcuminoids. (a) bisdemethoxycurcumin (t = 7.39 min, c = 0.567 g/100 g), (b) demethoxycurcumin (t = 7.83 min, c = 0.893 g/100 g), (c) curcumin (t = 8.32 min, c = 2.244 g/100 g).

Table 1. Column Performance Data for Individual Curcuminoids[a]

compound	A_s	k'	α	R_s	N
bisdemethoxycurcumin	0.92	2.80			50 882
demethoxycurcumin	0.93	3.04	1.08	3.39	50 830
curcumin	0.93	3.29	1.08	3.39	51 802

[a]Abbreviations: A_s, peak asymmetry; k', retention factor; α, separation factor; R_s, resolution factor; N, number of theoretical plates (efficiency).

Capcell Pak C18 UG120, Agilent Eclipse XDB C18, Waters Sunfire C18), three different lots of Shiseido column, mobile phase (A) variation depending on the acetic acid composition of 0.95, 1.0, and 1.05%, and column temperatures (20, 22.5, 25, 27.5, and 30 °C). These robustness results indicate that small variations were negligible in separating individual curcuminoids analysis.

Therefore, system suitability parameters related to the chromatographic separation were satisfactory, and total separation was highly efficient under the analytical conditions used.

c. Chromatograms, Qualitative, and Quantitative Analysis. A simple and rapid HPLC method for quantifying curcuminoids in turmeric samples was developed and validated. The chromatograms showed that the method can be used to successfully separate the three curcuminoids in a standard mixture of 25 mg/L (Figure 3). Retention times were 7.39 ± 0.08, 7.83 ± 0.08, and 8.32 ± 0.10 min, respectively for bisdemethoxycurcumin, demethoxycurcumin, and curcumin, and symmetric peaks were observed. The relative peak area and relative peak retention time of each curcuminoid were in the ranges of 0.02−1.18% and 0.33−2.54% RSDs, respectively. These results indicate that the chromatographic conditions used for the qualitative analysis were adequate. The parameters of the regression equations obtained for the calibration curves are shown in Table 2.

Method Validation. *a. Selectivity.* The selectivity of the HPLC method was estimated with the corresponding standards and peak retention time, peak purity, and resolution. Comparing retention times is one of the easiest measurements to make and identify in an HPLC run. Additionally, peak purity was confirmed by the DAD data while checking the purity of individual peaks. DAD facilitated the validation and development of the HPLC method based on spectra absorption and included effective data for verifying peaks.

b. LOD, LOQ, and Linearity. The LOD and LOQ were ranged from 0.03−0.04 mg/L and 0.10−0.14 mg/L,

Table 2. Calibration Parameters Obtained for Individual Curcuminoids from Three Different Calibration Curves Prepared in Triplicate

parameters	bisdemethoxycurcumin	demethoxycurcumin	curcumin
range of calibration (mg/L)[a]	0.20−100	0.20−100	0.20−100
retention time (min)	7.39 ± 0.08	7.83 ± 0.08	8.32 ± 0.10
slope (±S.D.)	60.32 ± 1.23	54.97 ± 0.66	52.15 ± 0.66
intercept (±S.D.)	−6.75 ± 7.19	4.52 ± 8.00	10.25 ± 15.48
regression coefficient (r^2)	0.99995 ± 0.00004	0.99992 ± 0.00005	0.99983 ± 0.00019
limit of detection (mg/L)[b]	0.03	0.04	0.04
limit of quantification (mg/L)[b]	0.10	0.14	0.14

[a]Assessed at five concentration levels. [b]Assessed by analysis of calibrated samples (n = 6).

Table 3. Validation Parameters for the Precision and Accuracy Assay of the Fortified Turmeric Solutions

parameters	bisdemethoxycurcumin	demethoxycurcumin	curcumin	total curcuminoids[c]
nominal concentration (g/100 g)	0.54	0.72	1.88	3.13
Intraday Validation[a]				
number of samples	6	6	6	6
concentration (g/100 g)	0.53 ± 0.01[d]	0.71 ± 0.01	1.87 ± 0.01	3.11 ± 0.03
precision (RSD %)	1.02	1.11	1.07	1.05
accuracy (%)	99.1%	99.2%	99.6%	99.4%
Interday Validation[b]				
number of samples	9	9	9	9
concentration (g/100 g)	0.54 ± 0.01	0.72 ± 0.01	1.88 ± 0.03	3.14 ± 0.05
precision (RSD %)	1.41	1.44	1.56	1.48
accuracy (%)	100.6%	100.6%	100.3%	100.4%

[a]Analysis was conducted six times/day for the repeatability test. [b]Analysis was conducted three times on three different days for the reproducibility test. [c]Sum of bisdemethoxycurcumin, demethoxycurcumin, and curcumin. [d]Data are mean ± standard deviation values.

Table 4. Recovery Method Results for Individual Curcuminoids Spiked in Turmeric Samples

compounds	present (g/100 g)	added (g/100 g)	found (g/100 g)	rsd (%)	recovery (%)
bisdemethoxycurcumin	0.567	0.05	0.618 ± 0.002	0.31	102.45 ± 4.61
		0.5	1.072 ± 0.002	0.22	101.04 ± 0.47
		2.5	3.062 ± 0.008	0.26	99.79 ± 0.32
		average		0.26	101.09
demethoxycurcumin	0.527	0.05	0.579 ± 0.002	0.37	104.01 ± 4.30
		0.5	1.013 ± 0.001	0.10	97.13 ± 0.20
		2.5	2.954 ± 0.012	0.41	97.08 ± 0.48
		average		0.29	99.41
curcumin	0.516	0.05	0.568 ± 0.001	0.15	103.66 ± 1.72
		0.5	1.007 ± 0.001	0.13	98.21 ± 0.27
		2.5	3.003 ± 0.016	0.53	99.48 ± 0.64
		average		0.27	100.45

Table 5. Intermediate Values and Uncertainties for Individual Curcuminoids Determination in Turmeric Sample

	concentration (g/100 g)	the combined uncertainty (g/100 g)	the expanded uncertainty (g/100 g)	relative uncertainty (%)
curcuminoids	C_{spl}	$u(Cs)$	$u(Cs) \times k$	$u(Cs) \times k/C_{spl}$
bisdemethoxycurcumin	0.56	0.02	0.04	6.60
demethoxycurcumin	0.87	0.03	0.05	5.87
curcumin	2.17	0.06	0.12	5.71
total curcuminoids	3.60	0.11	0.21	5.89

respectively. Linear regression was used to assess the calibration curves and correlation coefficients from seven different concentrations using five replicate injections. Regression coefficients were all >0.999 on each of the 3 days in which calibration curves were run. The calibration parameters, LOD, and LOQ of the three curcuminoids are shown in Table 2.

c. Precision and Accuracy. Table 3 shows the intraday and interday precision and accuracy values of the entire procedure for each curcuminoid. Excellent intraday precision data ($n = 6$) and interday precision data ($n = 9$) were obtained for the turmeric powder samples containing all analytes. The RSDs ranged from 1.02% to 1.11% for the intraday precision tests, and 1.41% to 1.56% for the 3-day interday precision tests. These values were considered in high compliance with FDA criteria (<15%) for bioanalytical method validation.[27] Accuracy estimates varied from 99.1% to 99.6% for the intraday accuracy tests, and 100.3% to 100.6% for the 3-day interday accuracy tests.

d. Recovery. The recoveries were 102.45−104.01%, 97.13−101.04%, and 97.08−99.79% for added level 0.05, 0.5, and 2.5 g/100 g (dilution factor was calculated in), respectively. The percentage recovery was acceptable at all added levels, and average recoveries were >97.08% with the RSD for each analyte <0.53%. Table 4 shows the recoveries (average 100.32%) that were close to 100%. This observation was similar to previous studies.[16,17,20] Further, recoveries of the method were in compliance with the FDA in the range of 80−120%.[27]

e. Stability. Lechtenberg et al.[14] and Gören et al.[21] reported that the main problem in curcumin analysis was that curcuminoids are light sensitive compounds. All experiments including stability test for curcuminoids analysis used light resistant amber colored glass vials to protect the curcuminoids from light. For the stability evaluation, the average and standard deviations between initial concentration and the found concentration of the solution were compared. The fortified matrix sample solution had an acceptable stability at room temperature for 12 h (SD < 3.26%), at −20 °C for 6 weeks (including standard stock solutions), postpreparative stability for 12 h, and three freeze−thaw cycles within 98.0−101.6% range based on initial concentration.

f. Measurement Uncertainty Assessment. This study was demonstrated to estimate the measurement uncertainty of

individual and total curcuminoids from turmeric powder by HPLC. The sources of measurement uncertainty (i.e., sample weight, final volume, standard weight, purity, standard solution, calibration curve, repeatability) in associated with the analysis of curcuminoids were evaluated. This approach has not yet been reported to curcuminoids determination in turmeric.

The content of total curcuminoids from turmeric powder was 3.6 g/100 g and the expanded uncertainty by multiplying coverage factor (k = 2) was 0.21 g/100 g at a 95% confidence level (Table 5). These expanded relative uncertainty values (5.71−6.60%) were considered in compliance with CODEX criteria (<8%).[33] The major contributors to the measurement uncertainty were identified in the order of calibration-curve (2.6%), standard stock solutions (1.7%), repeatability (1.4%), and sample pretreatment (0.2%) (Figure 4). Therefore, more careful experiments are required in these steps to reduce uncertainties of curcuminoids analysis with a better personal proficiency improvement.

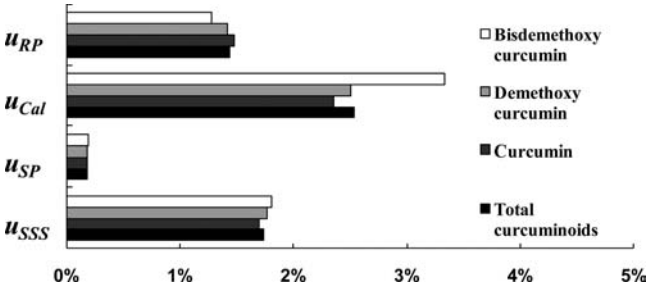

Figure 4. Uncertainty contributions of the curcuminoids determination in turmeric; standard stock solution (u_{SSS}), sample preparation (u_{SP}), calibration curve (u_{Cal}) and repeatability for the determination of curcuminoids in sample (u_{RP}).

Application of the Method to Determine Curcuminoids in Turmeric Samples. According to Korea's trade statistics from 2000 to 2011, turmeric was imported mainly from India (77.7%), followed by China (8.5%), Indonesia (4.6%), Myanmar (4.3%), and Vietnam (1.1%). A total of 610 t of turmeric were imported in 2011, which was 2.4 times more than in 2000.[34]

The 12 dried turmeric rhizome samples (originating from India, China, and Korea) were obtained from Kyeongdong oriental market in Seoul, Korea and tested for their individual curcuminoids. As shown in Table 6, all samples showed measurable concentrations of bisdemethoxycurcumin, demethoxycurcumin, and curcumin, but the results varied. The mean ± SD values of individual curcuminoids in the turmeric samples were 0.40 ± 0.34 g/100 g (range, 0.02−1.22 g/100 g), 0.60 ± 0.40 g/100 g (range, 0.06−1.10 g/100 g), and 1.31 ± 0.79 g/100 g (range, 0.18−2.29 g/100 g) for bisdemethoxycurcumin, demethoxycurcumin, and curcumin, respectively. The average total curcuminoid content in turmeric was 2.32 g/100 g with a range of 0.26−4.16 g/100 g. Among the turmeric sample tests, the relative percentage of each curcuminoid was as follows: curcumin (60.0%), demethoxycurcumin (24.8%) and bisdemethoxycurcumin (15.2%), respectively. The average total curcuminoid content of Indian turmeric (*C.longa*) was significantly (1.6−2.8 times) higher than that of the Chinese sample (*C. longa*) and 6.3−15.8 times higher than that of the Korean turmeric (*C. aromatica*) (p < 0.05). Jayaprakasha et al.[15] reported that Erode and Salem varieties of Indian turmeric have greater amounts of total curcuminoids content and may be good source for the isolation of curcuminoids. Difference of curcuminoids content from Korean and Indian varieties was probably due to varieties, geographic location, climate, and soil condition, which may affect the curcuminoid contents.

In addition, further studies were carried out on 107 Indian turmeric samples for a better understanding of curcuminoid content. Frequency distribution histogram of the individual curcuminoids in the samples used in this study showed that bisdemethoxycurcumin (Figure 5a), demethoxycurcumin (Figure 5b), curcumin (Figure 5c), and total curcuminoids (Figure 5d) exhibited normal distributions. Total curcuminoid contents in turmeric ranged from 2.70 to 4.41 g/100 g (average, 3.58 g/100 g) with a SD of 0.42 g/100 g. When curcumin was the main component it ranged from 1.58 to 2.81 g/100 g (average, 2.14 g/100 g) as determined by HPLC. The amounts of bisdemethoxycurcumin and demethoxycurcumin ranged from 0.41 to 0.88 g/100 g (average 0.58 g/100 g) and from 0.56 to 1.19 g/100 g (average 0.86 g/100 g), respectively. Our current study showed that curcumin had a higher percentile average, representing 59.9% of the total curcuminoids, and was followed by demethoxycurcumin (23.8%) and bisdemethoxycurcumin (16.3%) in Indian turmeric samples. These results are similar as Jayaprakasha et al.[15] They demonstrated that contributed percentile ranges of the total curcuminoids from turmeric were 45.1−79.6%, 11.7−36.9%, and 8.7−23.5% for curcumin,

Table 6. Quantification of Various Turmeric Samples Consumed in Korea[a]

	origin	bisdemethoxycurcumin (g/100 g)	demethoxycurcumin (g/100 g)	curcumin (g/100 g)	total curcuminoids (g/100 g)
1	India (*C. longa*)	0.66 (16%)	1.10 (26%)	2.28 (55%)	3.80 ± 0.19[a]
2		1.22 (31%)	1.01 (26%)	1.71 (43%)	
3		0.53 (14%)	0.94 (25%)	2.29 (61%)	
4		0.56 (15%)	1.00 (27%)	2.13 (58%)	
5		0.55 (15%)	0.95 (27%)	2.06 (58%)	
6	China (*C. longa*)	0.42 (19%)	0.64 (29%)	1.14 (52%)	1.91 ± 0.31[b]
7		0.29 (15%)	0.48 (24%)	1.21 (61%)	
8		0.29 (15%)	0.47 (24%)	1.22 (62%)	
9		0.25 (17%)	0.36 (25%)	0.85 (58%)	
10	Korea (*C.aromatica*)	0.04 (7%)	0.10 (19%)	0.42 (75%)	0.37 ± 0.17[c]
11		0.03 (9%)	0.07 (23%)	0.19 (68%)	
12		0.02 (9%)	0.06 (22%)	0.18 (69%)	

[a]Different letters in total curcuminoids are significant differences (p < 0.05), based on the ANOVA test. Values in parentheses indicate percentage contributed to total curcuminoids. Data are means of three replicates.

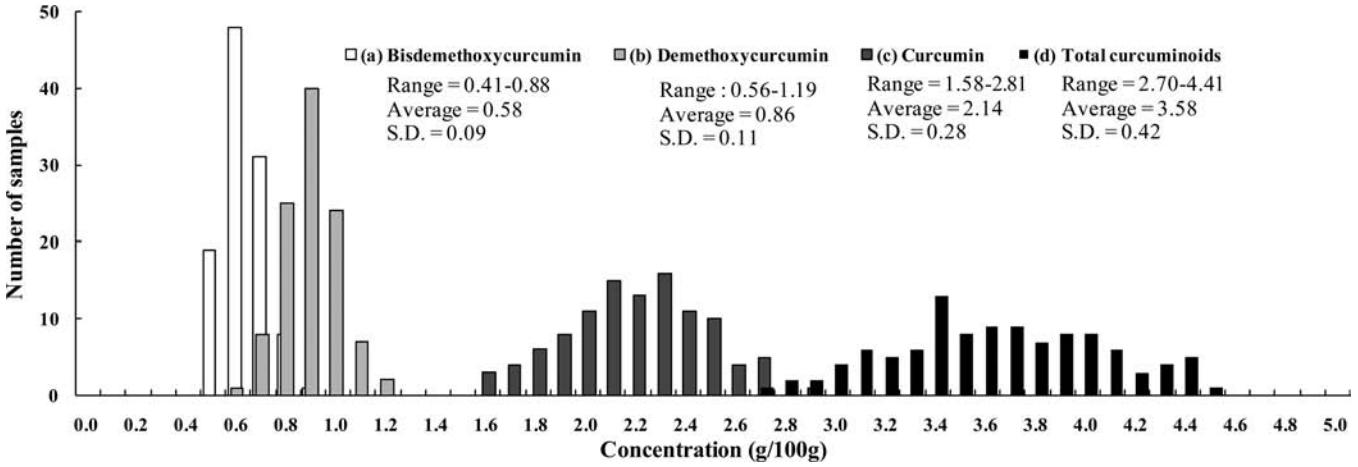

Figure 5. Frequency distribution histogram of the individual curcuminoid for the Indian turmeric consumed in Korea (n=107) (g/100 g). (a) Bisdemethoxycurcumin, (b) Demethoxycurcumin, (c) Curcumin, (d) Total curcuminoids.

demethoxycurcumin, and bisdemethoxycurcumin, respectively. Turmeric contains a maximum total curcuminoid level 1.63 times higher than the minimum level, and curcuminoids of turmeric consisted of curcumin (54.3−65.2%), demethoxycurcumin (19.3−29.3%) and bisdemethoxycurcumin (11.9−22.2%). The contents range of individual curcuminoids in Indian turmeric samples analyzed were similar to those reported previously. Total curcuminoid contents of turmeric are 2.3−9.2 g/100 g for Indian varieties[15] of *C. longa* (n = 4) and 0.80−1.0 g/100 g for *C. xanthorhiza*[14] observed that the curcuminoid contents from Indonesian turmeric[16] were 0.18−0.47 g/100 g for *C. mangga*, 0.98−3.21 g/100 g for *C. heyneana*, 0.02−0.03 g/100 g for *C. aeruginosa*, and 0.40 g/100 g for *C. soloensis*.

We have described the optimization and full validation procedure for curcuminoids in turmeric, and the HPLC-DAD method was more suitable for heat-refluxed sample extraction and excellent peak separation of bisdemethoxycurcumin, demethoxycurcumin, and curcumin in the turmeric samples. Heat-refluxed sample extraction can be easily carried out with excellent precision and accuracy. The validation procedure showed that the proposed method was selective, sensitive, accurate, and precise. The method was successfully applied to determine individual curcuminoids in samples among different turmeric origins. We expect that this method is acceptable for routine analysis of turmeric samples.

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