Optimization of the Conditions for the Analysis of Curcumin and a Related Compound in *Curcuma longa* with Mobile-Phase Composition and Column Temperature via RP-HPLC

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To efficiently extract the curcuminoids from turmeric, the extracted samples were simultaneously separated and analyzed via commercial C₁₈ reverse-phase high-performance liquid chromatography using combined water and acetonitrile as the mobile phase in the isocratic elution mode. The experiment results showed that the optimum mobile-phase composition of the water/acetonitrile was 60/40 vol % using the Knauer RP-column. The effect of the solvent composition (methanol in water) on the extraction yield, flow rate (1.0 mL/min), injection volume (10 μ L) and column oven temperature (40 °C) at a 425 nm wavelength was studied. It was evident that the amount of the curcuminoids that were extracted (extraction time: 4 h) with 100 % MeOH was higher than any aqueous MeOH composition. The correlation coefficients were greater than curcumin (1) 0.9985, demethoxycurcumin (2) 0.9982 and bisdemethoxycurcumin (3) 0.9990 in the regression analyses. The LC-MS data provided molecular weight information for the components in the curcumin standard (purity: 80 %). Thus, the optimum operating conditions were experimentally determined.

Key Words: Analysis, *Curcuma longa*, Curcuminoids, HPLC, Separation, Optimization.

INTRODUCTION

Curcumin is a yellow phenolic pigment derived from the powdered rhizome of *Curcuma longa*¹⁻⁵. It has been traditionally used in Asia for food and medicinal purposes². Turmeric (popularly known as curry), belongs to a group of aromatic spices, was originally used as a food additive in curry dishes to improve the food storage condition, palatability and preservation^{3,4}. Turmeric contains proteins, fats, minerals, carbohydrates and moisture. The essential oil which is obtained from it via steam distillation of rhizomes has α -phellandrene, sabinene, cineol, borneol, zingiberene and sesquiterpenes. Turmeric also has a water-soluble component, turmerin⁵. It has been reported to have a wide range of beneficial biological properties that include antitumor, anti-inflammatory, anti-oxidant and anti-angiogenic activities with low toxicity^{4,6} and its pharmacological activity has been attributed mainly to curcumin and two related compounds, demethoxycurcumin and bisdemethoxycurcumin⁷. Among these biological properties, the potent anti-oxidative effect of turmeric is well-known. Therefore, quantitative analysis of curcuminoids, especially of curcumin in turmeric, is very important to determine the quality of the plant material or of its processed products. For

commercial use, it is traded as a dye, spice and source of industrial starch. There have been many reports and several reviews of the analysis, identification and separation of individual curcuminoids⁸. The commercially available curcumin mixture contains 79.84-80 % major curcumin 15-16 % demethoxycurcumin, 2.96-3 % bisdemethoxycurcumin and 2.2 % other components⁹. Many methods of analyzing the curcuminoid contents of turmeric using HPLC, PTLC, LC/MS, GC and the spectrofluorimetric procedure have been described¹⁰. In this study, the conditions for the separation of the curcumin, demethoxycurcumin, bisdemethoxycurcumin from turmeric (*Curcuma longa*) were investigated and a method of its efficient separation and analysis was experimented on to find the optimum conditions with various parameters (column temperatures: 30, 40 50, 60, 70 and 80 °C; mobile-phase water A: 10, 20, 30, 40, 50 and 60 %; and acetonitrile B: 90, 80, 70, 60, 50 and 40 %) using reverse-phase high-performance liquid chromatography (RP-HPLC). Moreover, this study provides data on which a commercial process is based.

EXPERIMENTAL

The dried curcumin was purchased from a domestic market Hawaii at Hilo, USA in June 2011. Standard samples were

TABLE-1
DATA FOR QUANTITATIVE AND RETENTION TIME ANALYSIS PEAK AREA OF THREE CUMINOIDS

Mobile phase		Temp. (°C)	t_R (min) # 1	t_R (min) # 2	t_R (min) # 3	Peak area # 1 (%)	Peak area # 2 (%)	Peak area # 3 (%)	Peak area (mAU × min) # 1	Peak area (mAU × min) # 2	Peak area (mAU × min) # 3	Total Peak area (# 1, 2, 3)
Water (A)	ACN (B)											
40	60	30	3.33	3.07	2.84	82.23	14.05	2.33	255.71	34.97	5.79	296.48
		40	3.06	2.81	2.60	81.33	12.90	2.83	247.48	39.26	8.60	295.36
		50	2.77	2.54	2.35	80.79	13.45	2.95	242.48	40.38	8.85	291.71
		60	2.51	2.30	2.13	79.52	13.78	3.57	235.88	40.89	10.59	287.35
		70	2.32	2.13	1.97	73.21	14.86	4.51	209.57	42.53	12.89	265.10
		80	2.15	-	-	72.48	-	-	198.93	-	-	198.93
50	50	30	7.05	6.38	5.79	81.31	15.52	2.53	220.36	41.06	6.86	268.28
		40	6.02	5.40	4.86	77.75	15.13	3.11	214.64	41.77	8.54	264.95
		50	5.20	4.63	4.14	76.55	15.43	3.65	206.31	41.59	9.85	257.75
		60	4.56	4.05	3.60	72.99	16.53	3.83	194.36	44.01	10.21	248.56
		70	4.03	3.56	3.15	67.53	17.44	3.28	174.75	46.72	10.13	231.70
		80	3.40	-	-	64.09	-	-	168.71	-	-	168.71
60	40	30	22.46	20.03	17.94	80.76	16.43	2.81	197.30	40.14	6.87	244.31
		40	18.18	15.99	14.11	72.90	16.22	3.97	186.57	41.51	10.16	238.24
		50	14.76	12.79	11.12	66.92	18.09	5.21	163.45	44.19	12.72	220.36
		60	12.22	10.47	9.65	61.52	20.07	5.76	125.53	42.99	12.76	181.27
		70	10.29	8.77	7.45	51.38	20.82	5.91	116.76	40.82	13.23	170.81
		80	8.64	-	-	45.45	-	-	98.21	-	-	98.21

prepared by dissolving 4 mg of the standard chemical (curcumin: Sigma-Aldrich) in 20 mL of methanol and adjusting the concentration to 200 ppm before injected into the HPLC system. The HPLC-grade methanol, hexane, acetonitrile, dichloro-methane, ethyl acetate and acetic acid were purchased from J.T. Baker (USA). The double distilled water was filtered by a pump (Division of Millipore, Waters, Milford, MA, USA).

Solvent extraction: Using the curcumin standardize, dried samples without particles (sieving < 30 µm) was prepared and stored at low temperature and moisture content 10.30 % were used. Then 3 g of powdered curcumin was used for extraction using extraction solvent (200 mL) of 100 % methanol by dipping method for 4 h at 25 °C. Sample was filtered through a 0.2 µm membrane filter prior to HPLC analysis (under extract optimum conditions is shown not here).

HPLC and LC-MS analysis: The experiments were performed with a Dionex HPLC system equipped with an ultimate 3000 pump, ultimate diode array detector (DAD), injector 10 µL sample loop (Dionex, ID × L 0.18 × 550 mm Viper 550 mm USA) and Chromeleon data acquisition system (Dionex version 7.0.1.272). The standard curcumin was confirmed by LC-MS analysis (Varian: 500-MS IT Mass Spectrometer). Sufficient time was allowed for the stabilization of the C₁₈ column and detector signal after each injection. The adjustable experimental variables were the conditions of Isocratic modes and mobile phase compositions. The chromatographic columns used in this experiment are commercially available, obtained from Knauer RP-column (250 × 3.0 mm, 5 µm, C₁₈, with precolumn Eurospher 100-7, Germany). The injection volume was 10 µL and the flow rate of the mobile phase was 1.0 mL/min. The wavelength of the UV detector was fixed at 425 nm. The mobile phase solvents were A (water/ acetic acid = 99.9/0.1, v/v) and solvent B (acetonitrile/ acetic acid = 99.9/0.1, v/v). The run time was 30 min and isocratic method was applied variation mobile phase condition (solvent A : 40-60 % and B : 60-40 %) and using the best conditions analysis (solvent A : 60 % and B : 40 %).

RESULTS AND DISCUSSION

In this study, three curcuminoids from turmeric were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC). To efficiently extract the curcuminoids from turmeric, the variables that were involved in this procedure were optimized, including the extraction solvent methanol, the extraction dipping method and the extraction time (4 h). To extract the three curcuminoids, different analysis methods were used. Table-1 shows the data for the quantitative, retention time and peak area analyses of the three curcuminoids. Also, Fig. 1. [(a) and (b)] show the retention time behaviour and the resolution of the curcuminoids with a mobile-phase composition (%). To determine the optimum flow rate in this HPLC system, different mobile-phase compositions (%) were used. When the column temperature was 30 °C, (a) the mobile-phase A compositions were 90, 80 and 70 % and (b) the mobile-phase B compositions were 60, 50 and 40 %. When the column temperature increased, the retention time and the resolution decreased. Therefore, the mobile-phase compositions 60, 50 and 40 % were against the column temperature conditions. Fig. 2. (a) compares the chromatogram of the curcuminoids with the mobile-phase composition and the column temperature. When the mobile-phase B composition was 60 % and the column temperatures were 30, 40 50, 60, 70 and 80 °C, the peak area of the curcumin had a column temperature range of 30 °C (82.23 %, 255.71 mAU × min) to 80 °C (72.48 %, 198.93 mAU × min) and the column temperatures of the other two peak areas were 40 °C (12.90 %, 39.26 mAU × min) to 70 °C (14.86 %, 42.53 mAU × min) demethoxycurcumin and 30 °C (2.33 %, 5.79 mAU × min) to 70 °C (4.51 %, 12.89 mAU × min) bisdemethoxy-curcumin. Fig. 2(b) compares the chromatogram of the curcuminoids with the mobile-phase composition and the column temperature. When the mobile-phase B composition was 50 % and the column temperatures were 30, 40 50, 60, 70 and 80 °C, the peak area of the curcumin had a column temperature range of

Fig. 1. Comparison behaviour of retention time and resolution of curcuminoids with mobile phase composition (%). (Column temperature: 30 °C, (a) :mobile phase B : 90, 80 and 70 %, (b) : mobile phase B : 60, 50 and 40 %, flow rate : 1 mL/min, injection volume : 10 μ L/min)

Fig. 2. Comparison of chromatogram of curcuminoids with mobile phase and column temperature. (column temperature : 30, 40, 50, 60, 70 and 80 °C, (a) : mobile phase B : 60 %, (b) : mobile phase B : 50 %, flow rate : 1.0 mL/min, injection volume : 10 μ L/min)

30 °C (81.31 %, 220.36 mAU $\times$ min) to 80 °C (64.09 %, 168.71 mAU $\times$ min) of that of the total area and the column temperatures of the other two peak areas were 40 °C (15.13 %, 41.77 mAU $\times$ min) to 70 °C (17.44 %, 46.72 mAU $\times$ min) demethoxycurcumin and 30 °C (2.53 %, 6.86 mAU $\times$ min) to 60 °C (3.83 %, 10.21 mAU $\times$ min) bisdemethoxycurcumin. Fig. 3. [(a) and (b)] shows the chemical structure and the UV spectrum of the curcuminoids in this study. Fig. 3. (a) show the optimal resolution of the curcuminoids with the mobile-phase composition and the column temperature. When the mobile-phase B composition was 40 % and the column temperatures were 30, 40 50, 60, 70 and 80 °C, the peak area of the curcumin had a column temperature range of 30 °C (80.76 %, 197.30 mAU $\times$ min) to 80 °C (45.45 %, 98.21 mAU $\times$ min) of that of the total area and the other two peak areas were 40 °C (16.22 %, 41.51 mAU $\times$ min) to 70 °C (20.82 %, 40.82 mAU $\times$ min) demethoxycurcumin (DMC) and 30 °C (2.81 %, 6.87 mAU $\times$ min) to 70 °C (5.91 %, 13.23 mAU $\times$ min) bisdemethoxycurcumin. Curcuminoids 1, 2 and 3 were confirmed *via* both chromatographic and MS data identification (not shown here)

of the curcuminoid analogues in all the items using HPLC analysis, based on the retention times as well as on the comparison of their UV-visible spectra and maximum absorption wavelengths with the representative standards, which is the previously described method. Using this method, the optimum separation conditions were determined. By comparing the UV values of the curcuminoids at the 254-500 nm wavelength¹¹ the corresponding UV absorption maximum in methanol were 423-425, 420-421 and 413-418 nm for curcumin, demethoxycurcumin and bisdemethoxycurcumin, respectively^{7,10,11}. Quantification was performed on the basis of the linear calibration plots of the UV absorption peak area at 425 nm Fig. 3(a). Fig. 3 (b) shows the chromatograms with the turmeric extract (4 h at 25 °C), using the optimum separation conditions. Fig. 4. shows the calibration curve of the curcuminoids with the mobile-phase composition and the column temperature (column temperature: 40 °C; mobile-phase B composition: 40 %; flow rate: 1.0 mL/min; and injection volume: 10 μ L/min). The experiment parameters in the equilibrium curves were estimated using linear (equation $y = ax + b$) regression analysis, as follows:

Fig. 3. Optimal resolution of curcuminoids with mobile phase and column temperature. (column temperature : 30, 40, 50, 60, 70 and 80 °C, (a) : mobile phase B : 40 %, (b) : turmeric extraction : 4 h, flow rate : 1.0 mL/min, injection volume : 10 μ L/min

Fig. 4. Calibration curve of curcuminoids with mobile phase and column temperature. (column temperature : 40 °C, mobile phase B : 40 %, flow rate : 1.0 mL/min, injection volume : 3, 5, 10 and 15 μ L/min)

$$r^2 = 1 - \frac{\sum_{i=1}^n (y_i - y(x_i))^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (1)$$

wherein a and b are the parameters and r^2 is the regression coefficient (eqn. 1). The results showed that the calculated data fit well with the experimental data. The correlation coefficients were greater than curcumin (1) 0.9985, demethoxy curcumin (2) 0.9982 and bisdemethoxycurcumin (3) 0.9990, which showed good linearity within the test concentration range and the variation injection volume range. The results of this study showed the effect of the mobile-phase composition (%) and the column temperature (°C) on the total extraction yield and the resolution of curcuminoids. Based on the investigation, anyone can choose the best mobile-phase B composition of 40 % and the best column temperature of 40 °C for the best extraction and separation from turmeric, even if it is done the first time. When the solvent polarity and column temperature increase, the retention time, total yield and resolution decrease.

It was also found that the curcumin content was highest in the 100 % methanol extract and had a good resolution, although the total extraction yield reached its maximum value at the 60 % mobile-phase composition and the 30 °C column temperature. The ideal optimum conditions were a mobile-phase composition of 40 % and a column temperature of 40 °C. These results will be used to establish a database for the investigation of the constituents of natural products and the resources of pharmaceutical, nutrition and cosmetic products. Also, LC-MS analysis has been shown to be a powerful tool in metabolic profiling and metabolomics research. One study that used LC-MS to identify certain constituents of fresh turmeric has been reported⁶. Based on the simultaneous analysis of curcuminoids in this study, the components detected with mass spectra were correlated with the molecular mass information and the relative response. Each compound showed very intense protonated molecules $[M+H]^+$ at 369.3, 339.3 and 309 m/z , respectively (Fig. 5).

Conclusion

In this study, three major curcuminoids were efficiently extracted by optimizing the analysis conditions. The three major curcuminoids were simultaneously identified using the fast and reliable RP-HPLC diode-array method. The results of this study showed the effect of the mobile-phase composition and the column temperature on turmeric. Based on the investigation, anyone can choose the best analysis condition for extraction from turmeric. The results showed that the mobile-phase acetonitrile increased and the retention time decreased. The calculated data fit well with the experiment data. The correlation coefficients were greater than curcumin (1) 0.9985, demethoxycurcumin (DMC) (2) 0.9982 and bisdemethoxycurcumin (BDMC) (3) 0.9990 in the regression analysis. The LC-MS data provide molecular weight information for the components of the turmeric standard. Thus, the optimum operating conditions were experimentally determined. These results will be used to establish a database for the investigation of the constituents of natural products and the resources of pharmaceutical, nutrition and cosmetic products.

Fig. 5. MS spectra profile of the commercially available standard curcumin. (mobile phase MeOH : 100 %, flow rate : 1.0 mL/min, injection volume : 10 μ L/min)

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