

Quantitative Analysis of Multi-components in *Curcuma xanthorrhiza* by Single Marker

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ABSTRACT

A new simple and effective routine analytical method for quantification of curcuminoids in *Curcuma xanthorrhiza* was developed by high-performance liquid chromatography. This method based on chromatographic fingerprint combined with a quantitative analysis of multi-components by single marker (QAMS). Curcumin was selected as an internal marker for the determination of two other similar compounds, i.e. bisdemethoxycurcumin and demethoxycurcumin, by using the relative coefficient factor (RCF). Excellent linearity was obtained for each component ($r^2 > 0.9998$), and the recovery of extraction methods were within 100.23-103.95%. The precision of the method was good at inter-day and intra-day analysis (RSD < 4.0%). The stability of RCFs was good under various chromatographic conditions with RSD < 1%, and the ratio of retention time was used to locate each compound. The quantification of curcuminoids between QAMS and external standard method (ESM) proved the consistency and similarity of the two method (RSD < 2%). This study demonstrated that QAMS could be used as a routine method for quality control of curcuminoids in *C. xanthorrhiza*. This method successfully proved accurate, stable, more effective and simple than external standard method.

Keywords: *C. xanthorrhiza*, curcuminoids, QAMS, relative coefficient factor

INTRODUCTION

Curcuma xanthorrhiza belongs to the Zingiberaceae family and locally known as temulawak. This plant is origin and widely distributed in Indonesia. The dried rhizome of *C. xanthorrhiza* has been used for a long time as traditional medicine by Indonesian people, called jamu. Many pharmacological studies showed the efficacy of *C. xanthorrhiza*, such as anti-inflammatory and antihyperglycemic (Kim *et al.* 2014), antimicrobial (Mangunwardoyo *et al.* 2012), antioxidant and hepatoprotective activity (Devaraj *et al.* 2010). The pharmacological properties of *C. xanthorrhiza* are related to their chemical content, including curcuminoids. In the previous study, curcumin (CUR), bisdemethoxycurcumin (BDMC), and demethoxycurcumin (DMC) were found in *C. xanthorrhiza* with CUR content is higher than BDMC and DMC (Rafi *et al.* 2015). Curcuminoids are known for their pharmacological properties, i.e., antioxidant, antifungal, antiviral, anti-inflammatory,

and anticancer (Fadus *et al.* 2017; Kocaadam and Şanlıer 2015; Moghadamtousi *et al.* 2014). Curcumin has higher pharmacological activity than bisdemethoxycurcumin and demethoxycurcumin (Sandur *et al.* 2007; Jayaprakasha *et al.* 2006). However, the mixture of three curcuminoids has better activity compared only curcumin (Rege *et al.* 2014).

Evaluation of medicinal plant raw material commonly used a marker compound like curcuminoids in *C. xanthorrhiza*. The most analytical technique used for quantitative analysis of curcuminoids in *Curcuma* genus are liquid chromatography as reported by Osorio-Tobón *et al.* (2016), Erpina *et al.* (2017), Chao *et al.* (2018), and Poudel *et al.* (2019). However, with those method we must use the three curcuminoid standard with high purity which is expensive and not easy to get. So, we need a new method which is more efficient, precise, accurate, and inexpensive to determine curcuminoids for quality evaluation of *C.*

xanthorrhiza raw material. Wang *et al.* (2006) systematically proposed a quantitative analysis of multicomponent by single marker (QAMS). This method, based on the principle of the absorption (A), is linearly proportional to the concentration (W) of an analyte within a concentration range ($W = f.A$). It only uses one component to determine other components and use their internal relationship and proportion (Zhu *et al.* 2017). In recent years, this method has been widely used as an alternative method for quality control of many medicinal plants (Li *et al.* 2017; Peng *et al.* 2018; Wang *et al.* 2016; Xu *et al.* 2017). QAMS has been used for the determination of curcuminoids in *C. longa* (Chen *et al.* 2017). However, there is no paper has been reported regarding the use of QAMS in determination of curcuminoids in *C. xanthorrhiza*. Also, the mobile phase for the separation of curcuminoids in *C. longa* by HPLC was separated in low pH, and this is not safe for the durability of the stationary phase in the column. In this study, we proposed a new method using QAMS for quality control of *C. xanthorrhiza* based on the determination of curcuminoids content by only use curcumin as a single marker. By using only one component to determine other curcuminoids, it will increase the effectiveness, efficiency, and cost of analysis without sacrificing the quality of the analytical result.

MATERIAL AND METHODS

Chemicals and materials

Bisdemethoxycurcumin, demethoxycurcumin, and curcumin with the purity > 95% were purchased from ChromaDex Inc. (Santa Ana, CA, USA). Methanol, acetonitrile, formic acid of HPLC grade were obtained from Merck (Darmstadt, Germany).

Preparation of standard solution

Curcuminoids standard stock solution were prepared by appropriate amount of BDMC, DMC and CUR were dissolved with methanol in a 10mL volumetric flask to obtain 500 $\mu\text{g.mL}^{-1}$ respectively. An appropriate amount of stock solution was mixed with methanol to obtain six concentration range from 0.5 to 50 $\mu\text{g.mL}^{-1}$.

Preparation of sample solution

C. xanthorrhiza was collected from Bogor West Java and Surabaya East Java, Indonesia. The sample was cut into small pieces, dried, pulverized into powder and sieved. *C. xanthorrhiza* powdered

sample weighed accurately, then mixed with methanol and sonicated for 30min at room temperature. The extracts were filtered with a 0.45 μm membrane filter then diluted to 10mL with methanol.

Chromatographic conditions

Chromatographic conditions for the separation of the three curcuminoids were performed on an LC-20A series HPLC with a diode array UV-Vis detector system (Shimadzu, Kyoto, Japan). Separation of the analytes was achieved on the Zorbax Eclipse Plus C18 column (4.6mm x 150mm, 5 μm). The mobile phase consisted of acetonitrile (A) and 0.001% formic acid (B) in water, with a flow rate was 1.0mL. min⁻¹. The system of mobile phase applied was 0-20min, 42-58% A. The column temperature for this study was kept at 30°C, and the volume of the sample for separation was 20 μL . The curcuminoids detection wavelength was set at 425nm.

Calculation of relative correction factors

Determination of curcuminoids content in *C. xanthorrhiza* by QAMS was based on the comparison of each relative correction factor (RCF) to curcumin as an internal marker by following this equation (1):

$$f_{xc} = \frac{f_x}{f_c} = \frac{A_c \times C_x}{A_x \times C_c} \dots\dots\dots(1)$$

Where f_c is the correction factor of curcumin (CUR) as internal marker, f_x is the correction factor of analyte (BDMC and DMC), A_c is the peak area of curcumin, A_x is the peak area of analyte, C_x is the concentration of analyte ($\mu\text{g.mL}^{-1}$) and C_c is the concentration of curcumin ($\mu\text{g.mL}^{-1}$). The calculation of each f values was done under different volumes of injection (6, 8, 10, 12, 15 and 20 μL).

Calculation of relative retention time target compounds

Determination of BDMC and DMC in *C. xanthorrhiza* by QAMS is only using one component as an internal marker i.e., CUR. Therefore, to determine the location of the peaks of the BDMC and DMC accurately, the ratio (2) or the difference of retention time must be determined.

$$r_{x/s} = \frac{t_{R(x)}}{t_{R(c)}} \dots\dots\dots(2)$$

Where X is analyte (BDMC and DMC), and C is marker (CUR).

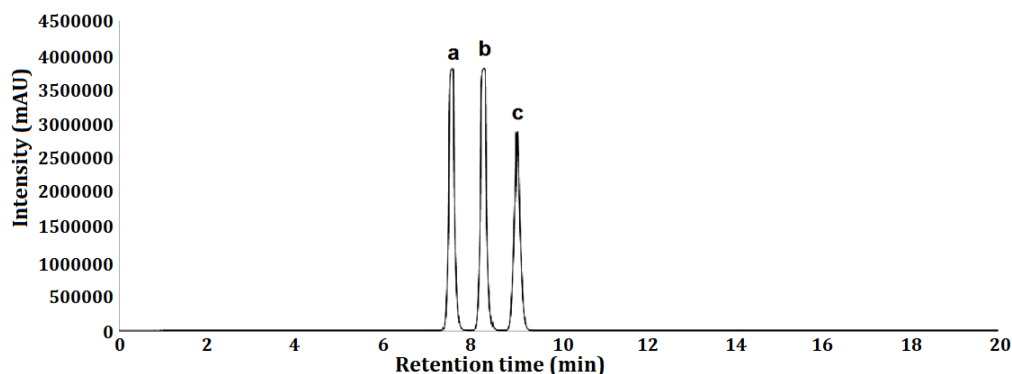


Figure 1 Chromatogram of standard of BDMC (a), DMC (b), and CUR (c).

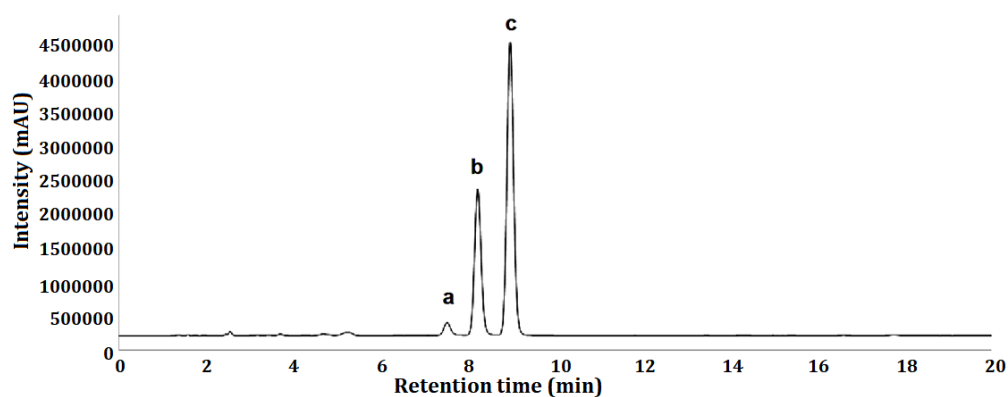


Figure 2 Chromatogram of BDMC (a), DMC (b), CUR (c) in sample of *Curcuma xanthorrhiza*.

Method validation

Validation of the method for determination of curcuminoids by QAMS based on High-performance liquid chromatography was evaluated following the guideline from the Association of Official Analytical Chemists (AOAC). The system suitability, linearity, precision, accuracy, and stability were evaluated in this study.

RESULT AND DISCUSSION

System suitability

In order to develop a new quality control method to determine curcuminoids in *C. xanthorrhiza* based on chromatographic fingerprint, this study was combined with QAMS and HPLC fingerprint analysis. The chromatographic condition needs to evaluate to determine system suitability and has met the minimum standard requirements before it used for quantitative analysis. This evaluation was conducted by the optimization of mobile phase composition and column temperature. The resolution of each analyte was primarily used as

the parameter for choosing the best chromatographic condition. The chromatograms of curcuminoids standard and sample, which has been optimized (Figures 1 and 2). The best separation was achieved when the column temperature was 30°C, and the mobile phase was 42-58% acetonitrile in 0.001% formic acid in water for 0-20min. The resolution of each peak was >1.5 (acceptance criteria for resolution > 1.5), and the total of analysis time for the quantitative analysis of curcuminoids was 37min. The detection wavelength for curcuminoids analysis was 425nm because it gave the highest sensitivity.

Linearity

Calibration curves are used to determine the linearity of the method. The mixed standard (Figure 1) solution was diluted in series with methanol to obtain six different levels in the concentration range 0.5-10 µg.mL⁻¹ for BDMC and 0.5-50 µg.mL⁻¹ for DMC and CUR. The calibration curve was plotted between the peak areas (Y) against the concentration (X) of the analyte.

Table I. Linearity of chromatographic method

Analyte	Regression equation	(r ²)	Linear range (µg/mL)
BDMC	y = 251291x - 13447	0.9999	0.50 - 10.00
DMC	y = 294272x - 74885	0.9999	0.50 - 50.00
CUR	y = 202545x - 37391	0.9998	0.50 - 50.00

Table II. Sample stability, precision and recovery of chromatographic method

Analyte	Stability ^a (RSD, %)	Precision		Recovery	
		Intra-day ^b RSD (%)	Inter-day ^c RSD (%)	Mean	RSD (%)
BDMC	2.00	0.60	1.83	100.23	0.06
DMC	2.14	2.66	1.65	103.95	0.15
CUR	4.11	3.16	1.10	100.26	0.81

a : Measurement at 0, 2, 4, 8, 12 & 24 hrs after extraction; b: n = 6; c : n = 3.

Table III. Determination of RCF with curcumin as internal marker in *C. xanthorrhiza*

Injection volume (µL)	6µL	8µL	10µL	12µL	15µL	20µL	Mean	RSD (%)
f _{BDMC}	0.886	0.861	0.855	0.849	0.841	0.832	0.854	1.71
f _{DMC}	0.715	0.709	0.707	0.703	0.701	0.697	0.706	0.58

The correlation coefficients (r²) of calibration curves for all analyte were >0.9998 (Table I). These high r² values indicate that the method has good linearity and stable at wide range concentration. The acceptance criteria from AOAC for linearity (r²) is above 0.99. So, the linearity result from our study have met the acceptance criteria of AOAC guidelines.

Stability

Stability was assessed by analyzing the solution of sample at 0, 2, 4, 8, 12 and 24h at room temperature after preparation of the sample. The result (Table II) showed that the RSD below 5%, it means the analyte was stable after 24h of extraction.

Accuracy

Recovery by the standard addition method was used to determine the accuracy of the method. The sample of *C. xanthorrhiza* was spiked by low, medium, and large amounts of three curcuminoids standards, and then these samples were extracted and analyzed for its recovery as described previously. Recovery of curcuminoids was in the range of 100.23-103.95% with the RSD was < 1% (Table II). It showed that the recovery of this method was not statistically different and in accordance with the acceptance criteria of recovery is 80-120%. So, the developed method was accurate.

Precision

The precision of this method was determined by intra-day and inter-day variations. Intra-day variation was assessed by six replications of the sample injection on the same day (Table II). Inter-day variation was assessed by injecting six replicates of samples on a different day. The RSD value is below 4.0% (acceptance criteria: RSD <4%), indicating the method was precise.

Quantitative analysis of multicomponent by single marker (QAMS)

Curcumin was chosen as a marker for quantitative analysis of two other curcuminoids, i.e., DMC and BDMC, due to its highest pharmacological activity, easy to obtain, commercially available, and highest content in *C. xanthorrhiza* (Figure 2). To determine BDMC and DMC in QAMS, we used f_{xc}. The RCF value of BDMC and DMC were determined by the variation of injection volume and then calculating used equation 1. The RCF of BDMC and DMC were 0.854 and 0.706, respectively (Table III). These RCFs were stable in various injection volume, with RSD 1.71% and 0.58%. These RCFs can be considered stable and can be applied in the QAMS method with a various injection volumes of sample extract.

Table IV. RCFs stability by some chromatographic factors

Chromatographic factors	BDMC		DMC	
	RCF	RSD (%)	RCF	RSD (%)
HPLC Column				
Agilent, Zorbax C18	0.843	0.21	0.701	0.00
Shimadzu, Simpack ODS C18	0.840		0.701	
Flow rate				
0.90 mL/min	0.841	0.59	0.700	0.21
1.00 mL/min	0.843		0.701	
1.10 mL/min	0.852		0.704	
Wavelength				
420 nm	0.843	0.11	0.701	0.00
423 nm	0.843		0.701	
425 nm	0.843		0.701	
427 nm	0.842		0.701	
430 nm	0.845		0.701	
Column temperature				
27 °C	0.843	0.15	0.701	0.00
30 °C	0.841		0.701	
33 °C	0.840		0.701	

Table V. Determination of BDMC and DMC peak by retention time difference and retention time ratio

Curcuminoids Standard (µg/mL)	BDMC			DMC		
	t _r (min)	t _r	t _r .BDMC/t _r .CUR	t _r (min)	t _r	t _r .BDMC/t _r .CUR
0.500	8.293	1.752	0.826	9.141	0.904	0.910
1.000	8.217	1.768	0.823	9.073	0.911	0.909
5.000	8.140	1.775	0.821	9.000	0.915	0.908
10.000	8.194	1.771	0.822	9.053	0.912	0.908
20.000	8.186	1.771	0.822	9.045	0.912	0.908
50.000	8.213	1.767	0.823	9.070	0.910	0.909
Sample A	7.700	1.599	0.828	8.473	0.826	0.911
Mean		1.767	0.823		0.911	0.909
RSD (%)		0.776	0.15		0.35	0.07

Stability test of RCFs in various chromatographic condition

The RCF stability is crucial for QAMS so that we could use this method in another column, instrument, or laboratories. The stability of BDMC and DMC were tested in a different column, column temperature, a flow rate of the mobile phase, and detection wavelength. Indicated excellent stability of RCFs under various conditions mentioned above (Table IV). The RSD values of all tests were <1%, so that we could use these RCFs for the simultaneous determination of BDMC and DMC along with CUR in *C. xanthorrhiza*.

Determination of curcuminoids peak

We will have difficulties determining BDMC and DMC by using only CUR as the reference

marker in external standard method. However, in QAMS, we could locate each peak by CUR peak without the reference compound of BDMC and DMC. There are two methods in QAMS for determining the location of each peak. The first one calculates the differences of the retention time and the second one, the use of ratio between CUR and the other curcuminoids (BDMC and DMC). Table V showed that the ratio of retention time has better RSD than the differences in retention time for each target compound (BDMC 0.15%; DMC 0.07%).

Comparison of QAMS and external standard method (ESM)

In order to validate QAMS feasibility for simultaneous determination of curcuminoids determination in *C. xanthorrhiza* using curcumin as

Table VI. Result comparison external Standard and QAMS for determination of Curcuminoids in *C. xanthorrhiza*

Samples	BDMC (µg/mL)		DMC (µg/mL)		CUR
	ESM	QAMS	ESM	QAMS	
U1	1.03	1.04	10.48	10.55	33.52
U2	1.02	1.03	10.53	10.60	33.56
U3	1.03	1.04	10.54	10.61	33.57
U4	1.04	1.05	10.52	10.59	33.54
U5	1.04	1.05	10.48	10.55	33.54
U6	1.13	1.15	10.55	10.62	33.51
U7	1.04	1.05	10.52	10.59	33.61
U8	1.04	1.05	10.51	10.58	33.65
U9	1.04	1.05	10.52	10.59	33.54
U10	1.12	1.13	10.68	10.76	33.74
Mean	1.05	1.06	10.54	10.60	33.58
RE (%)		1.12		0.65	
T. _{BGR} Local Market	1.01	1.03	6.59	6.57	17.76
RE (%)		1.40		0.35	
T. _{SBY} Local Market	0.69	0.69	5.03	4.94	18.65
RE (%)		1.22		1.65	

single marker, the contents of BDMC and DMC were determined by QAMS compared to ESM. Determination of BDMC and DMC, we used the following equation:

$$C_x = \left(\frac{A_x \times C_c}{A_c} \right) f_{xc} \quad (3)$$

The relative error (RE) of the two methods calculated by determining the deviation and similarity of each results. In this study, we used *C. xanthorrhiza* rhizome samples from two locations (Bogor and Surabaya). Table VI showed no significant difference between QAMS and external standard method. The RE of BDMC and DMC in *C. xanthorrhiza* was 1.12% and 0.65%, respectively, while BDMC and DMC from two local markets in Bogor and Surabaya were <1.40% and 1.65% respectively. Based on this RE value, QAMS can be applied as the new alternative method for routine analysis and quality control of *C. xanthorrhiza* in the difference chromatographic condition. The use of curcumin as a marker to determine the other two curcuminoids has reduced the cost of quality control analysis and made QAMS more effective and efficient than ESM.

CONCLUSION

In this study, QAMS was proven could be used as an alternative method of routine analysis of curcuminoids in *C. xanthorrhiza*, which is efficient,

precise, accurate, and reliable. The linearity of this method is > 0.9998 for each component. In this method, curcumin is used as an internal standard to determine two other components (BDMC and DMC) based on each RCF values, which proven stable at the various chromatographic condition. The ratio of retention time was used to determine the location of two other components and proved stable.

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