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Application of HPLC and response surface methodology for simultaneous determination of curcumin and desmethoxy curcumin in Curcuma syrup formulation

Niken K. Prabaningdyah[1,2], Sugeng Riyanto[1], Abdul Rohman[1,3*], Chairany Siregar[1,4]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281, Indonesia
[2]The National Agency of Drug and Food Control, District of Yogyakarta, Republic of Indonesia.
[3]Research Center of Halal Products, Gadjah Mada University, Yogyakarta, 55281, Indonesia.
[4]The National Agency of Drug and Food Control, District of Samarinda, West Kalimantan, Republic of Indonesia.

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ABSTRACT

The effectiveness of herbal medicine containing Curcuma extract depends on the active compounds of curcumin (CUK) and desmethoxycurcumin (DMCUR) present in formulation. The objective of this study was to optimize HPLC condition using experimental design of Box-Behnken design (BBD) for simultaneous determination of C and DMC in syrup containing curcuma extract. The column used was RP 18 Waters® X-Bridge (250 x 4.6 mm i.d.; 5 µm). Four variables namely column temperature, flow rate, solvent ratio of acetonitrile-acetic acid, and % acetic acid were used and subjected to RSM in order to get optimum condition. The predicted optimum assay condition consisted of mobile phase acetonitrile-acetic acid 4.08% (49: 51) delivered isocratically at flow rate 1.04 mL/min with column temperature of 40°C. Using this optimum condition, baseline separation of both compounds was achieved with good resolution. The optimized HPLC assay condition was validated according to International conference on Harmonization (ICH) guidelines by assessing several parameters such as specificity, linearity, accuracy, sensitivity and precision.

INTRODUCTION

The use of temula wak, also known as Java turmeric, with scientific name *Curcuma xanthorriza* as raw material in herbal medicines and food is increased 5.4% annually, which make high demand on this rhizome in herbal industries (Nihayati *et al*., 2013). Some pharmaceutical formulations such as syrup and tablet contain rhizome of temula wak intended to increase appetite (Oon *et al*., 2015). Several biological activities have been reported in *C. xanthorriza* for treatment of hepatitis, liver complaints, diabetes, cancer, rheumatism, hypertension, antioxidant, diuretic, and hepatoprotective effects (Devaraj *et al*., 2014). In addition, this rhizome was also used for anti-inflammatory, antibacterial, and antifungal effects (Hwang *et al*., 2000a; Hwang *et al*., 2000b; Rukayadi *et al*., 2013). The main component present in rhizome of *C. xanthorriza* is curcuminoid (mainly curcumin and desmethoxycurcumin), believed to be responsible for those biological activities, therefore, it is important to determine the levels of compounds or groups of compounds responsible for biological activities. Indeed, analytical techniques capable of quantifying curcuminoid (total and individual) should be developed. Several analytical methods have been developed and used for routine analysis of curcuminoid, namely Fourier transform infrared spectroscopy (Tanaka *et al*., 2008; Rohman *et al*., 2015), thin layer chromatography-densitometry (Péret-Almeida *et al*., 2005), high performance liquid chromatography using UV-visible detector and electrochemistry detector (Inoue *et al*., 2008; Syed *et al*., 2015), high performance liquid chromatography using

* *Corresponding Author*
Email: abdul_kimfar @ ugm.ac.id, Phone: +62274-543120

photodiode array detector (Korany *et al*., 2013), liquid chromatography-mass spectrometry (Asai and Miyazawa, 2000), and capillary Electrophoresis (Jiang *et al*., 2006). HPLC is method of choice for separation of curcuminoid in plant materials. Several parameters such as stationary phase, mobile phase composition, pH and flow rate of mobile phase, and organic modifiers contribute to HPLC separation (Snyder *et al*., 2010). Some authors use one variable of one time (OVAT) approach for HPLC separation which is ineffective due to elimination of interaction among variables, thus, the experimental design for identification of such interaction is needed. In our best knowledge, there is no report related to use of experimental design of Box-Behnken design (BBD) for simultaneous determination of CUR and DMCUR in syrup containing curcuma extract. Therefore, the objective of this study was to use BBD using four variables of column temperature, flow rate, solvent ratio of acetonitrile-acetic acid, and % acetic acid to get optimum HPLC condition for analysis of CUR and DMCUR in syrup formulation.

MATERIALS AND METHODS

Materials

Curcuminoid was purchased from E. Merck (Darmstadt, Germany). The standards of curcumin (CUR) and desmethoxycurcumin (DMCUR) were isolated from curcuminoid using column chromatography according to Péret-Almeida *et al.* (2005). The purity of CUR and DMCUR was checked using thin layer chromatography and high performance liquid chromatography. The solvents used for HPLC was indeed HPLC grade. The other solvents and reagents were of pro-analytical grade, unless otherwise specified. The syrup samples with batch number 17A0159 were claimed containing curcuminoid 2 mg from *C. Xanthorriza* were obtained around Yogyakarta, Indonesia.

Instrumentation and Software

A Shimadzu HPLC instrument- LC-20AD (Japan) equipped with Rheodyne 7725i injection valve with a 20 µL loop volume and Binary gradient pump was used. The system also includes detector of photodiode array (Shimadzu, SPD-M20A) operated at a wavelength of 425 nm. Data were acquired and processed by using LC-solution software. Chromatographic separation was performed using RP 18 Waters® X-Bridge (250 x 4.6 mm i.d.; 5 µm).The experimental design of Box-Behnken design and data analysis calculations were performed by using Design-Expert version 7.1.5.

Preparation of standard solution

An accurate weight of 5.075 mg of CUR and 3.270 mg of DMCUR were transferred into 100 mL and 50 mL for CUR and DMCUR respectively. The contents of the volumetric flask were dissolved in mobile phase to get 0.05 mg/mL of CUR and 0.06 mg/mL DMCUR. The working standard solution was obtained by diluting the standard stock solution with mobile phase during the analysis time. The stock solution and working standard solution were protected from light during analysis.

HPLC procedure

The condition of HPLC was optimized based on Wichitnithad *et al.* (2009) to get the best separation C and DMC. The Chromatographic separations were performed on Waters® X-bridge (250 mm x 4.6 mm i.d; 5 µm). A mixture of acetonitrile: acetic acid in aquadest was used as the mobile phase. The wavelength of detector PDA was set at 425 nm in which both compounds gave good response.Volume of injection was 20 uL.

Experimental design

The experimental design approach is useful for optimizing the chromatographic separation and helping out in the development of better understanding of the interaction of several chromatographic factors on separation quality (Karaliya *et al*., 2017). The mobile phase comprising mixture of acetonitrile-acetic acid (50: 50 v/v) with flow rate of 1 mL/min was selected in preliminary studies. The acetic acid concentration (X1), acetonitrile ratio (X2), flow rate (X3), and column temperature (X4) were selected as independent variable during Respon surface-Box-Behnken design, whereas peak area, retention time, tailing factor and resolution were chosen as response variable.

Validation of HPLC analysis

Before validation of HPLC, the system suitability test by injecting six replicate of analytes at a concentration of 3 µg/mL was performed. The acceptance criteria considered for the system suitability was taken as ±2% for the percent coefficient of variation (%CV) of the peak area and retention time (Khan *et al*., 2016).The validation of HPLC method was performed according to International Conference Harmonization (ICH, 2005) by assessing several parameters namely selectivity, linearity and range, limit of Detection (LoD), limit of quantification, precision, and accuracy.

Analysis of syrup formulation

A syrup sample equivalent to 5.0 mL syrup containing curcuma extract was accurately weighed into volumetric flask 100 mL. The sample was added with 50 mL mobile phase, shaken vigorously and made until volume with mobile phase. A-4.0 mL of this solution was taken and diluted until 20 mL, filtered using 0.45 µm filter, and injected into HPLC system.

RESULTS AND DISCUSSION

Experimental design optimization

In order to optimize HPLC method condition, the design of experimental of Box-Behnken design was used. The variables evaluated were acetic acid concentration (X1), acetonitrile ratio (X2), flow rate (X3), and column temperature (X4). The response variables were retention time (Y1), peak area (Y2), resolution (Y3) and tailing factor (Y4). The design showed a total of 29 experimental runs. Statistical analysis was performed using ANOVA to compute the significant differences among independent variables. The response surface methodology was

employed to analyze the effect of independent variables on the responses (Singh *et al*., 2005).

Using ANOVA results, the equation obtained using X1, X2, X3, and X4 as independent variables with retention time (Y1) as response was:

$\text{Log } Y_1 = 0.88 - 0.022X_1 - 0.23X_2 - 0.020X_3 - 0.29X_4 + 5.976 \times 10^{-6}X_1X_2 + 1.026 \times 10^{-4}X_1X_3 + 0.017X_1X_4 - 2.049 \times 10^{-4}X_2X_3 - 3.196 \times 10^{-3}X_2X_4 + 0.01X_3X_4 - 2.401 \times 10^{-3}X_1^2 + 0.062X_2^2 + 5.882 \times 10^{-4}X_3^2 + 0.087X_4^2$ (adjusted R^2 of 0.9999)

The statistical results revealed that adjusted coefficient of determination (Adj. R^2) obtained was > 0.8 was well within the acceptable limits (R^2> 0.8) that showed experimental model was good fit using polynomial equations (Karaliya *et al*., 2017). Based on ANOVA results, the variables of X1, X2, X3 and X4 as well as interaction between X1 and X4, X3 and X4 and quadratic form of X2 and X4 contributed significantly for response of Y1 ($P < 0.05$). Similarly, the equations for Y2, Y3, and Y4 were:

$\frac{1}{\sqrt{Y2}} = 1.904 \times 10^{-3} + 1.627 \times 10^{-5}X_1 + 4.596 \times 10^{-4}X_2 + 2.697 \times 10^{-5}X_3 - 5.058 \times 10^{-5}X_4 - 3.297 \times 10^{-6}X_1X_2 + 7.844 \times 10^{-6}X_1X_3 - 4.299 \times 10^{-6}X_1X_4 - 1.176 \times 10^{-5}X_2X_3 + 1.36 \times 10^{-5}X_2X_4 - 2.43 \times 10^{-5}X_3X_4 + 1.988 \times 10^{-5}X_1^2 - 6.028 \times 10^{-5}X_2^2 + 6.99 \times 10^{-6}X_3^2 + 4.268 \times 10^{-5}X_4^2$ (adjusted R^2 of 0.9992)

The variables of X2, X3, and X4 as well as quadratic form of X2 and X4 contributed significantly for response of Y2 ($P < 0.05$).

$\frac{1}{\sqrt{Y3}} = 0.62 + 4.861 \times 10^{-3}X_1 + 0.031X_2 - 0.017X_3 + 0.15X_4 + 1.091 \times 10^{-3}X_1X_2 + 2.394 \times 10^{-3}X_1X_3 + 3.038 \times 10^{-3}X_1X_4 - 1.445 \times 10^{-3}X_2X_3 + 0.014X_2X_4 + 4.066 \times 10^{-3}X_3X_4 + 4.7 \times 10^{-3}X_1^2 + 2.548 \times 10^{-3}X_2^2 + 3.087 \times 10^{-3}X_3^2 + 0.027X_4^2$ (adjusted R^2 of 0.99997).

The variables of X1, X2, X3 and X4 as well as interaction between X2 and X4 and quadratic form of X4 contributed significantly for response of Y3 ($P < 0.05$).

$\mathbf{Y_4} = 1{,}19 - 0{,}017X_1 - 1{,}583.10^{-3}X_2 + 2{,}25.10^{-3}X_3 + 0{,}092X_4 + 0{,}010X_1X_2 - 2{,}75.10^{-3}X_1X_3 + 8{,}75.10^{-3}X_1X_4 + 4{,}75.10^{-3}X_2X_3 + 0{,}012X_2X_4 + 6{,}75.10^{-3}X_3X_4 + 7{,}258.10^{-3}X_1^2 + 2{,}008.10^{-3}X_2^2 + 2{,}583.10^{-4}X_3^2 + 0{,}01X_4^2$ (adjusted R^2 of 0.9944).

Only variable X4 contributed significantly toward response of Y4 ($P < 0.05$). The complete results of response values of Y1, Y2, Y3 and Y4 using independent variables of X1, X2, X3 and X4 were compiled in Table 1.

Based on selected parameters, namely minimum values of retention time and tailing factor and maximum values of peak area and resolution, finally, HPLC optimum condition selected for prediction of CUR and DMCUR consisted of mobile phase composition of acetonitrile-acetic acid 4.08% (49: 51) delivered isocratically at flow rate 1.04 mL/min with column temperature of 40°C. Figure 1 exhibited HPLC chromatogram of CUR and DMCUR using optimized condition. This condition was used for system suitability test and method validation.

Table 1: Design of experiment based Box-Behnken design using dependent variables of acetic acid concentration (X1), acetonitrile ratio (X2), flow rate (X3) and column temperature (X4) with response variables of retention time (Y1), peak area (Y2), resolution (Y3) and tailing factor (Y4) used in HPLC method development for analysis of curcumin and desmethoxycurcumin.

Run	Independent variables				Responses			
	Acetic Acid Concentration (%) (X1)	Acetonitrile Ratio (%) (X2)	Flow rate (mL/min) (X3)	Column Temp. (° C) (X4)	Retention Time (Y1)	peak area (Y2)	Resolution (Y3)	Tailing factor (Y4)
1	2.6	60	0.5	35	9.439	536688	1.746	1.28
2	0.2	40	1	35	20.022	249992	3.969	1.144
3	5	60	1	35	4.62	267727	1.495	1.284
4	0.2	50	1	30	8.329	275717	2.399	1.205
5	2.6	50	1	35	7.652	270340	2.56	1.188
6	2.6	50	1.5	30	5.407	188639	2.167	1.186
7	0.2	50	1	40	7.604	269813	2.725	1.22
8	2.6	60	1	40	4.607	272410	1.588	1.306
9	5	50	0.5	35	14.259	496846	2.734	1.175
10	0.2	50	1.5	35	5.39	187219	2.329	1.201
11	5	50	1.5	35	4.92	183147	2.237	1.185
12	5	50	1	40	6.924	258570	2.597	1.187
13	2.6	40	1	30	19.852	266029	3.655	1.106
14	2.6	50	1	35	7.616	274488	2.564	1.189
15	2.6	50	1.5	40	4.929	184667	2.402	1.188
16	5	50	1	30	7.577	272835	2.361	1.183
17	5	40	1	35	16.444	238329	3.938	1.091
18	0.2	60	1	35	4.806	276572	1.548	1.302
19	2.6	50	0.5	40	14.319	488539	2.926	1.185
20	2.6	40	0.5	35	35.795	451365	4.222	1.119
21	2.6	50	0.5	30	15.678	541434	2.662	1.202
22	2.6	40	1	40	17.14	234583	4.33	1.104
23	2.6	50	1	35	7.614	278896	2.567	1.187
24	2.6	60	1	30	4.865	280748	1.488	1.281
25	2.6	40	1.5	35	12.443	177404	3.691	1.102
26	2.6	60	1.5	35	3.186	188192	1.394	1.312
27	2.6	50	1	35	7.615	277833	2.58	1.192
28	0.2	50	0.5	35	15.622	525134	2.817	1.232
29	2.6	50	1	35	7.615	278343	2.575	1.191

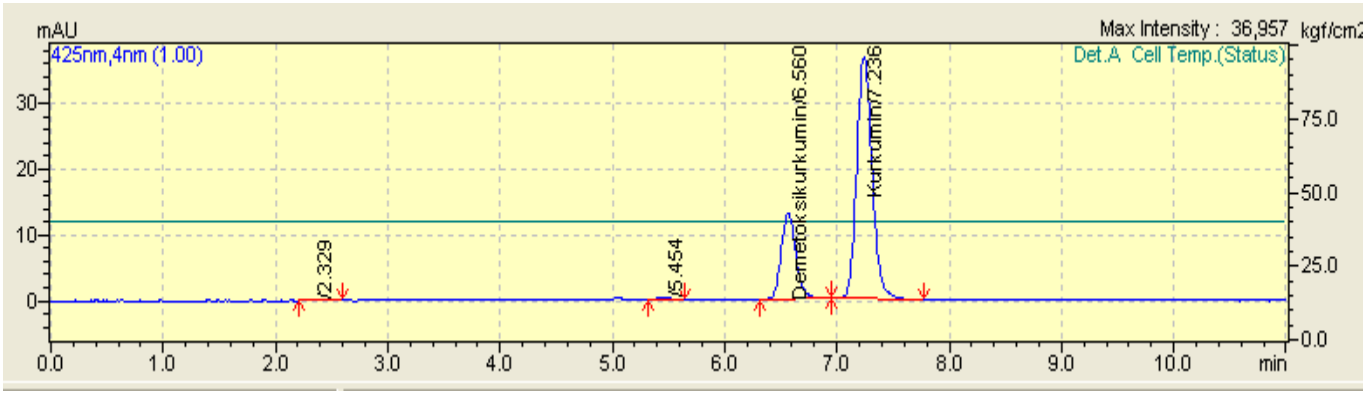

Fig. 1: HPLC chromatogram obtained during optimization using Box-Behnken design for analysis of curcumin and desmethoxycurcumin (see text for HPLC condition).

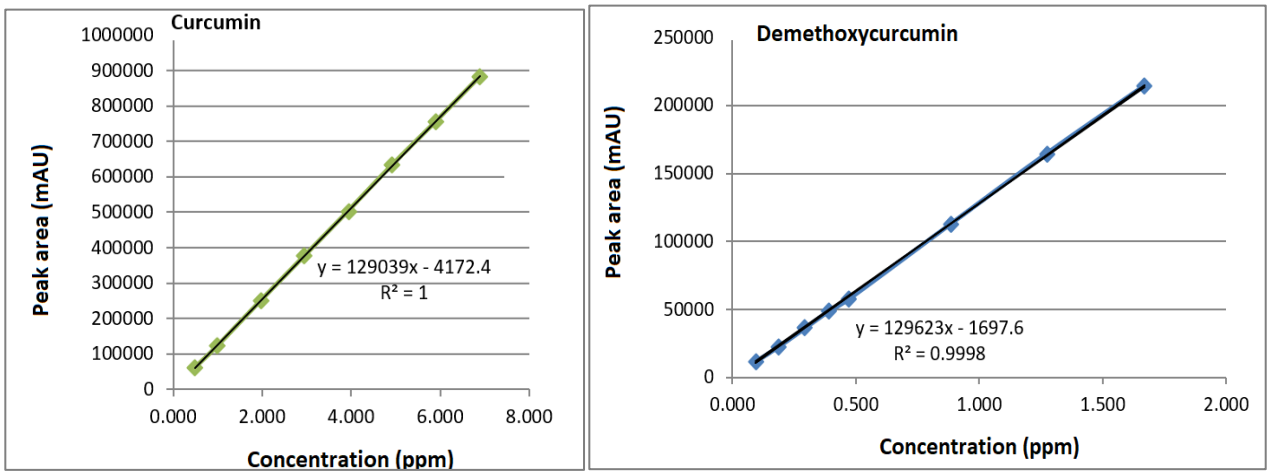

Fig. 2: The calibration plot for relationship between concentration of curcumin and desmethoxycurcumin (x-axis) and peak area (y-axis) along with linear regression equation and coefficient of determination (r^2).

Method validation

During system suitability test, the % relative standard deviation (RSD) of peak area and retention time was evaluated. RSD values were found to be less than 2%, i.e. 0.16% and 0.12% for peak area of CUR and DMCUR, respectively; and 0.07% and 0.06% for retention time of CUR and DMCUR, respectively indicating the suitability of HPLC system. The number of theoretical plates (N) and USP tailing factor (TF) for the six replicate injections were found to be 11121.8 and 1.24 for CUR, as well as 11935 and 1.214 for DMCUR, indicating the acceptable criteria for parameters of N and TF. HPLC which indicated system suitability was validated by determining several parameters namely

Selectivity

The selectivity of HPLC method was evaluated after injecting mobile phase, CUR at concentration 3.5 µg/mL, DMCUR at 0.22 µg/mL, and the mixture of CUR and DMCUR. Resolution (Rs) of ≥ 2.0 during chromatographic separation was used as acceptance criteria. Rs value obtained was > 2.0 indicating that HPLC was selective enough for analysis of CUR and DMCUR.

Linearity and range

The linearity of CUR and DMCUR was evaluated from coefficient of correlation (r-value) and intercept of linear regression describing the relationship between concentration of analytes (x-axis) and peak area (y). The concentration range used were 0.492-6.890 µg/mL for CUR and 0.094-2.452 µg/mL for DMCUR (Figure 2). The calibration plot demonstrated a good relationship with r-value of 0.9999 (CUR) and 0.998 (DMCUR). From linear regression, limit of detection (LoD) and limit of quantification (LoQ) were determined as:

$$LOD = \frac{3{,}3 \times Sy/x}{b} \quad LOQ = \frac{10 \times Sy/x}{b} \quad Sy/x = \sqrt{\frac{\Sigma(yi - yc)2}{n - 2}}$$

where

LoD and LoQ values obtained were 0.056 and 0.169 µg/mL for CUR, and 0.031 and 0.095 µg/mL for DMCUR, respectively.

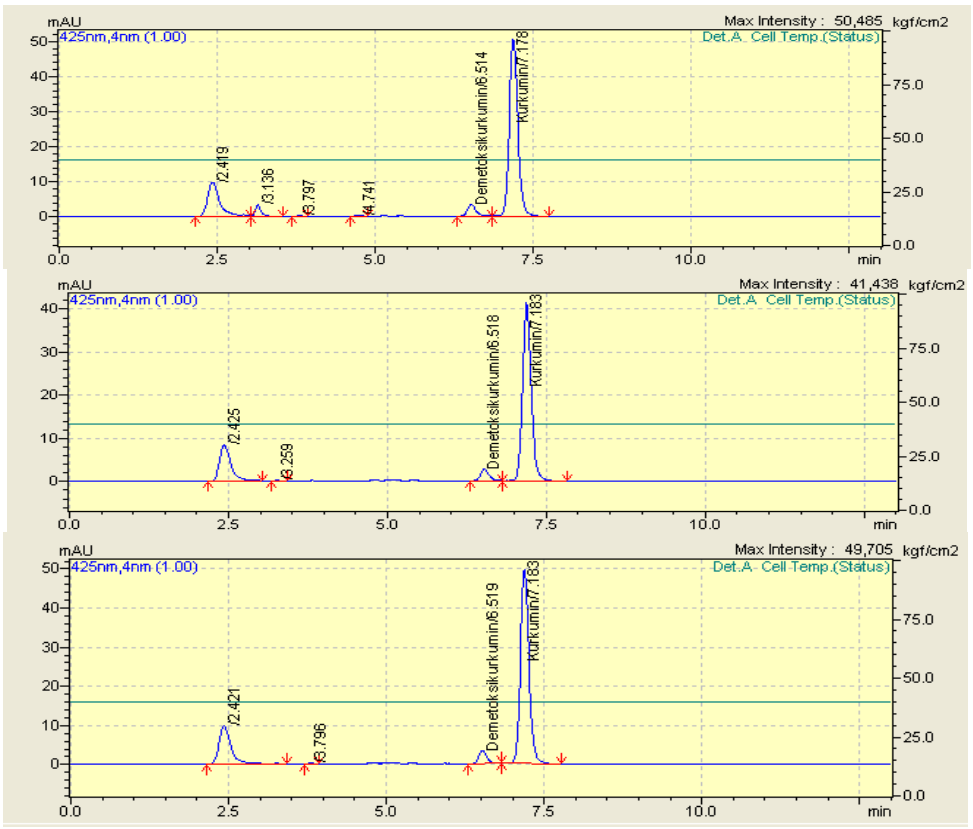

Fig. 3: The example of HPLC chromatogram of syrup samples using optimized condition. (see text for optimized HPLC condition). (tR = ± 6.514 min; Kurku = curcumin, tR = 7.178 min)

Precision

The precision of HPLC was evaluated by repeatability test (intra-day precision) and intermediate precision (inter-day precision) by analysis of eight replicates of homogenous syrup sample at concentration level of 1.827 mgCUR and 0.119 mgDMCUR in 5 mL syrup each. The intra-day RSD values obtained were 1.74% and 2.16% for CUR and DMCUR, respectively. While, inter-day RSD values of CUR and DMCUR were 1.73% and 2.15%, respectively. These RSD values were lower than those requested by Horwitz function (i.e. 4%) at concentration level of 1% of target analytes (Gonzalez and Herrador, 2007), indicating that HPLC at optimized condition was precise for simultaneous determination of CUR and DMCUR in syrup samples.

Accuracy

The accuracy of HPLC method was evaluated by standard addition method in which syrup samples were spiked with standard solution of CUR and DMCUR at 60, 80, 100, 120 and 140% from analyte concentration target and the recovery was calculated. The average recovery percentages for CUR and DMCUR were 97.04 and 100.51%, respectively. These values were in agreement with those specified in the Association of Official Analytical Chemists, as reported by Gonzalez and Herrador (2007). This indicated that HPLC method was accurate and systematic error could be negligible.

Analysis of CUR and DMCUR in syrup samples

The optimized and validated HPLC method was further used for simultaneous analysis of CUR and DMCUR in syrup samples. Figure 4 was an example of HPLC chromatogram obtained during analysis, which indicated selective separation. Table 2 compiled the analytical results of CUR and DMCUR in syrup samples. The concentration of CUR and DMCUR in 8 samples evaluated were in the range of 1.786-1.868 mg/5 mL syrup and 0.116-0.123 mg/5 mL syrup, respectively.

Table 2: The contents of curcumin (CUR) and demethoxycurcumin (DMCUR) in syrup samples containing curcuma extract.

Samples	Concentration (mg/5 mL syrup)	
	CUR	DMCUR
Sample 1	1.840	0.121
Sample 2	1.786	0.116
Sample 3	1.868	0.123
Sample 4	1.853	0.121
Sample 5	1.838	0.120
Sample 6	1.846	0.120
Sample 7	1.794	0.118
Sample 8	1.791	0.116

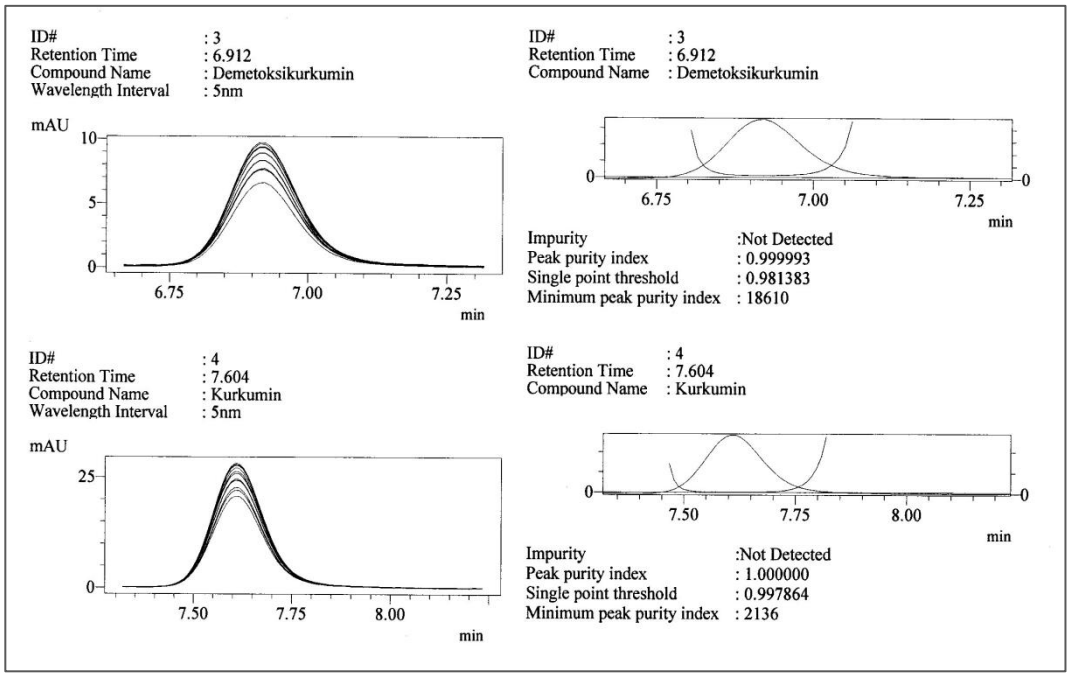

Fig. 4: one of peak purity data during optimization condition (acetic acid 0.2%, 1mL/min flow rate, 40 °C column temperature and 50 % acetonitril ratio to acetic acid.

Table 3: The composition of syrup formulation containing curcuminoid stated in label claim.

Composition **Each of 5 mL syrup contain :**	
Curcuminoid	2 mg
Vitamin B1	3 mg
Vitamin B2	2 mg
Vitamin B6	5 mg
Vitamin B12	5 mcg
Betacaroten 10%	4 mg
Dexpanthenol	3 mg
Lysine HCl	200 mg

CONCLUSION

The experimental design of Box-Behnken has been used successfully to optimize HPLC condition for simultaneous determination of CUR and DMCUR in syrup containing curcuma extract. The mobile phase of acetonitrile-acetic acid 4.08% (49: 51 v/v) delivered isocratically at flow rate 1.04 mL/min with column temperature of 40^{o}C was obtained during optimization. Using this optimum condition, the developed method was valid as appeared from acceptable criteria of validation parameters.

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