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Simultaneous analysis of Curcumin and demethoxycurcumin in *Curcuma xanthorriza* using FTIR spectroscopy and chemometrics

[1,2] Lestari, H.P., [1]Martono, S., [1,2]Wulandari, R., and [1,3*]Rohman, A.

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

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Abstract

Curcuma xanthorrhiza Roxb (Temulawak) contains curcuminoid with various pharmacological effects. To assure the quality and efficacy of *C. xanthorrhiza*, the content of curcuminoid in the product must be properly determined. This research aims to analyze the content of curcuminoid (curcumin and demethoxycurcumin) in ethanolic extract of *C. xanthorrhiza* using FTIR spectroscopy combined with partial least squares (PLS). The actual contents of curcuminoid in the ethanolic extract of *C. xanthorrhiza* were previously determined using HPLC with PDA detector. FTIR spectroscopy is validated by cross validation using leave-one-out (LOO) and external validation. The wavenumbers selected (4000-650 cm^{-1}) were relied on its capability to provide the correlation between the actual value and the predictive value of curcuminoid, indicated by the highest R^2 value with small PRESS and RMSEC values in the calibration model. During PLS calibration, the R^2 values of calibration model and LOO for curcumin (C), demetoxycurcumin (DM) and total curcuminoid (TC) were > 0.99 with RMSEC, RMSECV and PRESS values were relatively small, whereas the R^2 values for external validation were 0.98; 0.99; 0.98, with RMSEP and PRESS values were relatively small for C, DM and TC, respectively. Curcuminoid contents determined using FTIR spectroscopy combined with PLS were not statistically significant compared with HPLC method using independent sample t-test ($P > 0.05$). It can be concluded that FTIR spectroscopy combined with PLS can be used as an alternative method to determine the curcuminoid content in C. *xanthorrhiza.*

Introduction

One of the medicinal plant used popularly in Indonesia is *Curcuma xanthorrhiza* Roxb, or known as Temu lawak in Indonesia. *C. xanthorriza* is a well-known traditional medicine having some biological activities such as anti-inflammatory, antioxidant (Jantan *et al.*, 2012), anticancer activities (Park *et al.*, 2008), acetylcholinesterase inhibitors, as well as protective agents against neurodegenerative disorders (Zhang *et al.*, 2015), anti-metastatic due to xanthorrhizol (Choi *et al*., 2005), antimicrobial activity (Mary *et al.*, 2012), and hepatoprotective (Lin *et al.*, 1996).

Curcuminoid is believed to be responsible for some biological activities and is used as chemical marker in *C. xanthorriza.* Several methods, especially based on chromatographic and electrophoretic techniques, have been developed and used for analysis of curcuminoid in *C. xanthorrhizza* such as high performance liquid chromatography with various detection systems like PDA and or UV (Jadhav *et al*., 2007; Wichitnithad *et al*., 2009), and UV-mass spectrometer detector (Li *et al.*, 2011), thin layer chromatography (Paramasivam *et al.*, 2009; Phattanawasin *et al*., 2009), capillary electrophoresis (Lechenberg *et al.*, 2004), micellar electrokinetic chromatography (Watanabe *et al*., 2002; Unger *et al*., 2009). Rohman (2012) has reviewed some methods used for determination of total and individual curcuminoid. Chromatographic and electrophoretic methods are time consuming and need skilful analyst, therefore some simple techniques based on spectroscopic technique is developed.

UV-Vis spectroscopy has been used for analysis of curcuminoid, and the content of curcuminoid is expressed as total curcuminoid. The wavelength of 420 nn is used by Pothitirat and Gritsanapan (2006) for total curcuminoid determination. Near infrared and mid infrared spectroscopies are proposed for analysis of total curcuminoid and individual curcuminoid (Tanaka *et al*., 2008; Rohman *et al.*, 2015). Due to

*Corresponding author.
Email: *abdul_kimfar@ugm.ac.id*
Tel: +62274-543120

no separation nature, spectroscopic techniques are unsuitable for analysis of individual curcuminoid in the extract of *C. xanthorriza.* Fortunately, with the development of chemometrics software, it is possible to determine individual curcuminoid by selecting the specific wavelength (in UV spectroscopy) or wavenumbers (in infrared spectroscopy). In this study, we proposed FTIR spectroscopy combined with multivariate calibration of partial least square (PLS) for simultaneous analysis of individual curcuminoid (curcumin and demethoxycurcumin) and total curcuminoid in ethanolic extract of *C. xanthorriza* without any separation.

Materials and Methods

The rhizomes of *Curcuma xanthorriza* were obtained from several markets in Indonesia namely Bukit Duri, Harjobinangun, Imogiri, Johar Baru, Klaten, Kulon Progo, Kuningan, Parung Bogor, Plaza Purworejo, Purworejo, Sleman, Tegal, Temanggung, Wonosari, and Bandung during April-May 2015. The standard curcuminoid containing curcumin (C), demethoxycurcumin (DM) and bisdemethoxycurcumin (BDM) is obtained from E. Merck, Darmstat, Germany. The reagents and solvents used were pro-analytical grade. The solvents used as mobile phase during analysis with HPLC were HPLC grade.

Preparation of standards C, DM and BDM

The standards of C, DM, and BDM are isolated from curcuminoid by column chromatography using Silica Gel (230-400 mesh) from E. Merck and mobile phase of chloroform and methanol in gradient manner. The purity of C, DM and BDM was checked by thin layer chromatography using silica gel 60F254 from E. Merck with three different solvents systems having different polarity. The concentration of C, DM and BDM is determined using HPLC as in section of Analysis using HPLC.

Preparation of ethanolic extract of C. xanthorriza

The rhizomes of *C. xanthrorriza* is cleaned, cut into small using commercial cutter, and dried using sun drying with appropriate sealing. The cut rhizomes are powdered and then macerated using ethanol (1: 9) for 2 days. The macerate is filtered and the filtrate is evaporated using vacuum rotary evaporator. The extract is then subjected to partition with n-hexane in order to remove essential oil. The purified extract is then dried using oven at 40^{o}C and subjected to analysis with HPLC and FTIR spectrophotometer.

Analysis using HPLC

The purity of C, DM and BDM isolated from curcuminoid as well as the levels of C, DM and BDM is determined using HPLC using photodiode array detector at 425 nm. Separation of C, DM and BDM is carried using Waters X-bridge C_{18} column (250 mm x 4.6 mm i.d; 5 µm) maintained at temperature of 30oC. The mobile phase is the mixture acetonitrile: acetic acid 2% in aquadest (50: 50 v/v) delivered isocratically at 1.0 mL/min. The injection volume is 20 uL (Wichitnithad *et al*., 2009). Quantification is performed using external standard.

FTIR spectroscopy analysis

The samples of purified extracts of *C. xanthorriza* are made in good contact with ATR crystal and its spectra are scanned using FTIR spectrophotometer ABB MB3000 (Clairet Scientific, Northampton, UK), equipped with deuterated triglycine sulphate (DTGS) detector and beam splitter of germanium, and processed using Horizon MB FTIR software version 3.0.13.1 (ABB, Canada). The spectral FTIR are measured at mid infrared region corresponding to wavenumbers of 4000-650 cm^{-1} with resolution of 4 cm^{-1} and number of scanning of 32. All spectra were rationed against a background of air spectrum. After every scan, a new reference air background spectrum was taken. These spectra were recorded as absorbance values at each data point in duplicated.

Statistical analysis

Partial least square (PLS) calibration model used for making the correlation between the actual values of C, DM, and total curcuminoid is performed with the aid of Minitab software version 17. The values of R^2, root mean square error of calibration (RMSEC), root mean square error of cross validation (RMSECV), and predicted residual error of sum of squares (PRESS) are calculated using Minitab.

Results and Discussion

Analysis of Curcumin and Demethoxycurcumin using HPLC

The main curcuminoid in *C. xathorriza* is curcumin (C) and demethoxycurcumin (DM) with chemical structures in Figure 1. Demethoxycurcumin is more polar than curcumin, therefore, in HPLC analysis using C_{18} column, DM is less retained that curcumin. Table 1 compiled the levels of C and DM in purified extract of *C. xanthorriza*. In addition, the level of total curcuminoid (sum of curcumin and demethoxycurcumin) is also analysed. These values are used as actual values during analysis of C, DM

Table 1. The levels of curcumin and demethoxycurcumin in purified extract of *Curcuma xanthorriza* as analysed by HPLC.

Origin of samples	Level of curcumin	Level of demethoxycurcumin	The curcuminoid total
Bukit Duri	32.83	11.49	44.32
Harjobinangun	30.16	11.69	41.85
Imogiri	27.94	8.9	36.84
Johar Baru	26.5	9.87	36.37
Klaten	32.6	12.53	45.13
Kulon Progo	24.82	9.31	34.13
Kuningan	27.8	11.71	39.51
Parung Bogor	22.15	7.53	29.68
Plaza Purworejo	30.44	10.94	41.38
Purworejo	31.76	14.43	46.19
Sleman	32.37	9.3	41.67
Tegal	31.07	13.25	44.32
Temanggung	25.01	9.04	34.05
Wonosari	26.88	9.34	36.22
Bandung	27.68	10.69	38.37

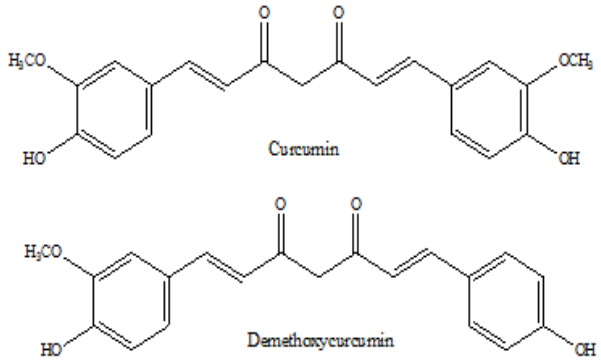

Figure 1. The chemical structure of curcumin and demethoxycurcumin

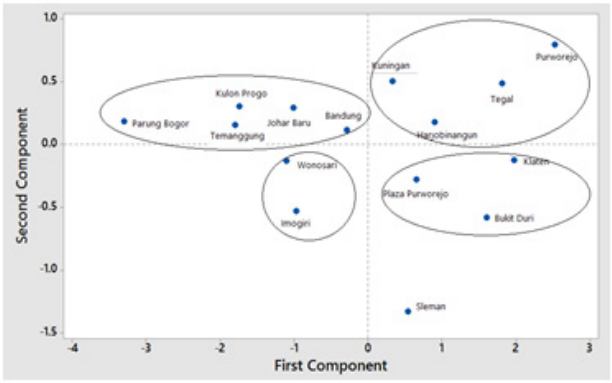

Figure 2. The score plot of principal component analysis for classification of purified extract of *Curcuma xanthorriza* using the levels of curcumin, demethoxycurcumin and total curcuminoid as variables

and total curcuminoid using FTIR spectroscopy. The highest level of curcumin is observed in *C. xanthorriza* from Bukit duri, while the highest level of DM is found in *C. xanthorriza* from Purworejo.

In order to make classification or grouping among *C. xanthorriza* from different region, principal component analysis (PCA) is explored. PCA is one of the classification technique with unsupervised category (Che Man *et al.*, 2011). Figure 2 revealed the PCA srore plot along the first principle component (PC1) and the second principle component using the levels of C, DM and total curcuminoid as variables. PC1 account for 90.6% variation (eigenvalue of 2.7174), PC2 account for 9.40% variation (eigenvalue of 0.2826), while PC3 has 0% variation (eigenvalue of 0.000). This confirmed that PCA is chemometrics technique used for reduction of variables. Based on PCA score plot, *C. xanthorriza* can be intentionally classified into 4 groups, according to quadrant in Figure 2. *C. xanthorriza* from Parung Bogor has the similar profile with that from Kulon Progo, Johar Baru, Temanggung and Bandung.

Analysis of Curcumin and Demethoxycurcumin using HPLC

The same purified extract of rhizomes of *C. xanthooriza* analysed using HPLC is then analysed using FTIR spectroscopy. Combined with multivariate calibration of partial least square (PLS), FTIR spectroscopy allows analyst to determine analytes in extract simultaneously without any preparation step (Bunaciu *et al*., 2011). Figure 3 revealed the typical FTIR spectra of purified extract of *C. xanthorriza.* Each peaks and shoulders correspond to functional groups present which are responsible for IR absorption. The main component in *C. xanthorriza* is curcumin, therefore, it is not surprising the appearance of –OH stretching vibration in 3450-3255 cm-1 which might from OH- curcumin. The first step is selecting the wavenumbers region or the combined wavenumbers region capable of providing the best prediction between actual values of C and DM with FTIR predicted values using PLS calibration model. Finally the whole mid infrared region (4000-650 cm-

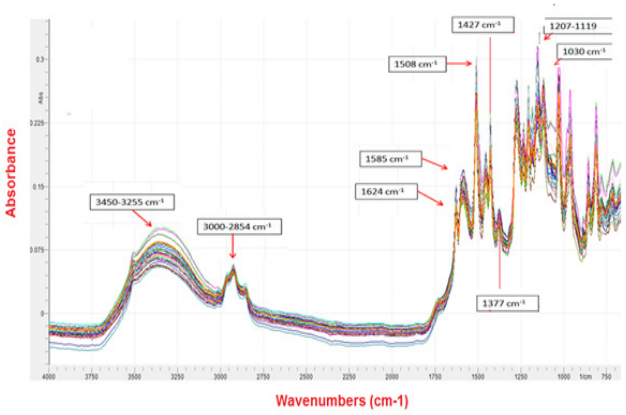

Figure 3. Typical FTIR spectra of purified extract of *Curcuma xanthorriza*, scanning using attenuated total reflectance mode at wavenumbers of 4000-650 cm^{-1}.

[1]) is selected for quantification of C, DM and total curcuminoid.

Figure 4 revealed PLS calibration model for the relationship between actual values of C, DM and total curcuminoid with FTIR spectroscopy-predicted values. The values of R^2 and RMSEC are used for evaluation of PLS model. The R^2 values obtained for such relationship are 0.9997, 0.9998 and 0.9996 for C, DM and total curcuminoid respectively. The R^2 value exceed 0.99 are acceptable for PLS model. Meanwhile, the RMSEC values are 0.0615%, 0.0296%, 0.0999% for C, DM and total curcuminoid respectively. The low values of RMSEC value indicated that PLS are precise enough. PLS calibration model is further validated using leave one out (LOO) technique, and the values of R^2 and RMSECV are used for evaluation model. During validation using LOO, the R^2 value of 0.9975, 0.9929, and 0.9951 are obtained with RMSECV of 0.1829%, 0.1814% and 0.3801% for C, DM and total curcuminoid, respectively.

The developed method is also validated using independent samples from six region, namely *C. xanthorriza* from Kulon Progo, Klaten, Kuningan, Plaza Purworejo, Wonosari and Imogiri. The selection of *C. xanthorriza* using these six regions is based on variety of curcuminoid contents as determined using HPLC (Table 1), therefore these are suitable for validation procedure. The output of validation showed that the values R^2, PRESS, and RMSEP obtained are 0.9774, 9.7312, and 1.3951 for curcumin; 0.9930, 0.5018, 0.3168 (for demethoxycurcumin) and 0.9839, 1.5516 and 0.5570 for total curcuminoid, respectively. FTIR spectroscopy and HPLC are finally compared for analysis of samples, and the results are statistically tested using independent sample t-test. The P value obtained is > 0.05, meaning that results obtained by HPLC and FTIR spectroscopy is not statistically different.

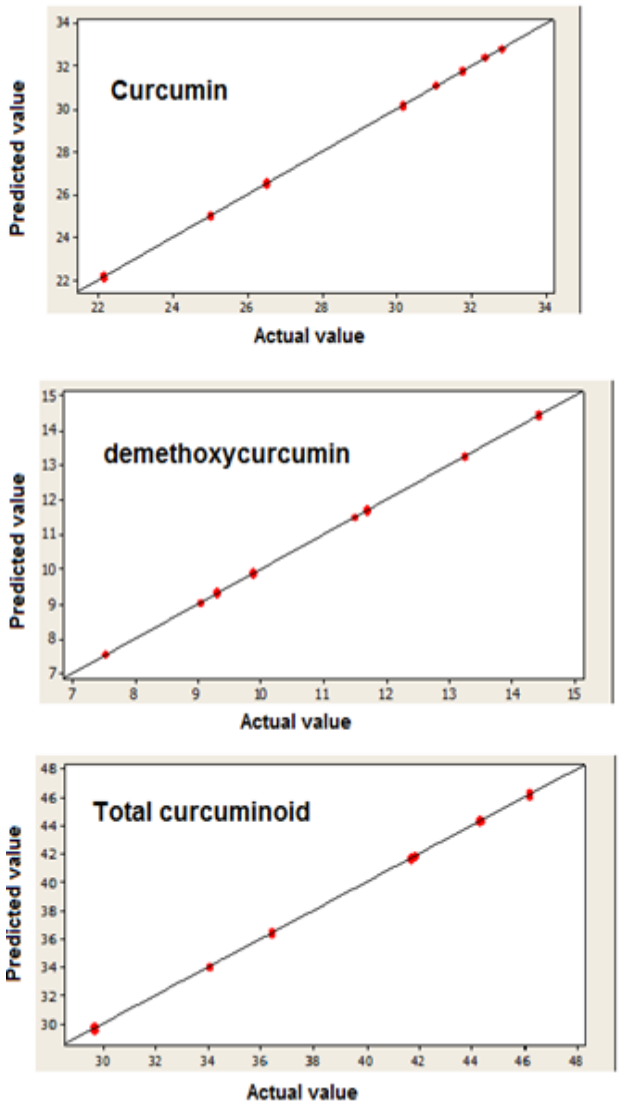

Figure 4. The relationship between actual values of C, DM and total curcuminoid with FTIR spectroscopy-predicted values.

FTIR spectroscopy in combination PLS calibration method is alternative official method (HPLC) for analysis of curcuminoid. This method is fast and no need to excessive sample preparation (Tanaka *et al.,* 2008; Rohaeti *et al*., 2015). However, FTIR spectroscopy developed can only be used for specific extract tested. If the composition of the extract samples to be analyzed is different, FTIR spectra of the samples are different. Consequently, new calibration model together with FTIR spectra optimization is needed (Rohman and Che Man, 2012).

Conclusion

FTIR spectroscopy at wavenumbers of 4000-650 cm^{-1} combined with partial least square (PLS) calibration is an alternative technique for analysis of curcumin, demethoxycurcumin and total curcuminoid

in purified extract of *Curcuma xanthorriza.* FTIR spectroscopic technique is fast, no excessive sample preparation and ideal for routine analysis.

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