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Liquid Chromatography and Fourier Transform Infrared Spectroscopy for quantitative analysis of individual and total curcuminoid in *Curcuma longa* extract

Ratna Wulandari[1,2], Sudjadi[1], Sudibyo Martono[1], Abdul Rohman[1*]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia.
[2]The National Agency of Drug and Food Control, Republic of Indonesia.

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ABSTRACT

Analysis of individual and total curcuminoid composed of curcumin (CUR), desmethoxycurcumin (DMCUR) and bisdemethoxycurcumin (BDMCUR) in Curcuma species is very important. Curcuminoids are frequently used as markers in herbal medicine involving the use of Curcuma species in its formulation. The objective of this study was to validate high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy-partial least square (FTIR-PLS) for quantitative analysis of total and individual curcuminoids in turmeric (*Curcuma longa* L.) extract. The turmeric powder was macerated using ethanol and evaporated to obtain an ethanolic extract. The actual contents of individual curcuminoids of CUR, DMCUR, and BDMCUR were determined using HPLC. FTIR-PLS can be used for quantitative analysis of individual and total curcuminoids using specific wavenumbers. The coefficient of determination (R^2) for the relationship between actual values and FTIR-PLS predicted values for calibration, internal, and external validation was >0.98 which indicated good accuracy. The relatively low values of errors in calibration, validation and external validation indicated good precision. FTIR spectroscopy-PLS can be used as an alternative technique over HPLC for determination of individual and total curcuminoid in turmeric extracts.

INTRODUCTION

The use of turmeric (*Curcuma longa* Linn) as a raw material in herbal medicines and food products has increased significantly (Liang *et al.*, 2004; Gad *et al.*, 2013). Because of its composition especially curcuminoid, turmeric is known as functional food components, turmeric has been reported to have antibacterial, antiinflammation, and antioxidant activities due to high contents of curcuminoid (Sing *et al.*, 2002; De *et al.*, 2009; Anubala *et al.*, 2014), neuroprotective (Issuriya *et al.*, 2014), immunomodulatory (Rogers *et al.*, 2010), antidiabetic (Wickenberg *et al.*, 2010), as well as antitumor and anticancer activities (Aggarwal *et al.*, 2003; Wilken *et al.*, 2011). Because of high antioxidant activities, turmeric has been widely used in cosmetics, nutraceuticals, and phytomedicines (Paulucci *et al.*, 2013).

In order to assure the quality of herbal materials from one batch to other batches, it is important to determine the levels of chemical compounds responsible for the biological activities. Curcuminoid in turmeric, especially curcumin, has been used as a chemical marker during biological activity evaluation (Gupta *et al.*, 1999). According to Indonesian Herbal Pharmacopeia, turmeric extract should contain minimum 33.90% of total curcuminoid with moisture content less than 10% (Ministry of Health, 2008). Indeed, analytical techniques capable of quantifying curcuminoid (total and individual) should be developed.

Some analytical techniques have been developed and used for quantitative analysis of curcuminoid, namely thin layer chromatography-densitometry (Péret-Almeida *et al.*, 2005), high performance liquid chromatography using ultraviolet-visible and electrochemistry detectors (Inoue *et al.*, 2008; Syed *et al.*, 2015), liquid chromatography-mass spectrometry (Asai and Miyazawa, 2000), and capillary electrophoresis (Jiang *et al.*, 2006). Due to its capability to provide separation among components in

*Corresponding Author
Abdul Rohman, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia.
E-mail: abdul_kimfar @ ugm.ac.id

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samples, chromatography-based methods are a method of choice for analysis of multicomponent such as in extract; however, these methods are time-consuming and involving the use of excessive chemicals and reagents. Therefore, there is a need to develop analytical techniques capable of quantifying analytes using some simple methods based on spectroscopy such as near-infrared spectroscopy (Tanaka *et al.*, 2008) and mid-infrared spectroscopy (Rohman *et al.*, 2015).

Fourier transform infrared (FTIR) spectroscopy is promising techniques for the quantification of curcuminoid due to its capability to provide fast and green analytical technique, with minimum sample preparation step (Bunaciu *et al.*, 2011; Rohman, 2012). Combined with multivariate calibration, FTIR spectroscopy can be used as an alternative technique for chromatographic techniques. Tanaka *et al.* (2008) have analyzed individual curcuminoid of curcumin (C), desmethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) and total curcuminoid (as a sum of C + DMC + BDMC) using near infrared at 1650-1780 nm. In this study, mid-infrared (4000-400 cm^{-1}) was used due to more peaks obtained, as consequence, the modeling of PLS using more peaks was more achievable (Bunacie *et al.*, 2011). Rohaeti *et al.* (2015) using FTIR spectroscopy in combination with principal component analysis (PCA) and canonical variate analysis (CVA) for identification and classification of turmeric, *temulawak* (*Curcuma xanthorhiza)* and *bengle* (*Zingiber cassumunar*). In our best knowledge, there is no report regarding the application of FTIR spectroscopy for analysis of individual and total curcuminoid using HPLC and FTIR spectroscopy combined with PLS. In this research, the individual curcuminoid was isolated from the mixture using column chromatography and the isolated compounds obtained was used for quantification using HPLC and FTIR spectroscopy combined with PLS regression.

MATERIALS AND METHODS

Curcuminoid containing CUR, DMCUR and BDMCUR was purchased from E. Merck (Darmstadt, Germany) with curcuminoid contents ≥ 94%. Turmeric was obtained from several regions of Java (East Java, Central Java, West Java, Yogyakarta, and Jakarta). The synthetic curcumin was kindly given by Prof. Dr. Sudibyo Martono from Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The solvents used for mobile phase were of HPLC grade. The other solvents and reagents were of pro-analytical grade unless otherwise was specified.

Isolation of C, DMC, and BDMC

Isolation of curcuminoid (with curcuminoid contents ≥ 94%) from E. Merck into CUR, DMCUR and BDMCUR was carried out according to Péret-Almeida *et al.* (2005) using column chromatography with stationary phase of silica gel 60 for column chromatography (230–400 mesh) (Merck, Darmstadt, Germany), with mobile phase of chloroform-methanol delivered in gradient manner. Each fraction obtained was subjected to TLC, and the fraction containing the same curcuminoid was compiled. The solvent was evaporated using a vacuum rotary evaporator, and the powder obtained was determined for its purity qualitatively using TLC with three different mobile phase systems. The purity of individual curcuminoids was also determined using HPLC with photodiode array detector at 425 nm using an internal normalization technique (relative percentage).

Preparation of ethanolic extract of turmeric

The samples of turmeric were cleaned from any dirty, cut into small using the commercial cutter, and dried using a conventional oven at 50°C. The dried turmeric was powdered using a grinder and subjected to sieving using mesh 40. A-50 gram of powdered turmeric was subjected to maceration using 500 mL ethanol 90% in aquadest for 24 hours (Tanaka *et al.*, 2008). The macerate was filtered and the supernatant was evaporated using a vacuum rotary evaporator at 50°C to obtain an ethanolic extract. The ethanolic extract was then fractionated using *n*-hexane two times, and the purified ethanolic extract was then added with amprotab (1 part extract:19 part amprotab) and subjected to FTIR spectroscopic measurement and HPLC analysis.

HPLC analysis and validation

The condition of HPLC was optimized based on Wichitnithad *et al.* (2009) to get the best separation among CUR, DMCUR, and BDMCUR. The final condition used during analysis of curcuminoid in ethanolic extract of turmeric was:

Column:	Waters X-bridge C18 (250 mm × 4.6 mm i.d; 5 µm)
Mobile phase:	acetonitrile : acetic acid 2% in aquadest (50:50 v/v) delivered isocratically at 1.0 mL/min
Detector:	PDA at 425 nm
Volume of injection:	20 uL
Column oven temperature:	30°C

Validation of HPLC method

The optimized HPLC condition was subjected to validation 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.

FTIR spectroscopy analysis

Analysis of ethanolic extract of turmeric added with amprotab was performed using the sampling technique of attenuated total reflectance (ATR) as in Rohman *et al.* (2015). The samples were directly placed on ATR crystal and its spectra were scanned using FTIR spectrophotometer ABB MB3000 (Clairet Scientific, Northampton, UK), equipped with deuterated triglycine sulfate (DTGS) detector and beam splitter of germanium, and processed using Horizon MB FTIR software version 3.0.13.1 (ABB, Canada). FTIR spectra were measured at mid-infrared (4000-650 cm^{-1}) using a 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 duplicate.

Statistical analysis

The analytical results obtained using HPLC was used

as actual content values of CUR, DMCUR, and BDMCUR and were correlated with predicted values of CUR, DMCUR, and BDMCUR using PLS calibration. PLS is calibration model used to solve problems involving high collinearity or to calculate correlated *Y* variables (Gad *et al.*, 2013). PLS has been associated with other mathematical methods and algorithms, and the most used algorithms to implement PLS regression is non-linear iterative partial least squares (NIPALS). The concentration variables (y-axis) are related to the predictor variables (*x-axis)* through auxiliary variables known as latent variables (factors or components), which are linear combinations of the variables x_1, x_2, …, x_K. These components are highly similar to principal components calculated by principal component analysis. Minitab software version 17 was used for statistical analysis during calibration, internal validation using leave one out technique, and external validation.

RESULTS AND DISCUSSION

The reference standard of CUR, DMCUR, and BDMCUR used for quantitative analysis was isolated from curcuminoid using column chromatography. The isolated compounds of CUR, DMCUR and BDMCUR were analyzed using TLC with three different polarity solvents. Figure 1 showed that CUR, DMCUR, and BDMCUR were well separated. Curcumin is the major component in curcuminoid and is obtained relatively easy compared to DMCUR and BDMCUR. The purity levels of CUR, DMCUR, and BDMCUR as determined using HPLC with a photodiode array detector at 425 nm were of 99.92%, 98.53% and 85.85%, respectively.

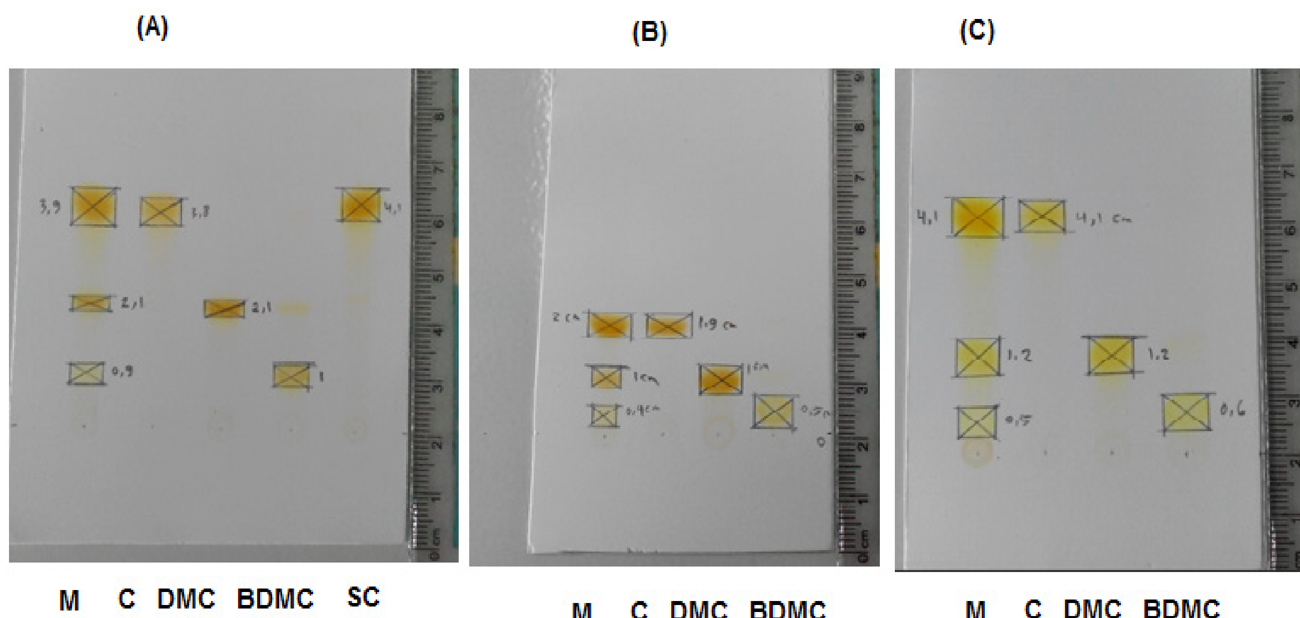

Fig. 1: Thin layer chromatography profile of curcumin (C), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) using three different solvents with stationary phase of silica gel GF254. System A = chloroform:methanol:acetic acid (95:3:2 v/v/v); system B = Toluene:acetic acid (9:1 v/v); and system C = chloroform:aquadest:Ethanol (25:0.04:0.96). M = Curcuminoid; SC = synthetic curcumin.

Due to its versatility, high-performance liquid chromatography (HPLC) was the method of choice for determination of curcuminoid because curcuminoid is relatively non-polar and suitable to be separated using reversed HPLC column (Prabaningdyah *et al.*, 2017). HPLC was reference method for quantitative analysis of CUR, DMCUR and BDMCUR in ethanolic extract of turmeric, and was optimized in order to meet the requirement of system suitability test, namely resolution >2; tailing factor <1.8 and number of theoretical plate >10.000. Two columns, namely Waters X-bridge C18 (250 mm × 4.6 mm i.d; 5 µm) and Waters Spherisorb, C_{18}, (250 × 4.6 mm, 5 µm) were compared, and finally Waters X-bridge C18 was chosen with mobile phase of acetonitrile:acetic acid 2% in aquadest (50:50 v/v) delivered isocratically at 1.0 mL/min. HPLC chromatogram of CUR, DMCUR, and BDMCUR obtained using optimum condition was shown in Figure 2. The elution order of curcuminoid was BDMCUR with t_R of ±6.795 min, DMCUR with t_R of ±7.43 min, and CUR with t_R of ±8.130 min. This order was in agreement with the polarity of curcuminoid, in which BDMCUR is more polar than DMCUR and CUR.

Validation of HPLC method for analysis of C, DMC, and BDMC

HPLC was validated for quantitative analysis of CUR, DMCUR and BDMCUR in turmeric extract by determining several parameters namely selectivity, linearity, limit of detection, limit of quantification, accuracy, and precision according to International Conference on Harmonization (2005). The system suitability test indicated that HPLC system used was precise enough because the relative standard deviation (RSD) values of retention times and a peak area of CUR, DMCUR and BDMCUR from six replicates were less than 2.0%. Besides, the resolution value (Rs) of >2, the number of a theoretical plate (N) > 10.000, and tailing factor (TF) ≤ 2 were achieved as requested by United States Pharmacopeia.

The selectivity of HPLC for separation of curcuminoid was expressed with resolution value (Rs). The obtained Rs values

of CUR, DMCUR and BDMCUR were 2.67, 2.59 and 15.56, respectively which indicated that the validated method was selective enough for separation of curcuminoid. The linearity of HPLC was assessed on concentration range of 0.4248-4.2484 μg/mL for CUR, 0.3139-1.8835 μg/mL for DMCUR, and 0.3288-1.644 μg/mL for CUR. The correlation coefficient (r) obtained for the relationship between concentration of CUR, DMCUR and BDMCUR and peak area (y-axis) were 0.9995, 0.9996 and 0.9996, with %y-intercept values of 3.43%, 3.21% and 6.17%, respectively. All r-values were higher than 0.999 indicating that the HPLC method was linear over the concentration range, which covers 50-150% from the target of analytes in the studied ethanolic extract of turmeric (ICH, 2005).

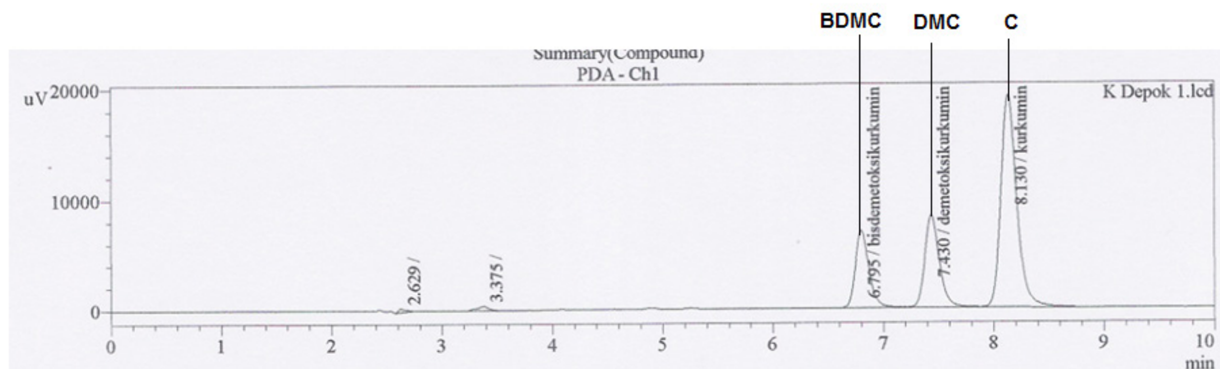

Fig. 2: HPLC chromatogram of ethanolic extract of turmeric. BDMC = bisdemethoxycurcumin with t_R 6.795, DMC = demethoxycurcumin with tR of 7.43 and C = curcumin with t_R of 8.130. Column = Waters X-bridge C_{18} (250 mm × 4.6 mm i.d; 5 μm), with column oven temperature of 30°C; mobile phase:acetonitrile:acetic acid 2% in aquadest (50:50 v/v) delivered isocratically at 1.0 mL/min; detector = photodiode array (PDA) at 425 nm.

The precision of developed HPLC was assessed by repeatability test and intermediate precision by analysis six replicates of homogeneous sample extract. RSD values of CUR, DMCUR, and BDMCUR were 1.34%, 1.42%, and 3.45%, respectively, lower than those requested by RSD Horwitz (i.e. 4%) at a concentration level of 1% of target analytes (Gonzalez and Herrador, 2007). In addition, the recovery values for accuracy studies of CUR, DMCUR, and BDMCUR were studied by standard addition methods by spiking CUR, DMCUR, and BDMCUR into the turmeric extract samples. The recovery values obtained were 98.05-100.38% for CUR, 99.30-101.60% for DMCUR, and 99.97-100.0% for BDMCUR. These values are in agreement with those specified in ICH (2005).

The sensitivity of HPLC was expressed by determining the limit of detection (LoD) and limit of quantification (LoQ). The LoD and LoQ values obtained were 0.0040 μg/mL, 0.0037 μg/mL, and 0.0049 μg/mL for CUR, DMCUR and BDMCUR, respectively, while LoQ values were 0.0162, 0.0104, and 0.0147 μg/mL respectively. The values of LoD were confirmed by injecting these concentrations 5 times, and RSD of 23.55% was obtained indicating that LoD values were not precise enough to be used as LoQ. In addition, the reported LoD and LoQ values of CUR were lower than those reported by Dandekar and Patravale (2009), i.e. 0.06 ± 0.01 μg/mL and 0.21 ± 0.045 μg/mL, respectively. However, LoD and LoQ values of this study were also lower (more sensitive) than those reported by Cheng *et al.* (2010) using ultra performance liquid chromatography (UPLC) with uv-vis detector. LoD and LoQ values were 0.040 and 0.134 μg/mL for CUR, 0.049 and 0.164 μg/mL for DMCUR, as well as 0.029 and 0.098 μg/mL for BDMCUR, respectively.

The validated HPLC method was subsequently used for quantitative analysis of CUR, DMCUR, and BDMCUR in the samples, and the results were shown in Table 1. The concentrations of CUR, DMCUR, and BDMCUR and curcuminoid total obtained from HPLC analysis was used as actual values in PLS modeling during prediction of CUR, DMCUR and BDMCUR using FTIR spectroscopy.

Table 1: The concentration of curcumin (CUR), demethoxycurcumin (DMCUR) and bisdemethoxycurcumin (BDMCUR) in ethanolic extract of Turmeric obtained from Java region, Indonesia.

Region	CUR	DMCUR	BDMCUR	Curcuminoid total*
Bandung (West java)	1.56	0.51	0.94	3.01
Jakarta	1.17	0.34	0.60	2.11
Cilacap (central Java)	1.40	0.55	1.52	3.47
Depok (west Java)	1.41	0.58	0.89	2.88
Magelang (central Java)	1.20	0.46	1.01	2.67
Bantul (Yogyakarta)	1.68	0.61	1.02	3.31
Karanganyar (central Java)	1.61	0.52	0.96	3.09
Kediri (East Java)	1.88	0.52	0.89	3.29
Kuningan (Jakarta)	1.41	0.47	0.98	2.86
Purworejo (central Java)	1.05	0.33	0.62	2.00
Sleman (Yogyakarta)	1.41	0.55	1.29	3.25
Sukaharjo (central Java)	1.19	0.52	0.92	2.63
Temanggung (central Java)	1.37	0.61	1.17	3.15
Wonogiri (central Java)	0.76	0.29	0.52	1.57

*Curcuminoid total is the sum of CUR + DMCUR and BDMCUR.

Analysis using FTIR spectroscopy

FTIR spectroscopy is taken into account as the fingerprint analytical technique used for qualitative and quantitative analyses of analyte(s) including *Curcuma longa* extracts (Prabaningdyah *et al.*, 2018). To facilitate FTIR spectroscopy as quantitative analysis method of curcuminoid in ethanolic extract of turmeric, PLS was employed for making the correlation between actual content values of curcuminoid as determined using HPLC and FTIR predicted

values using absorbance values at optimized wavenumbers. PLS is multivariate calibration technique based on inverse calibration in which concentration (y-axis) was modeled with factors (combination of absorbance variables) in the x-axis (Miller and Miller, 2005). Figure 3 exhibited FTIR spectra of ethanolic extract of turmeric from several regions of Java, scanned at wavenumbers of 4000-650 cm^{-1} along with main peaks corresponding to IR absorption at specific wavenumbers. All functional groups in Figure 3 were representative of those of curcuminoid. These peaks were in accordance with those reported by Rohman *et al.* (2015), Rohaeti *et al.* (2015) and Prabaningdyah *et al.* (2018).

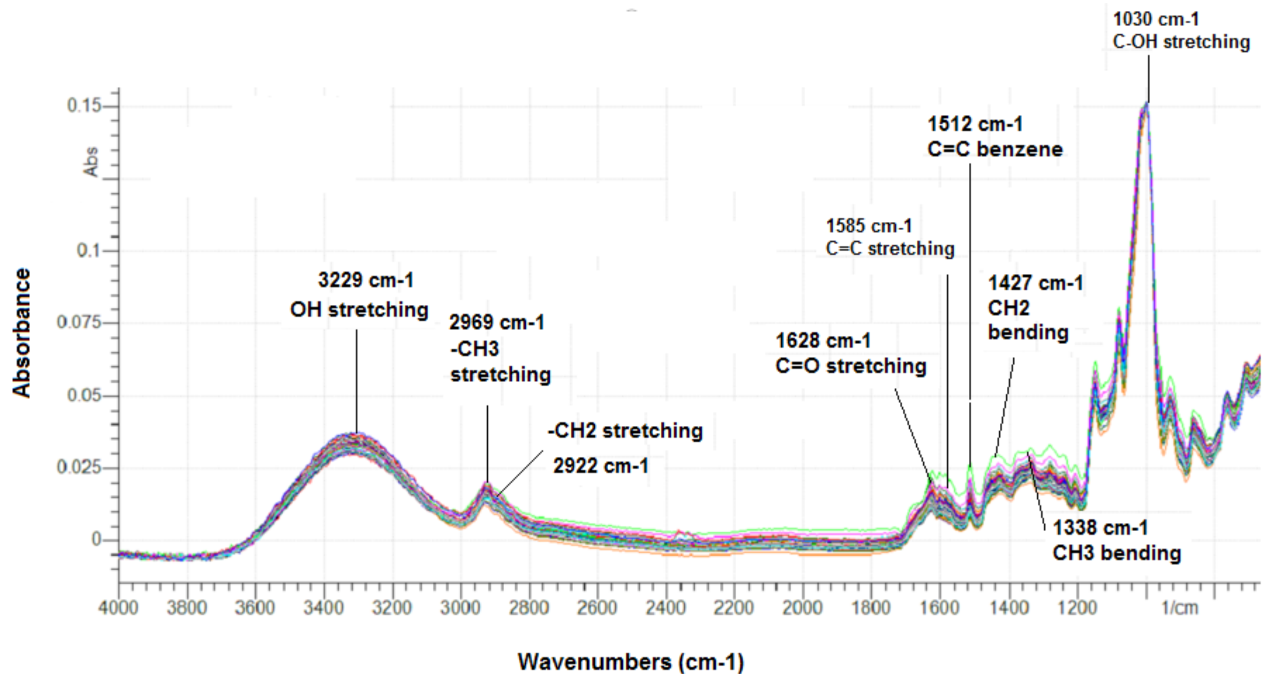

Fig. 3. FTIR spectra of ethanolic extract of turmeric from 14 regions of Java, scanned at mid-infrared region (4000-650 cm^{-1}) along with main peaks corresponding to IR absorption at specific wavenumbers.

Analysis of curcuminoid with FTIR spectroscopy coupled with PLS calibration was performed in three steps, namely calibration, validation, and analysis of unknown samples. Before calibration modeling, FTIR spectra of all samples were subjected to optimization in order to get the best region capable of providing the acceptable correlation between actual values and FTIR predicted values of a curcuminoid and the lowest errors. Optimization was performed by selecting the wavenumbers region and spectral treatments (Lestari *et al.*, 2017). Finally, the wavenumbers of 1400-1720 cm^{-1} were preferred for quantification of CUR, wavenumbers of 1481-1747 cm^{-1} used for quantitative analysis of DMCUR, the combined wavenumbers region of 1172-1226 cm^{-1} and 1469-1785 cm^{-1} used for analysis of BDMCUR, while the wavenumbers of 1407-1820 cm^{-1} were exploited for analysis of total curcuminoid. Figure 4 revealed the calibration model for the relationship between actual values of CUR, DMCUR, and BDMCUR and total curcuminoid and FTIR predicted values. The coefficient determination (R^2) obtained were 0.9998 (CUR), 0.9999 (DMCUR), 0.9996 (BDMCUR) and 0.9999 (total curcuminoid). The root mean square error of calibration (RMSEC) values obtained were 0.469, 0.0035, 0.0063 and 0.064 mg% for CUR, DMCUR and BDMCUR and total curcuminoid, respectively. The high value of R^2 and low values of RMSEC indicated that FTIR spectroscopy using these specific wavenumbers were accurate and precise for quantitative analysis of curcuminoid. From figure 4, it is clear that results obtained from HPLC were not significantly from those obtained by FTIR spectroscopy as indicated by the high R^2 value.

PLS calibration model was further validated using leave one out (LOO) technique. In LOO, one of the calibration samples was removed from the calibration model, and the remaining samples were used to make new PLS model. Subsequently, the removed sample was computed using the new PLS regression model. This manner was recurred, leaving each sample out in turn. The difference between the actual values and calculated values of CUR, DMCUR, and BDMCUR and total curcuminoid was calculated (Miller and Miller, 2005). The validation models of C, DMC, BDMC and total curcuminoid during LOO resulting the predicted residual error sum of squares (PRESS) of 1.4×10^{-6} for CUR, 2×10^{-7} for DMCUR, 4.4×10^{-6} for BDMCUR, and 2.6×10^{-6} for total curcuminoid. The low values of PRESS indicated that the calibration model was precise enough to be used for analysis of CUR, DMCUR and BDMCUR, and total curcuminoid in unknown samples. However, based on results obtained during external validation, RMSEP values of DMCUR and BDMCUR was too high, therefore, the developed model was not used for prediction of DMCUR and BDMCUR.

Analysis of CUR and total curcuminoid in unknown samples from Sleman and Sukoharjo, central Java using FTIR spectroscopy with PLS calibration model was performed. The results obtained were compared with those obtained using HPLC using statistical Tukey test at a significance level of 0.05. There was

no significant difference of results obtained by FTIR spectroscopy and HPLC (P > 0.05). The P values were 1.25 and 1.23 for CUR, as well as 3.25 and 2.63 for total curcuminoid of turmeric obtained from Sleman and Sukoharjo, respectively. FTIR spectroscopy combined with PLS calibration model offered alternative method over HPLC due to its simplicity and its capability to provide simultaneous analysis of curcuminoid without a separation step.

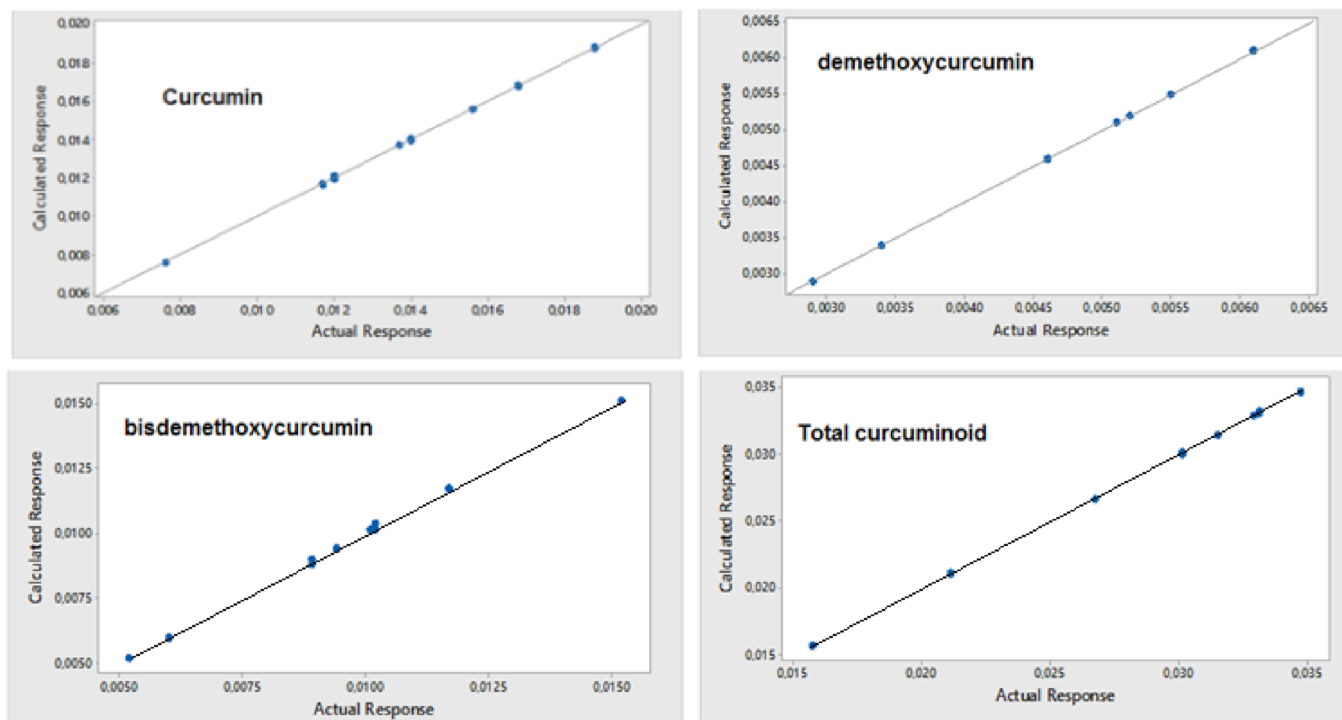

Fig. 4: PLS calibration model for the relationship between actual values of curcumin, demethoxycurcumin, bisdemethoxycurcumin and total curcuminoid with FTIR predicted/calculated values using specific wavenumbers.

CONCLUSION

FTIR spectroscopy combined with PLS calibration model is an alternative technique for HPLC due to the capability of FTIR spectroscopy-PLS to offer the close relationship between actual values as determined by HPLC and FTIR predicted values with low errors. CUR was determined at wavenumbers of the region of 1400-1720 cm^{-1}, while total curcuminoid was analyzed at 1407-1820 cm^{-1}. The R^2 value for the correlation between actual values of a curcuminoid and FTIR-PLS was >0.98 indicating the accuracy of the method with low errors. FTIR spectroscopy is fast, not destructive and no excessive sample preparation.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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