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Application of H-NMR metabolite fingerprinting and chemometrics for the authentication of *Curcuma longa* adulterated with *Curcuma manga*

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ABSTRACT

Curcuma longa or turmeric is known to have many functions in the field of foodstuff, cosmetics, and traditional medicines. The rhizome contains curcuminoid, especially curcumin which is believed to be the active compound responsible for pharmacological activities. The authenticity of *C. longa* is very important to avoid adulteration practices. The objective of this study was to develop thin layer chromatography (TLC) for curcumin analysis combined with H-NMR metabolite fingerprinting and multivariate analysis for the authentication of *C. longa* powder. Determination of curcumin in pure and adulterated powder of *C. longa* with *Curcuma manga* was performed using validated TLC method with the composition of the mobile phase of toluene: glacial acetic acid (82:18 v/v) and silica gel 60 F_{254} as a stationary phase with elution length 9 cm. The contents of curcumin in pure powder from several regions were in the range of 4.28-5.62%, while those in adulterated were of 5.25-1.35%. H-NMR metabolite fingerprinting combined with chemometrics of principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) using variables of the chemical shift was successfully used to classify the pure and adulterated powder of *C. longa*.

INTRODUCTION

Curcuma longa L. or turmeric is one of the herbaceous plants which is widely cultivated in tropical regions especially in India and Southeast Asia. This rhizome has been known to have many functions in foodstuff, cosmetics, and traditional medicine. Numerous pharmacological activities of the rhizome have been reported such as antioxidant, anti-inflammatory, antibacterial, hepatoprotective, cardioprotective, and anticancer activity (Mohanty *et al*., 2004; Cousins *et al*., 2007). The rhizome has the rich contents in curcuminoid, especially curcumin which is believed to be the main active component. Curcumin content is very important because it was used as one of the parameters in quality control of *C. longa* (Cheng *et al*., 2010). High-quality powder of *C. longa* will provide high-quality products. Because there are various products developed from turmeric and its demand is increasing, it is very important to ensure the authenticity of turmeric to avoid adulteration (Jurenka, 2009). Adulteration is a common practice in traditional medicine products. Adulteration poses a serious problem because it is related to the efficacy, safety, and quality of products. Turmeric is potential to be adulterated with other *Curcuma* species which are easy to obtain and have lower price such as *Curcuma manga* (Remya *et al*., 2004). Adulteration would affect curcumin content in the mixture because *C. manga* just has very low of curcumin. Therefore, it is very important to develop a fast and reliable method to detect the adulteration (Marikkar *et al*., 2001).

Several methods have been developed for authentication of turmeric powder such as thin layer chromatography (Pothitirat and Gritsanapan, 2005), high performance thin layer chromatography (Ashraf *et al*., 2012), high performance liquid chromatography (Jayaprakasha *et al*., 2002), and Fourier

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transform infrared spectroscopy (Rohman *et al*., 2015). Thin layer chromatography (TLC) has been developed for a long time for curcumin analysis in *Curcuma* species, especially *C. longa* powder (Paramasivam *et al*., 2009). However, the optimum TLC condition to obtain better resolution of curcumin separation and a good peak shape with minimum tailing still need to be explored. TLC offers many advantages for curcumin analysis, such as simple in preparation, use only small volume of organic solvent, can be used for qualitative and semiquantitative analysis, capable of analysis of several samples simultaneously, and required less time and cost (Phattanawasin *et al*., 2009). We developed a new method using mobile phase composition of toluene: glacial acetic acid (82:18 v/v) to obtain better resolution and a good of peak shape. Therefore, we performed method validation to prove our proposed TLC method.

H-NMR spectroscopy appears as the sophisticated method in metabolite fingerprinting for plant natural products (van der Kooy *et al*., 2009). It offers some advantages over other analytical techniques because of its fast analysis, simple in sample preparation, high reproducibility, and it can simultaneously detect the diverse group of primary and secondary plant metabolites (Dai *et al*., 2010). Chemometrics of multivariate analysis has a great capability to analyze the huge data generated from H-NMR measurement (Kim *et al*., 2011). The application of H-NMR spectroscopy and multivariate analysis has been successfully applied to determine the species or origin of dandelion (Jung *et al*., 2011), Italian sweet cherries (Longobardi *et al*., 2013), and American Ilex (Kim *et al*., 2010). In addition, H-NMR spectroscopy and multivariate analysis have been successfully applied to differentiate between the pure and adulterated saffron (Petrakis *et al*., 2015). However, using literature searching, there is no metabolite fingerprinting study has been conducted to detect the adulteration of C. longa powdered rhizome with C. manga using H-NMR spectroscopy method.

Rafi *et al.* (2011) have differentiated *C. longa*, *C. xanthorrhiza*, and *Zingiber cassumunar* using TLC fingerprint analysis. However, they just performed qualitative TLC analysis by inspecting the resulted TLC spot of each species. In this study, we developed TLC as a new method for the authentication of *C. longa* by quantitatively determining curcumin content in both pure and adulterated *C. longa* with *C. manga* in various concentrations. Gad and Bouzabata (2017) have also developed H-NMR spectroscopy and chemometrics for quality control of turmeric. However, the model for the authentication of *C. longa* adulterated with *C. manga* has not been developed. In this study, the use of H-NMR spectroscopy-based metabolite fingerprinting combined with chemometrics of principal component analysis (PCA) and a more powerful technique, orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were applied to differentiate between pure and adulterated powder of *C. longa* with *C. manga.*

MATERIALS AND METHOD

Rhizome sample collection and preparation

Rhizome samples of *C. longa* were collected from several regions in Yogyakarta and Central Java, while the rhizomes of *C. manga* were collected from Yogyakarta. Rhizomes were cleaned, chopped into small pieces, and air-dried. The dried rhizomes were ground into fine powder. The powder was used for analysis. The adulterated *C. longa* samples were prepared by adding *C. manga* in various proportions (5, 10, 25, 40, 50, and 75% wt/wt).

Standard and sample solution preparation

Curcumin standard solution was prepared by dissolving 10 mg of curcumin standard in 10 mL of methanol (1 mg/mL) as the stock solution.

For sample preparation, A-50 mg of pure and adulterated powder of *C. longa* was weighed accurately and put into 2 mL microtube. The powder was added with 1.5 mL methanol and then vortexed for 5 minutes. Subsequently, the samples were centrifuged for 5 minutes at 4000 rpm. The supernatant was used as the test solution.

TLC instrumentations and conditions

The standard and samples were spotted in the form of bands of width 5 mm with a Camag microliter syringe using a Camag Linomat V (CAMAG, Muttenz, Switzerland). The stationary phase used was precoated silica gel aluminum plate $60F_{254}$ (20 cm × 20 cm with 0.2 mm thickness; E.Merck, Darmstadt, Germany). The application rate was 150 nL/s and the distance between two bands was 15 mm. The mobile phase used was toluene: glacial acetic acid in a ratio 88:18 v/v. Plates were developed in ascending order in the chamber which was pre-saturated for 2 hours with the mobile phase. The elution length was 9 cm and the TLC was performed under temperature 25 ± 2°C and RH 60 ± 5%. After developing, the plate was dried and densitometric analysis was carried out at 427 nm using a Camag TLC scanner IV operated by WinCATS software.

Method validation of thin layer chromatography

Validation of TLC method was evaluated by assessing several performance characteristics namely specificity, linearity, accuracy, precision and sensitivity according to International Conference on Harmonization (ICH, 2005).

Specificity

The solutions of curcumin standard and sample were made and then spotted and eluated. The spectrum of standard and sample were observed. The spectrum and Rf (retardation factor) value of curcumin standard and curcumin in the sample must be identical.

Linearity

A 50 ppm of curcumin standard solution was prepared from the stock solution. This solution was spotted in a different volume (3, 4, 5, 6, and 7 µL) to obtain the concentrations of 150, 200, 250, 300, and 350 ng/spot of curcumin, respectively. The data of concentration versus peak area was observed. Linearity was evaluated by correlation coefficient (R) value. Linearity with R-value ≥0.997 was acceptable for analysis.

Accuracy

Accuracy was performed using standard addition method. During accuracy analysis, 120 ng, 150 ng, and 180 ng of curcumin standard were added to the samples as the low, medium, and high-level concentration, respectively. The amounts of curcumin added were

prepared from 60 ppm, 75 ppm, and 90 ppm of curcumin standard. The assay was performed in three replicates and recoveries value (%) of standard found in each concentration level were determined.

Precision

The intraday and interday precision were determined. Intraday precision was measured using samples in six replicates. The interday precision was performed by repeating the intraday assay in three different days. Precision was expressed as the percentage of coefficient variation (% CV).

Sensitivity

Sensitivity was evaluated by measuring limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated based on the following equations (LOD = 3.3σ/slope of curcumin calibration curve; LOQ = 10σ/slope of curcumin calibration curve). The LOD and LOQ found were performed using curcumin standard.

Assay of curcumin content

The sample test solutions were spotted on TLC plate, eluated, and detected. The percentage of curcumin content in each sample was determined by measuring the area under the curve (AUC) of each sample.

Sample preparation for H-NMR measurement

A-25 mg of pure and adulterated powder of *C. longa* was weighed and put into 2 mL centrifuge tube. The powder was added with 0.5 mL methanol D-4 (CD_3OD) and 0.5 mL KH_2PO_4 buffer pH 6.0 in D_2O contained TSP (trimethylsilyl propionic acid) 0.01%. The pH was adjusted to 6.0 using NaOD 0.1 M. The mixture was vortexed for 1 min, ultrasonicated for 20 min, centrifuged at 13500 rpm for 10 min, and then the supernatants (800 µL) were transferred into NMR tubes.

H-NMR measurement and multivariate analysis

The H-NMR spectra were recorded on a 500 MHz Jeol ECZ-R spectrometer. Each H-NMR spectrum acquired with the field strength of 11.74736 T (500 MHz), X_Offset 5.0 ppm, relaxation delay 5 s and 128 scans. The spectra were automatically and manually phase corrected. Baseline corrected was carried out using polynomial fit. The spectra were binned (bucketing) of equal width of 0.04 ppm in the range of 0.00-10.00 ppm excluding the regions of residual methanol (3.30-3.34 ppm) and water (4.71-5.10 ppm) using MestreNova 12.0.0.

Data analysis

Multivariate analysis was performed using PCA and OPLS-DA with Minitab 16 and SIMCA 14 software.

RESULTS AND DISCUSSION

Method validation

TLC used for quantitative analysis of curcumin was validated by determining specificity, linearity, accuracy, precision, and sensitivity. Specificity was measured by comparing the curcumin standard and samples for its Rf and UV spectra. A good resolved single spot of curcumin was observed at Rf value of 0.47 ± 0.3, either in standard or in evaluated samples (Figure 1). The densitogram of curcumin standard and curcumin in *C. longa* appeared at similar Rf value and a clear separation of curcumin from another component (demethoxycurcumin) in *C. longa* sample was obtained.

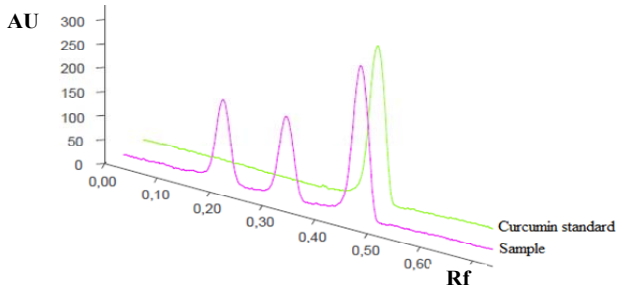

Fig. 1: The spectra of curcumin standard and curcumin in *Curcuma longa* sample.

Linearity was observed in the concentration range of 150-350 ng of curcumin standard. The calibration curve was linear with the regression equation of $y = 26.815x + 1954.4$, and the determination and correlation coefficient were 0.097 and 0.9985, respectively (Figure 2). The developed method showed a linear calibration curve. According to Chan *et al.* (2004), the models with a determination coefficient (R^2) ≥ 0.997 have a good linearity. Accuracy is the closeness of agreement between an accepted reference value and the value found in the measurement (ICH, 2005). Accuracy is reported as percent recovery. The recoveries value were evaluated using standard addition method. Samples were added with curcumin standard in three different level, namely 120 ng, 150 ng, and 180 ng, respectively. The recoveries of standard added were determined. The recoveries found were in the range of 99.06-101.10% (Table 1). The acceptance criteria for recoveries assay using concentration of analyte 60 ppm, 75 ppm, and 90 ppm was 90-107% (Gonzalez and Herrador, 2007). Our results meet the requirement for recoveries acceptance criteria. Therefore, this method has a good accuracy.

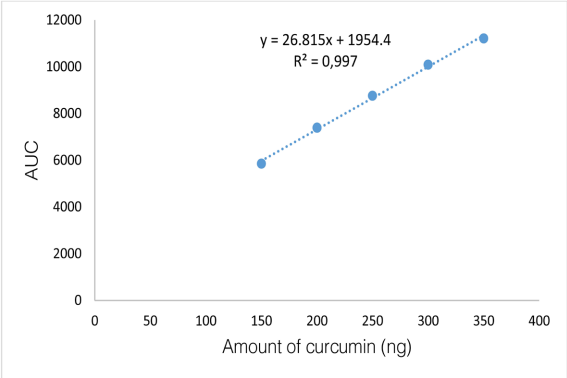

Fig. 2: Calibration curve of curcumin.

Table 1: A recovery study of curcumin using standard addition method.

Amount of curcumin added (ng)	Amount of curcumin found (ng)	Recovery (%)	Mean recovery (%)	RSD (%)
120	120.6116	100.51	100.01	0.88
120	118.7917	98.99		
120	120.6340	100.53		
150	151.9411	101.29	101.10	0.27
150	151.1803	101.79		
150	151.8068	101.20		
180	181.2605	100.70	99.06	1.43
180	176.6735	98.15		
180	177.0166	98.34		

The intraday precision was measured using *C. longa* sample. The precision was expressed as %RSD. The RSD values of intraday precision were ≤2% (Table 2). The interday precision was carried as in the intraday precision in three different days. The %RSD of interday precision was ≤2% (Table 2). For the precision assay, the acceptance criteria according to Horwitz for precision assay with analyte content (x) of $1\% < x \leq 10\%$ was not more than 2.8% (Gonzalez and Herrador, 2007). Our results fulfilled the requirement for precision acceptance criteria. Therefore, this developed method has a good precision.

Sensitivity was observed by measuring limit of detection (LOD) and limit of quantification (LOQ) of the developed method. The LOD found was 16.46 ng and the LOQ found was 49.89 ng. These results showed that this method has a good sensitivity. The low LOD and LOQ value showed the developed method has a good sensitivity. Curcumin contents were varied among several regions because metabolites formation was affected by their environmental conditions. The differences of the environmental condition among the regions may affect synthesis of metabolites including curcumin (Booker *et al*., 2014).

Table 2: Intraday and interday precision.

Replication	Day 1		Day 2		Day 3	
	AUC	Amount (ng)	AUC	Amount (ng)	AUC	Amount (ng)
1	8133.2	230.4233	8024.4	226.3658	8045	227.1341
2	8002.4	225.5454	8185	232.355	8177.9	232.0902
3	7903.8	221.8684	7966.7	224.2141	8107	229.4462
4	8057.7	227.6077	8107.2	229.4537	7971.7	224.4005
5	7926.3	222.7074	8264.9	235.3347	8262.8	235.2564
6	8111.2	229.6028	8017.9	226.1234	8049.2	227.2907
Mean (ng)		226.2925		228.9745		229.2697
SD		3.54		4.24		3.90
RSD (%)		1.57		1.85		1.70

The TLC validated method was used to measure the curcumin contents of *C. longa* powdered rhizome from several regions and also the curcumin contents in the series of adulterated *C. longa*. The curcumin found from several regions of *C. longa* was around 4.28%-5.62% (Figure 3). *C. longa* from Gunung Kidul was chosen in making series of adulterated samples because it has the highest curcumin content. The curcumin found in the adulterated powder of *C. longa* with *C. manga* was around 5.25-1.35% (Figure 4). Curcumin content decreased as the adulterants concentration increased. Based on the previous report, the curcumin content in *C. manga* is very low, not more than 0.05% (Policegoudra *et al*., 2011), therefore, when they were mixed with pure *C. longa* powder, it will decrease the curcumin content in the mixtures. Therefore, the developed TLC method can be used for the quality control of *C. longa* to ensure its authenticity.

H-NMR metabolite fingerprinting and chemometrics of multivariate analysis

Metabolite fingerprinting can be understood as the chemical patterns resulted from analytical machine output which contains many information, for example, the H-NMR spectra resulted from the measurement. H-NMR metabolite fingerprinting provides about 100-1000 variables that will be very useful for further analysis (Verpoorte *et al*., 2007). Metabolite fingerprinting involved sorting dataset and then each sample was classified. Chemometrics of multivariate analysis is a statistical method that can manage the complex data generated from H-NMR spectra. It is very useful in metabolite fingerprinting analysis. The spectra of *C. longa* and *C. manga* showed a specific spectrum for each sample (Figure 5a and 5b). There are some differences in certain chemical shift regions. These differences were caused by their metabolite contents which were different for each *Curcuma* species. *C. longa* has more signals with higher intensities in the regions of 0.00-3.00 ppm, while *C. manga* just had a few signals with low intensities. In the regions of 5.50-10.00 ppm, *C. longa* also has more signals and higher intensities than *C. manga*, but there were no signals after the region of 8.00 ppm. *C. manga* still have signal in the regions of 8.50 ppm and 9.50 ppm. Regions of 0.30-3.00 ppm, 3.00-6.00 ppm, and 6.00-10.00 ppm were associated with essential oils/ fatty acid, carbohydrates, and aromatic compounds, respectively (Booker *et al*., 2014).

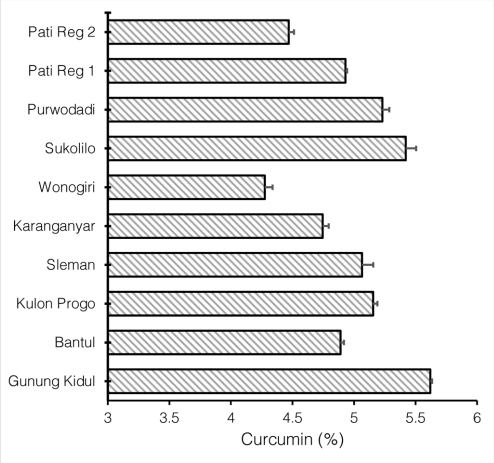

Fig. 3: Curcumin content in *Curcuma longa* from several regions.

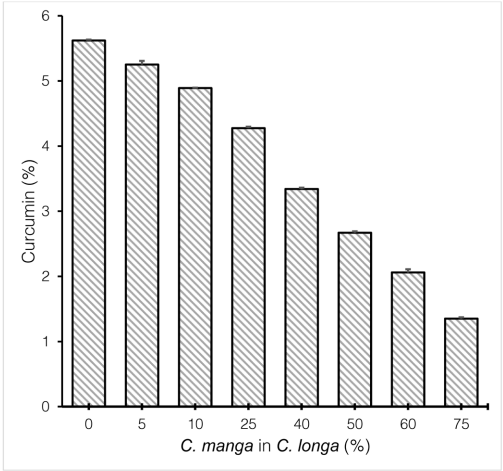

Fig. 4: Curcumin content in adulterated *Curcuma longa* with *Curcuma manga*.

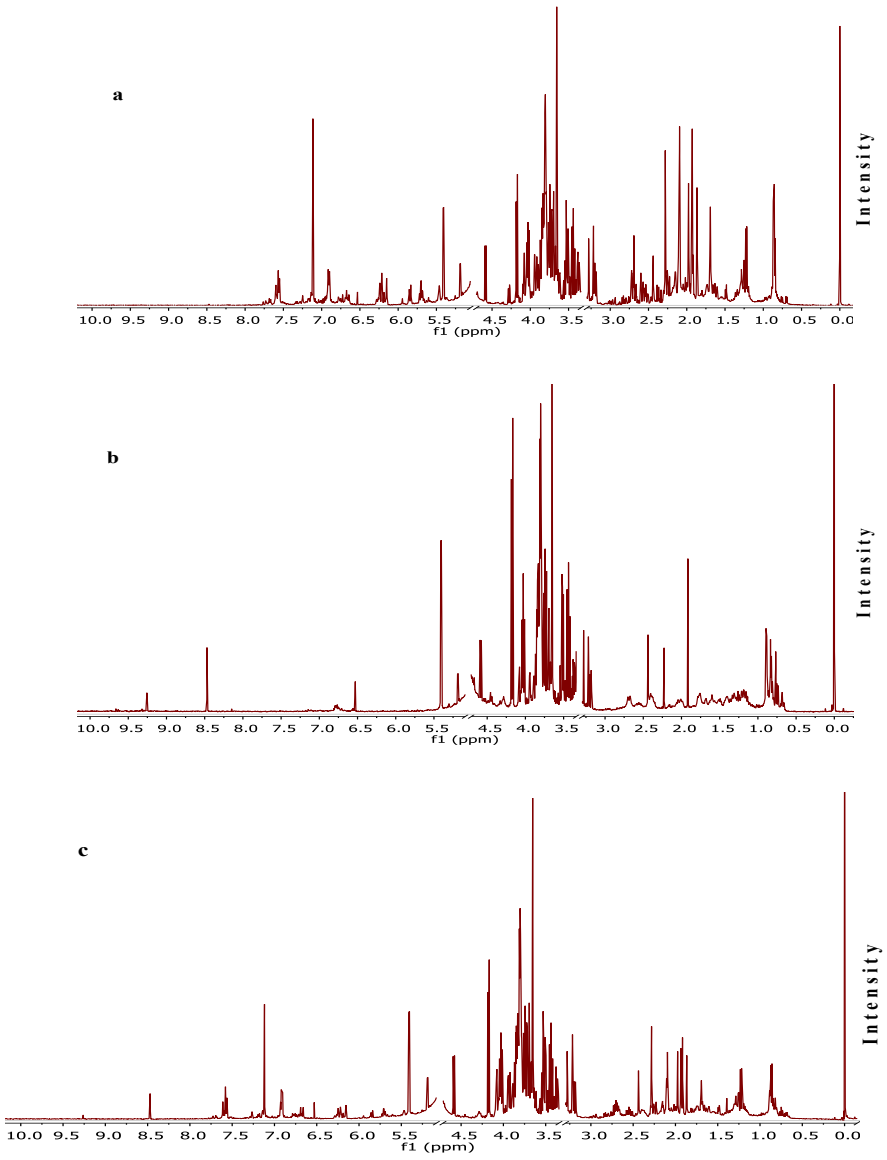

Fig. 5: H-NMR spectra of pure *Curcuma longa* (a), pure *Curcuma manga* (b), and adulterated *Curcuma longa* (50%) with *Curcuma manga* (c).

Curcuminoid is an example of metabolite contents in *Curcuma* species that will affect the profile of H-NMR spectra. Curcumin signals appeared in the regions of 7.28 ppm (singlet), 3.90 ppm (singlet), and 7.22 ppm (doublet), while demethoxycurcumin signals appeared in the regions of 5.89 ppm (singlet), 3.94 ppm (singlet), and 6.92 ppm (doublet) (Awin *et al*., 2016). Because *C. manga* just has very low contents of curcuminoid, the signals of curcuminoid appeared at lower intensities compared to *C. longa*. Therefore, it makes the spectra of *C. longa* had more signals with higher intensities in the regions of 6.00-8.00 ppm than in *C. manga*. The spectra of adulterated *C. longa* with 50% of *C. manga* showed a similar pattern with the spectra of pure *C. longa* (Figure 5c). It is difficult to distinguish by inspecting the spectra of pure and adulterated *C. longa* visually. Therefore, multivariate analysis is needed to resolve it.

PCA can be used to differentiate between pure *C. longa* and pure *C. manga.* The score plot of *C. longa* and *C. manga* appeared in the different area (Figure 6). Principal component analysis (PCA) is one of the multivariate analysis that can be used to classify and differentiate among a group of samples without any knowledge of their membership. Moreover, PCA also can be used to classify between pure and adulterated powder of *C. longa*. PCA was successfully distinguish between pure and adulterated powder of *C. longa* with *C. manga* in various proportions except in 5% of adulterant concentration (Figure 7). On the other hand, OPLS-DA is a supervised pattern recognition which allowed more powerful for classification compared to PCA. OPLS-DA allowed better separation of *C. longa* and adulterated *C. longa* with *C. manga.* All the series of adulterants were clearly separated from *C. longa* (Figure 8). OPLS-DA showed a good of fit (R2X = 0.912, R2Y = 0.795) and good predictivity (Q2 = 0.711). OPLS-DA was successfully distinguish between pure and adulterated *C. longa* with *C. manga* even in 5% of adulterant concentration (Figure 8). Validation of OPLS-DA by permutation test showed that the OPLS-DA model is robust and credible. Therefore, the combination of H-NMR spectroscopy and multivariate analysis method become a powerful method for the authentication of *C. longa*.

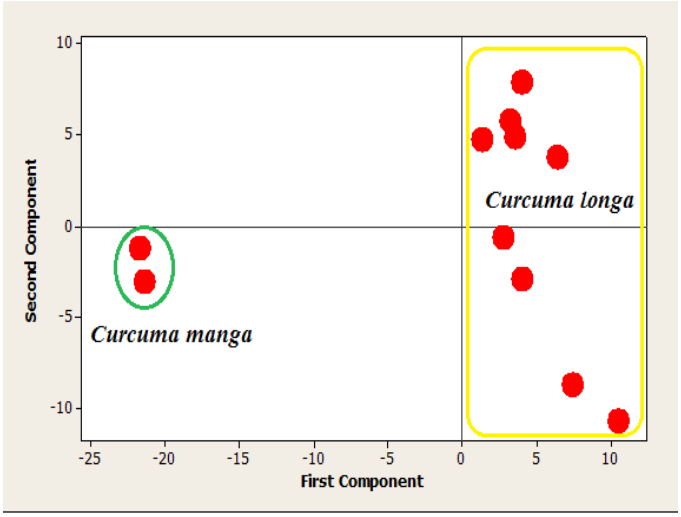

Fig. 6: PCA score plot of *Curcuma longa* and *Curcuma manga.*

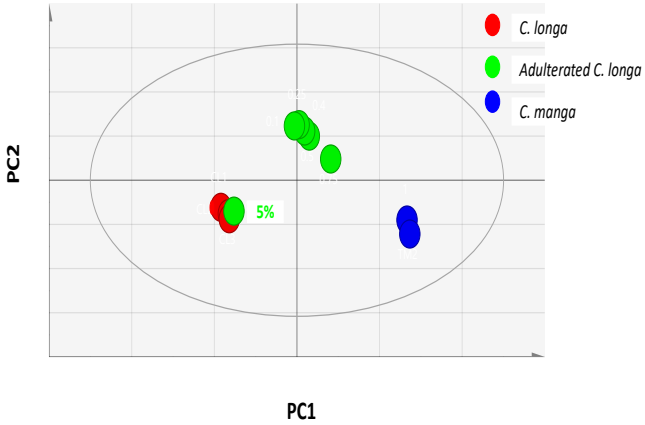

Fig. 7: PCA score plot of pure and adulterated *Curcuma longa* with *Curcuma manga*.

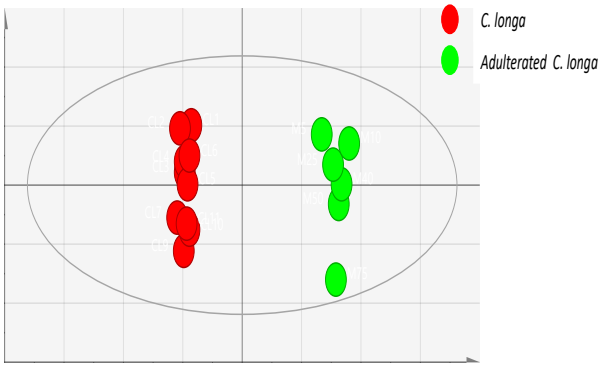

Fig. 8: OPLS-DA score plot of *Curcuma longa* from several regions and adulterated *Curcuma longa* with *Curcuma manga.*

CONCLUSION

In conclusion, the developed thin layer chromatography method for the estimation of curcumin content in pure and adulterated powder can be used for routine analysis of curcumin with good reproducibility. H-NMR spectroscopy method combined with chemometrics of PCA and OPLS-DA was a powerful method in metabolite fingerprinting and was confirmed successfully for classifying between pure and adulterated *Curcuma longa* powder with *Curcuma manga.*

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