The use of $^1$H-NMR spectroscopy and chemometrics of pattern recognition for authentication of *Curcuma xanthorrhiza* adulterated with *Zingiber montanum*

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**Abstract.** *Curcuma xanthorrhiza* is widely used in food and traditional medicine products. Due to its high demand, it is potential to be substituted or mixed with other species having similar appearance, therefore, rapid and reliable analytical method is highly required. The objective of this study was to develop $^1$H-NMR spectroscopy and chemometrics of pattern recognition as a metabolite fingerprinting technique for authentication of *C. xanthorrhiza* from *Zingiber montanum*. The powdered rhizomes were extracted using combination of methanol-D4 and phosphate buffer pH 6.0 in deuterium oxide (1:1 v/v) containing trimethylsilyl propionic acid (TSP) for chemical shift calibration. The variables extracted from $^1$H-NMR spectra were used for creating chemometrics models. Chemometrics of partial least square-discriminant analysis (PLS-DA) using 7 principal components (PCs) successfully classified between authentic and adulterated samples of *C. xanthorrhiza* with high value of R$^2_X$ (0.988), R$^2_Y$ (0.998), and Q$^2$ (0.993). Moreover, chemometrics of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) using 2 PCs and 4 orthogonal components perfectly discriminated authentic and adulterated samples of *C. xanthorrhiza*. The model showed high R$^2_X$ (0.965), R$^2_Y$ (0.976) as well as Q$^2$ (0.946) values. Validation using permutation test confirmed the validity both PLS-DA and OPLS-DA models. It suggested that combination of $^1$H-NMR and chemometrics method is promising for authentication of medicinal plants.

1. **Introduction**

*C. xanthorrhiza* is an herbaceous perennial plant belongs to Zingiberaceae family and it is widely cultivated in several countries in Asia especially in South East Asia. The rhizome is known to have several biological activities such as antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, and antibacterial activity. Some research has also explored regarding its anticancer activity [1-2]. The rhizome has been widely applied in several products including food, cosmetics as well as traditional medicine products [3]. Due to its high demand, the rhizome of *C. xanthorrhiza* especially in powder form is often adulterated with other rhizome which is widely available and having similar physical properties such as *Z. montanum* by unethical player to meet the demand [4]. Adulteration is a serious
problem because it is related to the quality, efficacy, and safety of the products. In a powder form, it is obviously difficult to differentiate between authentic and adulterated powder of C. xanthorrhiza [5]. The metabolite profile of genus Curcuma (C. mangga, C. zedoaria, and C. xanthorrhiza) and Zingiber (Z. officinale, Z. montanum, and Z. zerumbet) from three different locations has been determined using $^1$H-NMR spectroscopy [6]. However, it just measured the pure or individual samples, not in adulterated samples composed of the mixtures between different species. Therefore, the development of analytical method capable of detecting the presence of adulterant in binary mixtures with C. xanthorrhiza powdered rhizome is highly required for quality control.

Several analytical methods have been used for authentication of C. xanthorrhiza rhizome. Some of the methods focus on measuring the curcuminoid contents such as spectroscopy ultraviolet-visible [7], thin layer chromatography [6-7], ultra-performance liquid chromatography [10], capillary electrophoresis [11], and liquid chromatography tandem mass spectrometry [12]. However, these methods have disadvantages such as require long time, require high amount of solvent, and difficult in samples preparation. $^1$H-NMR spectroscopy is emerging as an analytical method in metabolite fingerprinting for authentication of medicinal plants [13]. It offers fast analysis, ease in sample preparation, highly robust and highly reproducible. Combined with chemometrics of multivariate analysis which can manage the complex data generated from $^1$H-NMR spectra, it becomes a powerful analytical technique for authentication [14]. Chemometrics of pattern recognition is aimed for classification and differentiation of samples. It has been widely used to analyze data generated from several instrumental methods including spectroscopy and chromatography for food authentication [14-15]. Therefore, the use of chemometrics pattern recognition is promising and potential to be applied for authentication of medicinal plants.

$^1$H-NMR spectroscopy and pattern recognition chemometrics of principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) have been successfully used for detection of C. heyneneana in C. longa powdered rhizome [17]. Adulteration of high quality Saffron has also been successfully detected using combination of $^1$H-NMR spectroscopy and chemometrics of OPLS-DA and bidirectional OPLS-DA [18]. To the best of our knowledge, there is no report regarding the use of $^1$H-NMR spectroscopy method in combination with chemometrics of pattern recognition for authentication of C. xanthorrhiza adulterated with Z. montanum. This research was aimed to develop $^1$H-NMR spectroscopy combined with chemometrics of pattern recognition for authentication of C. xanthorrhiza.

2. Material and Methods

2.1. Materials
Rhizomes of C. xanthorrhiza and Z. montanum were obtained from Yogyakarta, Indonesia. The solvents (methanol-D4, deuterium oxide) and chemicals (KH$_2$PO$_4$, Trimethylsilyl propionic acid/TSP) were purchased from Merck.

2.2. Sample preparation
Sample preparation was carried out according to Kim et al. [13] with slight modifications. Rhizomes were cleaned and chopped into small sizes approximately of 5 cm in length. Subsequently, rhizomes were dried and ground into powder. The series of adulterated samples of C. xanthorrhiza in binary mixtures with Z. montanum were prepared using several adulterant concentrations ranged from 0-100 % w/w. Amount of 25.0 mg samples were accurately weighed using analytical balance and placed into 2 mL microtube. Samples were extracted using NMR solvents consist of methanol-D4 and phosphate buffer pH 6.0 in deuterium oxide using ratio of 1:1 v/v. Methanol-D4 was chosen because it has an ability to extract wide range of metabolites. Whereas phosphate buffer was used to maintain the pH of metabolites in order to avoid significant shift in their chemical shift. Trimethylsilyl propionic acid (TSP) was added for chemical shift calibration. The mixtures were vortexed, ultrasonicated for 15 min and
centrifuged for 10 min at 13500 rpm. Supernatant was taken and transferred to NMR tube for NMR measurement.

2.3. $^1$H-NMR spectra measurement
The $^1$H-NMR spectra acquisition was carried out using JEOL ECZ-R 500 MHz NMR spectrometer. The samples were measured using field strength of 11.74 T with X_offset of 5.0 ppm and 5s of relaxation delay. Spectra were recorded in the range of 0-12 ppm consisting of 128 scans. Each sample was measured in three replicates.

2.4. Data analysis
Data pre-processing was performed prior to chemometrics analysis. $^1$H-NMR spectra were phase-corrected, then baseline correction was carried out using polynomial fit mode. To obtain data variables for multivariate analysis, binning of the spectra was performed in the chemical shift of 0.50-10.00 ppm for every 0.04 ppm. The binned data were subjected for chemometrics analysis of partial least square-discriminant analysis (PLS-DA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA).

3. Results and Discussion
The use of NMR spectroscopy for metabolite fingerprinting and metabolite profiling is getting increased in the past few years. $^1$H-NMR spectroscopy could be used as fingerprint method to differentiate samples because the spectra could be unique for each sample meaning that different sample will have different $^1$H-NMR spectra. The main factor contributed to the spectra differences is the difference in metabolite contents in every sample. $^1$H-NMR spectra of C. xanthorrhiza differs from the spectra of Z. montanum indicating they have different content in metabolite compounds. The secondary metabolites of C. xanthorrhiza are xanthorhizol, curcumin, demethoxycurcumin, phelandren, sucrose, borneol, sesquiterpenes, and flavonoids [3] while the secondary metabolites of Z. montanum are cassumunarins A, B, C, terpinen-4-ol, alpha and beta pinene, sabinene, myrcene, terpinene, and limonene [19]. The curcumin and demethoxycurcumin are the active compounds in C. xanthorrhiza which appeared at the chemical shift of 7.28, 7.17, 6.77, and 3.66 ppm for curcumin and 6.93, 5.88, and 3.66 for demethoxycurcumin [17]. Generally, $^1$H-NMR spectra of plant metabolites are divided into three categories namely amino acid and organic acid region (0.10-3.00 ppm), carbohydrate region (3.00-5.00 ppm), and aromatic region (6.00-8.00 ppm) [13]. The differences between C. xanthorrhiza and Z. montanum observed mainly in the region of amino acid and organic acid region (0.10-3.00 ppm) which the signals in C. xanthorrhiza are more complex with high intensities (Figure 1). It is presumed that the concentration of amino acid and organic acid in Z. montanum is low. A sharp signal appeared in the chemical shift of 5.45 ppm which is presumed to be the signal from sucrose [17] could be used as marker signal for C. xanthorrhiza because it is specific for C. xanthorrhiza. Eventhough the spectra of pure C. xanthorrhiza and pure Z. montanum could be clearly differentiated, however, in adulterated samples of C. xanthorrhiza, the spectra of authentic and adulterated samples were similar each other especially in the adulterant (Z. montanum) concentration of 10-40%. As a consequence, it is difficult to state whether the sample is authentic or adulterated if there is no information regarding the samples. Therefore, the use of advanced statistical analysis such as chemometrics of pattern recognition PLS-DA and OPLS-DA is highly required.
Chemometrics of pattern recognition is divided into unsupervised and supervised pattern recognition. Supervised pattern recognition such as PLS-DA and OPLS-DA has the potential for classification because of their high order in algorithm [20]. PLS-DA using 7 principal components has been successfully used to differentiate between authentic and adulterated powder of *C. xanthorrhiza* with *Z. montanum*. All samples containing *Z. montanum* were correctly classified as adulterated samples and clearly separated from authentic sample of *C. xanthorrhiza* as can be seen in PLS-DA score plot (Figure 2A). The PLS-DA model showed high value of R2X (0.988), R2Y (0.9980) and Q2 (0.993). The high values of R2X and R2Y (close to 1) indicated good of fitness of the model, whereas the high Q2 value (> 0.50) showed the good predictivity of the model. Another supervised pattern recognition method namely OPLS-DA using 2 principal components and 4 orthogonal component variables were also successfully applied for classification between authentic and adulterated samples of *C. xanthorrhiza*. The model has good of fitness indicated by the obtained value of R2X (0.965) and R2Y (0.976) and also good of predictivity demonstrated by its high Q2 (0.946). OPLS-DA showed better classification compared to PLS-DA model as shown in figure 2B. This might be caused by the different algorithm used in OPLS-DA. The OPLS-DA model used latent variables both in X and Y matrix, therefore resulting better separation than in PLS-DA which used latent variables only in Y matrix.
Figure 2. PLS-DA (A) and OPLS-DA (B) model of *C. xanthorrhiza* and adulterated *C. xanthorrhiza* with *Z. montanum*.

The PLS-DA and OPLS-DA models were validated using permutation test to confirm the validity of the developed models. Validation is required to avoid model overpredicting which tend to bias result. Permutation test is the common validation technique used for PLS-DA and OPLS-DA models which compared the value between original and permuted of R2 and Q2. Good permutation test is obtained when the original value of R2 and Q2 (in the right side) have the highest value compare to the all permuted R2 and Q2 (in the left side) as shown in Figure 3. Moreover, the intersection value of R2 and Q2 must between zero or less than zero. Result showed that the original value of R2 and Q2 has the highest value than the permuted ones both in PLS-DA and OPLS-DA models. Moreover, the obtained intersection of R2 and Q2 in PLS-DA model was (0.0, 0.24) and (0.0, -1.17) whereas the intersection of R2 and Q2 in OPLS-DA model was (0.0, 0.196) and (0.0, -0.871), respectively indicating the good validation model.

Figure 3. Permutation test of PLS-DA (A) and OPLS-DA (B) models of *C. xanthorrhiza* and adulterated *C. xanthorrhiza* with *Z. montanum*.
4. Conclusion

$^1$H-NMR spectroscopy provided fingerprint spectra which is very useful to differentiate between authentic and adulterated samples of *C. xanthorrhiza* powdered rhizome. Chemometrics of pattern recognition namely PLS-DA and OPLS-DA was successfully classified between authentic and adulterated samples of *C. xanthorrhiza* with *Z. montanum*. It can be concluded that $^1$H-NMR spectroscopy combined with chemometrics of pattern recognition is potential and promising to be used for authentication of medicinal plants.

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