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Determination of Total Phenolic Content and Classification Model of Local Variety Soursop (*Annona muricata* L.) Leaf Powder in Different Altitudes Using NIR and FTIR Spectroscopy coupled with Chemometrics

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ABSTRACT

Soursop (*Annona muricata* L.) leaves have been widely used traditionally to overcome health problems, this is related to its total phenolic content. This study was aimed to determine the classification model and total phenolic content of soursop leaf powder of local varieties, in different altitudes using Near Infrared (NIR) and Fourier-transform Infrared(FTIR) spectroscopy with chemometrics. Local variety soursop leaf samples that have been collected from low land (0-200 meter above sea level (masl)), medium land (201-700 masl) and high land (>700 masl) were prepared and then scanned using NIR and FTIR spectroscophotometers. Furthermore, the NIR spectra data from the samples were used as predictors on the LDA classification model of local and queen varieties soursop leaves to identify the sample varieties. Samples identified as local varieties, whose total phenolic content was determined using a comparative method (UV-Vis spectrophotometry). The highest mean of total phenolic content was observed in samples from medium land (Jember) of 5.72% w/w Gallic Acid Equivalent (GAE), followed by low land (Bangkalan) 2.95% w/w GAE and high land (Batu) 1.78% w/w GAE. NIR and FTIR spectra data belonging to the samples were analyzed by chemometrics qualitatively using LDA, SVM and SIMCA, and quantitatively using PLS, PCR and SVR. The best classification and calibration model are formed from the NIR spectra data, that are the LDA model with an accuracy of 100% and the PLS model with an R-square calibration value of 0.998071 and RMSEC of 1.2735631. The LDA and PLS models are applied to the real samples. The results of the sample's total phenolic content determination obtained from the NIR spectroscopy method and UV-Vis spectrophotometry method were then tested with Paired-Samples T Test and it can be concluded that the content obtained from the two methods did not have a significant different.

Keywords:*Annona muricata* L., total phenolic Content, NIR, FTIR, chemometric

INTRODUCTION

There are around 2300-2500 species in the Annonaceae family, and soursop (*Annonamuricata* L.) is one of the ethno-medically important species of this family (Kedari and Khan, 2014). An *in vitro* tests proved that the antioxidant activity of soursop leaves are stronger than those of *Annonasquamosa* and *Annonareticulata*(Baskar, *et al*., 2006). In Indonesia, soursop leaves are widely used by people for traditional medicine (HHS, 2012). The previous research conducted by Adewole and Ojewole (2009) showed that soursop leaves have antioxidant activity that can inhibit oxidative stress. That antioxidant activity is related to the highest phytochemical content in soursop leaves, namely phenolic compounds(Ibrahim and

Abdullahi, 2015). There are several factors that affected the phenolic content of a plant, such as variety and altitude (Ginting *et al.,* 2005). In Indonesia, two types of soursop varieties are grown, namely local (slightly sweet fruit sour taste) and queen (sweet fruit). The local and queen can only be identified based on the taste of the fruit, and cannot be identified based on their morphology (Sudjijo, 2014). The previous research conducted by Dewi(2018) and Juniarta (2018) has succeeded in identifying two varieties through its total phenolic content, that local soursop leaves contain a higher total phenolic content than queen soursop leaves. This identification has facilitated through the LDA classification model. Soursop that grows at different altitudes is also difficult to identify if only seen from its morphological form, and research to determine the total phenol content of soursop leaf powder that grows based on its altitude has never been done. Considering the uneven contour of Indonesian land, which are divided into low lands (0-200 masl), medium land (201-700 masl), and High lands (>700 masl) (Rukmana, 2002), this study was conducted to determine the total phenolic content and classification model of soursop leaf powder of local varieties at different altitudes using Near Infrared (NIR) spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy with chemometrics. Qualitative analysis using the classification method chemometrics (LDA, SVM, and SIMCA), while quantitative analysis using the regression method chemometrics (PLS, PCR, and SVR). Infrared spectra combined with the chemometrics method can be used to determine a plant from other plants(Sun, *et al.,* 2010).On the previous study conducted by the author, had succeeded classify medicinal plant extract using NIR spectroscopy coupled with LDA, SVM and SIMCA chemometric models on correct category (100% accuracy) (Wulandari, *et al.,* 2016).This study was aimed to gain the best calibration and classification models that can determine total phenolic content and classify local soursop powder at different altitudes.

MATERIAL AND METHODS

The materials used were soursop leaves of local varieties, gallic acid standard (Sigma), Folin-Ciocalteu reagent (Merck), Na_2CO_3, methanol 98%, and distilled water.

Preparation ofsoursop leaf powder

The training set and the test set sample of local variety soursop leaf were taken from the Bangkalan District (low land), Jember district (medium land), and the Batu city (high land). The real sample was taken from the Situbondo district (low land), Lumajang district (medium land), and Bondowoso district (high land). Google Earth Altimeter was used to determine sampling points. The samples were dried at room temperature (omitted from direct sunlight) and were grinded to form a powder followed by sieving with a 100 mesh sieve(Depkes RI, 2008). The gravimetric method was used to determine the water content of powder samples, in which the samples were resticted to <10% the moisture content. The moisture content were calculated as followed,

$$\text{Moisture content (\%)} = \frac{A - B}{A} \quad X\ 100\%$$

Where A is the weight of the initial sample (g) and B is the sample weight after drying (g).

AnalysisusingNIRandFTIRspectrophotometer

All samples and gallic acid standard were scanned by NIR spectrophotometer with five replications per replication of three shots for each sample and FTIR spectrophotometer with three replications for each sample(Lukman, *et al.,* 2016; Rahmawati, *et al.,* 2015), so that the NIR and FTIR spectra data are obtained. Through BRIMROSE (NIR) and OPUS (FTIR) software, each spectra data was codenamed.Gallic spectra data used as a comparison of the total phenolic content in the formation of a calibration model.

Identification of samples variety

Samples variety were predicted by using spectra data samples from NIR scanning as predictors on the LDA classification model of local and queen soursop (Dewi, 2018) in The Unscrambler X 10.2 software. Samples that had been identified as soursop leaf powder of local varieties can be used for further research process.

Determination of total phenolic content by UV-Vis spectrophotometry method

The overall sample of leaf powder was weighed 25mg each and replicated three times in each sample, and was then dissolved in methanol 98% to 10mL followed by 2 fold dilution. The standard gallic acid was weighed as much as 12.5mg and dissolved methanol to 25mL, so a gallic acid solution was formed with a concentration of 500ppm. Then, the solution was diluted into a series of concentrations of 5.12ppm, 10.24ppm, 15.36ppm, 20.48ppm, 40.96ppm, 51.20ppm, 81.92ppm, and 102.4ppm.

Furthermore, the maximum wavelength and operational time optimization were carried out. The gallic acid standard solution was piped 100µL from each series of concentrations and was added with 500µL Folin-Ciocalteu (1:10v/v water), this solution was then incubate for 6min, was then added with 400µL Na_2CO_3 (7.5%w/vwater) (Keskin-Šašić*etal.,* 2012) and was incubated for 90min of operational time (optimization results). The absorbance of each mixture was measured using a wavelength of 759nm (optimization results). The absorbance data and the concentration of gallic acid standard solution were processed into the standard curve of gallic acid to form regression equation of the standard gallic acid solution.

The sample solution was piped 100µL then followed by 500µL Folin-Ciocalteu (1:10 v/v water) addition prior incubation for 6min, 400µL Na_2CO_3 (7.5%w/v water)was added(Keskin-Šašić *et al.,* 2012) and was left for 100min operational time for samples from the high lands and 110min for samples from the low and medium land (optimization results). Absorbance was measured at 759nm (optimization results) using UV-Vis spectrophotometer (Hitachi U-1800). Absorbance value was proceeded in the regression equation gallic acid standard to generate total phenolic content as percentage of w/w equivalent gallic acid (% w/w GAE).

Determination and validation of the classification model

Samples were scanned using NIR spectrophotometer (Brimrose Corporation Luminar 3070) and FTIR spectrophotometer (Bruker Alpha). The NIR and FTIR spectra data of training set samples were processed using the Unscrambler X 10.2 software. Determination of the classification model was carried out by the LDA, SVM, and SIMCA methods. LDA, SVM, and SIMCA were supervised pattern recognition. This due to the analyst controlled the sample classification in wich the samples were previously group by the analyst and referred as the training set to facilitate the process of determining the classification (Enderle and Weih, 2005; Nurul, 2016).The best classification model indicated by 100% accuracy, which shows that the original prediction of the model was in accordance with the actual classification. The best classification model selected was then validated by Leave-One-Out-Cross-Validation (LOOCV) and 2-Fold-Cross-Validation (2FCV). LOOCV was done by taking a sample data set from the training set where the data was used as a validation set, while the remaining data was used to form a new model. 2FCV done by using the absorbance of the sample test set as a predictor on the selected model(Wulandari,*et al.,* 2016).

Determination and validation of the calibration model

The NIR and FTIR spectra data of the training set samples were analyzed quantitatively using PLS, PCR and SVR regression chemometrics through Unscrambler X 10.2 software. The absorbance value of the spectrum data wasused as a Y value (predictor) combined with the% w/w GAE value from a previously determined sample using the UV-Vis spectrophotometry method. The best calibration model was chosen based on the best predictive ability with the greater R-square value, the smaller the RMSE error value. The best calibration model selected was then validated by Leave-One-Out-Cross-Validation and 2-Fold-Cross-Validation(Lukman,*et al.,* 2016).

Selected and validated model applications on real samples

The selected and validated chemometric model applied to the samples of soursop leaf extract powder outside Bangkalan District, Jember District, and Batu City. Using spectrum data of real samples from Bondowoso District (high land), Lumajang District (medium land), and Situbondo District (low land) with absorbance as predictors for selected models for qualitative analysis (classification based on growing altitude) and quantitative (total phenolic content determination). The results of total phenolic content by UV-Vis spectroscopy method were used as a comparative data in determining total phenolic content(Wulandari *etal.,* 2016). In this study, samples were not used commercial samples, due to insufficient information regarding its altitude of the origin.

Data analysis

The results of the determination of the total phenolic content of real samples by the NIR method and UV-Vis spectrophotometry were compared through the Paired-Samples T-Test using SPSS 25 trial version. Data analysis was carried out at a confidence level of 99% with significance or Sig. (2-tailed) 0.005(Santoso, 2018; Wulandari *et al.*, 2016).

Table I. Samples identity, moisture content, and total phenolic content

Sample Group	Code name	Sampling Location*		Altitude (masl)*	Values are mean±SD of 3 replications	
		Districts	Sub- District		Moisture Content (%)	Total Phenolic Content (% w/w GAE) **
Training Set	BN1	Bangkalan	Arosbaya	7	4.41%±0.045	2.42±0.012
	BN2		TanjungBumi	30	4.31%±0.045	2.81±0.024
	BN3		Sepulu	28	3.81%±0.091	2.81±0.010
	JR1	Jember	Arjasa	232	4.91%±0.097	5.39±0.009
	JR2		Kalisat	274	4.32%±0.093	6.68±0.005
	JR3		Jelbuk	237	3.50%±0.057	5.89±0.009
	BU1	Batu	Batu	1019	4.39%±0.039	1.79±0.025
	BU2		Junrejo	747	4.90%±0.084	2.22±0.018
	BU3		Bumiaji	950	3.33%±0.051	1.61±0.016
Test Set	TS1	Bangkalan	Klampis	38	4.41%±0.036	3.75±0.026
	TS2	Jember	Batu	1021	4.31%±0.064	1.49±0.026
	TS3	Batu	Kalisat	206	3.81%±0.043	4.92±0.017
Real Sample	SN1	Bondowoso	Pakem	1123	4.91%±0.052	3.36±0.004
	SN2	Lumajang	Kedungjajang	202	4.32%±0.057	5.06±0.083
	SN3	Situbondo	Kapongan	23	3.50%±0352	3.63±0.003

*Google Earth Altimeter; ** Result of UV-Vis spectrophotometry method; masl: meter above sea level

RESULT AND DISCUSSION

Each of the entire sample were given with an identity (codename, sampling location, and altitude) and moisture content of <10% (Tabel I). Water content requirements of <10% were intended to eliminate water content interference in determination of the classification model and total phenolic content. According to (Agustina, *et al.,* 2015) the water content of the sample was inversely proportional to the reflectance in, which the higher the water content, the smaller the reflectance produced while the absorbance increased. All samples were identified as local varieties by the LDA soursop variety classification model. The results of NIR and FTIR spectra data (Figure 1) of all samples were used as predictors on the LDA soursop variety classification model. All samples have been identified as local varieties of soursop leaf powder.

Determination of total phenolic content by Uv-Vis spectrophotometry

The selected wavelength was 759nm because it produced the highest absorbance in the standard gallic acid. The operational time for the gallic acid standard was 90min, low and medium land samples are 110min, and 100min of high land samples. Previously, the operational time was determined so that different operational times for gallic acid and samples were obtained, in order to ensure that the total phenolic content determination was carried out accurately. Operational time was chosen when the absorbance of phenolic compounds from the sample was stable or there was no change, that was when the reaction between phenolic compounds in the sample and the Folin-Ciocalteu had run perfectly(Sari and Ayuchecaria, 2017). Based on the regression equation y=0.093x+0.058 obtained, the total phenolic content was then determined by using the results of absorbance measurements in the solution of soursop leaf powder samples which was previously reacted with Folin-Ciocalteu and Na_2CO_3 as values (x). The total phenolic content obtained was interpreted in percentage w/w equivalent of gallic acid (% w/w GAE) (Table I). The mean of total phenolic content of 5.72% w/w GAE belonging to the sample from Jember was the highest when compared to samples from Bangkalan (2.95% w/w GAE) and Batu (1.78% w/w GAE). This indicated samples from Jember had the highest potential sources of phenolic compounds.

Determination and validation of the classification model

The classification model of LDA, SVM, and SIMCA in this study used three types of categories, namely LOW, MEDIUM, and HIGH categories.

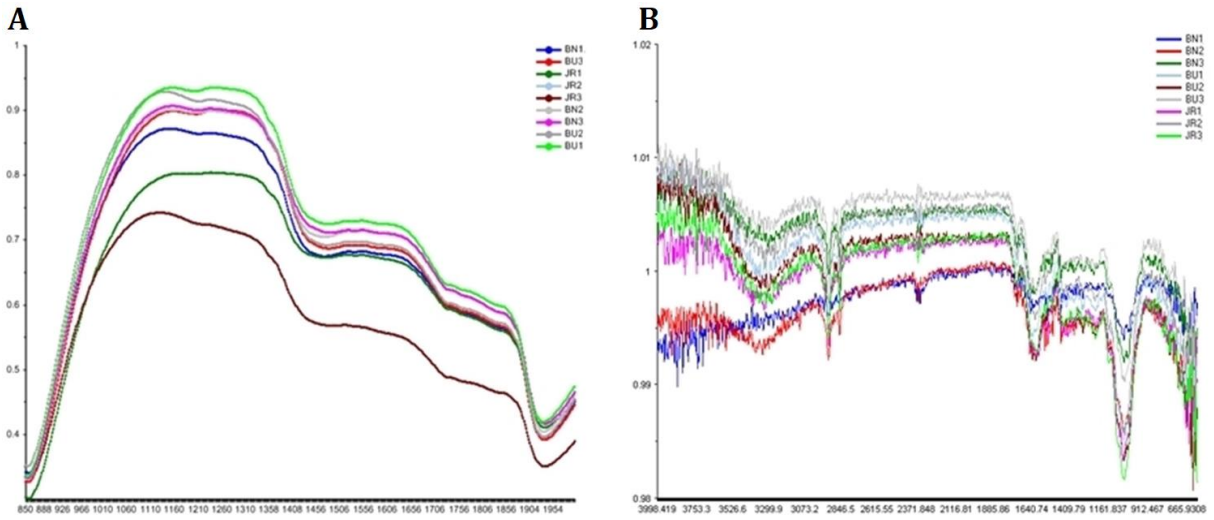

Figure 1. Spectra data A. NIR, B.FTIR of samples in different altitudes (BN1:7 masl; BN2:30 masl; BN3:28 masl; JR1:232 masl; JR2:274 masl; JR3:237 masl; BU1:1019 masl; BU2:747 masl; BU3:950 masl)

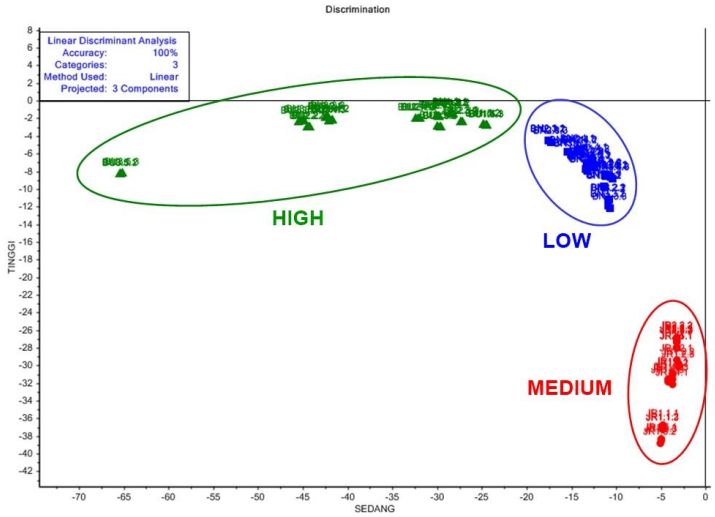

Figure 2. Mapping plot of the LDA model from NIR spectra

The three categories aimed to describe the classification of local varieties of soursop leaf powder based on the altitude of the growing area, which was LOW for samples from the low lands, MEDIUM for samples from the medium lands and HIGH for samples from the high lands (Figure 2). The accuracy of the LDA, SVM, and SIMCA classification models formed from NIR and FTIR spectra data (Table II). It can be seen that the best models were LDA and SVM which were formed from NIR, and also SIMCA spectra which are formed from FTIR spectra with 100% recognition ability. After cross-validation, the LDA was chosen as the best model because LOOCV and 2FCV results are 100%, while SVM and SIMCA when cross-validation were not 100% accurate.

Determination and validation of the calibration model

A total of 150 NIR and FTIR spectra data of the training set (samples and gallic acid standards) were analyzed quantitatively using the chemometrics of The Unscrambler X 10.2.The absorbance value of the spectrum data as a Y value (predictor) combined with % w/w GAE value of the predetermined sample with the UV-Vis

Table II. The classification ability of the classification model and the results of cross-validation of selected models

Model	NIR Classification Ability			FTIR Classification Ability		
	Accuracy (%)	Accuracy of Cross-Validation (%)		Accuracy (%)	Accuracy of Cross-Validation (%)	
		LOOCV	2FCV		LOOCV	2FCV
LDA	100	100	100	88.89	-	-
SVM	100	100	80	81.48	-	-
SIMCA	95.56	-	-	100	100	0

Table III. Results of R-square and RMSE value of the calibration model

Spectra	Model	R-square Calibration	R-square Validation	RMSEC	RMSECV
NIR	PLS	0.998	0.9979	1.2736	1.3133
	PCR	0.9969	0.9967	1.6022	1.6538
	SVR	0.9922	0.9920	3.1012	3.1094
FTIR	PLS	0.9981	0.9979	1.2736	1.3133
	PCR	0.9969	0.9967	1.6022	1.6538
	SVR	0.9922	0.9920	3.1012	3.1094

Table IV. The comparison of mean value of % w/w GAE results from the NIR spectroscopic method and the the UV-Vis spectrophotometry method

Real Samples	Mean of % b/b GAE±RSD(%)	
	NIR spectroscopic method	UV-Vis spectrophotometry method
SN1 Bondowoso	3.41±6.65	3.36±0.129
SN2 Lumajang	5.39±3.92	5.06±1.64
SN3 Situbondo	4.27±3.44	3.63±0.088

spectrophotometry comparison method (Table II) and gallic acid standard (100% w/w GAE) as the X (response) in PLS, PCR and SVR regression chemometric methods so that a calibration model was formed. The best calibration model was PLS which was formed from NIR spectra as it had the best R-squared and RMSE value among other calibration models formed (Tabel III). The PLS model has R-square calibration value of 0.998071 and R-square validation value of 0.9979144. This value indicated the PLS model provided a good regression linearity, which was the actual value, and predictive value composed a closed correlation. PLS model has RMSEC and RMSECV value of 1.2735631 and 1.3132828, respectively. This value shows that the deviation between the concentrations of the predicted results PLS model againts the actual concentration was minimal compared to the PCR and SVR models, in other words, the formed PLS model successfully predicted the total phenolic content of the sample with the same or close to the actual phenolic content.

The results of the LOOCV PLS model validation showed the R-square and RMSE values obtained were 0.9983148 and 1.2451497 respectively, while the results of 2FCV validation through the prediction of the test sample showed the R-square and RMSE values of 0.9827973 and 0.2830571, respectively. Based on the LOOCV and 2FCV validation, it can be concluded that the reliability or consistency of the prediction ability of the PLS calibration model was well-formed in which the model can be implemented in the actual sample.

Selected and validated model applications on real samples

The absorbance value of the NIR spectra data of the real sample was used as a predictor for the selected and validated model. The LDA classification model predicts all real samples in the right category, so the recognition ability obtained is 100%. The PLS calibration model was used as a quantitative analysis of NIR spectroscopic methods

for the real samples so that the total phenolic content of each real sample was obtained. The mean value of % w/w GAE results from the NIR spectroscopic method was compared with the results of the UV-Vis spectrophotometry method (Table IV).

Data from the determination of the total phenolic content of the NIR Spectroscopy method and the UV-Vis spectrophotometry method were analyzed by the Paired-Samples T-Test, the purpose of which was to determine whether the results of the total phenolic content of two different methods had the same or identical results. If the Sig. (2-tailed)> 0.005 then the two methods have identical results(Santoso, 2018). Table V showed analysis results of all real samples with the Sig. (2-tailed)> 0.005, which lead to the two methods to have insignificant differences.

Table V. The results of Paired-Samples T-Test of all real samples

Real Samples	Sig. (2-tailed)
SN1 Bondowoso	0.061
SN2 Lumajang	0.014
SN3 Situbondo	0.008

CONCLUSION

Local varieties of soursop leaves in the medium land (Jember) are the highest source of phenolic compounds, on mean a total phenolic content of 5.72% w/w GAE. NIR spectroscopic methods coupled with LDA and PLS chemometrics can be used to classify local varieties of soursop leaves based on their altitude, that is qualitatively through the LDA classification model and quantitatively through the PLS calibration model to determine the total phenolic content.

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