Analysis of flavonoid in extract of Rose Guava (Syzygium jambos (L.) alston) leaves using infrared spectroscopy and chemometrics

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Abstract. This present study was carried out to determine total flavonoid contents of ethanol extract of Rose guava leaves using combination of FTIR and Chemometrics. Flavonoid was extracted from Rose Guava leaves by maceration. IR spectra were correlated with flavonoid content using chemometrics. The chemometric method used for calibration analysis was partial Least Square Regression (PLSR). Partial least squares regression (PLSR) was used to build a prediction model based on the relationship between concentration of total flavonoids obtained from the reference method (AlCl3) and FTIR spectrum. The results of the combination of FTIR and chemometrics on the prediction of total flavonoid levels has resulted in a good predictive model with calibration values of R² 0.9999, RMSEC 0.0000637 and the results of PRESS value of 0.19225, R² 0.978 and RMSECV 0.041. From these results, combination of FTIR spectrum and PLSR can be used for the prediction of total flavonoids content of Rose guava leaves.

1. Introduction
The Rose Guava (Syzygium jambos Alston) is a plant from the Myrtaceae family. Traditionally the leaves and flower preserves are usually used as sedatives; the bark and seeds can relieve fever, diarrhea and dysentery. The fruit and bark contain saponins, flavonoids and tannins, besides its polyphenols contents [1,2]. Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photosynthesising plant cells [3]. Flavonoids have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, and anticarcinogen [4].

The analytical methods commonly used to determine flavonoid levels in medicinal simplicia includes ultraviolet spectrophotometry (UV) [5], high performance liquid chromatography (HPLC) [6], and capillary electrophoresis [7]. However, these methods require a series of time-consuming steps. Infrared (IR) spectrometry techniques combined with chemometrics can be used as an alternative method to measure flavonoid levels [8].

IR spectroscopy techniques have the potential as a fast analysis method because the analysis can be done directly on the dried powder samples without the stages of separation. The IR spectrum produced is the result of interactions amongst chemical compounds in a very complex sample matrix [9]. For this reason, a chemometric method is needed to obtain the qualitative and quantitative hidden information from the IR spectrum [10].

Chemometric is used to find a statistical correlations between spectrum data and information obtained from the sample [11]. Chemometric methods are very useful for processing of IR spectra. The
big advantage of chemometric methods is their capability to extract the information of IR spectra and explore this spectral information for qualitative or quantitative applications. The most frequently used of chemometric methods is Least Squares (PLS) regression [12]. The objective of this research is to develop a simple, rapid, and validated model of IR spectra for the determination of the flavonoid content. Furthermore, IR spectroscopy and chemometric methods were applied for determining flavonoid content in commercial samples.

2. Materials and methods

2.1. Tools and materials
The tools used in this research are Intel® CoreTM Processor i5-2520 CPU @ 250GHz 4.00GB RAM Dell Laptop, Minitab 18, OMNIC Spectra®, Thermo Scientific® Nicolet IS5 FTIR Spectrophotometer, and glass equipment. The materials used in this study were dried simplicia of rose guava leaves, 70% ethanol, KBr, AlCl3, quercetin, organic solvents for the extraction process and measurement of total flavonoids, acetic acid, NaOH, ammonia 30%, amyl alcohol, chloroform, HCL, Mayer and Dragendorff reagent, concentrated sulfuric acid, ether, hydrochloric acid, anhydrous acetic acid and magnesium powder.

2.2. Extraction method
The material used was Rose guava leaves (Syzygium jambos (L.) Alston) obtained from Kampung Sukalaksana RT 01/02 Manoko Cikahuripan Lembang West Bandung Regency, West Java. The simplicia was extracted by maceration method with 70% ethanol for 6 hours. Then the solvent was evaporated with a rotary evaporator to get thick extract to be dried.

2.3. Determination of total flavonoids content
The flavonoids content was determined by aluminum chloride method using quercetin as a reference compound [12]. Sample was prepared by mixing 0.5mL of 4mg/mL sample extract in ethanol with 3mL of ethanol, 0.2mL of 10% aluminum chloride, and 0.2mL of 1M potassium acetate and then diluted to25mL with distilled water. After incubation at room temperature for 30 min, the absorbance of the mixture solution was measured at 432 nm using spectrophotometer. Various standard solutions of quercetin (2000 up to 6000 ppm) were prepared from two stock solutions by dilution with ethanol.

2.4. FTIR spectrum preparation
The Samples were prepared in 5 different concentrations; 0.2%, 0.3%, 0.4%, 0.5% and 0.6%. Then the concentrations were homogenized with KBr, then FTIR measurements were carried out in the central IR region. Furthermore, the FTIR spectrum of each sample was processed with the PLSR chemometric program without derivatization.

2.5. Total flavonoid model preparation
The multivariate calibration model used the PLSR regression model. The formation of a prediction model for total flavonoids was completed by PLS involving the variable x (FTIR measurement results) and variable y (the results of standard method measurements). The model calibration and validation were evaluated by cross validation techniques. The accuracy of the model can be seen based on the results of the coefficient of determination (R2) and the precision was evaluated based on the RMSEC value (Root Mean Square Error of Calibration), RMSECV (Root Mean Square Error of Calibration Validation) and PRESS (Predicted Residual Error Sum of Squares). The acceptability of the model was determined by the low RMSEC and RMSECV values and a high coefficient of determination.

3. Result and discussion
The determination of total flavonoid levels was performed by the AlCl3 method. This AlCl3 method will form a complex between AlCl3 and C-4 and C-3 or C-5 ketone groups from the hydroxyl group of
flavonoids. The results for total flavonoids content in samples are presented in Table 1. The results of the spectra found that each extract had a different absorption peak because it was influenced by each concentration. The total flavonoids measurements were distributed around 67.17 up to 147.8 mg quercetin equivalence (QE)/g extract.

**Table 1.** Total flavonoids content in samples.

| Sample Concentrations (ppm) | Absorbance | Total Flavonoid (mgQE/L) |
|-----------------------------|------------|--------------------------|
| 2000                        | 0.295      | 67.17                    |
| 3000                        | 0.385      | 89.24                    |
| 4000                        | 0.492      | 115.21                   |
| 5000                        | 0.506      | 118.75                   |
| 6000                        | 0.625      | 147.78                   |

Figure 1 showed FTIR spectra of quercetin in various concentration. Those spectra have a different intensity and typical characteristic of absorption bands. In the PLS calibration models, the evaluation of the linearity method was carried out in order to show a proportional relationship between the absorbance of FTIR spectra versus the concentrations of flavonoid.

![FTIR spectra of quercetin](image)

**Figure 1.** FTIR spectra of quercetin A.2000, B. 3000, C.4000, D.5000 and D.6000 ppm.

In table 2, the results achieved a calibration model with concentration based on random numbers at the stage of the calibration set and the actual concentration. Furthermore, the concentration that can be predicted by the PLS calibration model was the observed concentration. The correlation data of PLS model showed good performance of PLS model, indicated by coefficient of determination (R2) higher than 0.99 and the low value of RMSEC (7). R2 and the root mean square error of calibration (RMSEC) were 0.978 and 0.0000637, respectively. Therefore, the calibration model can be used as a tool to predict the concentration of flavonoid content in medicinal plant.

**Table 2.** Actual and observed concentration values.

| No | Actual Concentration (mg/L) | Observed Concentration (mg/L) |
|----|-----------------------------|------------------------------|
| 1  | 0.671                       | 0.671                        |
| 2  | 0.892                       | 0.892                        |
| 3  | 1.152                       | 1.152                        |
| 4  | 1.187                       | 1.187                        |
| 5  | 1.477                       | 1.476                        |
In order to validate the developed model, leave-one-out cross-validation (LOOCV) was used. LOOCV was performed as follows: one sample was left out from the calibration set, a model was built with the remaining samples in the calibration set, then the left-out sample was predicted by this model, and the procedure was repeated by leaving out each sample in the calibration set. R2 and the root mean square error of validation (RMSEV) of LOOCV were 0.978 and 0.04029, respectively.

Precision and accuracy were obtained through multivariate analysis on validation parameters. Precisions can be described through RMSEC (Root Mean Square Error of Calibration), RMSECV (Root Mean Square Error of Cross Validation) and PRESS (Predicted Residual Sum of Squares). The better the ability of the model to predict or the better the precision is, the smaller the value and the smaller the prediction error will become. On the other hand, accuracy can be expressed in the coefficient of determination (R2), if the value (R2) is closer to 1, it indicates that there is a correlation between predictive and actual value and good predictive value. It means that the model has a good predictive capacity.

4. Conclusion
The application of the FTIR method combined with PLS (partial least square regression) chemometrics on the determination of the total flavonoids of 70% ethanol extract of Rose guava leaves (Syzygium jambos (L.) Alston) can provide an alternative in the quick analysis through total flavonoids prediction. The total calibrations are R2 = 0.9999 and RMSEC = 0.0000637. In addition, the results of the validation are R2 = 0.978 and RMSECV = 0.041.

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