Optimization of Flavonoid Extraction from Guava Leaves for Application in Reducing Urea Transformation in Soil

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Abstract. Flavonoid group is a versatile compound as it can be used in the food, pharmaceutical, and urea fertilizer industry. Five-main factors have been consistently studied for optimization, which are solid-to-solvent ratios, solvent-to-solvent ratios, the temperature of extraction medium, exposure time, and equipment power intensity. Factors Solid-to-Solvent Ratio and Time showed the greatest effects on the extraction. Response surface methodology is used to determine the best conditions for flavonoid extraction from guava leaves using an ultrasonic bath. There exist incongruencies between researchers in determining which solvent is best used for flavonoid extraction. Experimentally, the highest total flavonoid content (TFC) tested using Fe (II) colorimetry was 50.44 mg of Quercetin-equivalent per gram of dry weight leaves (mg QE / g d.w.) using 1:1:1 ratio of Water-Ethanol-Methanol (WEM), a first tri-solvent based extraction for flavonoid. Further testing via urea colorimetry shows that diluted compound of 8% of flavonoid extract can reduce urea transformation starting Day 3 of soil sampling but its performance is not at par with the commercial n-Butylphosphorothioic Triadmide (NBPT) compared in this study. Nevertheless, the effect shows a positive outlook on the extract’s potential as a urease inhibitor.

1. Introduction
Flavonoids are found in green leaves of most plants. Its strength as an antioxidant is known to be marketed as an effective food flavoring, health enhancer, and active compounds in medicine. One of the stable flavonoids abundantly found is Quercetin, which is frequently used as a reference in flavonoid based studies. Quercetin is easily sourced from high antioxidant fruits and leaves of apples, oranges, and guava trees. This brings to light on favorable ways to extract flavonoids. Conventional extractions use elevated temperatures and copious amounts of organic solvents. In addition, this causes prolonged exposure to light and oxygen, which in turn increases risk of flavonoid oxidation. Other techniques such as homogenization and subcritical solvent extraction have been implemented with promising results. Newer techniques such as the ultrasonic bath assisted extraction (UBAE) can be executed efficiently with minimal heating, low exposure times, and high reproducibility. This treatment is found to be effective due to the cavitation created from high frequency sound waves which cause breakage in tough membranes especially plant cell walls which ensure a high Total Flavonoid Content (TFC) extracted. Simple maceration does not break cell walls, which results in lower TFC within the same period. A recent study reported a 5-fold increase from simple maceration treatment to UBAE of radish leaves [1]. Along with the equipment, the type of solution plays a big role in TFC extracted. Alcohol aqueous solutions are the norm. Incongruencies can be found in which type of alcohol and solvent combination is best for flavonoid extraction. Frequent citations of Methanol solution yielding high flavonoid in a review study in 2012 [2] while multiple studies cite Water Ethanol as main solvent extractions.
Countless polyphenolic studies have identified the extraction factors for UBAE to be solid-to-solvent ratio, solvent to solvent ratio, temperature, exposure time, and ultrasound power intensity. The first part of the study addresses the optimization of flavonoid extraction by discussing the Central Composite Design (CCD) results of Water-Ethanol-Methanol system. An optimized method for flavonoid extraction is crucial as recent reports point towards flavonoid structures to inhibit enzymes urea nitrogen or urea-N (CON2H4). This property is useful in reducing urea-N losses which commonly occur when an uncontrolled reaction of urea-N applied as crop fertilizer produces high amounts of reduced nitrogen compounds like ammonia (NH3) and oxidized urea like nitrogen oxides (NOx). It was reported that up to 50% of urea will be lost per application. On average, 60 Tg of Urea-N per year with a 30% increment to 80 Tg by the year 2100 [3]. A large portion of urea is easily converted to NH3 via hydrolysis with the help of urease enzymes as per below simplified equation:

$$\text{H}_2\text{N} – \text{C(O)} – \text{NH}_2 + \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2$$  \hspace{1cm} (1)

The flavonoid structure was reported to carry a strong antiurease property. This occurrence is possible due to the chelation of either Nickel (Ni) base of urease enzymes or the enzyme’s vacant active sites. However, only the basic form of Quercetin has been studied. No study on a flavonoid extract has been done. To understand whether this is a consistent phenomenon, the second part of this study will discuss the results of the optimized flavonoid’s effects on soil-applied urea transformation.

2. Materials and Methods

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2.1. Chemical and Plant Materials

Solvants of 95% Ethanol (Puchem CID: 702), 95% Methanol (Pubchem CID: 887), Sulfuric Acid, Phosphoric Acid solutions and reagents namely Aluminium Chloride Hydrate (Puchem CID: 16211594), Potassium Acetate (Puchem CID: 517044), Potassium Chloride, Phenylmercuric Acetate, Diacetyl Monoxime, and Thiosemicarbazide were procured from local distributors. Quercetin (Puchem CID: 5280343) standard was acquired from Sigma Aldrich. Ready-for-pruning guava leaves were collected from the Taman Kekal Pengeluaran Makanan (TKPM) in Sungkai, Perak, Malaysia coordinated at 4°0’58’’ N, 101°16’48’’ E. The samples were oven-dried at 30 °C overnight and grounded.

2.2. Experimental Design

Throughout the experiment, the Design of Experiment (DOE) is set up using Stat Ease Design Expert (SEDX) statistical tool. Table 1 is the input parameters for the DOE of the first part of the study. SEDX tool that was generated using the study type of response surface methodology (RSM), an initial design of central composite (CCD), and the cubic design model. The produced levels of -2.34, 0, +2.34 are from rotational alpha ($\alpha$) value of recommended 2.34 in the statistical tool in efforts to avoid negative integers appearing.

2.3. Ultrasonic Bath Assisted Extraction

2.3.1. Extraction Procedure. The first (Run 1) was prepared using 1 g of grounded leaves. It was then mixed with 150 mL of 2:2:5 Water-Ethanol-Methanol solution in a 250 mL Erlenmeyer flask. It was then submerged, then subsequently filtered. The filtrate is then concentrated using a rotary evaporator until the solid residue remains.

2.3.2. Guava Leaf Extract (GLE) Analyses. The filtered concentrate undergoes Aluminium Chloride (AlCl3) Colorimetry to determine the flavonoid content [2]. The first dilution stage, the solid residue is diluted using 25 mL 80% Ethanol solution. A concentrate of 100 µL is put into a 15 mL test tube. 0.2 mL of 10% AlCl3 solution, and 0.2-mL of 1 M Potassium Acetate (KOAc) solution is added to the concentrate. The second stage dilution was done using 8.6-mL 80% Ethanol solution. It is then analyzed in a UV-Vis Spectrometer at wavelengths ranging from 414 to 416 nm. Formatting the text.
2.3.3. Application of GLE in Urea-N Analyses. Soil is collected from TKPM and it is air-dried and sieved. The refined soil is then put into 10 g containers. The rate of urea that is used is 21.74 mg per 10 g of soil. The soil sampling days were done at days 1, 2, 3, 5, and 7 each with triplicates [4]. The urea samples are analyzed using the Douglas-Bremner colorimetry method. Prior to analysing, each container is mixed with 100 mL of 2M KCL-PMA and the flask is shaken and filtered. The colorimetry effect is visible after reacting 1 mL aliquots from the mixture with the color reagent prepared using DAM, TSC, SA, and PA. Aliquots of 3 mL are placed inside cuvettes and the color change is recorded via UV-Vis Spectrophotometer at intensities measuring between 500-550 nm, with an absorption peak of 527 µm [5].

2.3.4. Statistical Analysis and Optimization An analysis of variance (ANOVA) was calculated for the study from the regression line produced. The R-squared (R2) value is acceptable to be > 0.9 to explain the results with a maximum error of 10 percent (%). Forecasting using the regression line focuses on the predictable R2 (Pred R2) value. Pred-R2 of the regression line is explained any predictions external to this study. If the Pred-R2 is within 0.2 units of the Adjusted R2 (Adj-R2), the two (2) values are within the reasonable agreement of each other and the regression line produced is significant to the study. The regression method is critical to the study as the R2 is dependent on the three (3) types of regression; forward, backward, and stepwise regression. In this study, all three were applied and stepwise was chosen as it fits the model.

3. Results and Discussions

Figure 1. Total Flavonoid Content of sample runs only by changing solvent-to-solvent factors B, C, and D.

Figure 2. Single factor perturbation of all studied factors

Figure 3. Urea transformation against sampling days.
### 3.1. Standard Curve

Absorbance (Abs) is the intensity of the spectrum absorbed in the sample while concentration (c) is the corresponding concentration of Quercetin equivalent. From 6 observations, the R\(^2\) value is found to be 0.9676 with an Adj-R\(^2\) of 0.9611. The F-value is found to be at 78.78 which is significantly higher than the significance F. A high R\(^2\), Adj-R\(^2\), and an F-value > (significance F-value) means the standard curve can explain a significant amount of the absorbance recorded within this study.

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Abs = (0.0007) \times c
\]

A standalone use of UV-Vis Spectrometry in flavonoid analysis in this study is enough as a previous study has found that a flavonoid extract, Kaempferitrin, yielded comparable R\(^2\) values of higher than 0.99 for both UV-Vis and a High-Performance Liquid Chromatograph (HPLC) [8].

#### Table 1. Design of experiment and corresponding total flavonoid content (TFC) via UBAE of guava leaves

| Run No. | A   | B  | C  | D  | E   | F   | G   | TFC  |
|---------|-----|----|----|----|-----|-----|-----|------|
| 31      | -\(\alpha\) | 0  | 0  | 0  | 0   | 0   | 0   | 50.44 |
| 41      | 0   | 0  | 0  | 0  | 0   | 0   | -\(\alpha\) | 10.82 |

\(\alpha\) Factors which are independently adjusted but falls under the same category

### 3.2. Total Flavonoid Content (TFC) Assay Results

Table 1 displays two extraction runs and their respective TFC collected. The highest TFC collected in the experimental run was in Run 31 with 50.44 mg of Quercetin-equivalent per gram of dry weight of guava leaves (mg QE / g d.w.). In comparison, the optimized predicted TFC using ultrasonic sonotrode assisted extraction (USAE) was at 13.00 mg QE / g d.w., which is proved to be at par with the lowest extracts in this study [6]. Run 31 was obtained with all factors set at midpoint except for factor A which was at its lowest limit. Figure 1 shows that the changes of solvent-to-solvent factors B, C, and D have significant fluctuations of TFC. Based on the cube model, the highest possible extraction only for solvent-to-solvent factor was found to be at an equal ratio of 1:1 Water-Ethanol-Methanol (WEM) at a TFC of 33.06 mg QE / g d.w.

### 3.3. Statistical Analysis

Using a Response Surface Reduced Quadratic Model which was modified using the backward elimination alpha=0.1, the Pred-R\(^2\) was calculated to be at 0.8522 which was within the tolerance of 0.2 of the Adj-R\(^2\) value of 0.9585. and the R\(^2\) value of 0.994. The Adeq Precision value is 22.327 which is well above the required value of 4.0 and the datasets were deducted to have a low influence from signal noise. In support of this experiment, the F-value for the model was found to be at 28.05 which implies the model is significant. It was calculated that there is only a 0.01% chance that this value to occur due to signal noise. Lack of Fit F-value was found to be 0.01 which implies the Lack of Fit is not significant relative to the pure error and the data fits the regression line. It was further analyzed that this Lack of Fit to occur 98.89% due to noise. Figure 2 shows the perturbation between 7 single-factors with reference to midpoints of each factor. Single factors which increase TFC are F > G > E > D while an increase in B > C > A will reduce TFC with A (solid-to-solvent ratio) giving the biggest effect as the reference points deviate from -2.43 to +2.43.

### 3.4. Urea Transformed Results

Figure 3 shows the results of the tested urea transformation for 3 different treatments; NBPT-treated, GLE-treated, and untreated. For GLE-treated, 5 different concentrations are use; GLE at 1wt% urea or 1% GLE, 4.5% GLE, 8% GLE, 11.5% GLE, and 15% GLE. NBPT-treated was tested using the concentration of of 50 mg kg\(^{-1}\) dry soil-applied [7]. The untreated control performed the worse as urea transformed was well above 50% by D5. On the same day, GLE treated soils did not overcome 50%.
The diluted compound of both 1% and 8% flavonoid extract can reduce urea transformation starting D3 of soil sampling. On the other hand, decreasing the concentration to 1% GLE resulted in the worst performance after D5. None of the GLE dilutions performed at par with the synthetic n-Butylphosphorothioic Triadmide (NBPT) which was compared in this study.

4. Conclusion
Out of the five factors, A (solid-solvent ratio) and F (exposure time) are the most crucial factors in UBAE while implementation of WEM solution as an extraction medium greatly boosted TFC yield as seen in the optimized Run 31 with 50.44 mg of QE extracted. The GLE treated soil starts to show stagnation of urea transformation hovering below 50% within Day 1 until Day 5 while the control continues to undergo urea transformation as high as 60%. Further isolation of GLE may greatly improve the reduction of urea transformation.

5. Acknowledgment
The authors wish to thank the Chem Eng Department, Universiti Teknologi PETRONAS for the financial support, and Mr. Supandi and Mr. Sulaiman from Jabatan Pertanian Daerah Batang Padang, Perak for their assistance in the fieldwork while conducting this study.

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