Optimization of batch extraction of functional ingredients from stevia leaves with response surface methodology

Hartini, Mega Renovasi Tara, Kun Harismah*, Ahmad Muhamad Fuadi
Department of Chemical Engineering, Faculty of Engineering, Universitas Muhammadiyah Surakarta (UMS), Surakarta, Indonesia
E-mail: *kun.harismah@ums.ac.id

Abstract. The aim of the study was to reveal an effective extraction procedure for maximization of the yield of stevioside, flavonoid, and total phenolic compounds as well as antioxidant activity in stevia extract. Extraction was carried out at temperature variations of $75 \degree C$, $80 \degree C$, $90 \degree C$ and variations of water solvents $280 \text{ mL/g}$, $290 \text{ mL/g}$, $300 \text{ mL/g}$. Stevioside and flavonoid analysis used spectrophotometer method, while phenol analysis used the Folin-Ciocalteau reagent method. The result is a change in temperature and solvent ratio affect the changes in stevioside, flavonoid, and phenol levels. Optimal conditions of stevioside, flavonoid, and phenol using response surface methodology (RSM) occur at a temperature range of $75 \degree C$ with a solvent ratio of $280-290 \text{ mL/g}$.

1. Introduction

Stevia rebaudiana Bertoni leaves are perennial herb that contain high sweetness (250-300 times greater than sucrose) due to the presence of diterpenic glycoside : stevioside, steviolbioside, rebaudioside (A, B, C, D, E, and F) and dulcoside A, which known as steviol glycoside is non-carcinogenic and no calorie content [1]. In addition to glycosides, the leaves of stevia contain also other phtytoconstituents, such as flavonoids, phenolic acids, fatty acids, proteins, and vitamins [2]. Stevioside and rebaudioside A are the main sweetening compound interest, they are thermostable even at temperatures of up to $200 \degree C$ [3]. It is known that stevia leaves possessed also antialergic, antimicrobial and detoxicative effect and antioxidant effect [4].

Extraction is the technique used for separation of therapeutically desired active constituent(s) and elimination of unwanted insoluble material by treatment with selective solvent [5]. Studied have evaluated different methods to extract the steviol glycosides using different techniques, from conventional alternatives such as hot water extraction or Soxhlet extraction, or even complex techniques such as supercritical CO2, ultrasonical or microwave-assisted [6]. Hot-batches extraction and cyclically pressurized extraction are also can be applied to extract stevia leaves. Hot-batch extraction (T) was more efficient than cyclically pressurized extraction (I). The dry material extracted using process T produced more dry residue than process I (58.2% with process T compared to 36.2% with process I) [7].

To get the optimum extraction conditions can be done using Response Surface Methodology (RSM). Response Surface Methodology is a collection of mathematical and statistical techniques
used for modeling and analyzing problems, where several variables affect a response, the aim is to optimize the response [8]. Recently, researchers across the world are focusing on the development of optimized extraction procedures aimed at economical, green, and efficient extraction of stevia, without compromising taste and extract recoveries [9]. The latter can be graphically represented with response surfaces that show to what extent the influence of parameters or their interactions are significant or not, and can then provide the optimal conditions to improve a process [10]. The main objective of this study was to determine the optimum value in extraction variable. The ingredients in stevia leaves that tested are stevioside, flavonoid, and phenol.

2. Materials and Method
Stevia plants are purchased in dry conditions from the Tawangmangu area, Central Java in September 2019. Extraction techniques using the method of Celaya & Kolb [11] with modification of the temperature and volume of the solvent. Stevia leaves were weighed, then washed, dried and mashed to 80 mesh. Add the solvent at a temperature of 75 °C, 80 °C, 90 °C with a volume ratio per volume of solvents 28, 29, 30 using a water solvent, then extracted for 30 minutes.

After the extract are obtained, the content of stevioside, flavonoids, and total phenols are tested. In the analysis of stevioside levels, extract stevia is diluted with distilled water and then measured using a spectrophotometer at a wavelength of 210 nm [12].

Flavonoid content analysis method is done by extract analysis and made a standard solution. The total flavonoid content was measured by the aluminum chloride colorimetry test. The reaction mixture consisted of 1 mL extract and 4 mL distilled water taken from a 10 mL volumetric flask. In the flask, 0.30 mL of 5% sodium nitrite and after 5 minutes, 0.3 mL of 10% aluminum chloride was added to the mixture. After 5 minutes, 2 mL of 1 M sodium hydroxide is added again then diluted to 10 mL with distilled water [13]. To made a standard solution, a set of quercetin standard solutions (20, 40, 60, 80 and 100 µg/mL) were prepared in the same manner as previously described. Absorbance for testing and standard solution was determined against the reagent residue at 425 nm with a spectrometer. The total flavonoid content is expressed as mg of quersetin/g extract [13].

Determination of total phenolic content using the method by Oksana [14] with modified solvent. It starts with making a standard solution of gallic acid. 1000 ppm gallic acid standard solution is made by weighing 10 mg of gallic acid dissolved with distilled water to a volume of 10ml. From the stock solution pipette 0.25 mL is diluted with water to a volume of 25 ml, resulting in a concentration of 10 ppm. From this solution pipette 1, 2, 3, and 4 mL and sufficient with water up to 10 mL, resulting in concentrations of 1, 2, 3, and 4 ppm. For the measurement of standard solution using the Folin-Ciocalteau method. Each concentration of 1, 2, 3, and 4 ppm was added with 0.4 mL Folin-Ciocalteau reagents, shaked, and left for 8 minutes. Add 4.0 mL of 7% Na2CO3 solution which is shaken until homogeneous. Add 10 mL of distilled water and let stand for 2 hours at room temperature. Measure the absorption at a wavelength of 730 nm, then make a calibration curve, connect the gallic acid concentration (mg/mL) to the absorbance. Determination of total phenolic from stevia extract by diluting the extract then pipette 0.5 mL, after that the test method is like measuring standard solution of gallic acid. Three repetitions are performed so that the phenolic content obtained as mg equivalent to gallic acid per 100 mg of sample.

3. Results and Discussion
This study uses the independent variable solvent ratio \((x_1)\) and temperature \((x_2)\). While the interactive variables analyzed were steviosida \((y_1)\), flavonoids \((y_2)\), and phenols \((y_3)\). Extraction efficiency is influenced by five parameters including temperature, time, solvent ratio, material
Table 1. Levels of Steviosides, Flavonoids and Phenols on Temperature Changes and Solvent Ratios

| T (ºC) | Solvent (mL/g) | Stevioside (mg GAE/mL) | Flavonoid (mg QC/mL) | Phenol (mg GAE/mL) |
|--------|----------------|------------------------|----------------------|-------------------|
| 75     | 280            | 0.60301                | 0.02207              | 0.01498           |
| 75     | 290            | 0.60307                | 0.02151              | 0.01502           |
| 75     | 300            | 0.60230                | 0.02155              | 0.01441           |
| 80     | 280            | 0.60295                | 0.02192              | 0.01456           |
| 80     | 290            | 0.60293                | 0.02182              | 0.01493           |
| 80     | 300            | 0.60298                | 0.02190              | 0.01482           |
| 90     | 280            | 0.60298                | 0.02242              | 0.01478           |
| 90     | 290            | 0.60296                | 0.02241              | 0.01483           |
| 90     | 300            | 0.60292                | 0.02241              | 0.01468           |

size and type of solvent [15]. Based on the analysis of stevioside, flavonoid and phenol levels the results of the data are obtained in Table 1.

3.1. Flavonoid

Table 1 showed that an increase in temperature causes an increase in flavonoid levels. Increasing the ratio of solvents causes changes in questionnaire to changes in flavonoid levels [8]. These results prove that not all antioxidant compounds are dissolved in water. From these results it can be said that an increase in temperature causes an increase in flavonoid levels, but an increase in the solvent ratio does not always cause an increase in flavonoid levels.

The second order polynomial regression method is used to determine the relationship between temperature, and the ratio of solvents to flavonoid levels. Temperature and solvent ratio simultaneously have a very strong influence on changes in flavonoid levels, this is due to the value of $R = 0.77342$. While the regression model shows that simultaneously the temperature and solvent ratio have a significant effect on the probability of 0.007. While in parallel the temperature and the ratio of solvent squared have a significant effect on changes in flavonoid levels. From this regression model the mathematical relationship between temperature and the ratio of solvent to flavonoid levels is as follows.

$$ y = -0.0001578 X + 0.000610495 Y - 2.14088889 E - 06 X^2 - 1.327000 E - 06 Y^2 + 0.5221986 $$ (1)

From the mathematical equation can be seen the relationship between temperature and solvent ratio of flavonoid levels in a 3-dimensional plot. The result is that the maximum extraction conditions occur at a temperature of 90 °C and a solvent ratio of 28 mL/g. These results indicate that an increase in temperature causes an increase in levels. While the increase in the solvent ratio does not cause an increase in flavonoid levels. Minimum flavonoid levels occur at 75 °C with a solvent ratio of 29 mL/g (Figure 1).

3.2. Phenol

Based on Table 1 it is known that the phenol content increases at the same temperature but different volume 280 mL/g to 290 mL/g, while the decrease in phenol content occurs at a solvent
290 mL/g to 300mL/g. This increase is due to the nature of phenols which are easily dissolved in the air. An increase in temperature causes a questionnaire change to changes in phenol [16].

The second order polynomial regression test was used to determine changes in temperature and the ratio of solvents to changes in phenol content. The release ratio has a significant effect on the probability of 0.014. While the temperature and solvent ratio did not have a significant effect, the square of the temperature and solvent ratio had a significant effect on changes in phenol content. The use of this regression model shows that temperature changes and solvent ratios have a very strong influence on changes in phenol content, what is meant by the double ratio value (R). The R value of this regression model is 0.7023, with the relationship between temperature and the number of sailors to the amount of phenol is written as follows.

\[
y_1 = -0.00036817 X + 0.001186 Y + 3.42222 E^{-07} X^2 -2.207333 E^{-06} Y^2 + 1.06542 E^{-06}
\] (2)

Based on these mathematical equations a comparison is obtained between phenol levels, temperature and ratio of solvent released three dimensions. Displays the analyzed variable, showing how much phenol has increased by the amount that can be dissolved.

These results shows that the maximum phenol conditions occur at a temperature of 75 °C and a solvent 280 mL/g. While the minimum content occurs at a temperature of 80 °C and a solvent ratio of 300 mL/g. Unlike the maximum conditions that exist in maximum operating conditions, the minimum requirements at a point in Figure 2.

3.3. Stevioside

Based on Table 1 it is known that the average of stevioside content in each temperature are increase. The solvent volume also influenced the increasing amount of stevioside content [17].

The second order polynomial regression test was used to determine changes in temperature and the ratio of solvents to changes in phenol content. The release ratio has a significant effect on the probability of 0.6029. While the temperature and solvent ratio did not have a significant effect, the square of the temperature and solvent ratio had a significant effect on changes in phenol content. The R value of this regression model is 0.7734. Figure 3 shows that the maximum stevioside conditions occur at a temperature of 80 °C and a solvent 290 mL/g. While the minimum content occurs at a temperature of 85 °C and a solvent ratio of 295 mL/g.
Figure 2. Relationship between temperature and solvent ratio to phenolic levels

Figure 3. Relationship between temperature and solvent ratio to stevioside levels

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