Analysis Study of Stevioside and Rebaudioside A from *Stevia rebaudiana* Bertoni by Normal Phase SPE and RP-HPLC

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Abstract: Solid Phase Extraction (SPE) method using silica as sorbent for stevioside and rebaudioside A analysis in *Stevia rebaudiana* Bertoni leaf have not been performed. The aim of this study is to develop SPE method using silica as sorbent for Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis of stevioside and rebaudioside A in *S. rebaudiana* leaf. The results of this study indicate that the optimal conditions for normal phase SPE (silica) are conditioned with 3.0 mL of hexane. The sample loading volume is 0.1 mL. Cartridge is eluted with 1.0 mL acetonitrile: water (80: 20, v/v) to separate both analytes. The cartridge is washed with chloroform and water of 0.3 mL respectively. The developed SPE sample preparation method meets the accuracy and precision test and can be used for the analysis of stevioside and rebaudioside A by RP-HPLC.

1 Introduction

Stevia rebaudiana is an herb plant originating from South America (Paraguay and Brazil). *S. rebaudiana* contains steviol glycoside compounds. Major steviol glycoside compounds in *S. rebaudiana* are stevioside (6-12%) and rebaudioside A (1-4%) [1]. Stevioside and rebaudioside A are widely used as a natural sweetener in some countries [2]. On the other hand, various regulations regulate the permissible levels of stevioside and rebaudioside A and its limits [3]. The economic value of the purified extracts of stevioside and rebaudioside A is viewed from the quantitative aspect of both analytical compounds [4]. Therefore, the quantitative aspect is very important in the development of studies related to the active compounds stevioside and rebaudioside A.

Various methods of stevioside and rebaudioside A analysis have been developed. Most of the quantitative methods of stevioside and rebaudioside A are based on the High Performance Liquid Chromatography (HPLC) method. The normal phase HPLC (NP-HPLC) method developed can completely separate the chromatograms of stevioside and rebaudioside A [5,6]. However, column conditioning takes a long time; in addition, method reproducibility is less due to repeating the retention time of the analysis so variably [7]. Reversed phase HPLC (RP-HPLC) method using gradient elution system can also separate both the stevioside and rebaudioside A chromatograms properly [3,4,8–10]. The RP-HPLC method using isocratic elution system developed is more practical in operation, efficient in acetonitrile consumption and rapid in running system [1,7,11].
Sample preparation is crucial step in the analysis of active compounds. Sample preparation methods employed in HPLC analysis include solid phase extraction (SPE) phase. The SPE method has advantage of removing impurities that can reduce complexity of sample matrix and preventing emulsion occurred in extract solution. Disadvantages of SPE method are more expensive and may occur biased by the solvent dilution factor [7]. The SPE method developed is all using reverse phase (C-18) (RP-SPE) [4,7,12]. Normal phase SPE (silica) method (NP-SPE) has not been developed for the analysis of stevioside and rebaudioside A compounds using RP-HPLC. In addition, every SPE method requires optimization of operational conditions because each method and sorbent will provide different characteristics and adsorption capacity. This research was aimed to develop sample preparation method using normal phase SPE for stevioside and rebaudioside analysis. The method developed was then applied for RP-HPLC analysis of both analytes in S. rebaudiana leaf.

2 Methods

2.1 Plant Materials

The leaves of S. rebaudiana (Bert.) are obtained from the Bandungan, Poloboga, Tajuk, Tawangmangu, and Pakis, Central Java, Indonesia. The identification of plant species of S. rebaudiana was done by the Faculty of Pharmacy UGM Pharmaceutical Biology Division under the supervision of taxonomist Drs. Djoko Santosa, M.Si.

2.2 Standard and Chemicals

The standard of stevioside and rebaudioside A used was purchased from WAKO, Japan that have purity grade of 99.0% based on Certificates of Analysis (CoA). Solvents for mobile phase of HPLC were acetonitrile (Liquid Chromatography grade), methanol (Liquid Chromatography grade). The solvents for extraction were ethanol (pro analysis grade). All these solvents were purchased from E. Merck, Germany. SPE cartridge used was Lichrolut Si (40-63 μm) purchased from E.Merck, Germany.

2.3 Conditioning and Optimization of sample loading

Silica sorbent of SPE was activated and conditioned using 3.0 mL of hexane. Standard solutions were used as sample loaded. Optimization of sample loading was performed based on volumes of standard. Standard solutions loaded were varying of 0.5; 0.2; and 0.1 mL. Each eluent volume of the standard solution adsorbed was analyzed using RP-HPLC method. The optimal volume loaded is a volume that gives a result of more than 99% of the adsorbed analyte compound in the stationary phase.

2.4 Optimization of Washing

A number of solvents involving chloroform, water, acetonitrile and mixed water: acetonitrile (8: 2, v/v and 9: 1, v/v) are used to wash impurities. A volume of solvent wash (0.3-5.0 mL) was eluted at stationary phase. The optimal solvent is a solvent which can remove the impurity compound without eluting the analytes. Each eluent optimization of the washing solution was analyzed by RP-HPLC method.

2.5 Optimization of Eluting

A number of solvent including pure methanol, mixtures of water: acetonitrile (2: 8, v/v), methanol: acetonitrile (1: 1, v/v) and mobile phase for eluting the analytical compound. Solvent volume of 1.0-5.0 mL is eluted on the stationary phase. The optimum solvent is a solvent which can remove the whole analytes without carrying the impurity compound. Each eluent optimization of the elution solution was analyzed by RP-HPLC method.
2.6 RP-HPLC Condition [1].

HPLC used was Knauer, GmBH, Germany. Stationary phase used was Eouroosphere C-18 (250 × 4.6 mm., 5 µm) with guard column. Mobile phase was mixture of methanol 10% in water : acetonitrile ( 65 : 35, v/v). TFA added into a mixture was 0.01% (v/v). Mobile phase was homogenized and degassed using ultrasonic. Flow rate of mobile phase applied was 0.6 mL/min. Separation was detected using UV detector at 210 nm. Sample volume injected was 20 µl using Rheodyne 7726i injector.

3 Result and discussion

Normal phase SPE using silica sorbent for sample preparation of stevioside and rebaudioside A analysis by HPLC has not been developed. The polar silanol group in silica interacts with the analytes through the primary interaction of hydrogen bonds of hydroxy groups of glycosides [13]. The interaction of the hydroxy group with a strong silanol group on the sorbent surface can retain the analytes. Elution strength and/or eluent polarity can be adjusted to break the interaction of the analyte compound with sorbent.

3.1 Optimization of Sample Loading

Silica as a stationary phase of normal phase SPE is conditioned prior to use. The solvent used for this conditioning is hexane. The sample loading volume of 0.1 mL is the optimal volume in which all compounds of the analyte are adsorbed in the stationary phase. In a 0.5 mL sample application, non-retained rebaudioside A and stevioside were 99.23% and 77.52% respectively. In the application of 0.2 mL of sample volume, rebaudioside A and stevioside which were not retained in silica were 67.25% and 63.40%, respectively. The results of this study indicate that each SPE material has a specific maximum adsorption capacity to the type of analytical compound and operational conditions of SPE (Sigma-Aldrich, 1998). The chromatogram profile of sample volume optimization applied to the normal phase SPE (silica) is shown in Figure 1.

3.2 Optimization of Eluting

In contrast to RP-SPE, the normal phase (SPE) phase separation mechanisms developed in this study are selective elution, which elutes the analytical compound and retains impurities [13]. After the sample is applied to the stationary phase, then the elution of the analytes is performed. Several solvents optimized for the elution of the analytes are methanol, acetonitrile : water (8 : 2, v/v), methanol :
acetonitrile (1:1, v/v) and mobile phase. The optimum solvent for the elution of a normal-phase SPE silica is acetonitrile: water (8:2, v/v) with 1.0 mL volume. The organic solvent-water mixture with a larger organic solvent ratio is the appropriate eluting solvent for the selective elution mechanism [13].

An acetonitrile: water (8:2, v/v) mixture has the most appropriate polarity and elution strength among other solvents to break the strong hydrogen bond interaction between the analytes and the sorbent without breaking the interaction of the impurity compound with the sorbent. These results indicate that a selective elution separation mechanism can be achieved for NP-SPE (silica). Important factor concerned is the suitability of the polarity and strength of the eluting solvent to be sufficiently strong to break the interaction of the analytes with sorbent but weak to break the interaction of impurity compounds with the sorbent. The elution optimization profile of the analyte compound with several solvents is presented in Figure 2.

![Figure 2. Chromatogram profile of analytes eluting optimization of normal phase SPE (silica). Eluting solvent optimized of (a) acetonitrile : water (8 : 2, v/v) (1. rebaudioside A, $t_R = 9,900$ min and 2. stevioside, $t_R = 10,850$ min); (b) methanol : acetonitrile (1 : 1, v/v) (1. rebaudioside A, $t_R = 9,933$ min and 2. stevioside, $t_R = 10,850$ min); (c) methanol (1. rebaudioside A, $t_R = 10,133$ min and 2. stevioside, $t_R = 10,833$ min); (d) mobile phase of RP-HPLC (1. rebaudioside A, $t_R = 9,850$ min dan 2. stevioside, $t_R = 10,767$ min).](image)

3.3 Optimization of Washing

The washing step after elution of the analytes is proposed to find out whether there are still compounds of analyte left in the stationary phase. In the initial optimization, the washing step is performed prior to the elution of the analyte compound. However, the analytes is still eluted in the water. In addition, the analytes interaction to the sorbent becomes stronger when the impurity compound is washed using chloroform.

Washing is done with a non-polar solvent chloroform, followed by acetonitrile : water (2:8, v/v) and followed by water. The result shows that no analytes are detected in the washing step. The chromatogram profile of washing step optimization in normal phase SPE (silica) is shown in Figure 3.
Chromatogram profile of washing optimization of normal phase SPE (silica). Washing solvent optimized of (a) 2.0 mL chloroform; (b) 1.0 mL acetonitrile: water (2:8, v/v); and (c) 0.6 mL water. There is no analytes detected in washing eluent.

3.4 Accuracy and Precision Test

Accuracy test is performed using the recovery test by standard addition method. Precision is determined on the basis of the relative standard deviation (RSD) of three levels replicated samples which have been spiked with the standard. Accuracy and Precision of developed NP-SPE method are presented in Table 1 and 2. The developed method is meet accuracy and precision and indicate that the SPE method can be used for RP-HPLC analysis. The NP-SPE (silica) method developed is more efficient in its implementation than RP-SPE (C-18) because the elution of the analyte compound is performed after loading stage. The developed NP-SPE method is become shorter in the process.

3.5 Method Application

The developed NP-SPE method is then applied to analyze ethanolic extract of *S. rebaudiana* leaf using RP-HPLC. The optimum normal phase SPE step is a sample application volume of 0.1 mL followed by eluting of the analytes compound using 1.0 mL acetonitrile: water (8:2, v/v). Chromatogram of sample analysis is shown in Figure 4.

The results of this study indicate that sample preparation in normal phase (silica) SPE was proven to clean the matrix complex of samples of ethanolic extract of *S. rebaudiana* leaf without loss of analytes (Figure 4). SPE normal phase (silica) developed method has a selective elution mechanism which is eluting the analytes first using particular solvent and holding the impurity compound in the stationary phase.

Profile of overlay chromatogram between non SPE (black line) and silica SPE (green line) RP-HPLC analysis of *S. rebaudiana* leaf.
Table 1. Accuracy and precision test of rebaudioside A determination using normal phase SPE (silica) method developed for RP-HPLC analysis

| average of standard spiked (mg) | Reb A (mg) founded in 500 mg of leaf extract | theoretical of Reb A concentration (mg) | Reb A founded in SPE sample | recovery (%) | RSD (n = 3) |
|--------------------------------|---------------------------------------------|----------------------------------------|-----------------------------|--------------|------------|
| 0.0215                         | 0.0052                                      | 0.0267                                 | 0.0268                      | 100.47       | 3.34       |
| 0.0493                         | 0.0112                                      | 0.0605                                 | 0.0618                      | 102.62       | 0.97       |
| 0.0766                         | 0.0066                                      | 0.0832                                 | 0.0838                      | 100.76       | 1.17       |

Reb A = rebaudioside A  
Stv = stevioside

Table 2. Accuracy and precision test of stevioside determination using normal phase SPE (silica) method developed for RP-HPLC analysis

| average of standard spiked (mg) | Stv (mg) founded in 500 mg of leaf extract | theoretical of Stv concentration (mg) | Stv founded in SPE sample | recovery (%) | RSD (n = 3) |
|--------------------------------|---------------------------------------------|----------------------------------------|-----------------------------|--------------|------------|
| 0.0294                         | 0.0142                                      | 0.0436                                 | 0.0442                      | 101.32       | 1.61       |
| 0.0643                         | 0.0100                                      | 0.0743                                 | 0.0737                      | 99.21        | 0.91       |
| 0.1104                         | 0.0158                                      | 0.1262                                 | 0.1321                      | 104.69       | 1.54       |

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References
1. Martono Y, Riyanto S, Rohman A, and Martono S. 2016 AIP Publishing p. 080001. Available from: http://scitation.aip.org/content/aip/proceeding/aipcp/10.1063/1.4958509
2. Liu J, Li J, and Tang J. 2010 Food Bioprod Process. 88 215.
3. Well C, Frank O, and Hofmann T 2013 J Agric Food Chem. 61 11312.
4. Gardana C, Scaglianti M, and Simonetti P 2010 J Chromatogr A. 1217 1463.
5. Kolb N, Herrera JL, Ferreyra DJ, and Uliana RF. 2001 J Agric Food Chem. 49 4538.
6. Dacome AS, da Silva CC, da Costa CEM, Fontana JD, Adelmann J, and da Costa SC. 2005 Process Biochem. 40 3587.
7. Bergs D, Burghoff B, Joehnck M, Martin G, and Schembecker G. 2012 J Für Verbraucherschutz Leb. 7 147.
8. Cacciola F, Delmonte P, Jaworska K, Dugo P, Mondello L, and Rader JI. 2011 J Chromatogr A. 1218 2012.
9. Jaworska K, Krynitsky AJ, and Rader JI. *JAOAC Int.* **95** 1588.
10. Zhao Y-G, Cai M-Q, Chen X-H, Pan S-D, Yao S-S, and Jin M-C. 2013 *Food Res Int.* **52** 350.
11. Martono Y, Riyanto S, Rohman A, Martono S. 2015 *Int J Pharm Clin Res.* **8** (5) suppl **397**
12. Woelwer-Rieck U, Lankes C, Wawrzun A, Wüst M. 2010 *Eur Food Res Technol.* **231** 581–8.
13. Supelco 1998 *Guide to Solid Phase Extraction* (Bulletin 910: Sigma-Aldrich).