Menara Perkebunan 2021, 89(2), 125-134 DOI: http://dx.doi.org/10.22302/iribb.jur.mp.v89i2.459
p-ISSN: 0125-9318/ e-ISSN: 1858-3768 Accreditation Number: 21/E/KPT/2018

Optimization on Na and Ca bentonite activation using response surface method for increasing selectivity of stevioside in stevia extract

Optimasi aktivasi bentonit Na dan Ca menggunakan metode respons permukaan untuk meningkatkan selektivitas steviosida pada ekstrak stevia

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Received 11 July 2021 / Accepted 22 October 2021

Abstrak

Permintaan konsumsi ekstrak stevia sebagai pemanis alternatif yang mengandung steviosida dan nol kalori semakin meningkat. Namun, warna gelap dan kandungan tanin yang tinggi dari ekstrak daun stevia menyebabkan aftertaste yang mengganggu dan mengurangi minat untuk konsumsi. Oleh karena itu, proses pemurnian wajib dilakukan. Beberapa penelitian tentang metode pemurnian menunjukkan pendekatan terbaik dengan adsorpsi menggunakan bentonit. Namun bentonit alam memiliki kapasitas adsorpsi yang terbatas dan selektivitas yang rendah. Aktivasi dengan asam dan suhu tinggi diharapkan dapat meningkatkan kapasitas adsorpsi warna dan selektivitasnya dalam mempertahankan steviosida pada ekstrak. Penelitian ini bertujuan untuk mendapatkan konsentrasi asam dan suhu optimum untuk aktivasi menggunakan rancangan percobaan Response Surface Method *(RSM) dan aplikasinya pada pemurnian ekstrak daun stevia. Berdasarkan parameter bilangan metilen biru, konsentrasi H_2SO_4 yang paling optimum digunakan untuk aktivasi adalah 0,17 N untuk kedua bentonit alam. Temperatur pemanasan adalah 358°C untuk Na-bentonit dan 481°C untuk Ca bentonit. Kapasitas adsorpsi maksimum Na dan Ca-bentonit teraktivasi masing-masing meningkat dari 15,65 dan 38,23 mg g^{-1} menjadi 197,72 dan 169,52 mg g^{-1}. Adsorben terbaik yang digunakan untuk pemurnian adalah Ca-activated, yang meningkatkan klarifikasi ekstrak hingga 81,37% pada 655 nm dan 86,64% pada 410 nm dibandingkan dengan bentonit Ca alami. Ini juga mengurangi tanin hingga 97,46% dan lebih selektif untuk memulihkan konten stevia 50,64% dalam larutan, yang lebih tinggi dari penelitian lain yang dilaporkan sebelumnya.*

[Kata kunci: bentonit teraktivasi, kapasitas adsorpsi, ekstrak daun stevia]

Abstract

The demand for stevia extract consumption as an alternative sweetener that contains stevioside with zero-calorie is increasing. However, dark color and high tannin content from stevia leaf extract causes a disturbing aftertaste and reduces interest in consumption. Therefore, the purification process is a mandatory step to be done. Several studies on purification methods show the best approach by adsorption using bentonite. However, natural bentonite has limited adsorption capacity and low selectivity. An activation with acid and high temperature is expected to increase the adsorption capacity of color and its selectivity on maintaining the stevioside at the extract. This study aimed to obtain the optimum acid concentration and temperature for the activation using the Response Surface Method (RSM) experimental design and its application to the purification of stevia leaf extract. Based on the parameter of methylene blue number, the most optimum concentration of H_2SO_4 used for activation was 0.17 N for both natural bentonite. The heating temperature was 358°C for Na-bentonite and 481°C for Ca bentonite. The maximum adsorption capacities of activated Na and Ca-bentonite were increased from 15.65 and 38.23 *mg* g^{-1} to 197.72 and 169.52 mg g^{-1}, respectively. The best adsorbent used for purification is Ca-activated, which increased extract clarification up to 81.37% at 655 nm and 86.64% at 410 nm compared to natural Ca bentonite. It also reduced tannin up to 97.46% and was more selective to recover 50.64% stevia content in the solution, which was higher than other previously reported studies.

[Keywords: activated bentonite, adsorption capacity, stevia leaf extract]

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Introduction

Stevia rebaudiana leaf extract is a sugar substitute for diabetic type II diet, a healthy and safe sweetener because of its 0 calories (Tiwari, 2010; Lemus *et al.*, 2012). Based on Liquid Chromatography (LC-QTOF/MSMS) analysis by Molina *et al.* (2017), both polar and non-polar extracts of stevia leaves contain more than 80 components of active ingredients, including terpenoid molecular groups, phenolic groups, quinic acid, caffeic, and their derivatives, sesquiterpenoids, fatty acid, and fatty acid amides. Stevioside and rebaudioside A in stevia leaf extract represent the sweet taste of stevia leaf extract and have a sweetness level of up to 300-450 times that of glucose (Characostas *et al*., 2008). Stevioside is found in a higher level as 4-15%, while rebaudioside A is 2-4% (Raini & Isnawati, 2011). Based on JECFA regulations (2007), the use of stevia as a sweetener must have steviol glycosides equivalent to 95%. This regulation makes the purification process of stevia leaf extract significant to produce stevia extract with good quality and safe to use as an alternative sweetener to replace sugar.

The purification process of stevia leaf has been studied with several methods such as filtration, adsorption, and membrane separation process. Among these, adsorption is most valuable for purification (Liu *et al*., 2019). Previous studies using the adsorption method with zeolite Ca increased solution clarity up to 80% but left a bitter taste from Ca dissolved (Morases & Machado, 2001). An approach on adsorption using activated carbon reduced the tannin content up to 39%, yet it did not calculate the stevioside level at the final product (Kusumaningsih *et al.*, 2015). Research related to optimizing contact time for purification of stevia extract using activated bentonite increased clarification of up to 95% by 30 minutes (Husada *et al.*, 2018). The weakness of the natural adsorbent used is its low adsorption capacity and selectivity, where the stevioside can be adsorbed at the same time with the color pigment. Therefore, we developed a pretreatment of natural Na and Ca-bentonite used with activation process to increase adsorption capacity and selectivity on maintaining stevioside in the extract.

The adsorption method for purification can be improved by using specific adsorbents or modifying the adsorbent by activation. Activation of adsorbent by chemical, physical, or mechanical means was used for better efficiency and effectiveness. This research determines two activating methods for natural Na and Ca-bentonite, using H_2SO_4 and high temperature. Acid can increase its active site (Dewi & Twilana, 2012), make it more hydrophobic, and reduce its adsorption power to water (Kurniasari *et al.*, 2011). According to Kurniasari *et al.* (2011), high temperatures cause recrystallization of the clay adsorbent to produce better crystals with large pores. These improvements of the active site and pores of bentonite used are expected to bind the most impurities in the form of pigments that have large sizes and contain Mg^{2+} in chlorophyll ion to bond with the active site formed in the activated bentonite used. This adsorbent would be more selective than adsorbing the steviol glycoside molecules, which is stevioside. These changes can improve the adsorbent capacity and selectivity. This research aimed to get the optimum concentration of H_2SO_4 and the temperature used for the activation of Na and Ca adsorbents. Activated Na and Ca bentonites used to purify the crude stevia extract are expected to improve the clarification and maintain stevioside from the extract.

Materials and Methods

Materials

Natural Na and Ca bentonites were purchased from PT Clariant. The dried stevia leaves were obtained from Stevia Indonesia under the trade name Stevi-Grow. Sulfuric acid (H_2SO_4 EMSURE®), methylene blue (CI. 52015) were purchased from Merck Millipore, stevioside analytical standard (≥95% HPLC grade) from Sigma Aldrich, and distilled water was produced from Direct-Q® 8 UV Remote Water Purification System.

Bentonite activation

The experimental design of bentonite activation was carried out using a Central Composite Design (CCD) with Response Surface Method (RSM) to determine the optimum activation conditions. The factors include sulfuric acid (H_2SO_4) concentration (1-5 N) and temperature (400-600°C), while methylene blue number was used as the response variable. Activation with H_2SO_4 refers to Rožić *et al.* (2010), with the ratio of bentonite: acid is 1:5 (w/v). A furnace was used for temperature conditioning for 3 hours. The activated bentonite was then pulverized to 100 mesh for further analysis.

Data analysis

In this research, the independent variables considered for the optimization process include the concentration of H_2SO_4 in N (X1) and heating temperature in degree Celsius (X2). Coded factor level used based on literature review shown the best-activated condition for the purification of stevia leaf extract. The experimental data obtained were analyzed its optimum response with methylene blue number determination and calculated by Minitab 11 to get the optimum condition. The optimum activated condition was then applied to Na and Ca-bentonite, respectively.

Methylene blue number determination

The analysis refers to Ramadhani *et al.* (2015) using a standard series of the curve. The linearity equation of the standard is $y = 0.19\,x - 0.2065$ with a regression of 0.9989. A small amount of bentonite was weighed and then added with 100 ppm methylene blue standard solution. The sample was allowed to react for 1 x 24 hours to obtain maximum adsorption. Measurements were carried out at a maximum λ of 664 nm for the standard and sample solution and then calculated using the linearity equation.

Analysis of bentonite crystal diffraction with x-ray diffraction (XRD)

Bentonite sample with 100 mesh size was analyzed for X-ray diffraction pattern using Rigaku XRD with Bragg-Brentano geometry. XRD measurements take in the range of 2θ 10°-90° with Co-K radiation used and operated at 40 kV and 25 mA. Scan Speed used is 5°/min with a step width of 0.01 deg. Diffractions were processed into a graph using Match 3 software to determine the majority of crystals contained in bentonite and their changes before and after activation.

Clarification measurement

Crude stevia leaf extract solution was adsorbed according to Husada *et al*. (2018) using a batch method with activated Na and Ca-bentonite at a ratio of 1:10 (w/v). Adsorption was carried out in the vial, which was stirred at a speed of 210 rpm for 3 hours. The extract was then separated from bentonite using a centrifuge at 6.000 rpm for 10 minutes. The absorbance of the stevia leaf extract solution before and after the adsorption process was measured by spectrophotometer at a λ of 665nm (for green pigment) and 410 nm (for yellow pigment) to obtain an increase in clarity value. The clarification of supernatant was analyzed with UV-VIS Spectrophotometer.

Tannin content analysis with folin-denis

The analysis followed the method of Kharismawati *et al.* (2009). The liquid sample was mixed with distilled water in a ratio of 1:7. After mixed well, 0.5 part of Folin Denis Reagent Solution was added. This solution was allowed to react completely for 3 minutes. One-part Na_2CO_3 was added to the mixture. Incubate the solution in a dark room for 30 minutes before reading it on a spectrophotometer at a wavelength of 745 nm. Tannin concentration (x) calculated by linear regression formula $y = 49.1003x - 0.0114$ and a regression of R^2 0.9951.

Stevioside analysis with high-performance liquid chromatography (HPLC)

A modified method by Chaturvedula & Zamora (2014) was used to analyze the stevioside concentration of stevia crude extract solution by using HPLC. The HPLC used was Waters with UV-VIS detector at the λ 202 nm and C18 OSD X-bridge™ column. The method used was a reverse-phase at 60°C with elution buffer Na-Phosphate and acetonitrile (68:32) pH of 2.63. The standard series was made from 200 – 1000 ppm of stevioside to verify the method. These standard series should have R^2 value between 0.995 – 1.00 to be qualified to calculate the stevioside content in the sample.

Results and Discussion

Bentonite activation

The activation process was aimed to obtain bentonite with a greater adsorption capacity and a better crystal to clarify the crude stevia leaf extract. Design of experiment using Central Composite Design (CCD) performed with setting the lowest and highest value used. In this research, the lowest H_2SO_4 concentration for activating bentonite is 1 N (Yildiz *et al.*, 2010), while the highest used is 5 N. Meanwhile, the temperature was set from 400-600°C. From this variable, Minitab 17 generated 13 combinations condition to be analyzed. The combination of experimental design and response can be seen in Table 1.

The Methylene Blue Number (MBN) is the maximum amount of dye absorbed by 1 g of adsorbent or color concentration in solids. MBN determines the adsorption capacity of adsorbents in the batch adsorption method (Raposo *et al.*, 2009). This adsorbent is expected to eliminate as much as pigment and tannin in stevia extract. Since pigment and tannin in stevia extract vary widely, we standardized the analytical parameters with methylene blue number.

The MBN response for activated Na and Ca bentonite increased compared to natural bentonite. These results align with previous research by Yildiz *et al.* (2004) and Noyan *et al*. (2008). However, the addition of H_2SO_4 concentration did not always positively correlate with adsorption capacity, as the table showed that higher concentrations have a lower MBN. Mohammad et al. (2020) reported a similar result that low concentrate H_2SO_4 produces the highest adsorption capacity. The cations in the dispersed double layer of bentonite are exchangeable with those in the free water. Therefore, methylene blue will exchange cations from both of these sources. To eliminate the free water cations, clay samples must be mixed with sufficient deionized water to dissolve the precipitated salts and cations in the free pore fluid. On these clay edges, the proton surface charge depends on the physico-chemical parameters of the solution (pH, ionic strength) that control the protonation state. The ion exchange must occur faster between the solution and the basal planes than between the solution and the interlayer flats.

The optimum condition for activating Na and Ca-bentonite was calculated using Minitab 17 software. A further test for suitability of the model formed is done by looking at the p-value of lack of fit (Table 2). This further test shows a not significant result in lack of fit and a significant result for the model used (p value >0.05). Therefore, this optimization model is acceptable for optimization of the bentonite Na and Ca activation conditions.

The optimum condition of H_2SO_4 concentration for activating Na and Ca-bentonite was calculated using Minitab 17 software. The optimum concentration is 0.17 N for both. While the optimum temperature for activating Na and Ca bentonite is 358°C and 481°C, respectively. These results are shown in Figure 1, where X-axis is acid concentration, Y-axis is the parameter calculated as adsorption capacity with MBN value, and Z-axis is for the temperature used. This result is in contrast to Husada *et al.* (2018), which was used H_2SO_4 at 3 N as a reference to determine 0 points in CCD-RSM experimental design. It is caused by the different sources of bentonite used, so they have other active groups and produce different optimum activation points. The Figure shows an optimum adsorption capacity at a low concentration of H_2SO_4. It means the active site of bentonite was already saturated and fully charged with H^+ from the acid at a low concentration. The temperature effect on adsorption capacity in Na bentonite significantly decreased along with the increase in temperature.

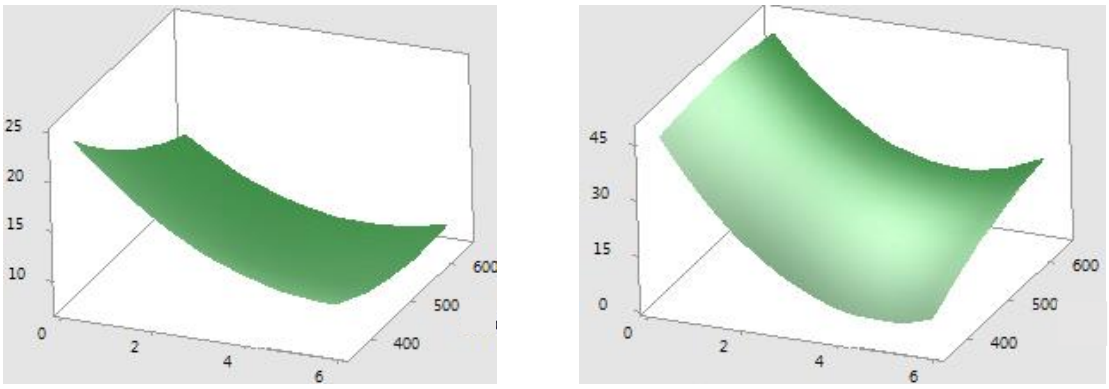
Optimized activated bentonite was then calculated its adsorption capacity using methylene blue solution. Methylene blue is often used as a reference analysis to identify adsorbent properties' initial identification (Nunes & Guerreiro, 2011). The comparison data of adsorption capacity on its equilibrium concentration is presented in Figure 2. The optimization process is used to maximize the adsorption capacity from bentonite. The adsorption capacity of activated Na and Ca-bentonite reached up to 197.72 *mg* g^{-1} and 169.52 *mg* g^{-1}, respectively. Each of these values is higher than the adsorption capacity of natural bentonite. This escalation proves that the activation process has been successful in increasing the adsorption capacity of natural bentonite.

Table 1. The combination of optimization treatment and the response of methylene blue number
Tabel 1. Kombinasi perlakuan optimasi dan respons terhadap nilai metilen biru

No	H_2SO_4 concentration *Konsentrasi H_2SO_4* (N)	Heating temperature *Suhu pemanasan* (°C)	Methylene blue number response (*mg* g^{-1}) *Respons nilai metilen biru (mg* g^{-1}*)*	
			Na Bentonite	Ca Bentonite
1	0.17	500	19.32	45.64
2	3.00	358	10.77	5.42
3	1.00	600	8.58	33.75
4	5.00	600	7.47	16.78
5	1.00	400	19.52	38.17
6	3.00	500	8.67	18.42
7	3.00	500	9.81	13.31
8	3.00	500	9.57	17.41
9	3.00	641	10.57	19.54
10	5.00	400	14.56	13.39
11	3.00	500	11.66	17.15
12	5.83	500	6.07	15.11
13	3.00	500	9.63	17.60
14	-	-	15.65	38.23

Table 2. Optimization of bentonite using Minitab 17
Tabel 2. Optimasi bentonit menggunakan Minitab 17

Parameter *Parameter*	Activated Na bentonite *Na bentonit teraktivasi*	Activated Ca bentonite *Ca bentonit teraktivasi*
p-value of Lack of fit	0.100	0.401
R-sq	88.39%	93.13%



(a) (b)

Figure 1. Optimization curve of adsorption capacity of (a) Na-Bentonite (b) Ca-bentonite
Gambar 1. Kurva optimasi kapasitas adsorpsi dari (a) Na bentonit (b) Ca bentonit

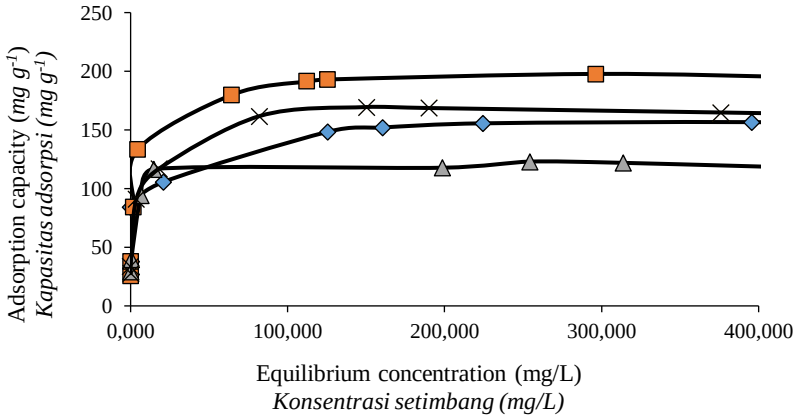


Figure 2. Equilibrium concentration comparison curve of activated and inactivated Na bentonite and Ca bentonite (Na inactivated, Na activated, Ca inactivated, Ca activated)
Gambar 2. Kurva perbandingan konsentrasi setimbang dari Na dan Ca bentonit teraktivasi dan tidak teraktivasi (Na inactivated, Na activated, Ca inactivated, Ca activated)

X-ray diffraction crystal analysis

Crystal formed in Na and Ca bentonite before and after activation is shown on Figure 3. The peak trend of the 2θ formed is pointed upwards with a low baseline in Na bentonite before and after activation. Sharp peaks were also seen in Ca bentonite before and after activation but tended to have a high baseline. This response did not affect the XRD readings, which were carried out qualitatively. The JCPDS 2θ value at 20º, 35º, 55º, and 63º shown in the graph represents *montmorillonite* crystal (Ruskandi *et al.*, 2020). Meanwhile, the quartz crystal shows a high d-spacing value at 2θ 26º, 39º and 42º. So, bentonite used in this research has a composition of *montmorillonite* and most *quartz* (SiO_2).

Based on the diffraction pattern, there is a left shift in 2θ axis while there is an increment of d-value (basal spacing), which indicates the formation of an active site due to the activation process. The entry of H^+ cations from acid between the layers of bentonite minerals replaces the charge balancing cations of the silicate layer in the mineral, causing an expansion of the distance between the existing silicate layers (Mahmudha *et al.*, 2016). The increase is expected to absorb more impurities in the leaf extract and be more selective to maintain the stevioside content.

Batch adsorption for stevia leaf extract

Activated bentonite was then used as an adsorbent for clarification of crude stevia leaf extract. The factor that affects the color of stevia leaf extract is chlorophyll pigment. There are two types of chlorophyll pigment in leaves: chlorophyll A with a dark green color tendency and chlorophyll B with a yellowish-green color (Chaturvedulla & Prakash, 2011). The chlorophyll pigment in stevia leaves contains Mg2+ ions, which bind to protein molecules in the plant matrix; during the extraction process by heating, these molecules are denatured

and unstable to be easily separated and replaced by hydrogen molecules (Arumsari *et al.*, 2019). This process makes a dark color at the extract used in this research. In previous studies (Mahl *et al.*, 2010; Gasamalla *et al.*, 2015), brown color positively correlates to flavonoids, phenols, tannins, and the unpleasant and bitter taste of the extract by separating these compounds.

Figure 4 presents the clarity of stevia extract in quantitative and qualitative. In general, both Na and Ca-bentonite can adsorb more pigments at lower wavelengths (410 nm) for yellow pigment. However, the application of activated Ca-bentonite produces higher clarification for stevia leaf extract compared with activated Na-bentonite. These results can be seen from the more amorphous crystals lost in activated Ca-bentonite so that it provides better clarification than activated Na-bentonite.

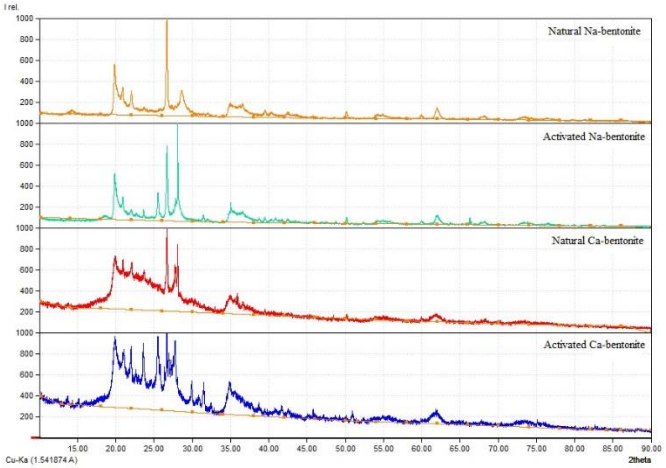
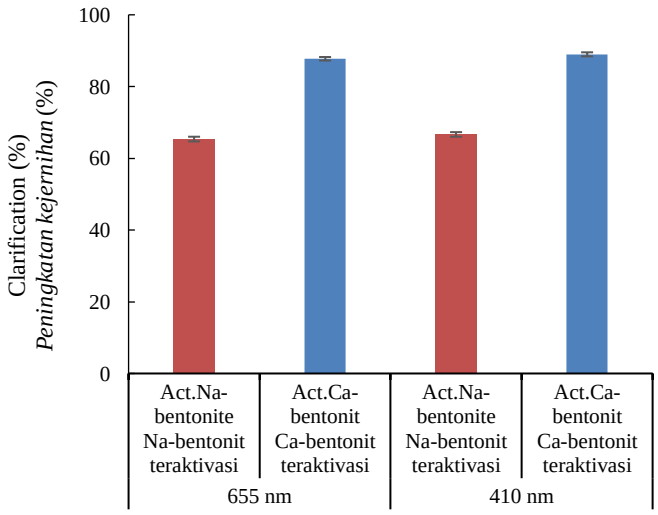
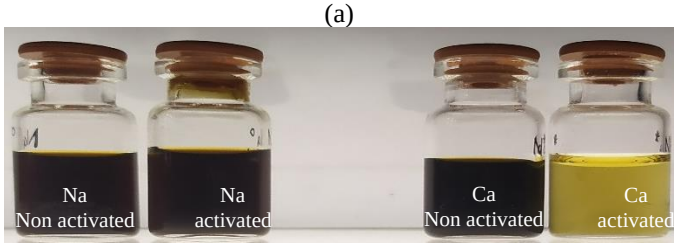


Figure 3. Bentonite diffraction by XRD
Gambar 3. Difraksi bentonit dengan XRD



(a)



(b)

Figure 4. Stevia leaf extract before and after adsorption process (a) quantitative (b) qualitative
Gambar 4. Ekstrak daun stevia sebelum dan setelah proses adsorpsi (a)kuantitatif (b)kualitatif

Tannin analysis with Folin-Denis at stevia leaf extract

Tannin concentration shows the greatest impact on taste and mouthfeel, significantly increasing sourness, bitterness, astringent texture, drying, and overall astringency (Frost *et al.*, 2017). Polyphenols as tannin may contribute directly to plant-based foodstuffs flavor, particularly astringency and bitterness (Soares *et al.*, 2020). For making a consumable stevia extract, this component must be eliminated.

Tannin content in stevia leaf extract before purification was 850 ppm. The tannin content in the extract makes the taste unpleasant, so that it reduces the desire to consume. Degradation of tannin can be seen in Figure 5 after the adsorption process carried out with activated and natural bentonite. Tannin levels in the solution after applying natural Na-bentonite decreased compared to the initial solution, but the levels were still high. Along with the decrease in tannins, it is hoped that the unpleasant taste in the extract will disappear. The application of activated and natural Ca bentonite significantly reduced tannin levels up to 97%. This research has a better result than Kusumaningsih *et al.* (2015) using activated charcoal only by 39.74%.

Stevioside content in stevia leaf extract

Water is a safe solvent used to extract stevia leaf for consumption. Besides, stevioside compounds, which are responsible for the sweet taste are more soluble in polar. Stevia extract in water has a dark green color that becomes brown due to the browning process caused by the heating during the extraction process. The main advantage of stevioside over other compounds in stevia extract is stable at 100°C (Buckenhuskers & Omran, 1997). The extract has about 6-10% stevioside content with up to 200 times the sweetness of glucose (Shamima *et al.*, 2019). The results of the analysis of stevioside levels in the extract used can be seen in Figure 6.

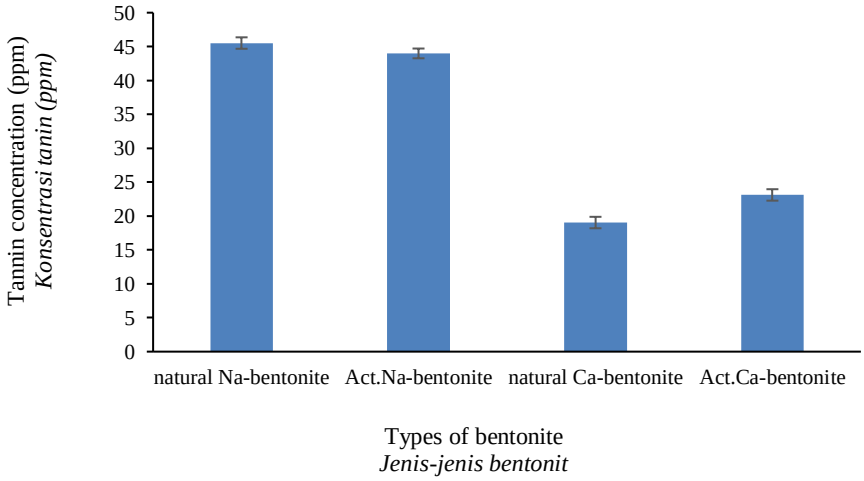


Figure 5. Tanin concentration after purification
Gambar 5. Konsentrasi tannin setelah pemurnian

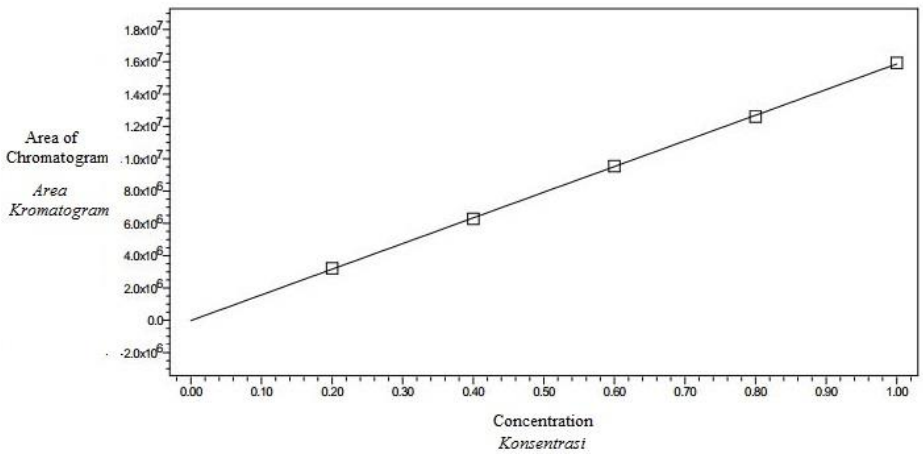


Figure 6. Standard series of stevioside
Gambar 6. Deret standar steviosida

The more water used for extraction, the more stevioside is extracted from stevia leaves, but the concentration of stevioside in the extract decreased (Refai *et al.*, 2020). Pigments present in the crude extract affected the purification process and affected the purity of the produced stevioside. Moreover, de-pigmentation as indication on the removal of pigments (carotenoids and chlorophylls a and b) were increased during this process. This means at the same time that the natural stevioside sweetener was extracted from the dried ground leaves by water was rich in pigments. These results are in line with those mentioned by Abdel-salam *et al.* (2009).

Before injecting the sample, verification of the modified method used had been carried out. A series of stevioside standard was analyzed at 200, 400, 600, 800, and 1000 ppm. Then the correlation between concentration and area read from HPLC is depicted in graphical form, which can be seen in Figure 6. The linearity of the curve is then determined and produces the equation of $y = 1.59e^{7}x - 1.54e^{4}$ with R^2 0.999798. By using this linear equation, x (concentration) of stevioside in the solution can be calculated.

Quantitative calculation of stevioside using linier equation from standard series mentioned before. Steviosides in stevia leaves have varying levels based on the extraction technique and solvent used (Kartikasari *et al*. 2018; Wuryanto & Wahono, 2014). In this research, the crude extract having a stevioside concentration of 10.04 ± 0.26 g/100 mL. From Figure 7, Ca bentonite resulting a slightly similar concentration of stevioside after the adsorption process between crude extract, natural and activated bentonite. Meanwhile, Na bentonite shows a different level of stevioside resulted from the adsorption process, which produce the lowest concentration at adsorption with natural Na bentonite. Stevioside level after treatment with activated Na bentonite is 1.02 ± 0.37 g/100 mL extract. At the same condition, Ca bentonite adsorption for the stevia extract resulting the stevioside level at 4.19 ± 0.88 g/100 mL. Activated Ca bentonite can make a recovery level at as much as 41.75 % of stevioside from the crude extract.

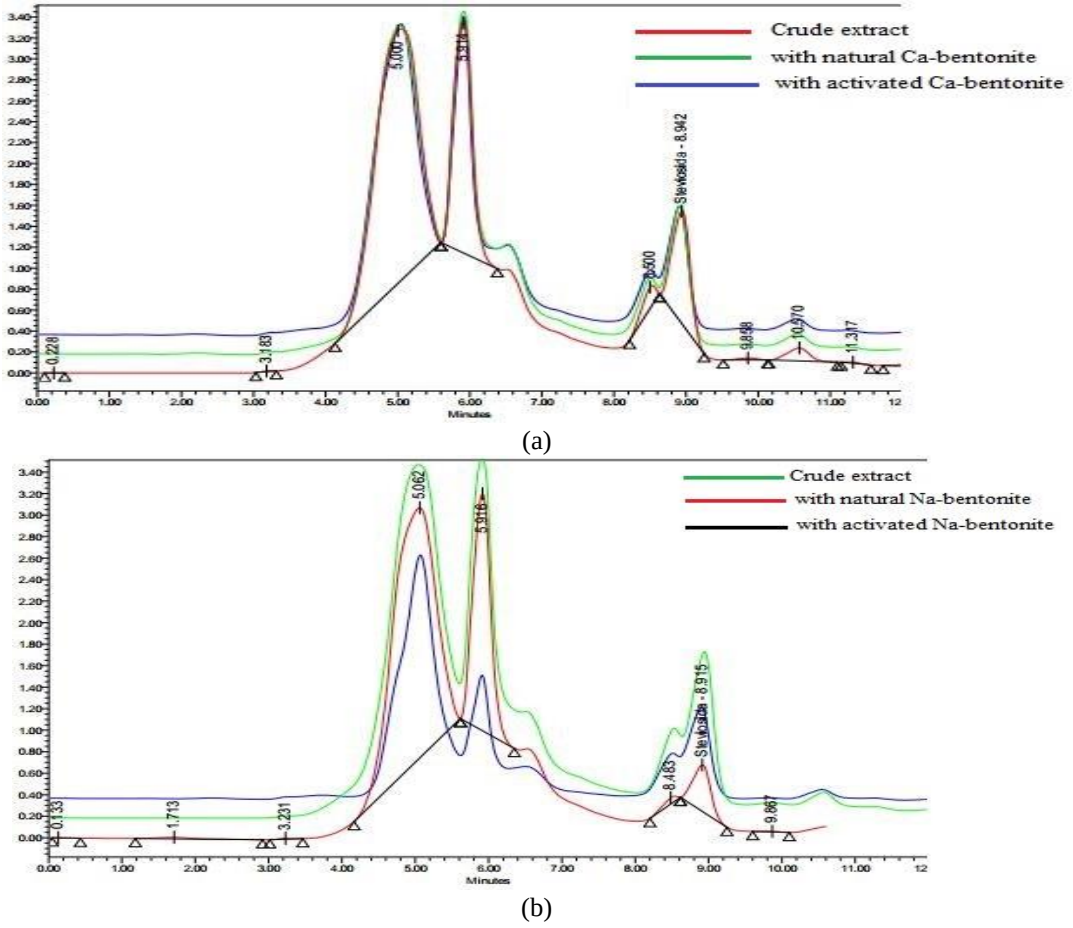


Figure 7. HPLC for stevia extract (a) using Ca-bentonite (b) using Na-bentonite

Gambar 7. KCKT untuk ekstrak stevia (a) dengan Ca-bentonit (b) dengan Na-bentonit

Conclusion

The best optimization conditions for the activation process of Na bentonite were 0.17 N H_2SO_4 and 358°C furnace heat, while for Ca bentonite were 0.17 N H_2SO_4 and heat of 481 °C. The maximum adsorption capacities of bentonite Na and Ca were 197.72 *mg* g^{-1} methylene blue and 169.52 *mg* g^{-1} methylene blue, respectively. The adsorbent application to the crude extract of stevia leaves resulted in a promising purification step with an increase of 86.64% in clarity and an increase of 50.64% in recovery of stevioside content using activated Ca-bentonite.

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