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Research Article

SEPARATION OF REBAUDIOSIDE A FROM *Stevia rebaudiana* EXTRACT USING LOW POLARITY RESIN AB-8

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ABSTRACT

There are many methods to separate or purify the rebaudioside A compound from *Stevia rebaudiana* extract. However, the ion-exchange chromatography using macroporous resin is still the most popular among those methods. The separation of rebaudioside A from stevia crude extract by macroporous resin AB-8 was optimised in this adsorption separation study. This approach was applied to evaluate the influence of four factors such as the adsorption temperature, desorption time, elution solution ratio, and adsorption volume on rebaudioside A yield of the purified stevia extract. The results showed that the low polarity resin AB-8 is able to separate rebaudioside A from stevia extract with 0.601 in yield compared to the high polarity resin HPD 600 with 0.204 in yield used in Anvari and Khayati study. The best conditions for rebaudioside A separation by macroporous resin AB-8 were at 35°C of adsorption temperature, 30 min of desorption time, elution solution ratio 2:1, and 50 mL of adsorption volume.

Keywords: sweetener, *Stevia rebaudiana*, rebaudioside A, Steviol glycoside, adsorption separation method, macroporous resin

INTRODUCTION

Stevia rebaudiana Bertoni from Asteraceae family is a natural non-caloric sweetener native to Paraguay. This natural sweetener contains 11 main steviol glycosides which give the sweet taste in stevia included stevioside, rebaudioside A to F, rubusoside, steviolmonoside, steviolbioside, and dulcoside A (Geuns, 2003). The chemical structures of stevioside and rebaudioside A are very similar to each other but they showed some differences in sweetness (Wheeler et al., 2008). Stevioside tastes about 150 – 300 times sweeter than sucrose and rebaudioside A tastes about 200 – 400 times sweeter than sucrose. In addition, rebaudioside A has the bitter after taste. Meanwhile, stevioside has no bitterness (Geuns, 2003).

Rebaudioside A and stevioside are the major compounds in steviol glycosides, both of the compounds added up to more than 75% of the total steviol glycosides in stevia extract. Moreover, rebaudioside A has the best quality for sweetness amongst the other steviol glycosides, close to that of glucose (Chatsudthipong & Muanprasat, 2009). Therefore, rebaudioside A is considered to be the major component in stevia, and the demand for rebaudioside A is increasing in the stevia industry.

In the previous study, a few methods have been reported to extract rebaudioside A from *S. rebaudiana* (Zhang, Chen, Shi, & He, 1998; Liu, Li, Xu, & Zhou, 2007). Among all these methods, the adsorption separation technique using macroporous adsorption resin is a relatively new and popular separation method used for rebaudioside A at present. Since this macroporous resin has a special selectivity and good stability, some more low cost and easy regeneration which attracted the industry to use this method in rebaudioside A separation (Babic, Van der Ham, & De Haan, 2006; Liu et al., 2006).

From the previous study, the macroporous resin type HPD 400 and HPD 600 used for the separation of rebaudioside A has previously been studied by Anvari and Khayati (2016). Both resin type HPD 400 and HPD 600 are middle and strong polarity resin, respectively. Based on the results found from the study, the resin HPD 600 has a lower adsorption capacity for rebaudioside A compared to HPD 400. This is because rebaudioside A has higher polar hydrocarbon molecules, therefore, the low polar resin has shown better adsorption capacity than high polar resin for rebaudioside A (Anvari & Khayati, 2016). In this study, resin type AB-8 with low polarity was used to optimise the factors such as desorption time, adsorption volume, elution ratio, and adsorption temperature that affected the efficiency of rebaudioside A separation.

MATERIAL AND METHODS

Materials

New *S. rebaudiana* variety S10A produced through mutation breeding using gamma irradiation in Malaysian Nuclear Agency was planted by Duta Nusajaya Sdn. Bhd. in Penampang, Sabah, Malaysia. The leaves were harvested, washed, and dried at 40°C for 24 h then stored at room temperature. The macroporous resin AB-8 was used in the experiment and its physical properties as listed in Table 1. The resins were pre-treated by dipping them in ethanol for 48 h, then washing with ultrapure water thoroughly to remove the monomers and porogenic agents trapped inside the pores during the synthesis process.

Table 1 Physical properties of the macroporous resin

Resin type	Functional group	Average pore (nm)	Particle diameter (mm)	Polarity
HPD 600	Acylamino polystyrene	7.1	0.3 – 1.25	High polarity
HPD 400	Polydivinyl benzene acrylic ester	8.3	0.3 – 1.20	Middle polarity
AB-8	Polystyrene	13 – 14	0.3 – 1.25	Low polarity

Preparation of Crude Sample

Approximately 40 g of the *S. rebaudiana* leaves were extracted three times, with 1 h for each time in 4 L of boiling water, and all of the clear extracts were combined. The extracts were evaporated in a heating mantle boiler at 100°C to achieve the final volume of 2 L. The concentrated raw solution was used as the feed in all the following experiments.

Experimental Design

Taguchi experimental method with L16 array was used to optimise the separation of steviol glycoside (Sorana & Lorentz, 2007). The factors included desorption time, adsorption volume, elution solution ratio, and adsorption temperature were studied. The experimental factors are given in Table 2. For each experimental trial, the rebaudioside A yield was determined.

Adsorption/Desorption Experiments

The extraction process was conducted on stevia variant produced by Malaysian Nuclear Agency. Different volumes of crude extract solution were added into 5 g of macroporous resin in separate screwed cap bottle and shook in an incubator shaker for 4 h at 35, 40, 45, and 50°C. After that, the resins were washed 5 times with ultrapure water, then desorbed it in 100 mL ethanol-ethyl acetate solution at 30, 60, 90, and 120 min in the incubation shaker (Table 2). Finally, the desorption solution was evaporated and dried in an oven at 100°C to constant weight. All the experiments were carried out in triplicate.

Table 2 L16 array experimental design and the results of rebaudioside A yield

Sample no.	Factor levels				Average rebaudioside A yield
	Adso. temp (°C)	Deso. time (min)	Elu. ratio	Adso. vol. (mL)	
1	35	30	1	50	0.601
2	35	60	2	100	0.711
3	35	90	3	150	0.579
4	35	120	4	200	0.438
5	40	30	2	150	0.595
6	40	60	1	200	0.507
7	40	90	4	50	0.715
8	40	120	3	100	0.661
9	45	30	3	200	0.484
10	45	60	4	150	0.580
11	45	90	1	100	0.645
12	45	120	2	50	0.687
13	50	30	4	100	0.650
14	50	60	3	50	0.667
15	50	90	2	200	0.489
16	50	120	1	150	0.574

The initial concentration of the solution was 1.3 mg/ml.

Analysis of Samples

The amount of rebaudioside A in each sample was analyzed using high-performance liquid chromatography (HPLC). The sample was diluted in 70% (v/v) ethanol-water solution. The rebaudioside A standard was purchased from Sigma-Aldrich and used in this experiment.

$$\text{Rebaudioside A yield (µg/mL)} = \frac{\text{rebaudioside A (purified extract)}}{\text{rebaudioside A (present in the feed)}}$$

RESULTS AND DISCUSSION

The results showed rebaudioside A yield in the range from 0.438 – 0.715 (µg/mL)/(µg/mL) corresponding to the four factors (Table 2). Tables 3 – 6 showed the effects of each factor, which determined the contributions of individual variables on the rebaudioside A yield. Measurement averages were made at the level of each factor.

Table 3 Adsorption temperature effect on the rebaudioside A yield

Sample no.	Adso. temp (°C)	Yield [ug/ug]	Average yield [ug/ug]
1	35	0.601	0.582
2	35	0.711	
3	35	0.579	
4	35	0.438	
5	40	0.595	0.620
6	40	0.507	
7	40	0.715	
8	40	0.661	
9	45	0.484	0.599
10	45	0.580	
11	45	0.645	
12	45	0.687	
13	50	0.650	0.595
14	50	0.667	
15	50	0.489	
16	50	0.574	

Table 4 Desorption time effect on the rebaudioside A yield

Sample no.	Deso. time (min)	Yield [ug/ug]	Average yield [ug/ug]
1	30	0.601	0.583
5	30	0.595	
9	30	0.484	
13	30	0.650	
2	60	0.711	0.616
6	60	0.507	
10	60	0.580	
14	60	0.667	
3	90	0.579	0.607
7	90	0.715	
11	90	0.645	
15	90	0.489	
4	120	0.438	0.590
8	120	0.661	
12	120	0.687	
16	120	0.574	

Table 5 Elution solution ratio effect on the rebaudioside A yield

Sample no.	Elu. ratio	Yield [ug/ug]	Average yield [ug/ug]
1	1:1	0.601	0.582
6	1:1	0.507	
11	1:1	0.645	
16	1:1	0.574	
2	2:1	0.711	0.621
5	2:1	0.595	
12	2:1	0.687	
15	2:1	0.489	
3	3:1	0.579	0.598
8	3:1	0.661	
9	3:1	0.484	
14	3:1	0.667	
4	4:1	0.438	0.596
7	4:1	0.715	
10	4:1	0.580	
13	4:1	0.650	

Table 6 Adsorption volume effect on the rebaudioside A yield

Sample no.	Adso. vol. (mL)	Yield [ug/ug]	Average yield [ug/ug]
1	50	0.601	0.668
7	50	0.715	
12	50	0.687	
14	50	0.667	
2	100	0.711	0.667
8	100	0.661	
11	100	0.645	
13	100	0.650	
3	150	0.579	0.582
5	150	0.595	
10	150	0.580	
16	150	0.574	
4	200	0.438	0.480
6	200	0.507	
9	200	0.484	
15	200	0.489	

The study of adsorption temperatures on the separation of rebaudioside A found that the yield was slightly increased when the adsorption temperature increased from 35°C to 40°C but decreased with the increased of adsorption temperature above 40°C (Table 3). This is because of the macroporous resin adsorption process was an exothermic process. In this experiment, any increasing of temperature more than 40°C was adverse to adsorption (Fu, Shen, & Yao, 1990). The desorption time effect on rebaudioside A yield is shown in Table 4. It showed that rebaudioside A yield increased with the addition of desorption time from 30 min to 60 min and achieved the highest yield at 60 min then started to decrease after 60 min. The results showed an extended time beyond 60 min only caused a reduction in the yield.

The results of elution solution ratios showed no significant differences between the ratios. However, the highest rebaudioside A reading, 0.621 (µg/mL)/(µg/mL) was obtained at the ratio 2:1 (Table 5). A decreased in yield was obtained when the elution solvent ratio was extending the optimum level. This is because macroporous resins did not swell completely in the high ethanol contained a desorption solution. According to the experiment, the increase of elution ratio will cause the increase of ethanol in desorption solution, which made the adsorbed rebaudioside A not easily desorbed from macroporous resins. Table 6 showed the correlation between rebaudioside A yield and adsorption volume. The yield of rebaudioside A decreased with the increase

of adsorption volume. It is probably due to the macroporous resin had a different affinity toward stevia solution. The resin becomes saturated at the volume of 50 to 100 mL, further increase of the adsorption volume caused a significant reduction in yield.

The analysis of variance for the experiment is shown in Table 7. The degree of significance of each factor was represented by its *P*-value. A significance level of 0.05 indicates a 5% risk of concluding that a difference exists when there is no actual difference. In this study, the adsorption volume factor has a *P*-value of less than 0.05. It means the differences between some of the means are statistically significant. However, the other factors showed no significant differences between the means with *P*-value greater than 0.05. The results showed that all factors included adsorption temperature, desorption time and elution solution ratio except adsorption volume were no significant.

Table 7 Analysis of variance of the parameters for the rebaudioside A separation experiment

Source	D.F.	Sum of square	Mean square	*F*-value	*P*-value
Adsorption temperature (°C)	3	0.002	0.001	0.1606	0.9199
Residual error	8	0.036	0.005	–	–
Total	11	0.038			
Desorption time (min)	3	0.002	0.001	0.3563	0.7862
Residual error	8	0.015	0.002	–	–
Total	11	0.018			
Elution solution ratio	3	0.002	0.001	0.4267	0.7394
Residual error	8	0.015	0.002	–	–
Total	11	0.017			
Adsorption volume (mL)	3	0.072	0.024	10.0386	0.0044
Residual error	8	0.019	0.002	–	–
Total	11	0.091			

Based on the results from these four factors, we can determine that the adsorption temperature at 35°C, desorption time at 30 min, elution solution ratio at 2:1 and adsorption volume of 50 ml stevia extract for every 5 g of resin used were the best conditions optimised in this study. Under the optimised conditions, the low polarity resin AB-8 is more efficient to separate rebaudioside A from stevia extract compared

to the high polarity resin HPD 600 used in Anvari and Khayati (2016) study. The rebaudioside A yields were 0.601 and 0.204 in resin AB-8 and HPD 600 respectively. Two of the *S. rebaudiana* marker compounds, rebaudioside A and stevioside were detected and identified in the HPLC chromatogram with comparison to the standard rebaudioside A and stevioside (Figure 1). We ensure that the adsorption separation technique using macroporous resin AB-8 can be applied to separate the rebaudioside A and stevioside from *S. rebaudiana*.

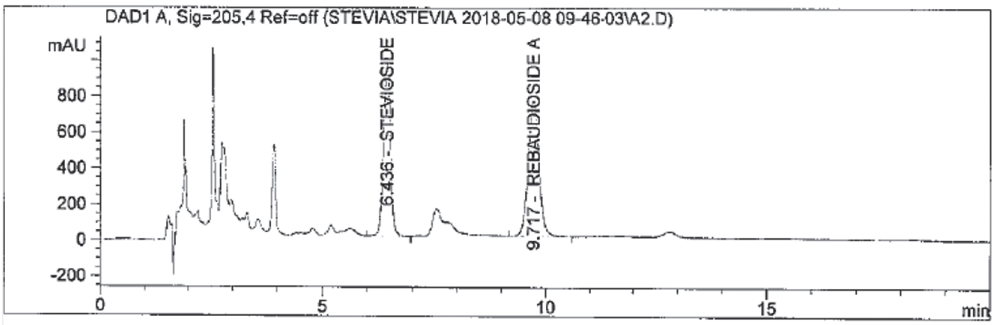

Figure 1 Two of the major marker compounds for *S. rebaudiana*, the rebaudioside A and stevioside were detected in the purified stevia extract in HPLC analysis

CONCLUSIONS

The adsorption separation process of rebaudioside A with macroporous adsorption resin AB-8 was studied and optimised in this work. A low polarity resin AB-8 was used to replace the high polarity resin in this study to see the affinity of steviol glycosides towards different polarity resin. The optimised separation process will be used as the reference for the establishment of a pilot-scale extraction plant for stevia.

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