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Optimization of *Rebaudioside A* extraction from stevia leaves using response surface methodology

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Abstract. Rebaudioside A or Reb A is an extractable component from stevia leaves or scientifically known as Stevia rebaudiana. Reb A is famous for its exceptional sweetness and widely used as a non-caloric sweetener. Its potential widespread use requires an easy and effective extraction method. In this study, extraction of Reb A from stevia leaves with parameters such as temperature, material ratio and extraction time were investigated. Time of extraction were 1, 1.5, 2, 2.5 and 3 hours. Temperature was varied at 40^0C, 45^0C, 50^0C, 55^0C and 60^0C. Material ratio was 1:5, 1:10, 1:15, 1:20 and 1:25. Then, using Design Expert software, the ranges of the parameters were entered in the Central Composite Design (CCD) to create 20 different combinations of parameters for extraction. After executing the experiments, the yield of Reb A obtained was keyed in Response Surface Methodology (RSM) for optimization. Finally, the optimum condition was tested and validated by calculating the percentage error. Since the percentage error was less than 10%, the optimum condition, which is, 35^0C, 1:6 material ratio and 5 hours of extraction was accepted.

1. Introduction

The expanding usage of sugar has brought about a few nutritional and medical issues such as obesity which leads to diabetics and eventually cardiovascular diseases with more attention on dental caries issue. Increase in the number of the diseases can be related to uncontrolled intake of glucose and high-calorie sweeteners [1]. World Health Organization (WHO) detailed that 371 million individuals worldwide have diabetes in 2011. The assessed number of diabetics increased in 2014 to 422 million. There are around 1.6 million people passed away due to diabetes in 2016 [2].

The food industry customarily uses table sugar as the main source of sweetening agent. However, there is an expanding interest among customers for Stevia-based natural source sweeteners [3]. Also, the sweetener is safe to be consumed, has almost zero calorie, unaffected by heat and pH changes and last but not least, tastes exactly like sugar. Stevia is a pleasant tasting herb and a member of the daisy family [4]. There are many types of stevia such as *Stevia eupatoria*, *Stevia rebaudiana* and *Stevia salicifolia*. *Stevia rebaudiana* is one of the 154 members from the genus stevia and one of just two that produce sweet glycosides [5].

In extraction processes, where there are various independent variables affecting the reaction, it is likely that the operational factors interface and influence each other's effects on the reaction. The customary way of investigating one factor at one time (OFAT) may be suitable in certain processes, however it failed to consider the combined effects of different components included. Hence, it is essential to utilize an optimization method that can determine many elements and significant interactions between these elements, so that an arrangement of ideal experimental conditions can be established [6]. RSM is a combination of statistical and mathematical methods commonly used in the food industry to quantify the impacts of a few factors and to optimize conditions [7]. This approach has been effective for increasing yields of enzymes [1] and therefore applied in this study to optimize the production of Reb A.

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The objectives of this research are to find out maximum yield of Reb A from stevia leaves under optimum extracting condition, to conduct statistical analysis of the parameters affecting the extraction process using RSM and to model the data obtained from the experiment.

2. Methodology

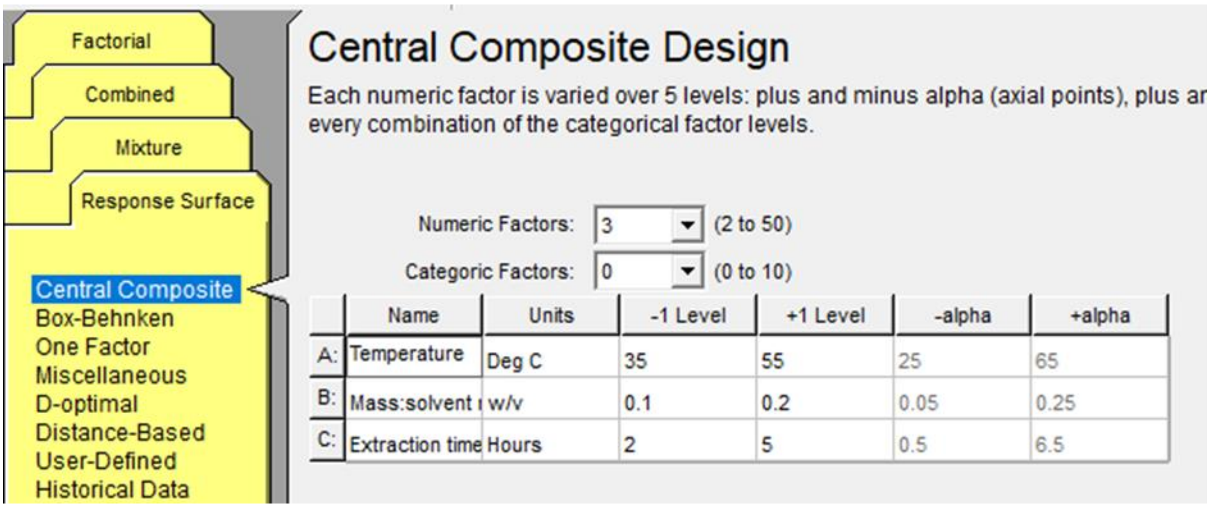
2.1 Materials & Apparatus
The apparatus and material used in this work are listed in table 1.

Table 1. List of apparatus & materials.

Apparatus/Material	Quantity	Source
Stevia leaves	1*kg*	Kuala Kangsar, Perak
Ethanol abs	1*L*	Fisher Scientific
Acetonitrile	1*L*	Fisher Scientific
Stevioside standard	10mg	Nano Life Quest
Oven	1	
Blender & sieve set	1	
Conical flask	10	
Incubator shaker	2	
Vacuum filtration set	1	
Filter paper(110*mm*)	40	
Centrifuge tubes	30	
Centrifuge machine	1	
Culture tubes	25	
Eclipse Plus C18, 5μm, 4.6 x 250*mm*	1	
HPLC machine	1	
HPLC vials	20	
Disposable syringe with needles	20-25	

2.2. Planning design of experiment
The design of the experimental work is shown in table 2 and table 3.

Table 2. Central Composite Design (CCD).


Factorial
Combined
Mixture
Response Surface
Central Composite
Box-Behnken
One Factor
Miscellaneous
D-optimal
Distance-Based
User-Defined
Historical Data

Central Composite Design

Each numeric factor is varied over 5 levels: plus and minus alpha (axial points), plus ar
every combination of the categorical factor levels.

Numeric Factors: 3 (2 to 50)
Categoric Factors: 0 (0 to 10)

	Name	Units	-1 Level	+1 Level	-alpha	+alpha
A:	Temperature	Deg C	35	55	25	65
B:	Mass:solvent r	w/v	0.1	0.2	0.05	0.25
C:	Extraction time	Hours	2	5	0.5	6.5

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Table 3. Design of experiment (DOE).

Run	Temperature (^0C)	Stevia: Ethanol Ratio	Time (Hours)	Reb A Yield (%)
1	55.00	1:10	5.00	
2	45.00	1:6	3.50	
3	55.00	1:5	2.00	
4	45.00	1:6	3.50	
5	55.00	1:10	2.00	
6	25.00	1:6	3.50	
7	35.00	1:10	2.00	
8	45.00	1:20	3.50	
9	45.00	1:6	3.50	
10	45.00	1:4	3.50	
11	45.00	1:6	6.50	
12	65.00	1:6	3.50	
13	45.00	1:6	3.50	
14	35.00	1:5	5.00	
15	55.00	1:5	5.00	
16	45.00	1:6	0.50	
17	35.00	1:10	5.00	
18	45.00	1:6	3.50	
19	45.00	1:6	3.50	
20	35.00	1:5	2.00	

2.3. Sample Preparation and Extraction

The dried stevia leaves were further dried in an oven at 60^0C for 1 hour to remove any moisture left in them [8]. Then, the leaves were grinded to powder form in a heavy duty blender and sieved using 0.5*mm*, 0.2*mm* and 0.1*mm* sieves. The powder was collected in a sealable polyethylene bag and stored in a 4^0C chiller. 5*g* of the powdered stevia and 50*ml* of absolute ethanol were measured and put in a conical flask. The conical flask was then left for extraction in an incubator shaker for 5 hours at 55^0C and 150 rpm as shown in run 1. These 2 steps were repeated according to the remaining 19 runs in Table 3.

2.4. Centrifugation, Filtration and HPLC Analysis

After all 20 runs were completed successfully, the liquid phase containing the analyte was filtered from the solid phase by using filter paper (110*mm*). Then, the liquid phase was filled in separate centrifuge tubes and labelled. They were centrifuged at 1000 rpm for 1 hour. After centrifugation, the liquid phase separated into supernatant and precipitate. Vacuum filtration was done to separate supernatant from precipitate. The supernatant was filled in separate test tubes, labelled and sealed properly to avoid contamination. HPLC analysis was carried out using Eclipse Plus C18 (25*cm* × 4.6*mm* I.D., 5μm). The column temperature was at 27–28°*C* and UV detection was adjusted at 210*nm*. The injection volume was set to 10μL at a flow rate of 1*ml*/min. HPLC column was equilibrated by pumping mobile phase through it until a drift-free baseline was obtained. The chromatograms of the sample solution and the standard solution were recorded in 10 minutes. The chromatogram of each sample was compared to the standard to find the retention time of Reb A. The peak areas (mAU*s) of Reb A were calculated automatically by a solutions software equipped with HPLC [8].

2.5. Optimization and Validation

The percentage of Reb A yield was calculated using the formula:

$$\%\ Reb\ A = [Wr/W] \ x\ Ac\ x\ [1.20/Ar]\ x\ 100 \quad (1)$$

where Wr = weight (*mg*) of Reb A in the standard solution, W = weight of sample (*mg*), Ac = Peak area of Reb A from the sample solution and 1.20 = relative molecular weight of Reb A, Ar = Peak area of Reb A from the standard solution [8].

The percentage yields of Reb A for all 20 runs were keyed in the RSM. Analysis such as ANOVA was conducted, final model was formed and 3D model graph was plotted by RSM. In the model graph, the points where the maximum yield of Reb A produced were noted and the optimum values of the three parameters were recorded. In the optimization part, the values of the parameters and percentage yield of Reb A were set at optimum and maximum level respectively. RSM proposed an optimum condition with expected yield value for extraction of Reb A.

Extraction was conducted once again under the proposed optimum condition. After centrifugation and filtration, HPLC analysis was conducted on the supernatant to obtain the peak area of Reb A. The peak area was then substituted into equation (1) to calculate the percentage yield of Reb A. The percentage error in the yield value was calculated using the formula:

$$\%\ error = (\%\ yield_{EXP} - \%\ yield_{RSM}) \times 100\% / \%\ yield_{RSM} \qquad (2)$$

3. Results and discussion

3.1. Determination of optimum condition

Experiments were conducted according to the conditions in the DOE (Table 3) using ethanol and peak area of Reb A was obtained from HPLC analysis for each sample. From the peak area, percentage yield of Reb A was calculated using equation (1). Table 4 shows the percentage yield for Reb A in all 20 samples. The percentage yields of Reb A in table 4 were inserted in RSM and optimization was done. An optimum extraction condition was then proposed by RSM with expected percentage yield of Reb A. After extraction and HPLC analysis of optimum sample, the peak area of Reb A was used to calculate its yield value. The yield value of Reb A came to 0.164%. Then, the percentage error in the yield value was calculated. Since the percentage error was only 6.50% which is less than 10%, the proposed optimum condition and final model were accepted to be used for large scale production of Reb A.

Table 4. Percentage yield of Reb A in all 20 runs.

Run	Temperature (^{0}C)	Stevia: Ethanol Ratio	Time (Hours)	Reb A Yield (%)
1	55.00	1:10	5.00	0.142
2	45.00	1:6	3.50	0
3	55.00	1:5	2.00	0
4	45.00	1:6	3.50	0
5	55.00	1:10	2.00	0.1768
6	25.00	1:6	3.50	0.1158
7	35.00	1:10	2.00	0.1441
8	45.00	1:20	3.50	0.1938
9	45.00	1:6	3.50	0.21
10	45.00	1:4	3.50	0
11	45.00	1:6	6.50	0.1912
12	65.00	1:6	3.50	0
13	45.00	1:6	3.50	0.1628
14	35.00	1:5	5.00	0.1821
15	55.00	1:5	5.00	0
16	45.00	1:6	0.50	0
17	35.00	1:10	5.00	0.1345
18	45.00	1:6	3.50	0.1683
19	45.00	1:6	3.50	0.1676
20	35.00	1:5	2.00	0.1867

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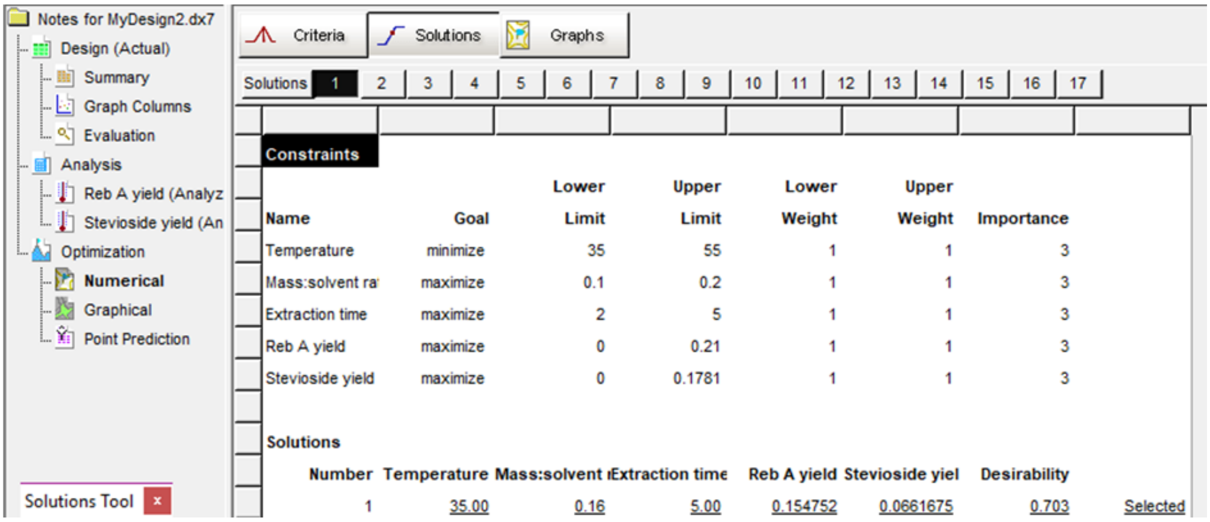
Table 5. Proposed optimum condition for extraction of Reb A.



Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	minimize	35	55	1	1	3
Mass:solvent ra	maximize	0.1	0.2	1	1	3
Extraction time	maximize	2	5	1	1	3
Reb A yield	maximize	0	0.21	1	1	3
Stevioside yield	maximize	0	0.1781	1	1	3

Solutions

Number	Temperature	Mass:solvent r	Extraction time	Reb A yield	Stevioside yiel	Desirability	
1	35.00	0.16	5.00	0.154752	0.0661675	0.703	Selected

3.2. *Interaction Effect of Factors on Reb A Yield*

3.2.1. Temperature with extraction time. As shown in figure 1, there was no significant interaction between temperature and extraction time. The percentage yield of Reb A increased with time as temperature decreased. The maximum yield of Reb A produced was 0.146018%.

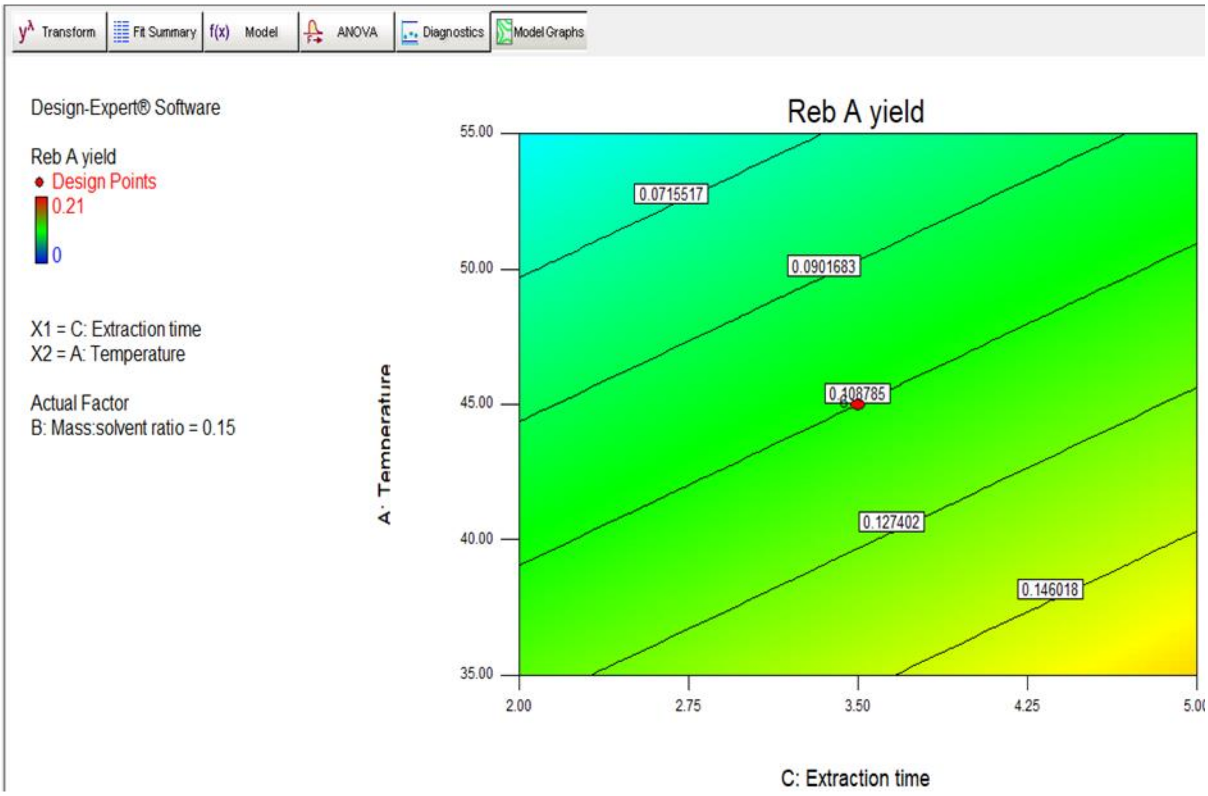


Figure 1. Model graph on interaction between temperature and extraction time.

3.2.2. Temperature with material ratio. As seen in figure 2, there was no significant interaction between the solute: solvent ratio and temperature. The yield value of Reb A increased with the material ratio, but the temperature continued to decrease. Maximum yield of Reb A produced was 0.157802%.

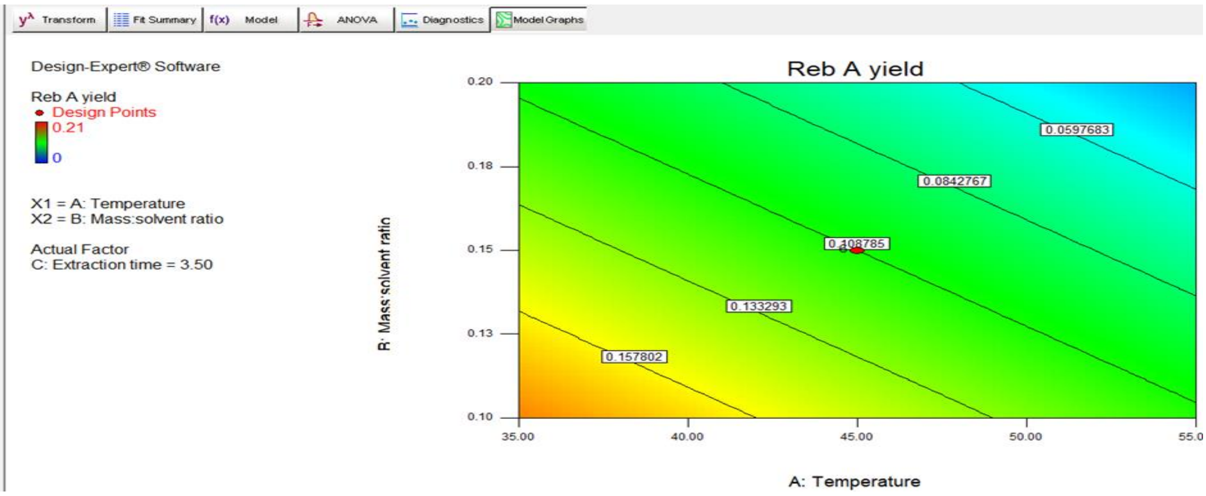


Figure 2. Model graph on interaction between stevia: ethanol ratio and temperature.

3.2.3. Extraction time with material ratio. As shown in figure 3, there was a notable interaction effect between extraction time and material ratio. The yield value of Reb A increased with material ratio and extraction time. Maximum yield of Reb A produced was 0.148352%.

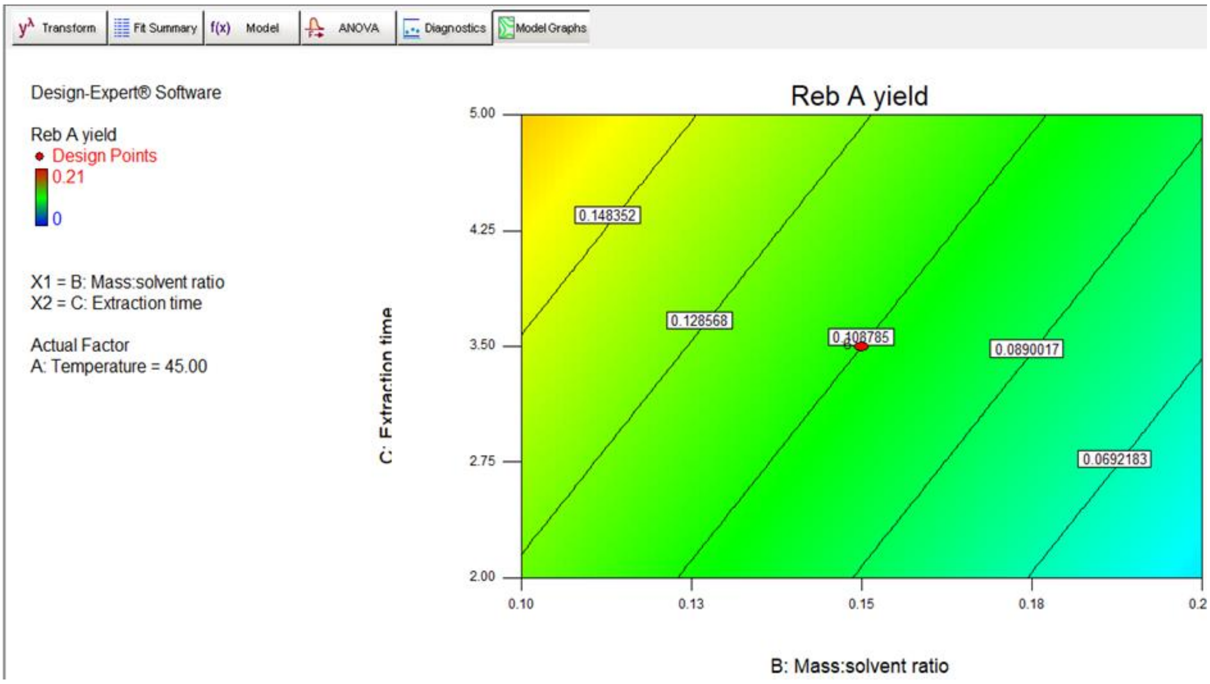


Figure 3. Model graph on interaction between material ratio and extraction time.

3.3. Hypothesis testing of the interaction effects
The ANOVA is shown in table 6.

Table 6. ANOVA table.

ANOVA for Response Surface 2FI Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.030	6	4.995E-003	1.49	0.2551	not significant
A-Temperature	*6.891E-004*	*1*	*6.891E-004*	*0.21*	*0.6573*	
B-Mass:solven	*1.823E-006*	*1*	*1.823E-006*	*5.451E-004*	*0.9817*	
C-Extraction ti	*0.013*	*1*	*0.013*	*3.92*	*0.0691*	
AB	*3.125E-004*	*1*	*3.125E-004*	*0.093*	*0.7647*	
AC	*1.191E-003*	*1*	*1.191E-003*	*0.36*	*0.5609*	
BC	*0.015*	*1*	*0.015*	*4.38*	*0.0565*	
Residual	0.043	13	3.344E-003			
Lack of Fit	*0.019*	*8*	*2.379E-003*	*0.49*	*0.8256*	*not significant*
Pure Error	*0.024*	*5*	*4.887E-003*			
Cor Total	0.073	19				

3.3.1. Hypothesis testing for interaction effect of temperature with extraction time (τγ).

H_0: $(\tau\gamma)_{11} = (\tau\gamma)_{12} = (\tau\gamma)_{13} = (\tau\gamma)_{14} = (\tau\gamma)_{15} = (\tau\gamma)_{21} = \ldots = (\tau\gamma)_{55} = 0$ (no interaction effect) Vs H_1: $(\tau\gamma)_{ik} \neq 0$; for at least one interaction of i and k (have interaction effect)

F_{cal} from the ANOVA table became 0.36.

$F_{\alpha,(a-1)(c-1),N-1}$ ($F_{0.05,16,19}$) from F distribution table became 2.2149.

Decision: Since F_{cal} (0.36) is lesser than ($F_{0.05,16,19}$) (2.2149), H_0 is accepted.

Conclusion: The interaction between temperature and extraction time does not have a significant effect on the yield of Reb A. This conclusion is supported by the p-value for the interaction effect of temperature with material ratio from the ANOVA since the p-value is 0.5609 which is higher than the type one error (0.05).

3.3.2. Hypothesis testing for interaction effect of temperature and material ratio (τβ).

H_0: $(\tau\beta)_{11} = (\tau\beta)_{12} = (\tau\beta)_{13} = (\tau\beta)_{14} = (\tau\beta)_{15} = (\tau\beta)_{21} = \ldots = (\tau\beta)_{55} = 0$ (no interaction effect) Vs H_1: $(\tau\beta)_{ij} \neq 0$; for at least one interaction of i and j (have interaction effect)

F_{cal} from the ANOVA table became 0.093.

$F_{\alpha,(a-1)(b-1),N-1}$ ($F_{0.05,16,19}$) from F distribution table became 2.2149.

Decision: Since F_{cal} (0.093) is lesser than ($F_{0.05,16,19}$) (2.2149), H_0 is accepted.

Conclusion: The interaction between temperature and material ratio does not have a significant effect on the yield of Reb A. This conclusion is supported by the p-value for the interaction effect of temperature with material ratio from the ANOVA since the p-value is 0.7647 which is higher than the type one error (0.05).

3.3.3. Hypothesis testing for interaction effect of material ratio with extraction time (βγ)

H_0: $(\beta\gamma)_{11} = (\beta\gamma)_{12} = (\beta\gamma)_{13} = (\beta\gamma)_{14} = (\beta\gamma)_{15} = (\beta\gamma)_{21} = \ldots = (\beta\gamma)_{55} = 0$ (no interaction effect) Vs H_1: $(\beta\gamma)_{jk} \neq 0$; for at least one interaction of j and k (have interaction effect)

F_{cal} from the ANOVA table became 4.38.

$F_{\alpha,(b-1)(c-1),N-1}$ ($F_{0.05,16,19}$) from F distribution table became 2.2149.

Decision: Since F_{cal} (4.38) is higher than ($F_{0.05,16,19}$) (2.2149), H_0 is rejected.

Conclusion: There is a significant effect of interaction between material ratio and extraction time on yield of Reb A.

3.4. Model development of experimental data

The statistical model for the general factorial design of three factors with two factor interaction as follows:

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\beta)_{ij} + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijkl} \quad (3)$$

i = 1, 2, ..., 5; j = 1, 2, …, 5; k = 1, 2, …, 5 and l = 1, 2, …, 20

where Y_{ijkl} is the response variable in this case the yield of Reb A, μ is the grand mean, τ_i is treatment one effect on the response variable in this case temperature effect on the yield of Reb A, β_j is treatment two effect on the response in this case material ratio effect on the yield of Reb A, γ_k is treatment three effect on the response in this case extraction time effect on the yield Reb A, $(\tau\beta)_{ij}$ is the interaction effect of temperature and material ratio on the yield of Reb A, $(\tau\gamma)_{ik}$ is the interaction effect of temperature and extraction time on the yield of Reb A, $(\beta\gamma)_{jk}$ is the interaction effect of extraction time and material ratio on the yield of Reb A and ε_{ijkl} is the random error term.

Table 7 and 8 shows the final model for the extraction in terms of coded factors and actual factors respectively.

Table 7. Final model in terms of coded factors.

Reb A yield =	
+0.052	
-6.563E-003	* A
+3.375E-004	* B
+0.029	* C
-6.250E-003	* A * B
+0.012	* A * C
-0.043	* B * C

where A stands for temperature, B stands for material ratio and C stands for time.

Table 8. Final model in terms of actual factors.

Reb A yield =	
-0.24172	
-1.62792E-003	* Temperature
+2.56658	* Mass:solvent ratio
+0.068092	* Extraction time
-0.012500	* Temperature * Mass:solvent ratio
+8.13333E-004	* Temperature * Extraction time
-0.57067	* Mass:solvent ratio * Extraction time

The error term (ε_{ijkl}) could not be estimated. Therefore, the reduced final model will be:

$$Reb\ A\ yield = 0.052 + 0.029C + 0.012AC - 0.043BC \quad (4)$$

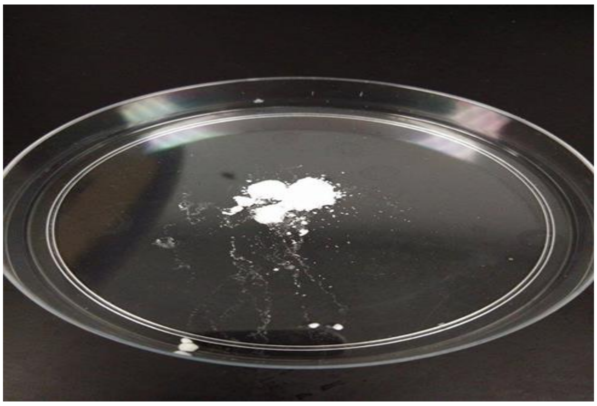


Figure 4. Pure Reb A.

4. Conclusion

In this study, RSM was used to conduct statistical analysis of the parameters involved in extraction process of Reb A. From the analysis, the optimum condition obtained was 35^0C, 1:6 material ratio and 5 hours while maximum yield retrieved was 0.164%. Data collected from the experiment was reduced to a model as Reb A yield = 0.052 + 0.029C + 0.012AC – 0.043BC. Although the optimum condition was accepted, only 0.164% of Reb A yield is produced. This study can be more improvised to obtain better extracting condition and higher yield of Reb A. For instance, the range of the parameters tested can be increased so that the response data (yield values) will be more varied. The number of parameters tested can be increased to determine their effects as well on the yield of Reb A. Number of extraction is a parameter that might have a significant effect on the yield of Reb A.

Acknowledgements

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Appendices

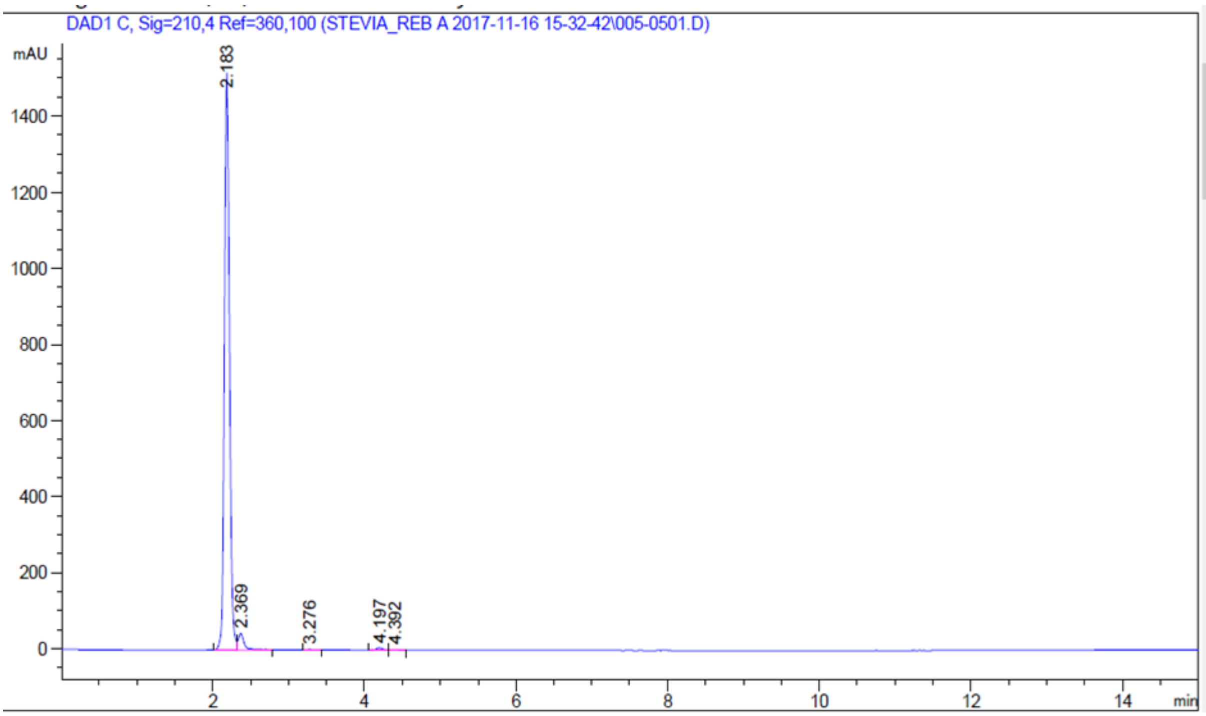

Figure A1. Standard curve for Reb A.

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References

[1] Puri M, Kaur A, Barrow C B and Singh R S 2015 *Applied Microbiology and Biotechnology* **89** 715
[2] Devi N 2019 https://www. medicalnewstoday.com/articles/323627.php
[3] Leung A Y and Foster S 2014 *Encyclopedia of common natural ingredients used in food, drugs and cosmetics* **2** 589
[4] Lewis W H 2016 *Economic Botany* **46** 336
[5] Soejarto D D, Compadre C M, Medon P J, Kamath S K and Kinghorn A D 2015 *Economic Botany* **37** 71
[6] Cui W, Mazza G, Oomah B D and Biliaderis C G 2015 *LWT-Food Science and Technology* **27** 363
[7] Li Q H and Fu C L 2014 *Food Chemistry* **92** 701
[8] Afandi A, Sarijan S and Shaha R K 2015 *Journal of Tropical Resources and Sustainable Science* **1** 62