RECOVERY OF REBAUDIOSIDE A AND STEVIOSIDE FROM STEVIA REBAUDIANA PLANT USING SOLID LIQUID EXTRACTION

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ABSTRAK

Pengekstrakan pepejal-cecair berlaku apabila cecair sebagai larutan pengeksrak akan mengambil komponen dari pepejal. Dalam pembelajran ini, tiga larutan pengekstrak telah digunakan iaitu ethanol mutlak, acetone dan air suling. Larutan itu akan mengambil dua agen pemanis, iaitu rebaudioside A dan stevioside dari tumbuhan stevia rebaudiana. Agen pemanis itu amat dikenali sebagai pemanis semulajadi tanpa kalori dan tidak meniggalkan kesan buruk terhadap kesihatan manusia. Objektif kajian ini dijalankan ialah untuk mengnalpasti keadaan ekstrak terbaik untuk memperoleh stevioside dan rebaudiside A yang paling banyak. Kaedah pengektrak dilakukan dalam tiga fasa. Fasa pertama iaitu untuk mengenal pasti nisbah terbaik diantara serbuk stevia dan larutan pengekstrak, fasa kedua untuk mengnalpasti durasi ekstrak terbaik dan fasa ketiga untuk mengenalpasti suhu terbaik. Ethanol merupakan larutan pengekstrak terbaik berbanding acetone dan air. Pengekstrakan stevioside dan rebaudioside A menggunakan ethanol dalam parameter terbaik (nisbah 1:25, 1 jam dan 45 ℃) dapat menghasilkan 12.48% stevioside dan 0.57% rebaudioside A.

ABSTRACT

Solid-liquid extraction is a recovery process which the extracting solvent will recover component from solid. In this study, three extracting solvent been used which are absolute ethanol, acetone and distilled water. The solvent will extract two sweetening agents, rebaudioside A and stevioside from stevia rebaudiana plant. The sweetening agent was well known as natural sweetener with zero calorie and does not give adverse effect on human health. The objective of this study was to determine the best parameters to extract the highest amount of stevioside and rebaudioside A. The extraction was done in 3 phase. First phase to determine the best ratio between stevia and extracting powder, second phase to determine the best extracting time and third phase was to determine the best temperature. Ethanol was the best extracting solvent compared to acetone and water. Extraction of stevioside and rebaudioside A using ethanol in the most preferable parameter (ratio 1:25, 1 hour and 45°C) produced 12.48% yield of stevioside and 0.57% yield of rebaudioside A.

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LIST OF SYMBOLS

℃	Degree Celsius
%	Percent
w/v	Weight over volume
g	Gram
ml	Millilitre
mg	Milligram
cm	Centimetre
mAU*s	milli Absorbance Unit times second
℃	Degree Celsius
%	Percent

LIST OF ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
Reb A	Rebaudioside A

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Stevia Rebaudiana, a small plant that famous with its sweet leaves that contain a group of sweetening agent called diterpenenic glycoside. Eight diterpenenic glycoside that been found in stevia leaves are identified as stevioside, steviolbioside, dulcoside, rebaudioside (reb) A, reb B, reb C, reb D and reb E. These 8 glycoside contribute in the sweet taste that come from the leaves. In most researches and study, the components that often been extracted are stevioside and reb A. This both component, stevioside and reb A can be found up to (6-17% w/w) of the leaf extract (Lemus-Mondaca, 2011). Both glycosides have become the highlight of study on stevia leaves.

This sweetener gives a bunch of interests to the consumer due to its natural origin, contain zero calorie and also does not contain any side effect after consumption on human health (Gupta, 2013). According to the World Health Organization, there are over 346 million diabetic populations across the world. People are looking forward for alternative sweetener (natural) as the refined sugar are contributing in the diabetic disease and bad for human health. Artificial sugar was said to be bad for the teeth because it provides easily digestible energy for the bad bacteria in the mouth (Touger-Decker, 2003). Many alternative low calorie sweeteners can be found in market but most of them are artificial sweetener Stevia is a natural zero calorie sweetener and the sweet compound able to pass through digestive process of the body without chemically breaking down results in making it a safe food substance for consumption by people who need to regulate their blood glucose level (Abdullateef, 2012).

The sweetening agent can be found in the leaves part of the plant and various type of extraction has been done to obtain that component for instant enzymatic extraction

(Puri, 2011), solid liquid extraction (Afendi, 2013), pressurized hot water extraction (Rao, 2012) and many more. In this study, extracting solvent which are absolute ethanol, acetone and distilled water been used to extracting reb A and stevioside from dried green stevia leaves.

1.2 Problem Statement

The common extraction process that most researcher done require a tremendous amount time followed with complex series of steps. In addition of the problem here, there are exact information about the effect of the extraction methods on the sweetener content, the stevioside. The long isolation procedures of steviol glycoside known to produce noxious residue and bitter alkaloids harmful to human health (Rao, 2012). This study is made to construct a simple and convenient extraction process of stevia leaves to get high number of sweetener agent which are the stevioside and reb A. Note that the elimination of noxious residue and bitter alkaloids need to be eliminated in further purification process.

Abundant article or research made on extracting this leaves, such as extraction of stevioside using polar solvent, solvent plus water, supercritical fluid extraction, microwave assisted extraction, separation by chromatography and many more. All this studies were made to determine and achieve high yield of stevioside and reb A as to increase the production.

Some of the extraction process might using dangerous solvent as an extracting solvent. The solvent may harm in the way it been handle, thus require certain skill to prevent any unwanted things to happen or perhaps some special equipment to contain the solvent. The other ways might be the way the solvent reacts with component in the stevia leaves and perhaps produce harmful side product even in small amount.

What important in achieving high yield of stevioside and reb A are the selection of extracting solvent and also the suitable parameter during the extracting process. For example, in this study, the extracting solvent been used are acetone, water and absolute ethanol. These three solvent have its own different boiling point. Thus the optimum temperature whereas the solvent will extract the most amount of stevioside and reb A is different and more unlikely is they are all unknown. This study is made to layout the process environment in achieving highest yield of stevioside and reb A.

1.3 Research Objective

1.3.1 General Objective

The objective of this research is to determine the optimum parameters and conditions during the extraction process to achieve high yield of stevioside and reb A from stevia rebaudiana leaves.

1.3.2 Specific objective

- To determine the highest yield of stevioside and reb A using different type of extracting solvent, which are absolute ethanol, acetone and water.
- To study the effect of temperature during extracting on yield of stevioside and reb A
- To determine the best ratio of extracting solvent and weight of stevia powder in order to produce highest yield of stevioside and reb A
- To determine the best duration time of extracting in order to get highest yield of stevioside and reb A

1.4 Scope of Study

In this study, highest amount of stevioside and reb A obtained by adjusting the parameter during solid-liquid extraction. To achieve the study's objective, the following scope have been identified

- Different types of solvent used for recovering stevioside and reb A from stevia leaves in solid liquid extracting process. The solvents are ethanol, acetone and water. All three extracting solvent have different polarity index and polar function (Afendi, 2013).
- Temperature (40°C, 45°C, 50°C, 55°C and 60°C) of extracting solvent during the extraction process on yield of stevioside and reb A. Different temperature will give different rate of reaction and too high temperature will accelerate all unwanted side reaction (Afendi, 2013).

- Ratio between stevia powder (g) to extracting solvent (ml) (1:5, 1:10, 1:15, 1:20, and 1:25) on the yield of stevioside and reb A during the extraction process. Various ratio to determine whether the extracting solvent is enough to recover all the stevioside and reb A.
- Extraction time (1, 1.5, 2, 2.5 and 3 hours) for recovering stevioside and reb A from stevia leaves. Long extracting time only will waste of time thus knowing the suitable period of time for the extraction process is important.

CHAPTER 2

LITERATURE REVIEW

2.1 Stevia Rebaudiana

Stevia rebaudiana (Bertoni) is well known as sweet herb from Paraguay. Mostly country like China, South Korea, Japan and Brazil apply this natural sweetener on their food and beverages. Nowadays the evolution of stevia rebaudiana product has reached to different type such as stevia-added herbal teas and soft drinks. Further study on this unique plant as antimicrobial and antitumor activities as prove that this plant has a lot potential in industry (Chatsudthipong, 2009).

Rao (2012), reported that on these recent years, the demand of natural non-nutritive high intensity sweeteners with low-calorie value as an alternative to sucrose. People tend to choose this 100% natural sweetener instead of sugar or other artificial sweetener due to increase of the obesity among the people nowadays. Obesity has led to other chronic diseases like cardiovascular disease, particular cancer types, type 2 diabetes, and hypertension (Ameer, 2017).

Saccharose has been used for treatment of diabetes mellitus, obesity and hypertension, also provide prevention. Stevioside in the leaf of this plant also give same therapeutic effect and help fight against diseases stated before. Several study has been made and proves that the components in the leaf gives other therapeutic effect as they have anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumour, anti-diarrheal, diuretic, and immunomodulatory actions (Chatsudthipong, 2009).

Figure 2.1: Leaves of stevia rebaudiana plant (Bhutia, 2012)

2.2 Rebaudioside A and Stevioside

The presence of special component which is diterpenoid glucoside make this plant special. The major component from the group diterpenoid glucoside are stevioside, dulcoside A, steviolbioside, rebaudioside (reb) A, B, C, D and E. All these isolated diterpenoid glycoside have same chemical backbone structure 'steviol' (Chatsudthipong, 2009).

Comparing the sweetness between these glycoside and sucrose, dulcoside A is 50-120 times sweeter, reb A is 250-450 times sweeter, B is 300-350 times sweeter, C 50-120 times sweeter, D 250-450 times sweeter, E 150-300 times sweeter, steviobioside 100-125 times sweeter and stevioside 300 times sweeter. All the component of diterpenoid glucoside in the stevia leaves are varies. The major components of the leaf are stevioside (5-10% of total dry weight) and reb A (2-4%). This study is more focused on extracting reb A from the stevia leaf. Even the sweetness of reb A is high compared to the diterpenoid glycoside, its amount in the leaf is quite small. Reb A only consist about 3-4% rather than stevioside 6-10% in the sweetener mixture (Singla, 2015).

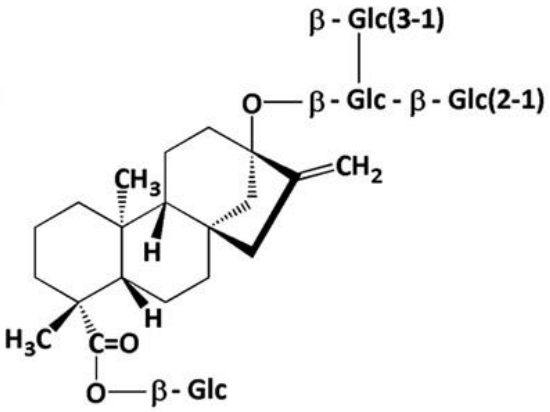


Figure 2.2: The chemical structure of Reb A and its related component.

Source from (Chatsudthipong, 2009).

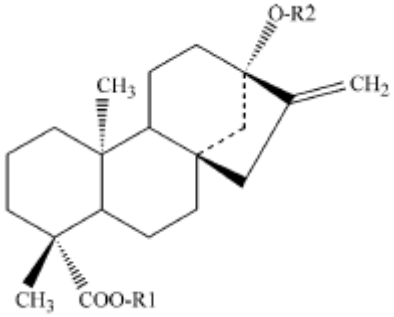


Figure 2.3: The chemical structure of stevioside and its related component.

Source from (Geuns, 2003)**.**

The chemical name for this component is 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester, and its chemical formula is C44H70O23, (JCEFA, 2007).

The reb A shows great stability toward microbial activity, pH treatment and heat. Reb A also show no significant degradation in carbonated beverages that been keep at 60°C for 137 hours. Reb A was found to be more superior than stevioside in terms of sweetness and lack of a bitter after-taste (Adari, 2015).

The separation of reb A from plant leaves impeded by some impurities such as resins, organic acid, protein, and especially the pigments which are chlorophyll, carotene

and xanthophyll (Martins Paula, 2016). Thus further purification process needed to be done in order to remove these impurities up to 95%.

2.3 Extraction Process

Table 2.1 shows several type of extraction processes that can be done to extract the sweetener agent from dried stevia leaves. The purpose of making that tabulation is to compare each type in several aspects. For example, the equipment that needed to run the extraction process, extracting solution used suitable according to the types of extraction, parameter and most important is the yield of reb A.

Table 2.1: Comparison between several types of extraction with different method

Method	**Material**	**Condition**	**Recovery**	**Sources**
microwave-assisted extraction	Ethanol 75%	4 min 160W microwave-power	Reb A - 15.3 mg/g	(Ameer, 2017)
Pressurized hot water extractor		100 kPa 100-110°C 10 min	Stevioside – 6.5 μg/ml	(Rao, 2012)
Enzymatic extraction	Hemicellulose Cellulose pectinase	1 hour 60°C	Stevioside – 369 ± 0.11 microg	(Puri, 2011)
Solid liquid extraction in soxlhet	Methanol	1:10 3 times extraction 50°C 1 hour	Reb A – 1.84 g/100g	(Afendi, 2013)
Solid liquid extraction in incubation shaker Centrifuge for further clarification	Distilled hot water	50°C 3 hours 1:10	STEVIA SWEETENER - 49.90%	(Dilnessa, 2014)

The first consideration that must be made to choose suitable method of extraction is the equipment availability. It is impossible to do the extraction if accordance equipment is unavailable. Besides that, borrowing or renting an equipment from outsource will cost a lot. Comparing all extraction process, the simplest and convenient method is the solid liquid extraction. This is because this method does not need specific equipment.

According to (Afendi, 2013), in his journal stated that methanol was the best solvent for the extraction or reb A from stevia leaves in terms of high extract and component yield. Besides that, ethanol and aqueous acetone were also found to be effective on extracting reb A. This bring to next consideration which is the extracting solvent that can be used. All three solvent mentioned before are polar organic solvent that contain hydroxyl group. This polarity will make the extraction process more efficient.

Next consideration is the conditions during the extraction process. To satisfy the objectives, several condition during the extraction must be prepared which are the extracting time, ratio between dried stevia leaves and extracting solvent and temperature of the solvent. There are also critical criteria to be make in choosing the parameter condition during extraction. For examples the selection of temperature during the extraction process. Ethanol have boiling point at 78.37°C and the temperature variant for the extraction process that using ethanol is must be below its boiling temperature. Or else during the extraction, the ethanol will evaporate and extraction will not happen. The boiling point of acetone is 56°C and for water is 100°C. Noted that the temperature value must below than the boiling temperature of extracting solvent.

Mainly in this project, the method of extraction from (Dilnessa, 2014)'s journal will be apply. It uses solid liquid extraction method which is the extracting solvent as the liquid and dry powder of stevia rebaudiana as the solid. This extraction principle is manipulating the solubility properties and transfer the solute or desired component from one phase to another. This can be achieving only if the solute (reb A) have higher solubility in the second phase than the first phase. The solid phase (dry leaf powder), contain the solute (reb A), dispersed into the liquid phase (ethanol, acetone, and water) and mix. Later on, the solute will extract to solid phase due to solubility different. And lastly, the solid phase is removed by using the filtration method.

The solute and liquid phase that obtained in previous method need to be further clarify by using centrifugation process. The extract which coloured dark brown kept well in bottle. That extract contains not just reb A but also other sweetener component such as stevioside and others. These components in extract can be separated by using High Performance Liquid Chromatography. The major component when using this method are the pure standard, mobile phase, and type of column.

The pure standard that will be use is reb A (>97% purity). The mobile phase is HPLC grade acetonitrile with water ratio (80:10) and at pH 3.0. This pH can be adjust using phosphoric acid (85% reagent grade). Lastly the column that will be use is Eclipse Plus C18 (25cm x 4.6mm I.D., 5µm).

2.4 Parameters of Extraction Process

2.4.1 Temperature

The first condition or parameter are temperature. The rate of transfer of rebaudiana from solid phase to liquid phase is said to be optimum at 50°C, as the rate increase from 25°C to 50°C and decrease later on, (Afendi, 2013). From that information, the selected temperatures for extracting of are 40°C, 45°C, 50°C, 55°C and 60°C. Temperature of 50°C must be around at the middle as we need to obtain the curve from yield vs temperature graph and see the increase and decrease of the yield. This can be proven by Figure 2.4 below.

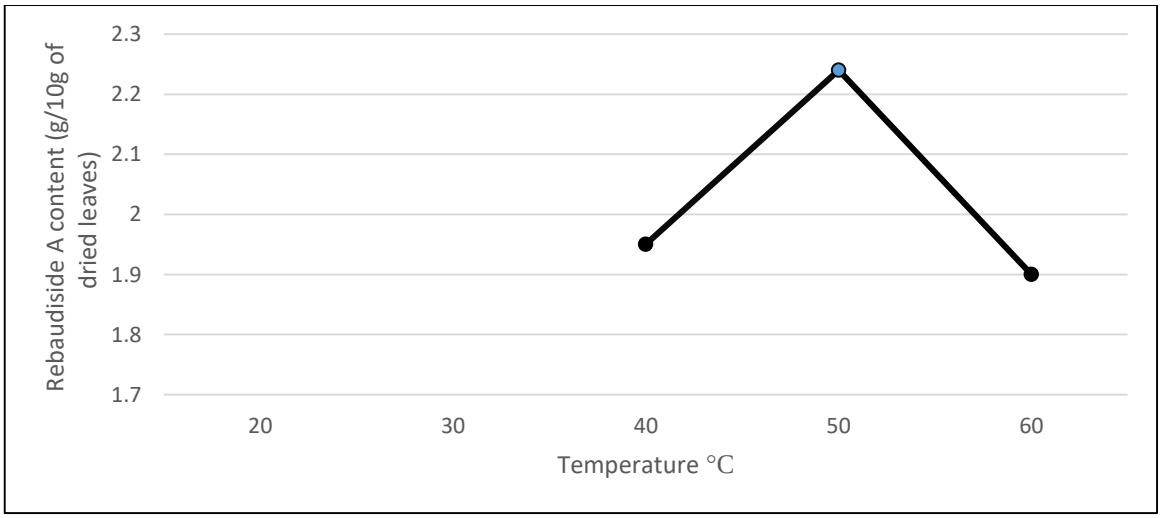


Figure 2.4: Graph of yield of reb A against Temperature (°C) (Afendi, 2013)

2.4.2 Ratio

Table 2.2 shows the ratio of extracting solvent and powder and their yield. For all type of extracting solvent, the ratio to dried stevia leaves are 1:5, 1:10, 1:15, 1:20 and 1:25, as 1 unit is equal to 2. And the final condition is contact time of the solid and liquid. The optimum contact time was said to be optimum if it reached 1.5 hours and prolong the contact time will not increase the yield of reb A (Afendi, 2013). Thus, the desired contact time for this experiment are 1 hour, 1.5 hours, 2 hours, 2.5 hours and 3 hours.

Table 2.2: Relation between ratio of dried leaves (g) to extracting solvent (ml) towards yield of reb A (Afendi, 2013).

Proportion mass: solvent ratio (w/v)	**Reb A % (g/100g dried leaves)**	**Total Soluble Solid (TSS) %**
1.5	1.53	9.0
1.10	2.24	8.3
1.15	2.20	8.0
1.20	2.05	7.2
1.25	1.93	6.0

2.4.3 Time

Figure 2.5 shows the comparison between different extracting time and yield of reb A, thus the third parameters is extraction time. The highest yield of reb A was recorded at time 1.5 hours and 2 hours. This non-increment on value of yield indicates that no more reb A will be extracted after that period of time.

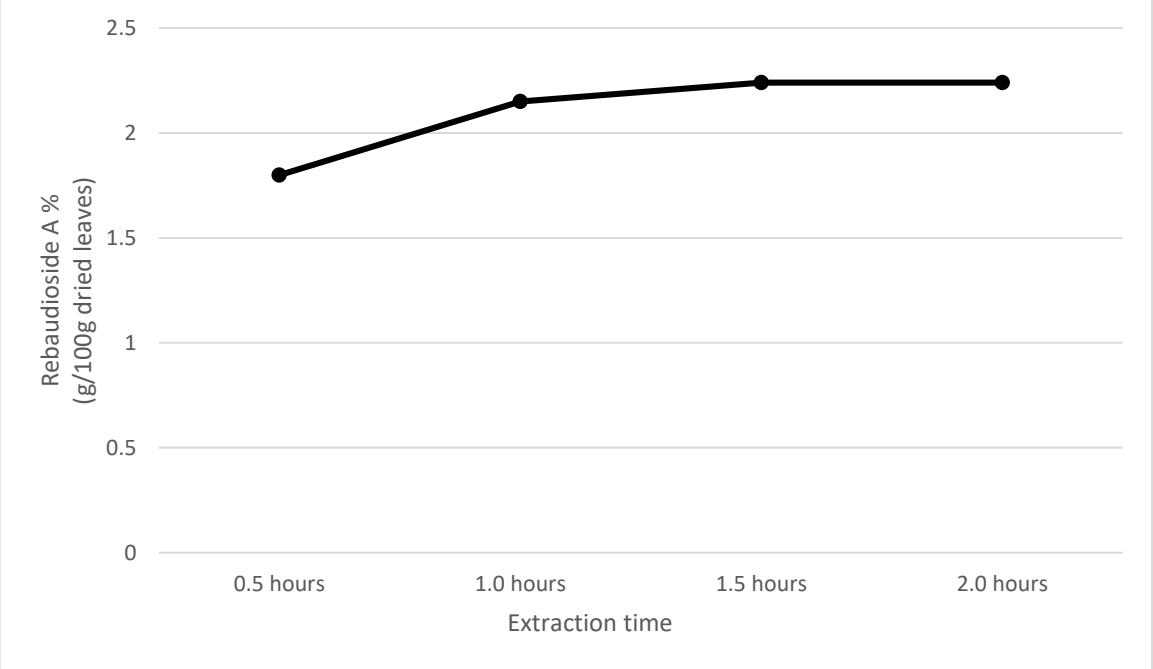


Figure 2.5: Graph of yield of Reb A against extraction time (hour), (Afendi, 2013)

CHAPTER 3

METHODOLOGY

3.1 Materials and Chemicals

Distilled water, acetone, absolute ethanol, stevia rebaudiana leaves (fresh or dried), phosphoric acid, HPLC standard reb A (>97 purity), HPLC grade acetonitrile.

3.2 Equipment and Apparatus

A 45 unit test tube, 45 unit 50ml beaker, 45 unit petri dish, 45 unit centrifugation tube, aluminium foil, 0.2µm milipore filter (Whattman No. 1), 1 unit of Cheese-cloth, 0.22 µm Millipore filtration membrane, vacuum filtration unit, incubation shaker, water bath, magnetic stirrer (Model: SP131320-33, Brand: Thermo), pH meter (Model: S220, Brand: Streamline), centrifuge (Model: 5810R, Brand: Eppendorf), analytical HPLC (Model: Agilent technologies 1200 infinity series, Brand: Agilent Technologies), HPLC column Ecplise Plus 100 C18 (25 cm × 4.6 mm I.D., 5µm), weighing balance (Model: JP1203C, Brand: Mettler-Toledo) and refrigerator.

3.3 Methodology

3.3.1 Sample Preparation

Fresh mature leaves of stevia rebaudiana was separated from the stem by using hand. The leaves were weighed 30 g using analytical balance spread on a sheet of aluminum foil. Then the leaves undergone drying process to decrease the moisture content by placed in a 60 ℃ heated oven for 1 hour (Dilnessa, 2014). After the leaves been heated in oven, all the leaves been put in grinder and became fine powder. In order to get uniform powder size, all the powder was meshed. The smallest fine powder was kept in polyethylene bags and stored.

Figure 3.1: Separation of leaves from stem

Figure 3.2: Leaves been spread before the drying process

Figure 3.3: Grinder that been used for grinding the dried leaves into a fine powder

Figure 3.4: Sieves that been used to get consistent powder size of leaves powder

Figure 3.5: Fine powder of stevia leaves been kept in polyethylene bag for storage

3.3.2 Extraction Process

Stevia powder was weighed 2 g using microbalance and placed in conical flask or 50ml beaker. Extracting solvent water, ethanol and acetone was poured into the conical flask and beaker according to their respective volume to make the ratio between these two substances 1:5, 1:10, 1:15, 1:20 and 1:25 (Afendi, 2013). All 15 conical flask and beaker was covered with aluminum foil and placed in incubation shaker. The parameters in the incubation shaker was set 45 ℃ with 150 rpm for 2 hour (Dilnessa, 2014). Noted

that this round of extraction process for manipulated variable. The result from HPLC analysis will show the best ratio and second round of extraction with manipulated variable, temperature and proceeded with third manipulated variable which is temperature.

Figure 3.6: Mixture after the extraction occurred in the incubation shaker

Simple filtration method was used to separate the solid and liquid after the extraction process. The residue of powder that left on the filter paper was disposed.

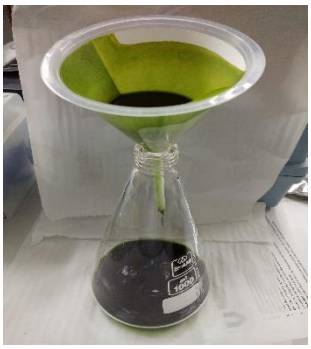


Figure 3.7: Filtration using filter paper to separate the solid from the liquid

Figure 3.8: Unwanted solid residue of the powder

The liquid part was undergone further separation by using centrifugation method. All the solvent was transferred into centrifuge tube and the centrifugation process took places approximately 1 hour in 1000rpm. The outcome of centrifugation shows 2 different layer and the bottom part which is solid residue was put to waste and the upper part was poured into test tube for storage purposes.

3.3.3 HPLC Analysis

3.3.3.1 Sample preparation

The sample was filtered using membrane filtration. Firstly, the sample was sucked into syringe. The membrane filtration was put on and the sample run through the membrane and into the HPLC vial. Only 1.5 ml of sample per vial. The purpose for this sample preparation is to eliminate any small particle that contained in the sample.

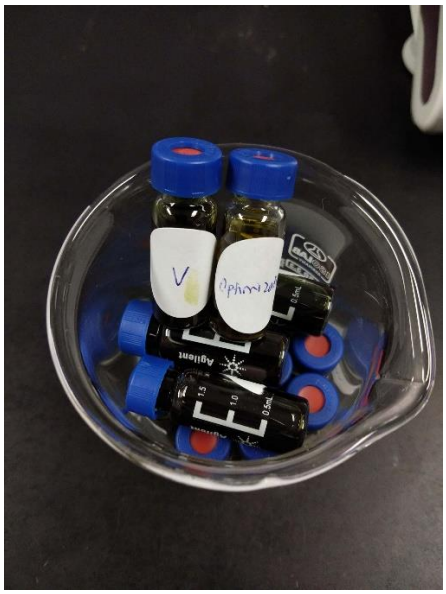


Figure 3.9: Sample inside HPLC vial.

3.3.3.2 Mobile phase preparation

The mobile phase that been used is acetonitrile plus distilled water with ratio 80:20 respectively and pH of 3 (Afendi, 2013). Firstly, 800ml of acetonitrile HPLC grade was measured using measuring cylinder and 200 ml of distilled water also measured using measuring cylinder. The distilled water was poured into schott bottle and followed by acetonitrile. The reaction between these two solvent will make the solution became slightly cold and was left to room temperature for the pH measurement.

The initial pH for the mobile phase was 6.5. Acetic acid has been used to decrease the pH of mobile phase until 3. 5 µm of acetic acid was dropped into the solution and was mixed well. The new pH of mobile phase was measure using pH meter and if the pH is not satisfied, the adding of acetic acid was repeated until the pH reach 3.

After obtained the pH 3 of mobile phase, the mobile phase was undergone vacuum filtration in order to eliminate any impurities or small particle in the mobile phase. The filtered mobile phase was placed back into clean schott bottle and placed in sonicator. The sonication of mobile phase occurred for 30 minutes with room temperature along with the sample.

3.3.3.3 HPLC

The parameter and condition for HPLC was adjusted to the desired. Temperature of column was set at 18℃ and th UV detection was 210 nm. The injection of sample volume was set at 0.1µl and the flow rate was 1ml/min. The standard stevioside (>99.3% purity) and reb A (>97% purity) were used. HPLC column was equilibrated by pumping mobile phase through it until a drift-free baseline was obtained. The chromatograms of the sample solution and of the standard solution were recorded in 10 minutes. The peak areas of reb A and/or stevioside were calculated automatically by solutions software equipped with HPLC. Percentage of reb A and stevioside was calculated using below formula 3.1 (Afendi, 2013).

Where,

$$\%X = \left[\frac{Ws}{Wx}\right] \times Fx \times \left[\frac{Ax}{As}\right] \times 100 \quad (3.1)$$

X = sample of stevioside or rebaudioside A

W_s = weight (mg) of X in the standard solution

Wx = weight (mg) of X in sample solution

As= Peak area of X from the standard solution

Ax = Peak area of X from the sample solution

Fx = formula weight of X

3.3.4 Tabulation of Data

Table 3.1 shows the first round extracting process is about study the effect of ratio between solid and liquid on the yield of reb The variable here is the ratio between the dry leaf solid and extracting liquid. While the constant parameters are the temperature which is 50°C and contact time 2 hours. The highest yield of this round for each extracting liquid been use for the next round as constant.

Table 3.1: Data from first round of extracting process

Ratio	**Yield of reb A using respected extracting solvent**		
	Ethanol	**Acetone**	**Water**
1:5			
1:10			
1:15			
1:20			
1:25			

In this second round of extracting process shown in table 4 above, the purpose is to study the effect of contact time between solid and liquid on yield of reb A. the constant parameter is contact time between solid and liquid while the constant parameters are temperature 50°C and optimum ratio that we obtained from first round of extracting process. Same as before, the optimum contact time will be constant for the next round of extracting process. This second round can be referred to Table 3.2 below.

Table 3.2: Data from second round of extracting process

Time (hours)	**Yield of reb A using respected extracting solvent**		
	Ethanol	**Acetone**	**Water**
1			
1.5			
2			
2.5			
3			

Table 3.3 which is the last round of extracting process is to study the effect of temperature on the yield of reb A. As the variable here is the temperature and the constant is optimum ratio from first round and optimum contact time from second round for each extracting solvent.

Table 3.3: Data from third round of extracting process

Temperature	**Yield of reb A using respected extracting solvent**		
°C	**Ethanol**	**Acetone**	**Water**
40			
45			
50			
55			
60			

The results from third and last round of extracting process will give the highest yield for respective temperature, solid liquid ratio and contact time. Comparing data from each extracting solvent will answer the objective of determining the suitable extracting solvent on extracting reb A from stevia leaves.

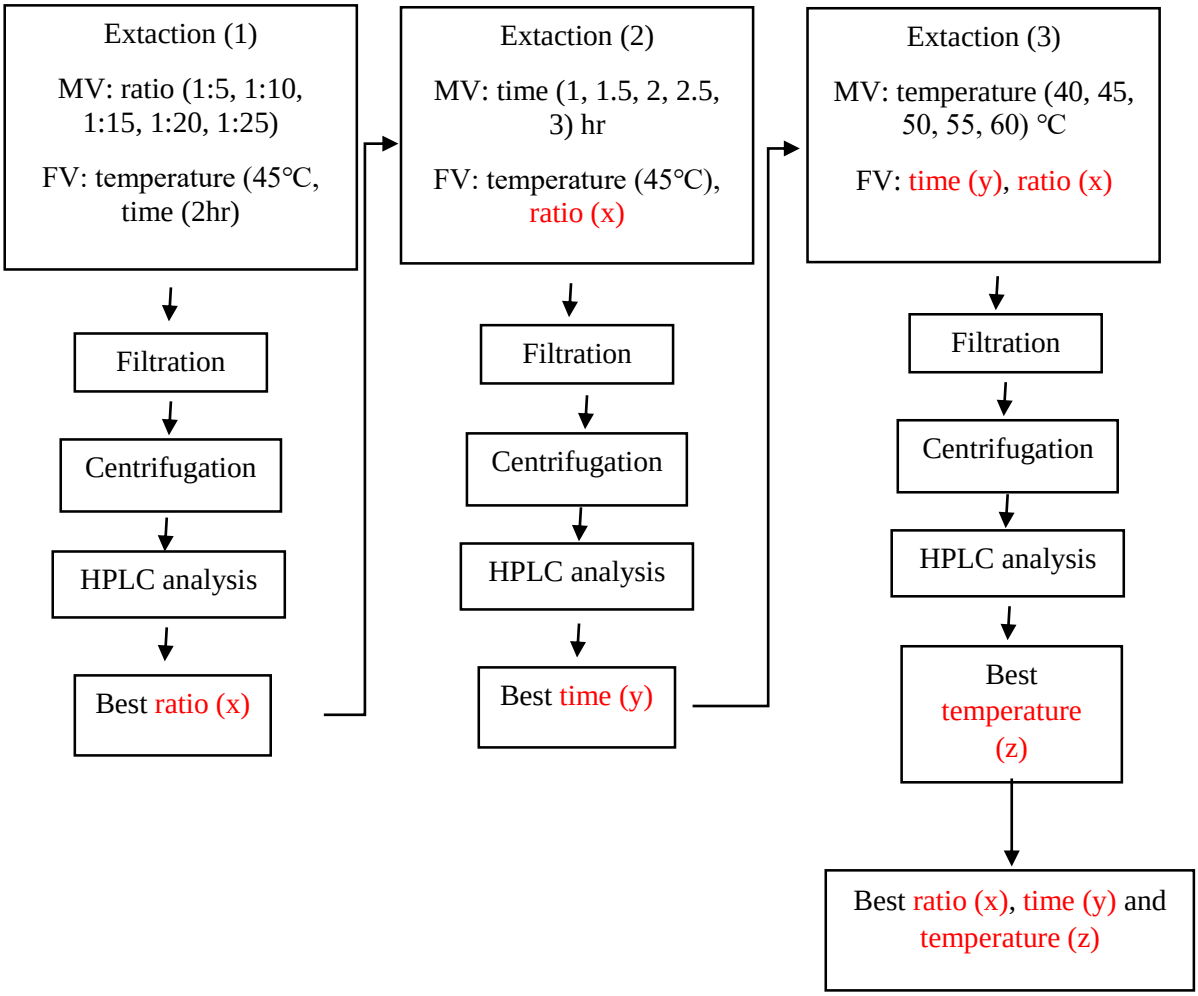


Figure 3.10: Work flow for all three extraction phases

CHAPTER 4

RESULTS AND DISCUSSION

The yield value of reb A and stevioside was calculated using formula 1. The value that needed by the formula was obtained by the chromatogram from the HPLC analysis.

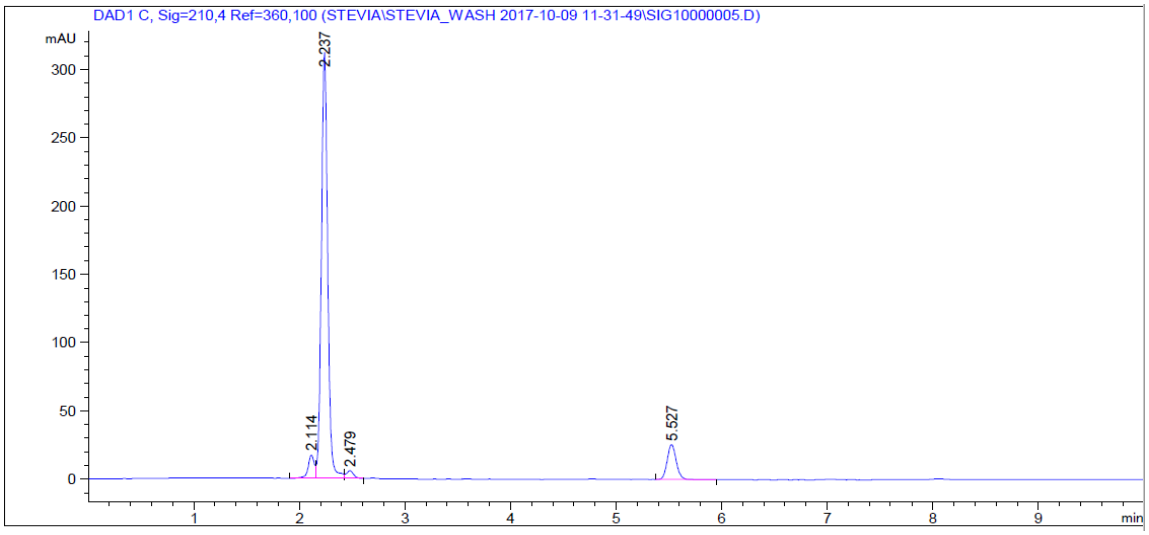


Figure 4.1: Chromatogram of standard stevioside with concentration of 0.5 mg/ml

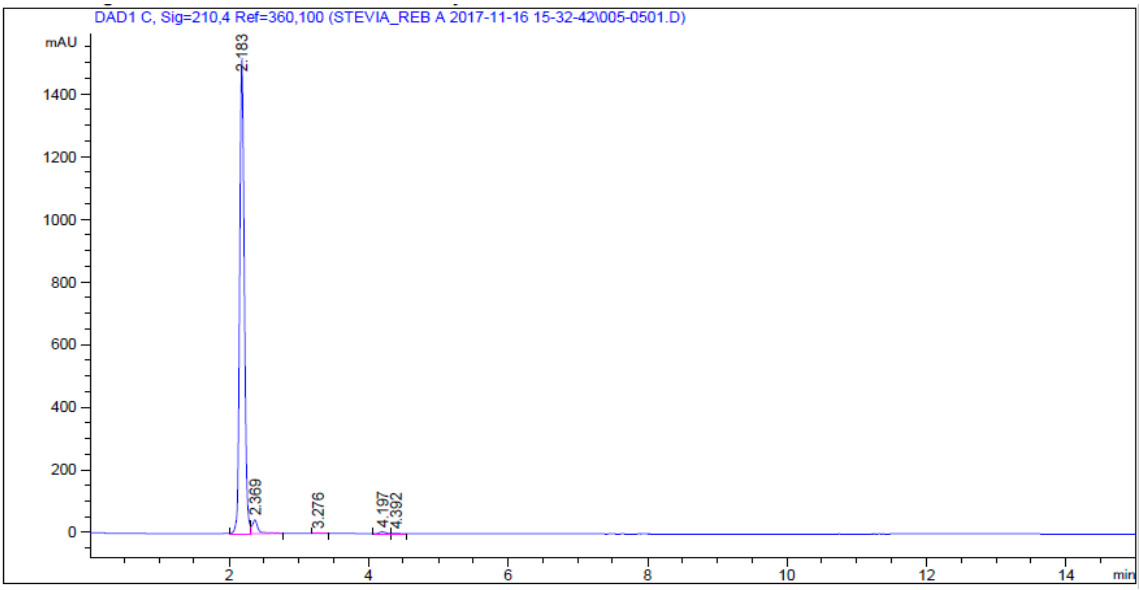


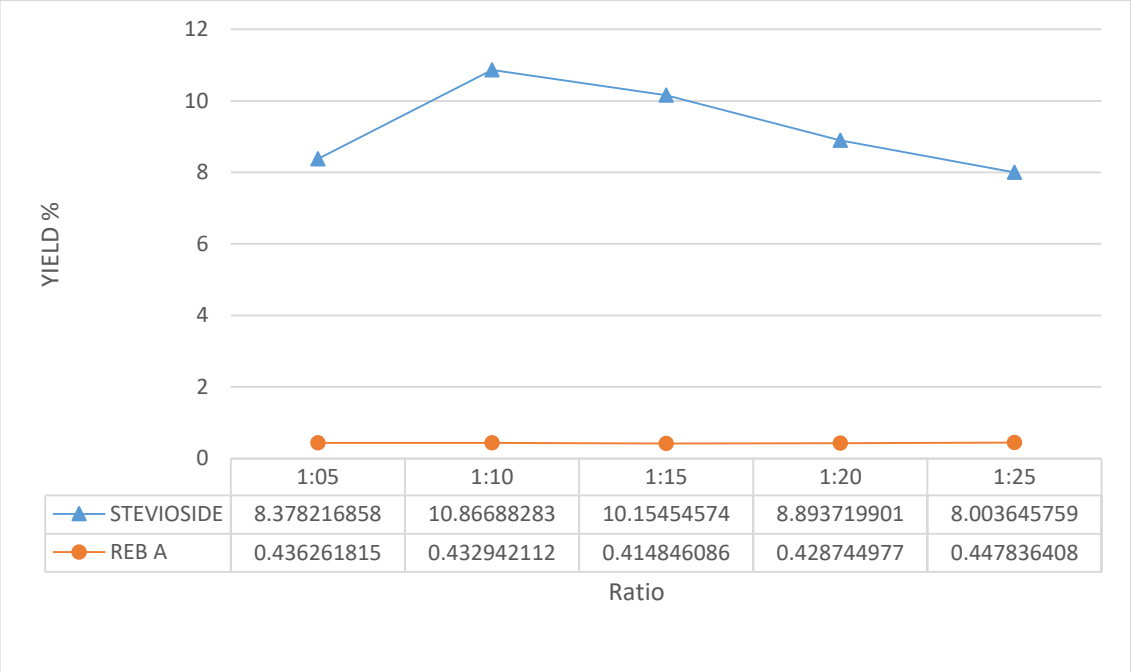
Figure 4.2: Chromatogram of standard reb A with concentration of 0.5 mg/ml

Figure 4.1 shows the peak of standard stevioside with peak at retention time of 2.237. This peak area was 1296.02 mAU*s. While figure 4.2 shows the peak of standard reb A at peak of 2.183. The peak of reb A have are of 7484.87 mAu*s. This standard indicated that every peak at 2.1 was peak for reb A and peak at 2.2 was peak for stevioside.

4.1 Effect of material ratio on Yield of rebaudioside A and stevioside

The extraction process was made to determine the best parameter in order to achieve high yield of sweetening agent which are stevioside and rebaudioside A from stevia leaves. The first phase of extraction have time and temperature as the fixed variable and ratio as the manipulated variable. All 5 ratios between stevia leaves powder with extracting solvent are 1:5, 1:10, 1:15, 1:20 and 1:25 whereas 1 unit equal to 2. The fixed parameters were 60℃ and extraction of 1 hour. All three extracting solvent used were ethanol, acetone and water.

4.1.1 Extracting solvent: Ethanol



	1:05	1:10	1:15	1:20	1:25
STEVIOSIDE	8.378216858	10.86688283	10.15454574	8.893719901	8.003645759
REB A	0.436261815	0.432942112	0.414846086	0.428744977	0.447836408

Figure 4.3: Yield of reb A and stevioside during extraction by ethanol in multiple ratios.

Figure 4.3 shows that the effect of yield for the sweetening agent rebaudioside A and stevioside when extracted using ethanol in five different ratio which are (1:5, 1:10, 1:15, 1:20 and 1:25). In overall, the yield of rebaudioside A is quite low compared to stevioside. This is because (Lemus-Mondaca, 2011) mentioned that the constituent of reb A only (2-4%).

Yield of reb A does not significantly affected by the ratio between stevia powder and extracting solvent ethanol. Increasing of ratio from 1:5 to 1:15 shows slightly decrease in the yield of Reb A but the further increase of ratio to 1:25 shows constant increment that lead to the highest yield of reb A recovered, 0.45%. Due to little amount of reb A in the leaves, 10 ml of extracting solvent was enough to extract all the reb A.

The yield of stevioside increase as we increased the ratio between stevia powder and extracting solvent. This increment happen because at ratio 1:05, there was not enough solvent to extract the stevioside and some of the stevioside were still in the cell. Further increasing of material ratio to 1:15, 1:20 and lastly 1:25. The yield of stevioside shows slightly decrease. This result indicates that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract to become saturated and prevent further increase (Xu, 2005). To conclude, the best ratio to extract reb A for extraction using ethanol was 1:25.

4.1.2 Extracting solvent: Acetone

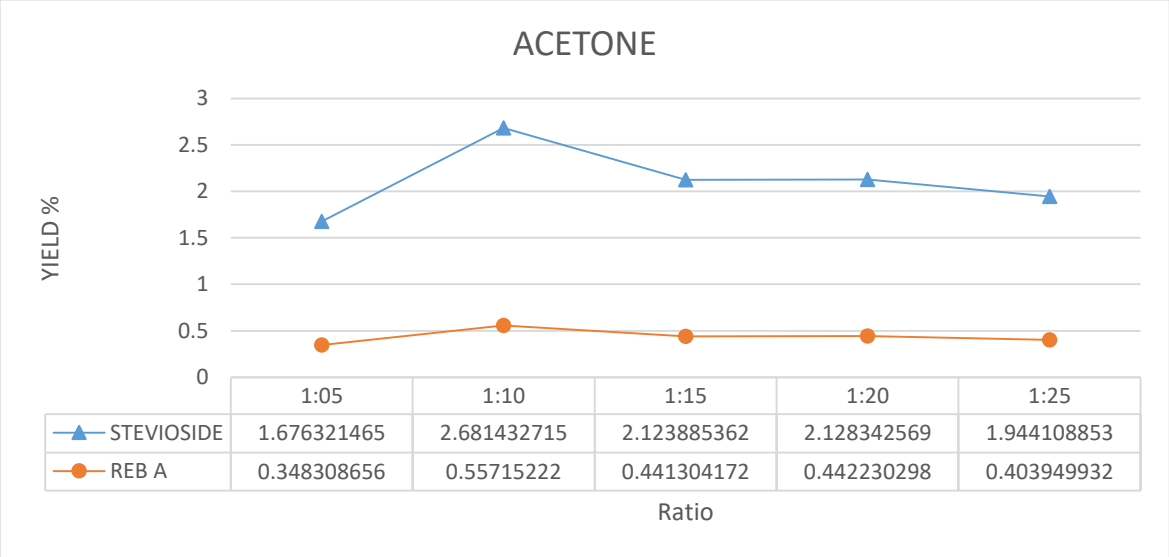


Figure 4.4: Yield of reb A and stevioside during extraction by acetone in multiple ratios.

Figure 4.4 indicates the yield of stevioside and reb A after the extraction of acetone with multiple ratios. Yield of reb A increase as the ratio between stevia and solvent increase from 1:5 to 1:10. Further increase in ratio only decrease the yield of reb A because. This is because the reb A might has fully extracted and excess in the solvent will dissolve the extract and make the solvent saturated.

Highest yield for stevioside can be obtained in 1:10 with the yield of 2.68%. Increase in the ratio does not increase the yield of stevioside but only decrease it. We can conclude that at any ratio, extraction of stevioside under 60℃ for 2 hours only can get around 2.68% of stevioside yield. Increase in temperature or extracting time might able to increase the yield. This will be answered in second phase and third phase of extraction.

Therefore, the best ratio to extract reb A using extracting solvent acetone is 1:10 because increase in extracting solvent does not increase the yield of reb A.

4.1.3 Extracting solvent: Water

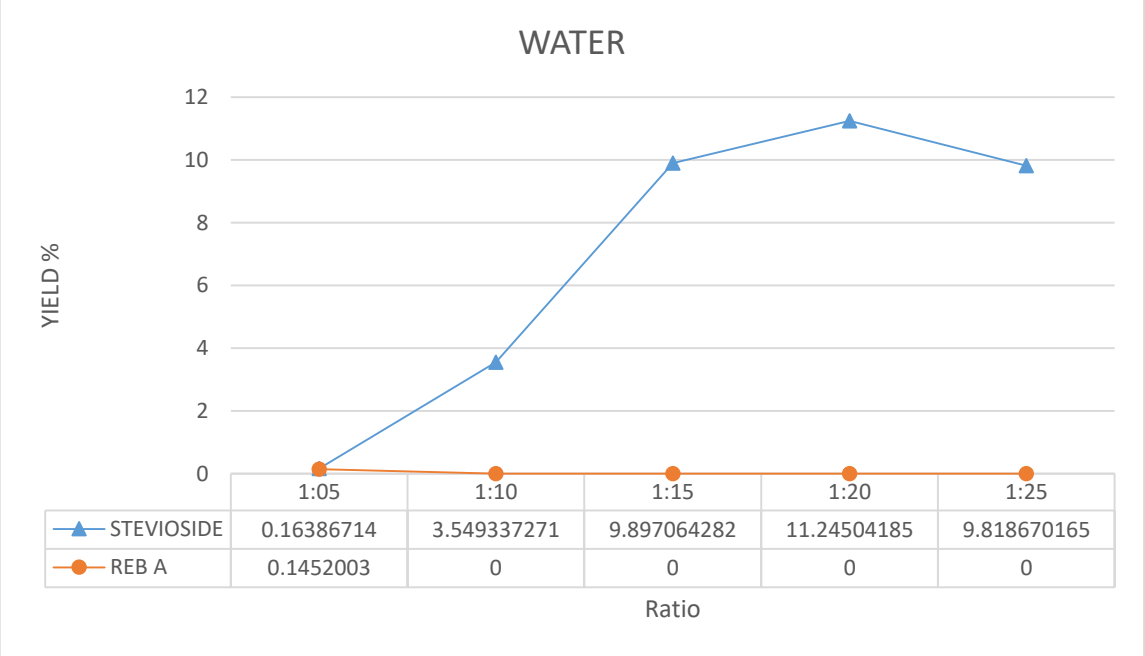


Figure 4.5: Yield of reb A and stevioside during extraction by water in multiple ratios.

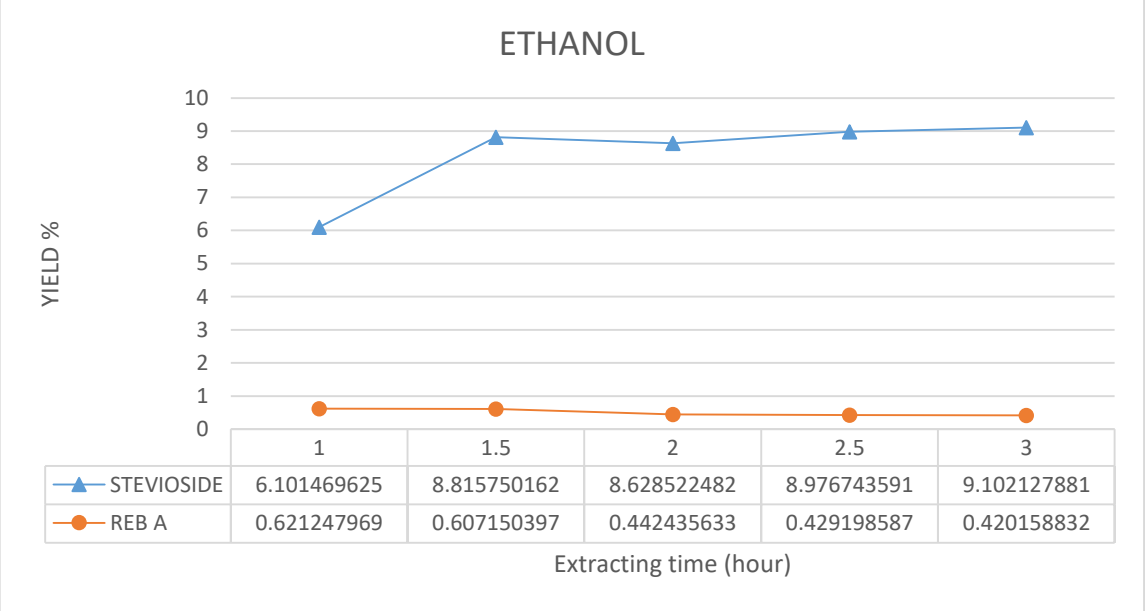
At 1:05, the yield of stevioside was very low compared to other ratio. Increase in ratio between stevia and solvent will increase the yield of stevioside until 1:20. As the highest yield obtained at ratio 1:20 with yield of 11.25%. Increase the ratio to 1:25 only show less yield because at ratio 1:20, the material ratio increase its limit (Xu, 2005).

Extracting reb A using water was not very good because as yield of reb A (0.15%) only appeared when extract under ratio 1:5. Increasing the extracting solvent does not increase in yield but only make the reb A disappeared. This might due to reb A in the solvent became too dilute and cannot be quantified. We can conclude that most preferable ratio when extracting reb A is 1:5 and increasing the ratio will not give higher yield of reb A.

4.2 Effect of extracting time on Yield of rebaudioside A and stevioside.

In second phase of extraction, the manipulated variable was extracting time within the range from 1 hour to 3 hour and 0.5 hour as the interval. The fixed parameter were temperature 60°C and powder to solvent ratio, ethanol with 1:25, acetone 1:10 and water 1:5.

4.2.1 Extracting solvent: Ethanol



	1	1.5	2	2.5	3
STEVIOSIDE	6.101469625	8.815750162	8.628522482	8.976743591	9.102127881
REB A	0.621247969	0.607150397	0.442435633	0.429198587	0.420158832

Figure 4.6: Yield of reb A and stevioside during extraction by ethanol in 5 different extracting time.

Figure 4.6 illustrate the relationship between yield of reb A and stevioside and different extracting time. Highest yield of reb A was achieved when the stevia was extracted for 1 hour. Increase in extracting time will decrease the yield of reb A. According to (Afendi, 2013), reb A can be oxidize easily when in ethanol thus increase in extracting time in high temperature will cause loss of reb A yield.

Opposite with reb A, yield of stevioside increase as the extracting time increased. Huge difference in yield of stevioside between extracting time 1 hour and 1.5 hour. And further increase in time only shows slight increase of the yield. One hour of extraction to recover stevioside was not enough. In this results, most preferable extracting time to recover reb A was at 1 hour because further the extracting time will cause loss of the yield.

4.2.2 Extracting solvent: Acetone.

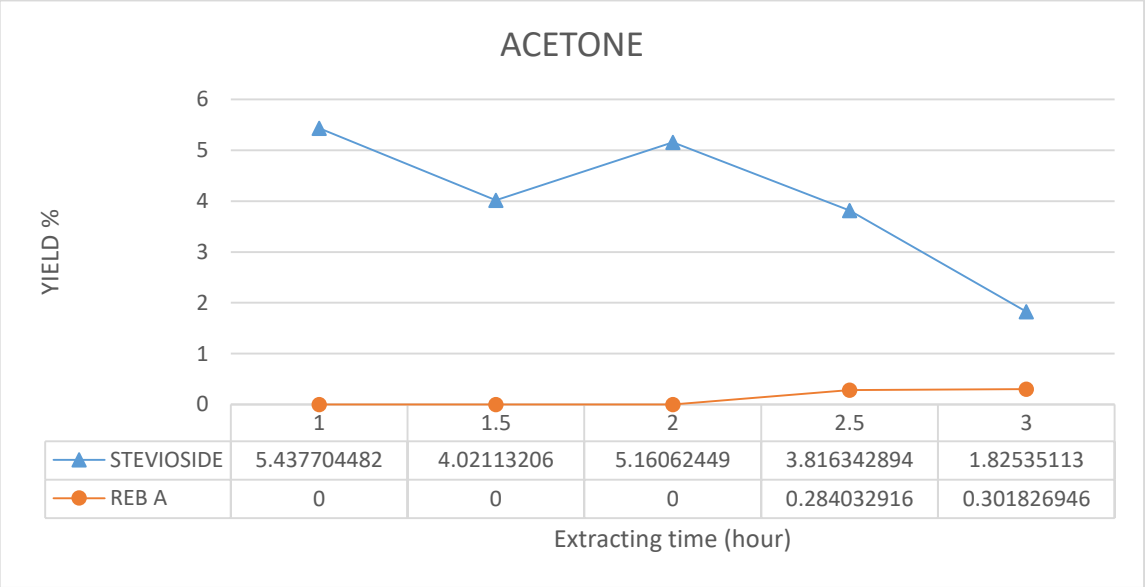


Figure 4.7: Yield of reb A and stevioside during extraction by acetone in 5 different extracting time.

One hour of extraction gave the highest yield of stevioside with yield value of 5.44%, the yield decrease when extracting time increase to 1.5 hour. The yield increase back at 2 hour of extraction, and then further increase in extracting time only will decrease the yield of stevioside.

On the other hand, highest yield of reb A obtained at extraction time of 3 hours. The line of yield for reb A had increased trend from 2 hour moving to 2.5 then 3 hour. Two hours was not enough to extract reb A from stevia when using acetone as the extracting solvent. Thus, most preferable extracting time in term of yield of reb A was 3 hours.

4.2.3 Extracting solvent: Water

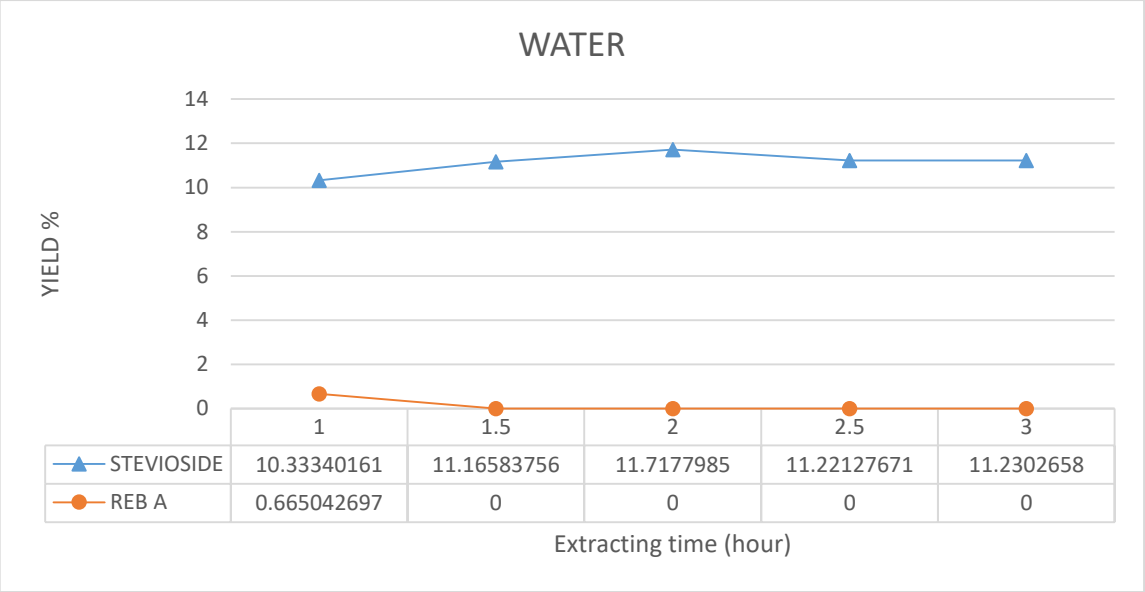


Figure 4.8: Yield of reb A and stevioside during extraction by acetone in 5 different extracting time.

Extracting time did not affect greatly on the yield of stevioside. The yield had increasing trend from 1 to 2 hour of extraction as the highest yield was at 2 hour with 11.72%. Then, the trend decreased. Extracting for 1 hour only gave the value of reb A, 0.67%. The zero value of yield when extracted for 1.5 to 3 hours might due to poor separation in HPLC quantification. Form the values in the figure 4.6, most preferable extracting time was 1 hour and the reb A yield was 0.67%.

4.3 Effect of temperature on Yield of rebaudioside A and stevioside.

This third and final phase answer the second specific objective which is the effect of temperature on yield of reb A and stevioside. In this phase, the manipulated variable was temperature with values of 40℃, 45℃, 50℃, 55℃ and 60℃. The fixed ratio for ethanol was 1:25, acetone 1:10 and water 1:5. While the fixed extracting time for ethanol was 1 hour same like water and for acetone, 3 hours.

4.3.1 Extracting solvent: Ethanol

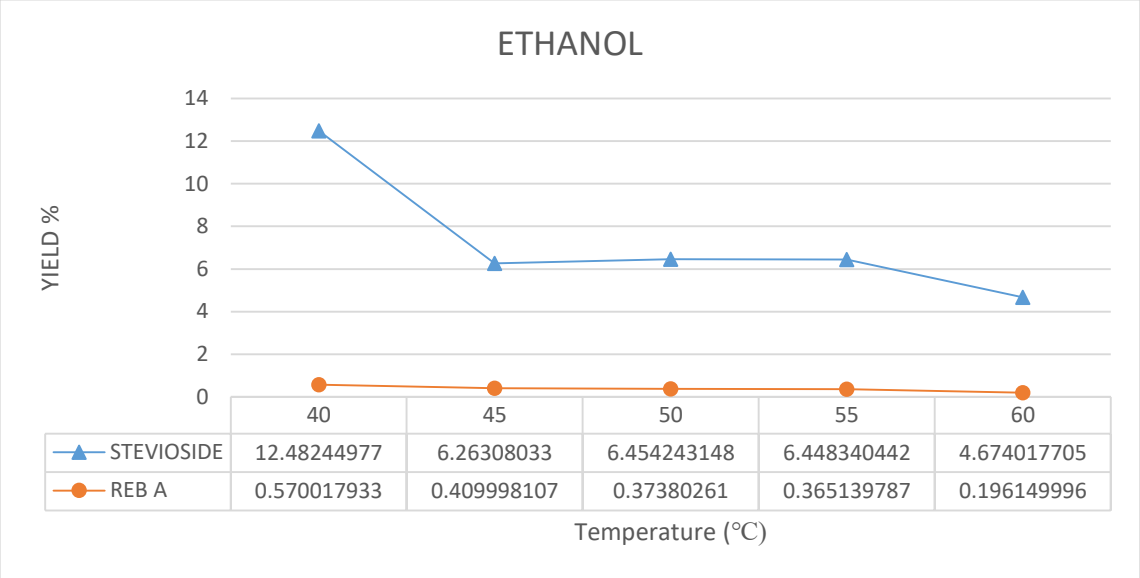


Figure 4.9: Yield of reb A and stevioside during extraction by ethanol in 5 different temperature.

Figure 4.9 is an illustration of yield of stevioside and reb A from 5 different temperature of extraction. Highest yield of stevioside obtained in extraction under 40℃. The difference in yield between 40℃ and 45℃ was 6.22 %. This drastic decrease in temperature indicates how significant to extract stevioside in lower temperature.

Almost the same trend as the yield of stevioside, yield of reb A was at highest when extracted under lowest temperature, 40℃. Increase in temperature only slightly decrease the yield of reb A. (Afendi, 2013) stated that higher temperature can accelerate the solvent flow and thus increase the reb A yield. But, extracting in higher temperature also decrease the fluid density that may reduce the extraction efficiency (Guo-qing, 2005). Thus, most preferable temperature for extracting solvent ethanol was 40℃

4.3.1 Extracting solvent: Acetone

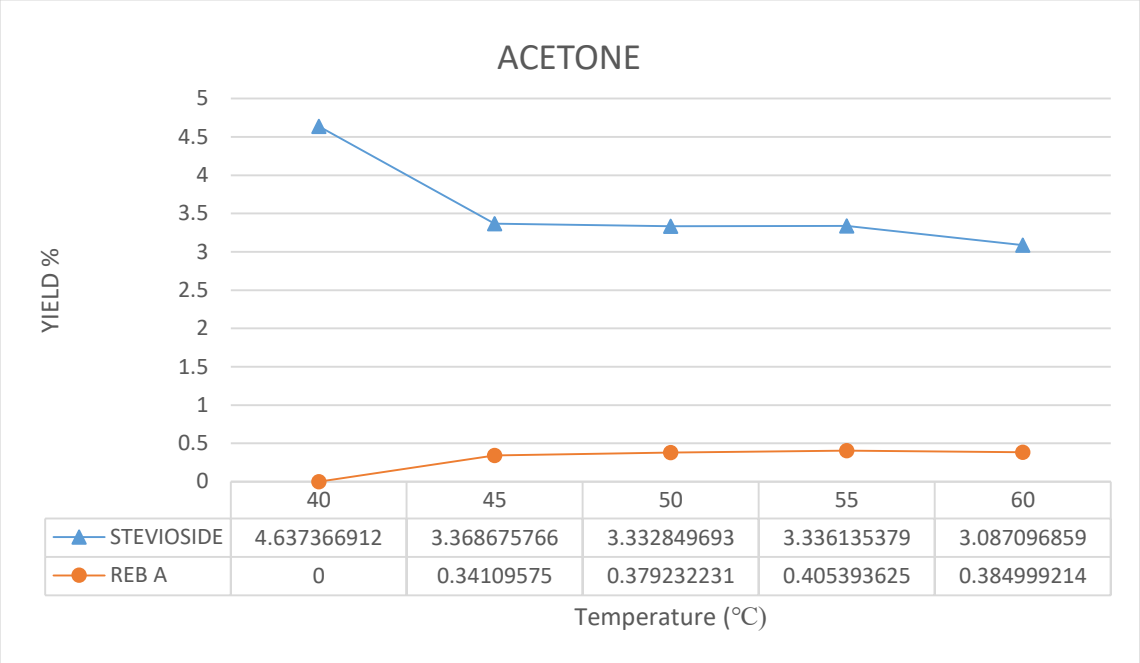


Figure 4.10: Yield of reb A and stevioside during extraction by acetone in 5 different temperature.

In above figure, yield of stevioside was the highest when extracted under 40℃. Increase in temperature to 45℃ drastically decrease the yield for 1.27%. Increasing in temperature to 55℃ does not affect the yield of stevioside but at highest temperature, 60℃, the yield drop to 3.08%. Increase in temperature will accelerate the reaction but also trigger any unwanted side reaction (Afendi, 2013). The drop in yield of stevioside at 60℃ might due to the solvent was saturated with other impurities.

Yield of reb A was zero when extracted at 40℃. Increase in temperature triggered the recovery of reb A using acetone. Means that acetone need more heat energy so that extraction of reb A can take places. For acetone, the best temperature to recover reb A was 55℃.

4.3.1 Extracting solvent: Water

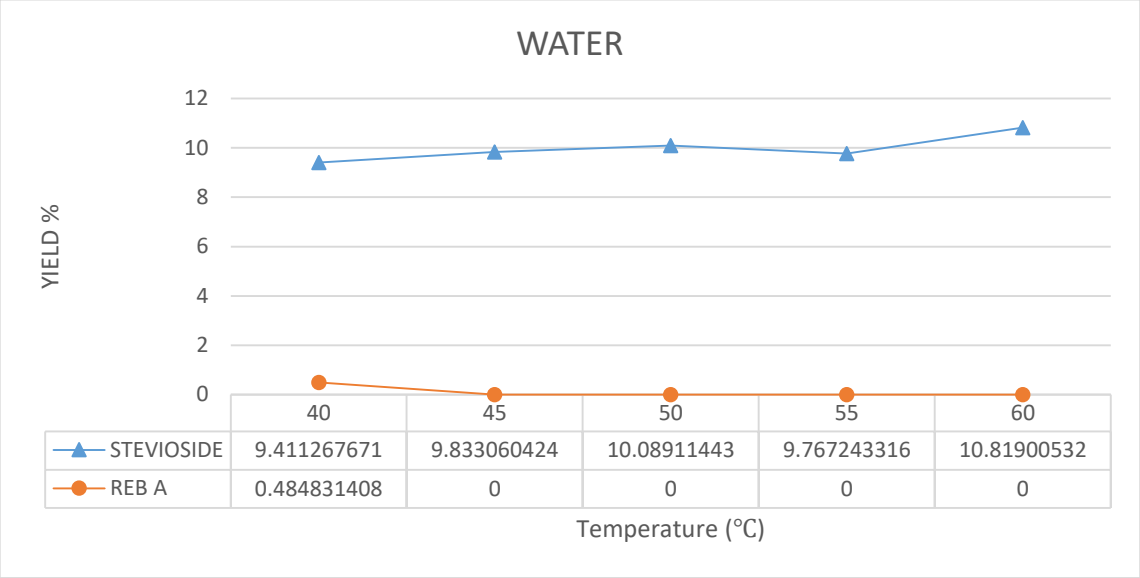
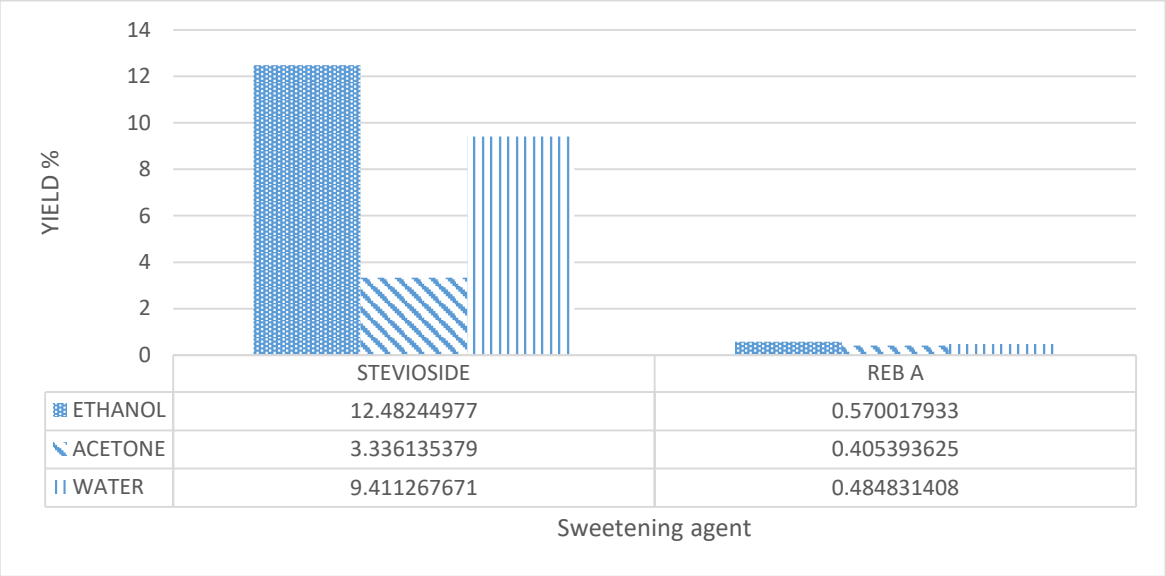


Figure 4.11: Yield of reb A and stevioside during extraction by acetone in 5 different temperature.

The yield of stevioside for water were almost as high as ethanol, indicates that water is good at extracting stevioside. As the temperature increase, the yield of stevioside also increase and the highest yield was 10.82%. This increasing yield was because increase in temperature will accelerate the recovery of stevioside process and make the reaction more active. On the other hand, the highest yield of reb A obtained in 40℃, 0.48%. Extraction in 45℃ until 60℃ showed zero values. In higher temperature, more stevioside obtained but zero yield of reb A recovered. This results indicate that most preferable temperature for extraction using water was 40℃.

4.4 Comparison between extracting solvent

Extracting solvent ethanol, acetone and water has been used to extract stevioside and reb A. Most preferable parameter was obtained after finished all the phase of extraction. For ethanol, the best parameter was 1:25 ratio between stevia and solvent, 1 hour of extraction in 40℃. For acetone, the parameters are 1:10 for ratio, 3 hour of extraction in 55℃. And lastly, most preferable parameters for water are 1:5, 1 hour and 40℃.



	STEVIOSIDE	REB A
ETHANOL	12.48244977	0.570017933
ACETONE	3.336135379	0.405393625
WATER	9.411267671	0.484831408

Figure 4.12: Yield of stevioside and reb A extracted from the preferable condition of ethanol, acetone and water.

Extracting solvent ethanol shows the highest yield for reb A and stevioside compared to acetone and water. This is because ethanol contain hydroxyl group which is hydrophilic. Rebaudioside A, which is a large and polar hydrocarbon molecule, dissolves better in ethanol although it has more polar function (Afendi, 2013). Water was not preferable because extraction in phase 1,2 and 3, most of them failed to recover reb A although the yield of stevioside were high. Acetone was not good in extract stevioside as the yield value is very low compared to ethanol and acetone.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

This study showed stevia rebaudiana, a natural sweetener containing stevioside and reb A as its main sweetening agent. All three extracting solvent able to recover stevioside and reb A. But, the best extracting solvent was ethanol. Water can extract stevioside almost as much as ethanol but in term of extracting reb A, it was poor. Meanwhile, acetone can extract reb A not as much as ethanol can but the yield of stevioside was very low.

Besides determining the best extracting solvent, the yield of reb A and stevioside can be improved by adjusting the ratio between stevia and extracting solvent, extracting time and also temperature. For extracting using ethanol, the most preferable parameters were 1:25, 1 hour and 40℃.

5.2 RECOMMENDATION

The determination of preferable parameter was been done by method one factor at time. But the parameters value was not accurate as the value only limited to the only value that we tested. As to obtain the correct value and combination, it is recommended to use alternative optimization process such as Response Surface Methodology (RSM) (Puri, 2011). The RSM able to determine the optimum combination for extraction parameter for extracting stevioside and reb A.

The second recommendation is, chromatogram from the sample show some numbers of impurities means that further clarification and purification is important to produce highly pure stevioside and reb A.

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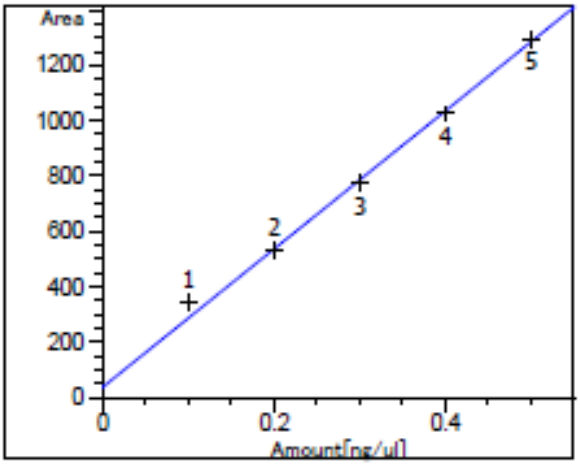
APPENDIX




```
  at exp. RT: 2.237
DAD1 C, Sig=210,4 Ref=360,100
Correlation:              0.99779
Residual Std. Dev.:    34.85890
Formula: y = mx + b
      m:    2505.20547
      b:      35.83491
      x: Amount
      y: Area
```

Appendix A Calibration curve of stevioside standard

```
Acq. Operator    : SYSTEM                      Seq. Line :   2
Acq. Instrument  : HPLC 1260                    Location : Vial 2
Injection Date   : 9/11/2017 3:07:30 PM            Inj :   1
                                               Inj Volume : 10.000 µl
Method           : C:\CHEM32\1\DATA\STEVIA_9.11.17 2017-11-09 14-50-25\STEVIA251017.M (
                   Sequence Method)
Last changed     : 9/11/2017 2:50:25 PM by SYSTEM
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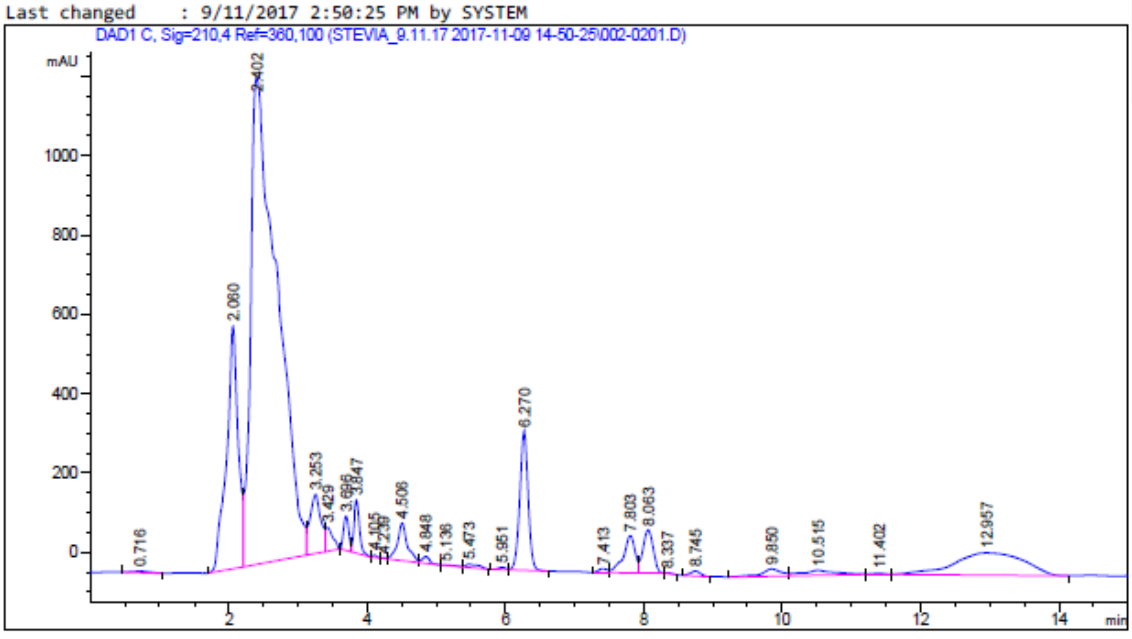
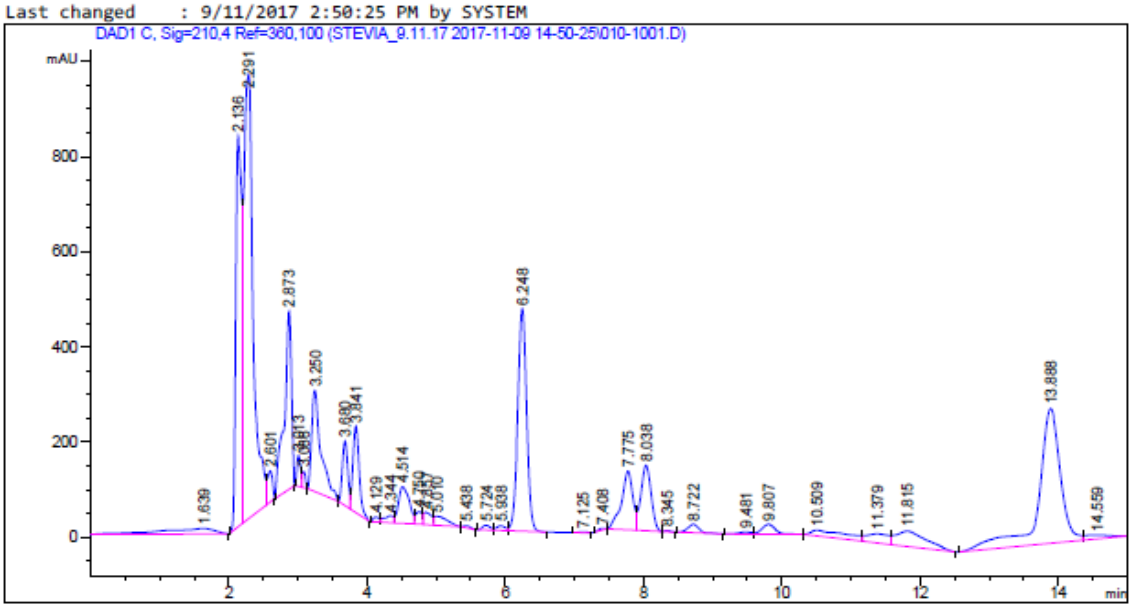


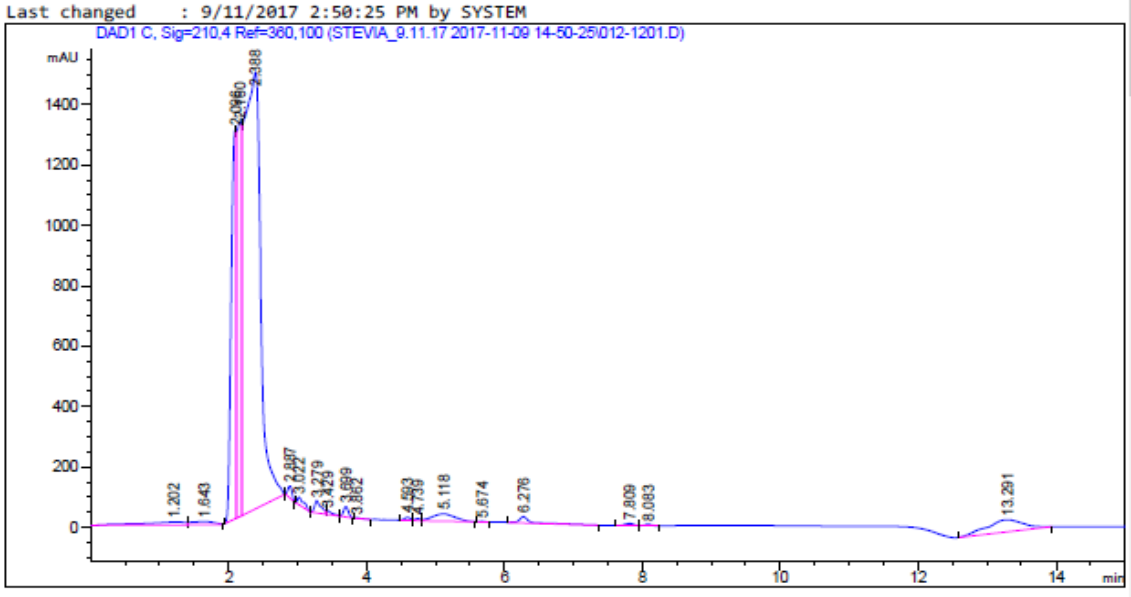
Appendix B chromatogram of the most preferable parameters (1:25, 1 hour, 40℃), extraction by ethanol

Acq. Operator : SYSTEM Seq. Line : 10
Acq. Instrument : HPLC 1260 Location : Vial 10
Injection Date : 9/11/2017 5:15:51 PM Inj : 1
Inj Volume : 10.000 µl
Method : C:\CHEM32\1\DATA\STEVIA_9.11.17 2017-11-09 14-50-25\STEVIA251017.M (Sequence Method)
Last changed : 9/11/2017 2:50:25 PM by SYSTEM



Appendix C chromatogram of the most preferable parameters (1:10, 3hour, 55℃), extraction by acetone

Acq. Operator : SYSTEM Seq. Line : 12
Acq. Instrument : HPLC 1260 Location : Vial 12
Injection Date : 9/11/2017 5:47:58 PM Inj : 1
Inj Volume : 10.000 µl
Method : C:\CHEM32\1\DATA\STEVIA_9.11.17 2017-11-09 14-50-25\STEVIA251017.M (Sequence Method)
Last changed : 9/11/2017 2:50:25 PM by SYSTEM



Appendix D chromatogram of the most preferable parameters (1:5, 1 hour, 40℃), extraction by water

```
Acq. Operator   : SYSTEM                        Seq. Line :  10
Acq. Instrument : HPLC 1260                      Location : Vial 10
Injection Date  : 9/10/2017 1:12:09 PM               Inj :   1
                                               Inj Volume : 10.000 µl
Acq. Method     : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M
Last changed    : 9/10/2017 11:31:49 AM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M (Sequence
                  Method)
Last changed    : 10/10/2017 2:32:26 PM by SYSTEM
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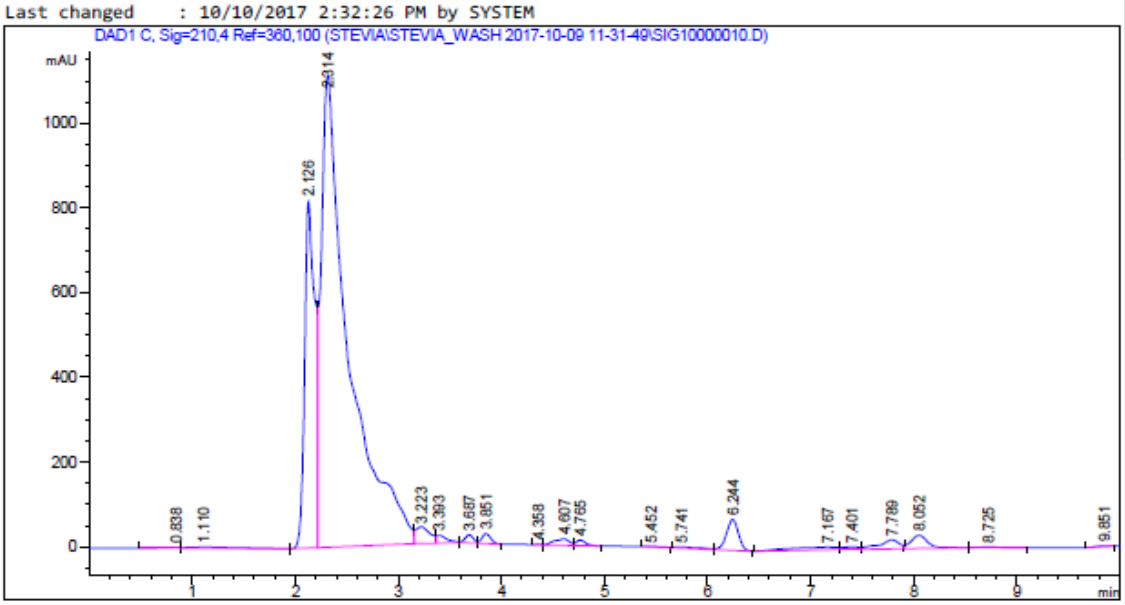

Appendix E, chromatogram of best ratio (1:25) extraction using ethanol.

```
Acq. Operator   : SYSTEM                        Seq. Line :  16
Acq. Instrument : HPLC 1260                      Location : Vial 16
Injection Date  : 9/10/2017 2:18:17 PM               Inj :   1
                                               Inj Volume : 10.000 µl
Acq. Method     : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M
Last changed    : 9/10/2017 11:31:49 AM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M (Sequence
                  Method)
Last changed    : 10/10/2017 2:32:26 PM by SYSTEM
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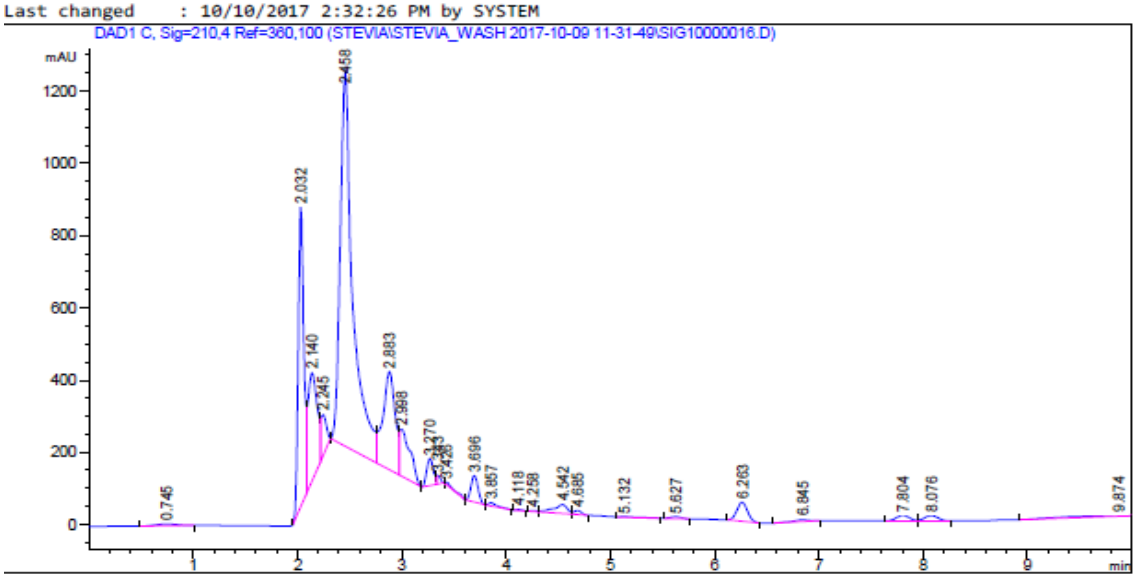
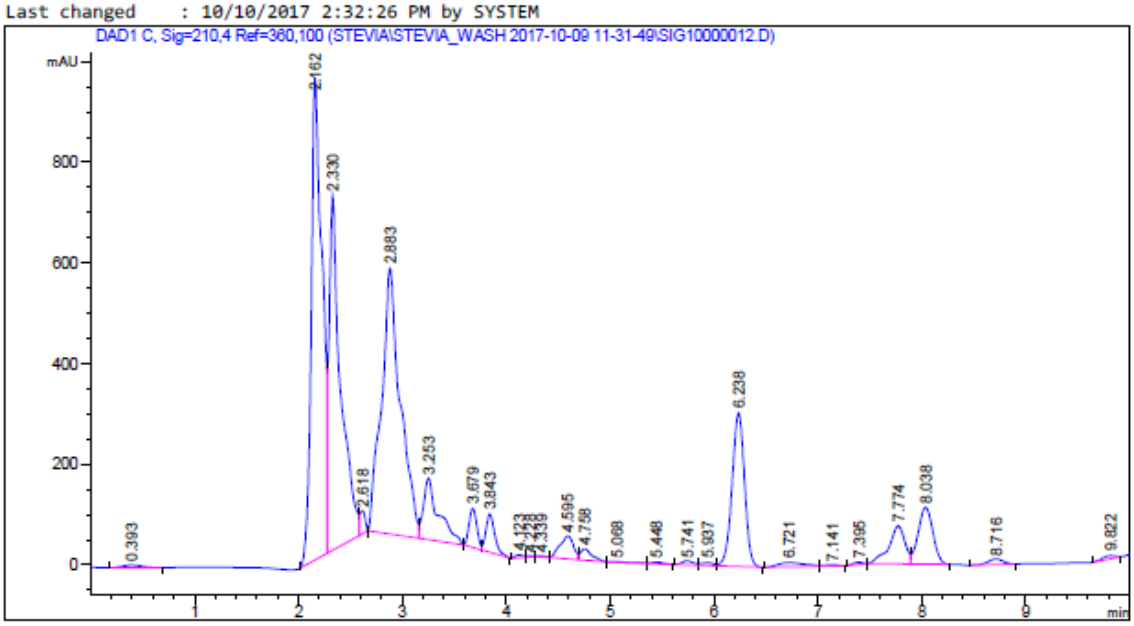


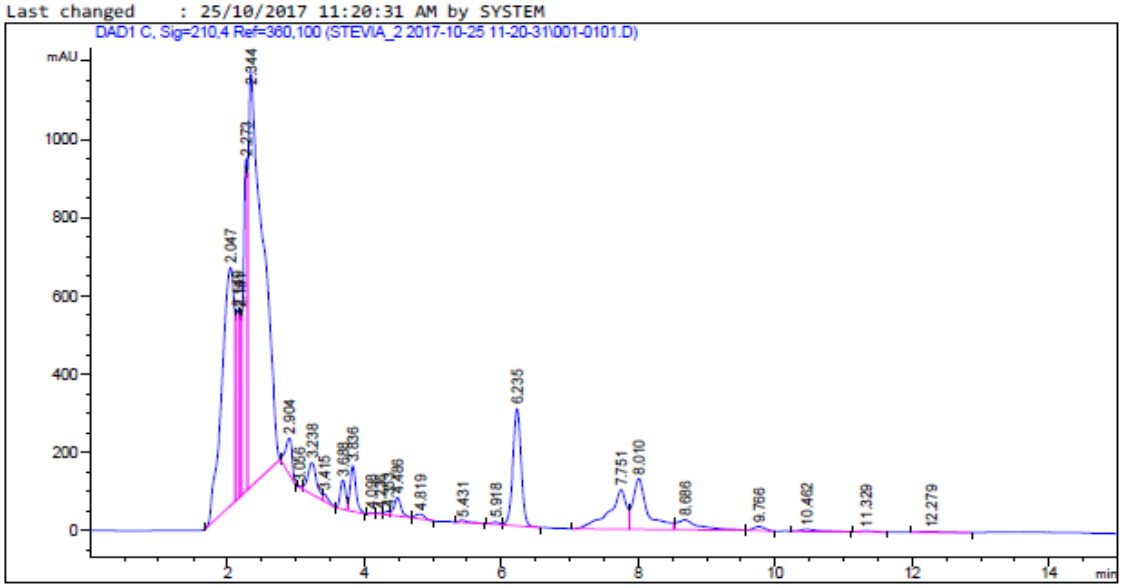
Appendix F, chromatogram of best ratio (1:5) extraction using water

Acq. Operator : SYSTEM Seq. Line : 12
Acq. Instrument : HPLC 1260 Location : Vial 12
Injection Date : 9/10/2017 1:34:13 PM Inj : 1
Inj Volume : 10.000 µl
Acq. Method : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M
Last changed : 9/10/2017 11:31:49 AM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\STEVIA\STEVIA_WASH 2017-10-09 11-31-49\STEVIA.M (Sequence Method)
Last changed : 10/10/2017 2:32:26 PM by SYSTEM



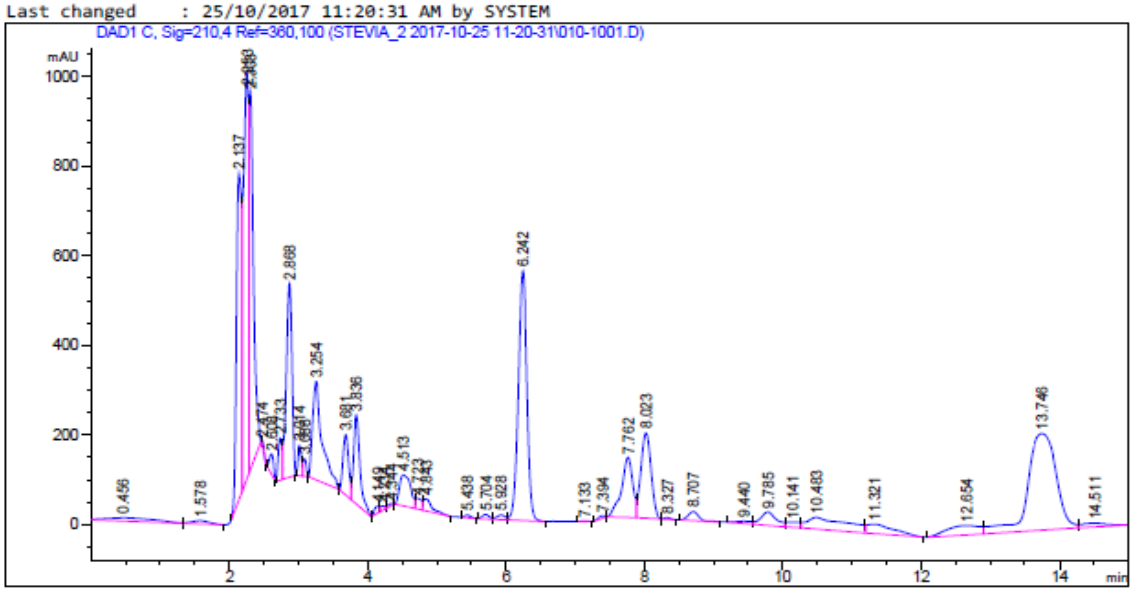
Appendix G, chromatogram of best ratio (1:10), extraction using acetone.

Acq. Operator : SYSTEM Seq. Line : 1
Acq. Instrument : HPLC 1260 Location : Vial 1
Injection Date : 25/10/2017 11:21:28 AM Inj : 1
Inj Volume : 10.000 µl
Method : C:\CHEM32\1\DATA\STEVIA_2 2017-10-25 11-20-31\STEVIA251017.M (Sequence Method)
Last changed : 25/10/2017 11:20:31 AM by SYSTEM



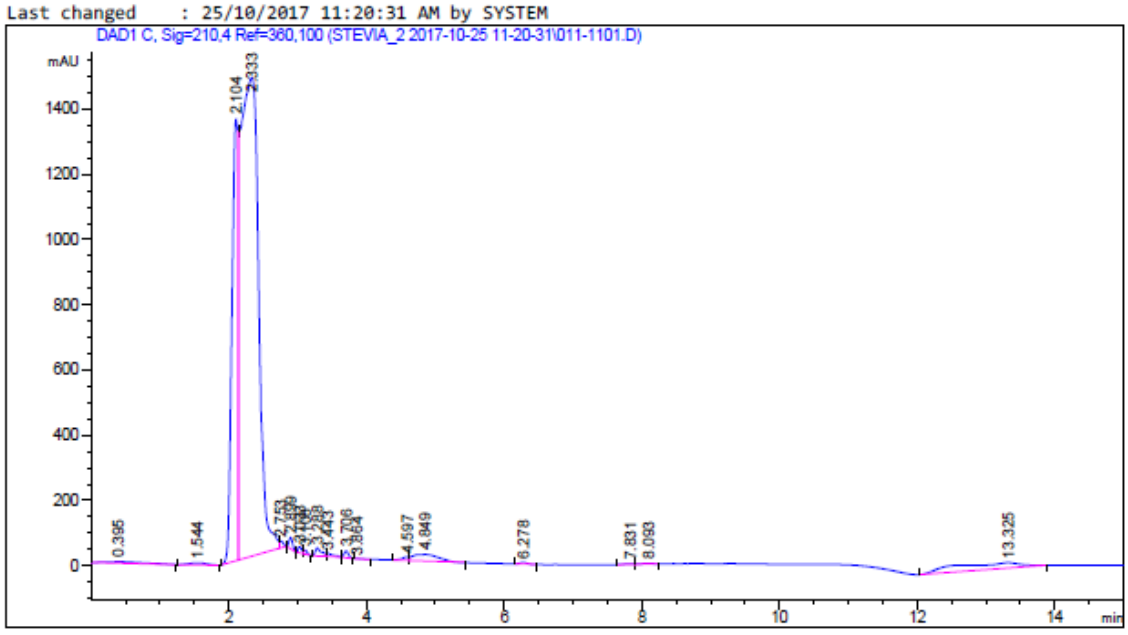
Appendix H, chromatogram of the best time (1 hour), extraction using ethanol.

Acq. Operator : SYSTEM Seq. Line : 10
Acq. Instrument : HPLC 1260 Location : Vial 10
Injection Date : 25/10/2017 1:45:57 PM Inj : 1
Inj Volume : 10.000 µl
Method : C:\CHEM32\1\DATA\STEVIA_2 2017-10-25 11-20-31\STEVIA251017.M (Sequence Method)
Last changed : 25/10/2017 11:20:31 AM by SYSTEM



Appendix I, chromatogram of best time (3 hour), extraction using acetone

Acq. Operator : SYSTEM Seq. Line : 11
Acq. Instrument : HPLC 1260 Location : Vial 11
Injection Date : 25/10/2017 2:02:03 PM Inj : 1
Inj Volume : 10.000 µl
Method : C:\CHEM32\1\DATA\STEVIA_2 2017-10-25 11-20-31\STEVIA251017.M (Sequence Method)
Last changed : 25/10/2017 11:20:31 AM by SYSTEM



Appendix J, Chromatogram of best time (1 hour), extraction using water