RECOVERY OF REBAUDIOSIDE A FROM STEVIA REBAUDIANA (BERTONI) EXTRACTS USING TWO DIFFERENT PURIFICATION TECHNIQUES

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Bachelor of Manufacturing Engineering Technology (Pharmaceutical)

Faculty of Engineering Technology
UNIVERSITI MALAYSIA PAHANG

January 2018

ACKNOWLEDGEMENTS

First of all I am grateful to The Almighty God who allow for me to alive and for establishing me to complete this work.

I would like to give my special thanks to my supervisor Prof. Datin Dr. Mimi Sakinah Binti Abdul Munaim who has supplied me invaluable construction ideas and suggestions on my thesis work. I also would like to thank her for encouraging me and give moral support during conducting an experiment and during writing this thesis.

I take this opportunity to record my sincere thanks to my group mates, all lecturers and laboratory staffs of Faculty of Engineering Technology (Ftech) for their collaboration and willingness to achieve my laboratory work in the specified schedule successfully.

My special thanks to my father, Abdul Wahid Bin Mohammad and my mother, Roshidah Binti Mohd Arshad who always encourage and support me in term of moral support and financial support.

Last but not least, I also place on record, my sense of gratitude to one and all who, directly or indirectly, have lent their helping hand in this venture.

ABSTRAK

Stevia adalah pemanis rendah kalori yang diekstrak daripada daun stevia rebaudiana (Bertoni). Dua komponen utama yang bertanggungjawab terhadap rasa manis stevia adalah stevioside dan rebaudioside A (Reb A). Reb A boleh dianggap sebagai komponen terbaik dari daun stevia kerana ia lebih manis daripada stevioside dan ia tidak mempunyai rasa pahit selepas pengambilannya. Walau bagaimanapun, Reb A sangat sukar untuk dipisahkan daripada steviol glycosides lain seperti stevioside dan rebaudioside C (Reb C) kerana struktur kimia stevioside, Reb A, dan Reb C sangat serupa. Objektif kajian ini adalah untuk mengenal pasti kaedah terbaik yang akan menghasilkan kuantiti and kualiti Reb A yang tinggi daripada ekstrak stevia mentah. Terdapat dua jenis kaedah pemurnian yang telah dijalankan dalam kajian ini iaitu pengekstrakan cecair-cecair (LLE) menggunakan dietil eter dan butanol dan penjerapan menggunakan arang aktif. Pelbagai parameter telah dijalankan seperti nisbah dietil eter dan ekstrak stevia, peratusan arang aktif dan peringkat pengekstrakan. Kuantiti Reb A ditentukan oleh kaedah kromatografi berprestasi tinggi (HPLC) dengan menggunakan lajur Eclipse Plus C18 (250 × 4.6 mm I.D., 5 μm). Dalam kajian ini, LLE merupakan kaedah pemurnian yang terbaik kerana ia boleh mengekstrak Reb A sehingga 0.29% berbanding dengan kaedah penjerapan yang hanya boleh mengekstrak Reb A sebanyak 0.01%. Analisis menunjukkan bahawa Reb A yang mempunyai tinggi kuantiti dan kualiti telah diperolehi pada nisbah bahan 1:2 dan pengekstrakan perlu diulang sebanyak 3 kali untuk mendapatkan jumlah Reb A yang maksimum. Kesimpulannya, LLE merupakan kaedah yang terbaik untuk mengekstrak Reb A daripada ekstrak stevia.

ABSTRACT

Stevia is a low calories sweetener extracted from the leaves of stevia rebaudiana (Bertoni). Two main compounds responsible for the sweet taste of stevia are stevioside and rebaudioside A (Reb A). Reb A can be considered as the best part of stevia leaves because it is sweeter than stevioside and it has no bitter aftertaste. However, Reb A is very difficult to be separated from other steviol glycosides such as stevioside and rebaudioside C (Reb C) because the chemical structures of stevioside, Reb A, and Reb C are very similar. The objective of this study is to identify the best method that will produce a high yield and purity of Reb A from crude stevia extract. Two types of purification methods were studied in these researches which are liquid-liquid extraction (LLE) using diethyl ether and butanol and adsorption using activated charcoal. Various parameters were carried out such as the ratio of diethyl ether and stevia extract, the percentages of activated charcoal and the extraction stages. The quantification of Reb A was determined by analytical high-performance liquid chromatography (HPLC) using Eclipse Plus C18 (250 × 4.6 mm I.D., 5 μm) column. In this study, LLE was found to be the best purification method because it can recover up to 0.29% of Reb A compared to adsorption method which only can recover 0.01% of Reb A. The analysis showed that the highest yield and purity was obtained at material ratio 1:2 and the extraction needs to be repeated for 3 times in order to recover maximum amount of Reb A. As a conclusion, LLE is the best purification method for recovery of Reb A from stevia extracts.

TABLE OF CONTENT

CHAPTER 5 CONCLUSION

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

^{o}C	Degree celcius
%	Percentage

LIST OF ABBREVIATIONS

bw	Body weight
cm	Centimetre
DE	Diethyl ether
EDI	Estimated daily intake
g	Gram
GC	Gas chromatography
GRAS	Generally Recognized As Free
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-Performance Liquid Chromatography
HSCCC	High-speed counter-current chromatography
ICH	International Council for Harmonization
LC	Liquid chromatography
LLE	Liquid-liquid extraction
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
NF	Nanofiltration
nm	Nanometre
PHWE	Pressurized hot water extractor
Ppm	Part per million
PAD	Pulsed Amperometric Detection
Reb A	Rebaudioside A
Reb C	Rebaudioside C
RPM	Revolution per minutes
SLE	Solid-liquid extraction
UF	Ultrafiltration
µL	Microliter
µm	Micrometre

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In 2016, New Straits Times reported that diabetes is one of the top 10 deadliest diseases in Malaysia. Statistical data from National Health and Morbidity Survey 2015 revealed that the number of diabetics in Malaysia have increased to 17.5% from 15.2% in 2011 (Bernama, 2016). One of the major factors that contribute to diabetes is obesity. The British medical journal, The Lancet, show that 49% of women and 44% of men in Malaysia were found to be obese (Idris, 2016). Obesity is closely related with insulin in human body. The main function of insulin in the human body is to allow the sugar present in the blood to enter the muscle and tissue cell. When the fat in the body is increased, the muscle and tissue cells become resistance to insulin, leading to high blood sugar level in blood (hyperglycemia) and finally lead to diabetes (Czech, 2017). However, in a modern lifestyle era, people are getting concerned about their health and try to avoid taking too much sugar and high-calories food in their daily diet. This result in the high demand for stevia sweeteners in the foods market because it has no calories and not harmful as other chemical sweeteners like Aspartame and Saccharin.

Stevia rebaudiana (Bertoni) or commonly known as stevia or “sugar leaf” is a small perennial herb belonging to the sunflower family (Asteraceae). It was discovered by indigenous people in South America who used the stevia leaves to sweeten beverages and chew for the sweet taste. The compound responsible for the sweet taste in the stevia leaves is steviol glycosides. This includes stevioside, Reb A, Reb B, Reb C, Reb D, Reb E and Reb F, steviolbioside and dulcoside A (Anvari, 2016). Stevioside tastes about 300 times while reb A tastes about 400 times sweeter than 0.4% sucrose solution. The best part of stevia extract is Reb A because it has no aftertaste but stevioside has a bitter aftertaste.

Basically, there are two steps involved in the isolation of Reb A. Firstly, dried ground stevia leaves were extracted using various techniques such as solvent extraction method, supercritical fluid extraction and enzymatic extraction. Then, the stevia extract undergoes a purification process to remove color and other impurities to produce high purity of stevia. There are a lot of studies on purification method such as membrane filtration, column chromatography, preparative HPLC, liquid-liquid extraction (LLE) and adsorption using activated charcoal. The yield of Reb A was determined using analytical HPLC.

1.2 Problem Statement

Stevioside, Reb A and Reb C are the major constituents of steviol glycosides and they are responsible for the sweet taste of stevia. Among them, Reb A has the highest sweetness and high quality of taste. Nevertheless, it is very difficult to isolate Reb A from stevioside and Reb C because the chemical structures of stevioside, Reb A, and Reb C are very similar. There are a lot of purification methods used to isolate Reb A from crude stevia extract, for examples Ultrafiltration (UF) and Nanofiltration (NF) membrane, column chromatography, preparative HPLC, and crystallization technique. However, these methods might take a longer processing time, low yields, complicated process and difficult to scale up. Due to several limitations in this experiment such as availability of apparatus and equipment, time and cost, LLE method and adsorption method have been selected in this present study. The yield of Reb A was compared to identify the best method that will provide the highest extraction yield and purity of Reb A.

1.3 Research Objectives

General objective

The objective of this study is to identify the best purification method that will produce high yield and purity of Reb A from crude stevia extract.

Specific objectives

i. To determine the yield and purity of Reb A using different ratio of diethyl ether and crude stevia extract in LLE method.
ii. To study the effect of number of purification towards the yield of Reb A in LLE method.
iii. To determine the yield and purity of Reb A using different percentages of activated charcoal in adsorption method.
iv. To compare the yield of Reb A between different number of extraction in adsorption method.

1.4 Scope of the Study

In order to achieve research objectives, the following scopes have been identified:

i. To determine the effects of different ratio of diethyl ether (ml) and crude stevia extract (ml) on the yield and purity of Reb A in LLE. The ratios are 1:2, 2:2, 3:2, 4:2 and 5:2. The yield of Reb A was analysed using analytical HPLC.
ii. To study the effects of different percentages of activated charcoal (5, 10, 15, 20 and 25%) on the yield and purity of Reb A in adsorption method. The concentration of Reb A was determined by analytical HPLC.
iii. To evaluate the effect of extraction stages (stage I, II and III) on the yield of Reb A in LLE and adsorption method. The quantification of Reb A was analysed using analytical HPLC
iv. The yield of Reb A in both purification techniques were compared to determine the best method which provided high yield and purity of Reb A.

CHAPTER 2

LITERATURE REVIEW

2.1 Stevia rebaudiana (Bertoni)

Stevia plant and it sweet taste profile were first described by Swiss botanist Moises Santiao Bertoni in 1899 while he was conducting a research in Eastern Paraguay. Initially, it was called *Eupatorium rebaudianum* but in 1905 it was changed to Stevia rebaudiana (Bertoni). Stevia is a small herbaceous plant which is originating from South America (Brazil and Paraguay). It is a low calorie natural sweeteners and it has been widely applied to food and pharmaceutical industries (Lemus-Mondaca, 2012). Stevia has approximately 230 species but only Stevia rebaudiana (Bertoni) have the highest sweetness taste.

Some other related species of stevia are *Stevia dianthoidea, Stevia anisostemma, Stevia lemmonii, Stevia bertholdii, Stevia enigmatica, Stevia eupatoria, Stevia micrantha, Stevia crenata, Stevia salicifolia, Stevia ovata, Stevia plummerae, Stevia serrata, Stevia Phlebophylla* and *Stevia viscida* (Yadav, 2011) (Bhutia, 2016). Figure 2.1 shows Stevia rebaudiana (Bertoni) plant.

Figure 2.1 Stevia rebaudiana (Bertoni) (Source: Lemus-Mondaca, 2012)

2.2 Compositions of stevia extract

The major components of steviol glycosides in stevia leaves are stevioside, Reb A, B, C, D, E and F, steviolbiodise as well as dulcoside A (Purkayastha, 2014). Typical dry weight for the four major glycosides are 9.1% stevioside, 3.8% reb A, 0.6% reb C and 0.3% dulcoside (Gallo, 2017). Meanwhile, for the sweetener properties, Reb C taste about 50-120 times, stevioside tastes about 300 times while Reb A tastes about 400 times sweeter than 0.4% sucrose solution. The degree of sweetness of the steviol is determined by the number of carbohydrate units at the C-13 and C-19 positions. Reb A taste sweeter than stevioside because it has an extra glucose unit at the C-13 position (Adari, 2016). Figure 2.2 demonstrate the molecular structure of stevioside and Reb A.

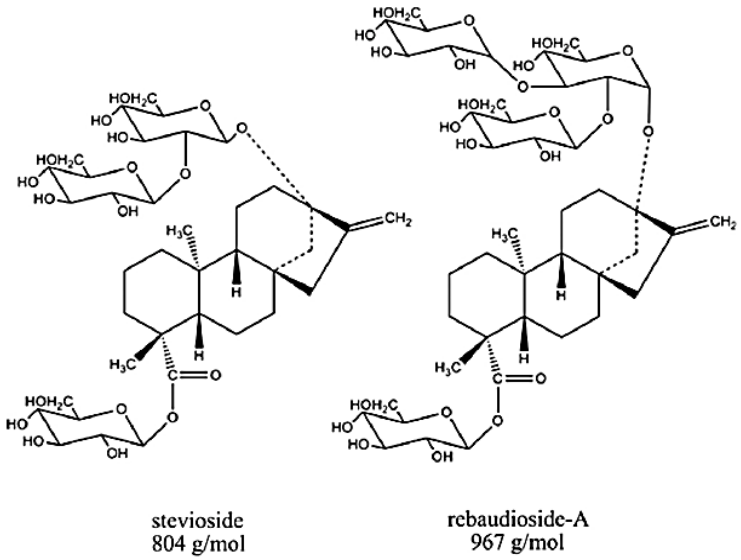


Figure 2.2: Molecular structure of stevioside and Reb A (Source: Vanneste, 2011)

In the United State, Reb A is Generally Recognized As Safe (GRAS) to be used as a sweetener in beverage including meat and poultry. An estimated daily intake (EDI) of Reb A is 2 milligrams per kilogram body weight per day (2mg/kg bw/day) (Larowe, 2011). Furthermore, various toxicological studies on stevioside and Reb A found that they do not have mutagenic, carcinogenic and allergic reactions to the consumers when it is used as a sweetener in a beverage (Lemus-Mondaca, 2012). In addition, abundant literatures reported that stevioside and Reb A have an antibacterial, antioxidant, antimicrobial, anti-retroviral and hypoglycemic activity (Deshmukh, 2014).

2.3 Purification process

Purification is a process of removing undesirable contaminants such as chemical contaminants, biological contaminants, and suspended solids. Purification process is very important in the production of stevia extract because it may affect the quality, concentration, characteristics and taste of stevia. Up to now, there are many purification methods of stevia extract containing Reb A in varying degrees of purity have appeared in the published and patented literature. Highly purified Reb A possesses an improved taste profile and eliminated undesirable aftertaste of stevia.

In the previous studies, membrane filtration method is often adopted to separate Reb A from stevioside and other compounds. A dried stevia leaves are subjected to pressurised hot water extractor (PHWE) followed by isolation and purification of main glycosides through Ultrafiltration (UF) membrane. Then, the main glycosides are concentrated by using Nano filtration (NF) membrane. However, the yield of Reb A obtained at the end of the process decreases significantly from the initial concentration. It was reported that the initial concentration of Reb A in 100gram (g) stevia leaves was 2.6g but after final purification process, Reb A content was only 0.2g per 100g stevia leaves (Rao, 2012). This problem may occur due to deposition of Reb A on a membrane filtration unit that may has caused membrane fouling (Guo, 2012).

Huang (2010) isolated and purified three major steviol glycosides which are stevioside, Reb A and Reb C using high-speed counter-current chromatography (HSCCC). HSCCC is a liquid-liquid partition chromatography technique that is free to solid support matrix and it can produce high yield of product. In this study, the yield of stevioside, Reb A and Reb C are 54mg, 36mg and 13mg from 200mg of the crude extract. Even though liquid-liquid partition chromatography can produce a high recovery of Reb A, it is difficult to scale up and not suitable for industrial production (Lv, 2014).

In other literature, Lv (2014) separated and purified steviol glycosides from crude stevia extract by macroporous resin and preparative HPLC under hydrophilic interaction liquid chromatography (HILIC) mode. Using this method, 79.2mg of stevioside, 33.7mg of Reb A and 9.4mg of Reb C were obtained from 200mg of the crude extract. The major disadvantages of purification by HPLC under HILIC mode is it needed a high volume of aprotic solvent acetonitrile. Moreover, it is a less flexible technique (Heaton, 2012).

Besides that, Reb A can be separated from stevioside by recrystallization method. However, this method has several limitations such as complicated operation, environmental contamination and the massive consumption of materials and reagents (Chen, 2012). The comparison of Reb A yield using different purification methods were shown in Table 2.1.

Table 2.1 Comparison of different purification methods

Method	**Product yield**	**Percentage yield (%)**	**References**
Ultrafiltration (UF) and Nanofiltration (NF) membrane	0.2g of Reb A per 100g of extract	0.20	Rao, 2012
High-speed counter-current chromatography (HSCCC)	36mg of Reb A per 200mg extract	18.00	Huang, 2010
Macroporous resin and preparative HPLC under HILIC mode.	33.7mg of Reb A per 200mg extract	16.85	Lv, 2014
Adsorption using activated charcoal and celite	Stevioside	12.00	Mohankumar, 2011
LLE using diethyl ether and butanol	Stevioside	6.51	Abou-arab, 2010

2.4 Adsorption method using activated charcoal and celite

Adsorption is one of the methods used to purify, concentrate or separate different types of components. In the United States, approximately 350 million pounds of activated carbon are used annually in adsorption processes (M.Roy, 1995). Activated charcoal or also known as activated carbon is a fine black odourless and tasteless powder made from wood or other materials (Pillay, 2013). It has a small, low-volume pores that increase the surface area available for adsorption or chemical reactions. In the purification of stevia extract, activated charcoal acts as a decolourizer agent. It removed plants pigment, carotenoids, chlorophylls, tannins and phenolic substances which are responsible for giving a dark green or brown colour to the stevia extract.

In the previous study, Rajab (2009) treated crude green stevia extract with 10% activated charcoal for 20 minutes. In the second steps of purification, the extract was treated with 5% of celite for 20 mins. The concentration of stevioside obtained was 11.6%. In Indian Patent No. 245903, 5% of activated charcoal and 5% of celite has been used to purify stevioside and the final concentration of stevioside obtained was 12% (Mohankumar, 2011). However, the previous study only provides information about the concentration of stevioside and less information about the yield and purity of reb A obtained using this method. Therefore, the present study will investigate the effects of different concentration of activated charcoal and the effects of purification stages on the final yield and purity of reb A.

The advantages of this method are low cost, simple operation and shorter processing time compared to other methods such as membrane filtration and preparative HPLC. The most important are, this method is considered as a ‘green method’ and environmental friendly because it does not use any dangerous chemical or reagent and it is safe for human consumption (Adari, 2012). Table 2.2 shows the comparison between different percentage of activated charcoal, celite and yield of the product obtained using adsorption method.

Table 2.2 Comparison between the different percentages of activated charcoal and celite with the yield of stevioside

Title	Percentage of activated charcoal (%)	Type of charcoal	Percentage of celite (%)	Yield of stevioside (%)	References
Simple extraction and membrane purification process in isolation of steviosides with improved organoleptic activity	20	Animal charcoal	5	N/A	Rao, 2012
Purification and toxicity studies of stevioside from Stevia rebaudiana (Bertoni)	10	N/A	5	11.6	Rajab, 2009
A process for production of steviol from stevia species (Indian patent)	5	N/A	5	12.0	Mohankumar, 2011

2.5 LLE method using diethyl ether and butanol

LLE is a mass transfer process that separate compounds or solutes based on their relative solubility in two different immiscible liquids, usually water and an organic solvent (Pohorecki, 2010). LLE considered as a simple or partial purification process. A feed solution containing a different type of solutes mixed with an immiscible solvent having a different density. The mass transfer of the desired solute occurs between the two immiscible liquids until they reach an equilibrium stage. Then, the mixture is then separated by gravity or by a centrifugal force depends on the degree of separation or the types of solvent use.

In the purification of stevia extract, the feed solution (crude green extract) was mixed with diethyl ether. Diethyl ether is an organic compound that acts as a reagent to remove the green colour from the crude green extract. Diethyl ether will float above water because it is less dense and slightly soluble in water. A lower transparent layer rich with reb A is collected and extracted with butanol. According to International Council for Harmonization (ICH) Harmonised Tripartite Guideline, Class 3 residual solvents such as butanol are limited to not more than 50mg per day or 5000 part per million (ppm) (USP 30, 2007). Even though LLE method uses alcohol to extract the desired product but later it will be evaporated to the surrounding. Moreover, it is a simple technique and only used basic laboratory equipment.

The liquid-liquid extraction method is not limited to the purification of stevia only, but it is also used in purification of other herbs plants. Kim (2007) purified two monoterpene glucosides which are paeoniflorin and albiflorin in *Paeonia lactiflora* plant using LLE method. Meanwhile, Kim (2001) purified saponin compounds in *Bupleurum falcatum* by LLE technique and preparative liquid chromatography (LC). Table 2.3 shows the parameters involved in the purification of different types of plants using liquid-liquid extraction.

Table 2.3 Parameters involved in the purification of different types of plants by liquid-liquid extraction technique

Types of plant	Ratio of diethyl ether and extractant	Number of extraction stages	Volume of butanol	Purity (%)	Yield (%)	References
Bupleurum falcatum	150ml:50ml distilled water + herbal extract	2	100ml	13.70	62.80	Kim, 2001
Paeonia lactiflora	150ml:50ml distilled water + herbal extract	N/A	100ml	N/A	95.40	Kim, 2007
Stevia rebaudioside (Bertoni)	25ml:50ml distilled water + crude extract	N/A	N/A	N/A	N/A	Afandi, 2013
Stevia rebaudioside (Bertoni)	N/A	2	N/A	81.53	6.51	Abou-arab, 2010

2.6 Analysis

Separation and Detection of Steviol Glycosides

Generally, the content and the composition of steviol glycosides were analysed by analytical HPLC. The first chromatograms of steviol glycosides were produced on amino-based or reversed-phase columns (C18) in combination with UV detection mode. The advantage of amino-based stationary phases is it has a high selectivity for all steviol glycosides. Apart from that, it also has the ability to separate the most abundant isomer pair such as reb B with stevioside and reb A with reb E. In general, HPLC separation of chemical compounds can be divided into three modes; polarity, electrical charge, molecular size and solubility (Chatrabhuji, 2015). In this case, the separation of steviol glycosides depends on the molecular polarity in which the more glucose units attached to the *ent*-kaurene backbone, the higher the retention time on the column. Stevioside has 3 glucose moieties meanwhile reb A has 4 glucose moieties, so that reb A has higher retention time and elutes after stevioside (Wölwer-Rieck, 2012). However, amino-based column have several disadvantages. One of them is poor reproducibility and long equilibrium times. (Karásek, 2007)

Afandi (2013), Zllabur (2015) and Carbonell-Capella (2017) analysed the steviol glycosides using reversed-phase columns (C18). Normal phase HPLC is used for the separation of the lipids into classes lipid. Meanwhile, reversed-phase HPLC is used to separate each lipid class into individual species. Reversed-phase HPLC consists of non-polar stationary phase (hydrophobic) and polar mobile phase (Cazes, 2001). Reverse phase HPLC column has several positive feature, which is very high resolution and robust. It is also possible to detect steviol glycosides but the selectivity of C18 column is low especially in the separation of stevioside and rebaudioside A. However, this problem could be solved by using two columns in series or by gradient elution. After HPLC separation, steviol glycoside is often determined by UV detection at 200 or 210 nm (Wölwer-Rieck, 2012). Table 2.4 below shows an overview of separation and detection of steviol glycosides using HPLC analysis.

Table 2.4 Separation and detection of steviol glycosides using HPLC

Separation mode	Columns	Detection mode	References
HPLC	Nucleosil C18	UV	Afandi, 2013
HPLC	Nucleosil C18	UV	Zllabur, 2015
HPLC	Nucleosil C18	UV	Carbonell-Capella, 2017
HPLC	Amino (NH2)	UV	Karásek, 2007
HPLC	Amino (NH2)	UV	Wölwer-Rieck, 2012
HPLC	Amino (NH2)	Pulsed Amperometric Detection (PAD)	Ahmed, 2002

CHAPTER 3

METHODOLOGY

3.1 Plant material/sample

The mature dried Stevia rebaudiana leaves were obtained from Kuala Kangsar, Perak.

3.2 Materials/Chemicals

n-Butanol, activated charcoal powder (-100 particle size, decolorizing) and diethyl ether (≥99.9%) were purchased from Sigma Aldrich (Malaysia). Acetonitrile for HPLC grade (≥99.9%) were obtained from Thermo Fisher Scientific. Acetone was obtained from Avantor Performance Materials. 85% phosphoric acid and absolute ethanol were purchased from HmbG Chemicals. Standard stevioside hydrate (≥95% purity) was obtained from Sigma Aldrich and standard reb A (≥98% purity) was obtained from Tokyo Chemical Industry.

3.3 Equipments and apparatus

Separatory funnel, conical flask, beaker, measuring cylinder, laboratory funnel, HPLC vials, Whatman No. 1 filter paper (Brand: Whatman), 5ml syringe (Brand: Terumo), 0.45 μm Millipore filtration membrane, water bath (Model: D09906, Brand: Memmert), centrifuge (Model: 5810R, Brand: Eppendorf), analytical HPLC (Model: Agilent technologies 1200 infinity series, Brand: Agilent Technologies), HPLC column Eclipse Plus C18 (250 × 4.6 mm I.D., 5 μm), magnetic stirrer (Model: SP131320-33, Brand: Thermo), pH meter (Model: S220, Brand: Streamline), weighing balance (Model: JP1203C, Brand: Mettler-Toledo), incubator shaker (Brand: INFORS HT Ecotron), rotary evaporator (Model: N-1200B, Brand: Eyela commercial blender (Brand: Waring), sieves (mesh hole 2.0, 1.0 and 0.5mm in diameter), refrigerator and vacuum filtration unit.

3.4 Preparation Method

Preparation of Stevia leaves

The woody stems and the brown leaves were removed from the dried dark green leaves. The leaves once again undergo drying process to remove excessive water content in the leaves. 30g of leaves were weight and spread on the aluminium foil as shown in Figure 3.1. The leaves were dried in the oven for 1 hour at 60^{o}C.

Figure 3.1 Dried stevia leaves before oven drying

After that, the leaves were crushed in a commercial blender until it becomes a powder. The powder was separated by sieving through mesh hole 2.0, 1.0 and 0.5mm diameters to obtain very fine powder. Stevia powder was kept in a sealed polyethylene bags and stored properly (Afandi, 2013).

(a) 

(b) 

Figure 3.2 (a) Commercial blender used to crush dried stevia leaves into powder. (b) Sieves with mesh hole 2.0, 1.0 and 0.5mm diameter.

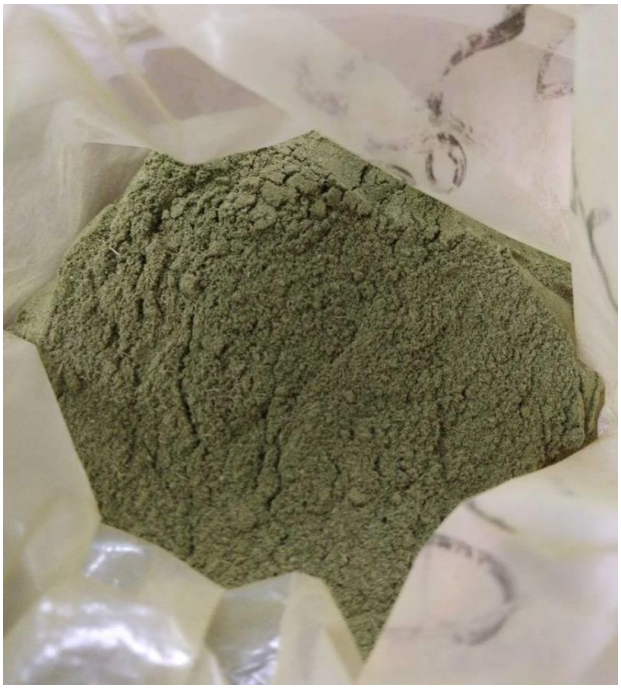


Figure 3.3 The powder form of dried stevia leaves

Extraction (Scale up)

100g of dried stevia powder was placed in the 1000ml conical flask along with 600ml of absolute ethanol. Another 100g of dried stevia powder was mixed with 600ml of absolute ethanol in the 1000ml conical flask. When absolute ethanol was added to the stevia powder, the colour of the solution was immediately turns dark green as shown in Figure 3.4. The top (opening) of the conical flask was wrapped with aluminium foil to prevent evaporation of ethanol during the extraction process (Afandi, 2013).

Figure 3.4 Mixture of 100g stevia powder and 600ml absolute ethanol

Then, both conical flasks were placed in the incubator shaker. The temperature of incubator shaker was set at 35ºC and the extraction time was 5 hours. The conical flask was removed from the incubation shaker and let to cool down to room temperature about 30 minutes (Afandi, 2013). The mixture was filtered through Whatmann No.1 filter paper as shown in Figure 3.5. The filtrate was collected and then it was centrifuged for 1 hour at 1000rpm to remove other impurities and the supernatant was filtered using vacuum filtration unit. The pure stevia extract which coloured dark green was kept in a 1 liter Scott bottle for the further purification process (Afandi, 2013).

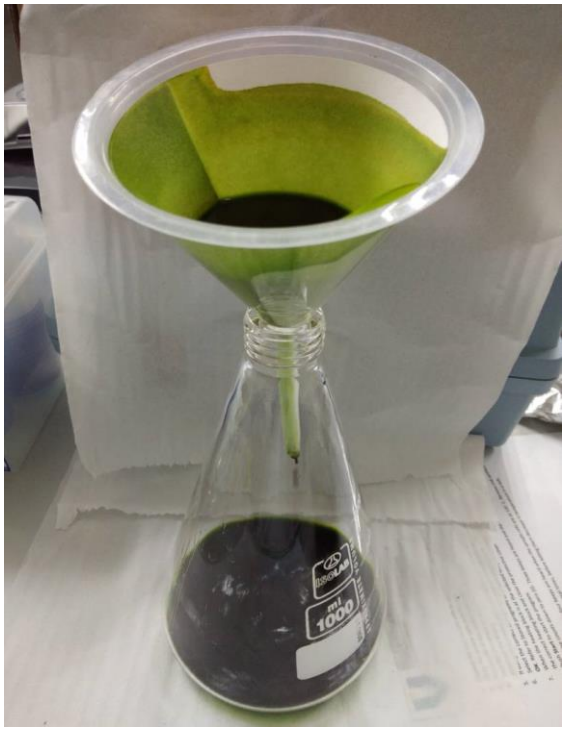


Figure 3.5 Filtration process using Whatmann No.1 filter paper

500ml of stevia extract was concentrated in rotary evaporator at 60^{o}C as shown in Figure 3.6. After the sample become concentrated, 500ml of water was added to dissolve the crude extract (Afandi, 2013).

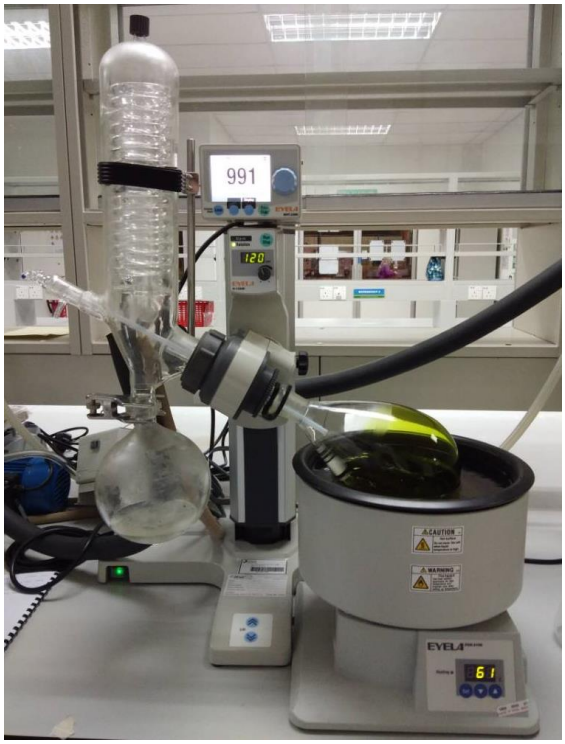


Figure 3.6 Ethanol was removed from stevia extract using rotary evaporator

Purification of reb A using liquid-liquid extraction method

40 ml of crude extract was mixed with 20 ml of diethyl ether in the 100ml separatory funnel. The mixture was allowed to form two different layers as shown in Figure 3.7 before it was separated (Afandi, 2013).

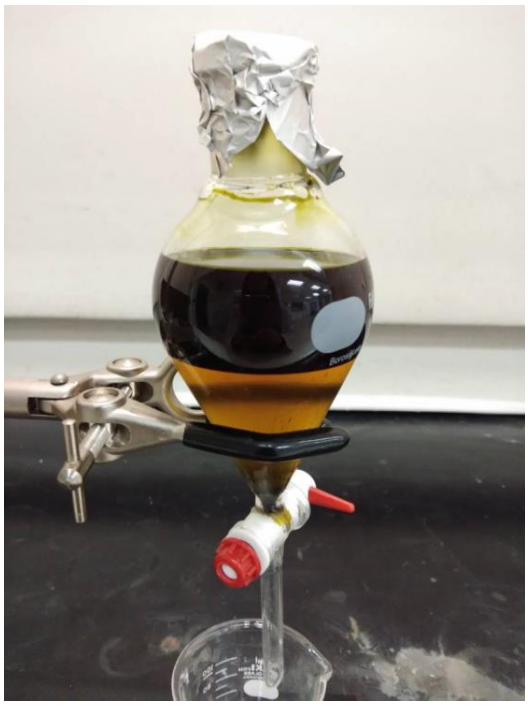


Figure 3.7 Two different layers were form after diethyl ether was added into separatory funnel containing crude stevia extract.

Diethyl ether acts as a reagent to remove the green color of the crude extract. The lower layer rich with Reb A was collected into a beaker (Stage I). The upper layer containing diethyl ether and chlorophylls remaining in the separatory funnel was mixed with 20ml of diethyl ether. The mixture was allowed to form two different layers. The lower layer containing Reb A was collected (Stage II) and mixed with the lower layer collected during the first stage of purification. These steps were repeated for the third stage (Stage III) of extraction to determine the effect of extraction stages on the yield of Reb A. Then, lower layer from stage I, stage II and stage III were mixed and extracted again with butanol using a 100ml separatory funnel. The upper layer containing butanol extract was collected and refrigerated overnight at 4^{o}C to get fine crystals (Afandi, 2013).

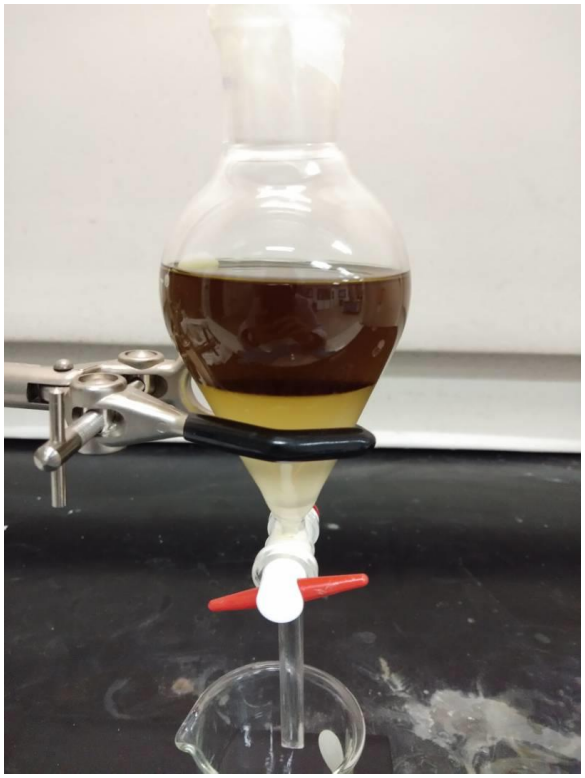


Figure 3.8 Two different layers were formed when butanol was mixed with lower layer from previous extraction stage.

The concentration of Reb A was determined by HPLC analysis. The experiment was repeated by using different ratio of diethyl ether and stevia extract as shown in Table 3.1.

Table 3.1 Ratio of diethyl ether (ml) and crude stevia extract (ml)

Ratio of diethyl ether and crude stevia extract	**Volume of diethyl ether (ml)**	**Volume of crude extract (ml)**
1:2	20	40
2:2	40	40
3:2	60	40
4:2	80	40
5:2	100	40

Steps involved in liquid-liquid extraction using diethyl ether and butanol was summarized and shown in Figure 3.9.

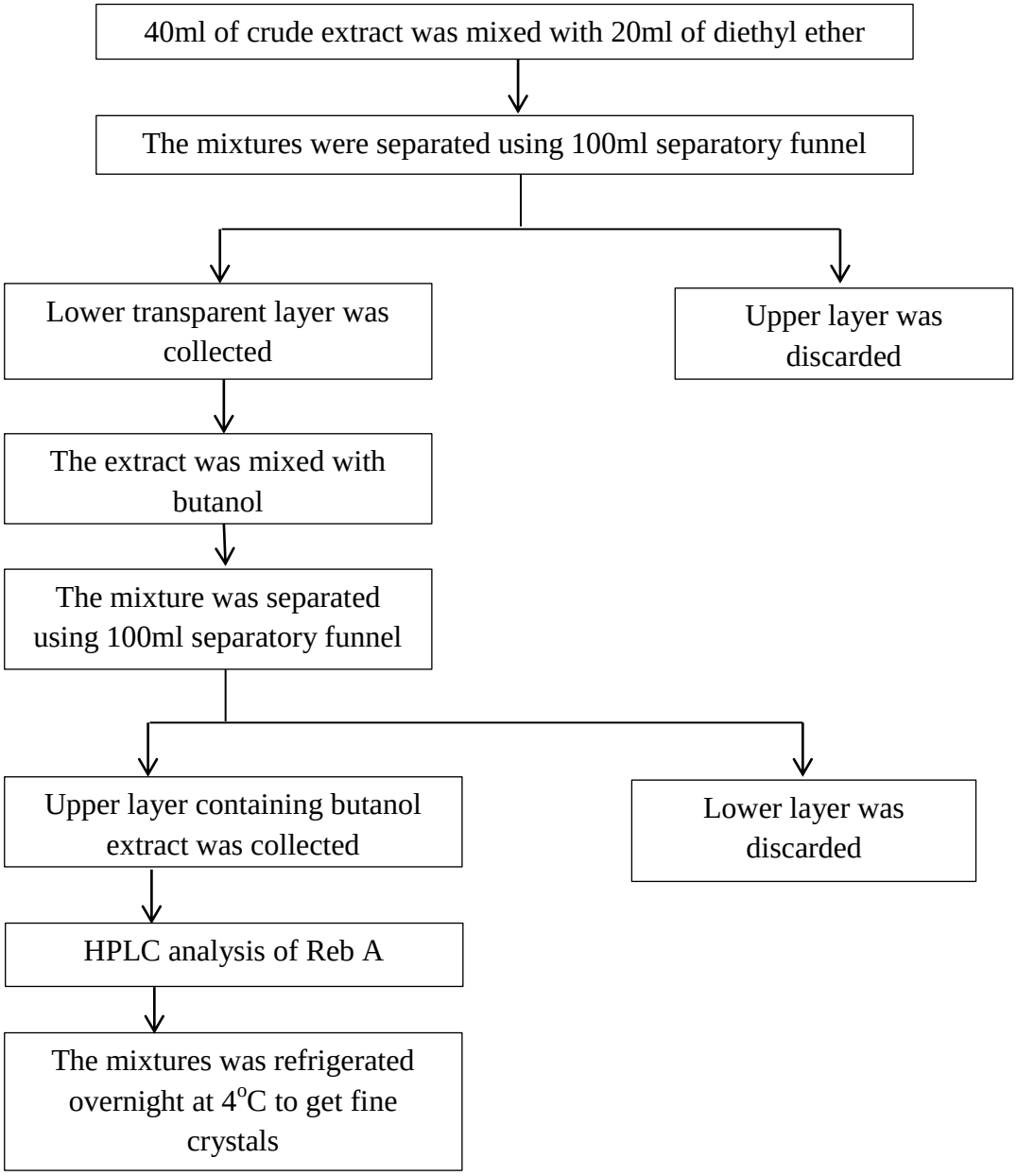


Figure 3.9 Steps involved in purification of Reb A using LLE method.

Purification of Reb A using adsorption method.

40ml of crude stevia extract rich with Reb A was mixed with 5% of activated charcoal in a beaker. The solution was stirred with a magnetic stirrer for 20 minutes. The mixture was filtered through Whatman No.1 filter paper and the filtrate was collected in the 100ml volumetric flask (Stage I) as shown in Figure 3.10. The residual charcoal was mixed with boiling water for 10 minutes and filtered to recover the remaining Reb A content in the residual charcoal. The process was repeated twice (Stage II and Stage III) to determine the effect of different purification stages toward the recovery of Reb A (Rajab, 2009).

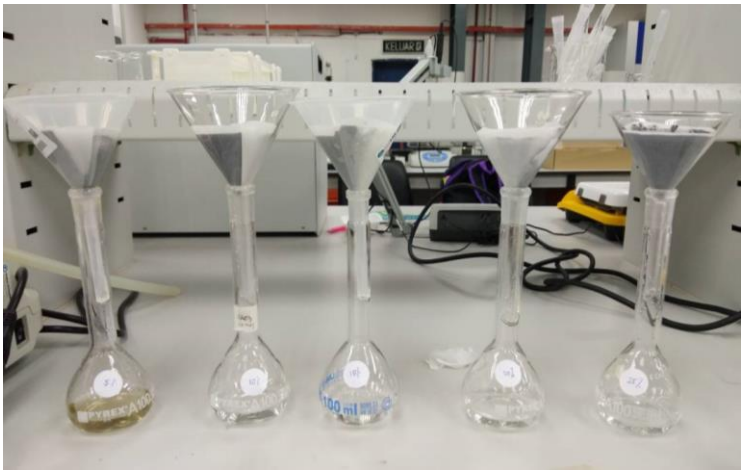


Figure 3.10 Mixture of stevia extract and activated charcoal were filtered through Whatmann filter paper.

The solution was spray dried at 180^{o}C inlet temperature, 9^{o}C outlet temperature, 500psi pump pressure to form a white rebaudioside powder (Rajab, 2009). Step I as shown in figure 3.11 were repeated using a different percentage of activated charcoal (5%, 10%, 15%, 20% and 25%). The yield of Reb A was determined by analytical HPLC. Table 3.2 shows the percentage of activated charcoal and it actual weight.

Table 3.2 Percentage of activated charcoal (g) and crude stevia extract (ml)

Percentage of activated charcoal (%)	Weight of activated charcoal (g)	Volume of crude extract (ml)
5%	2	40
10%	4	40
15%	6	40
20%	8	40
25%	10	40

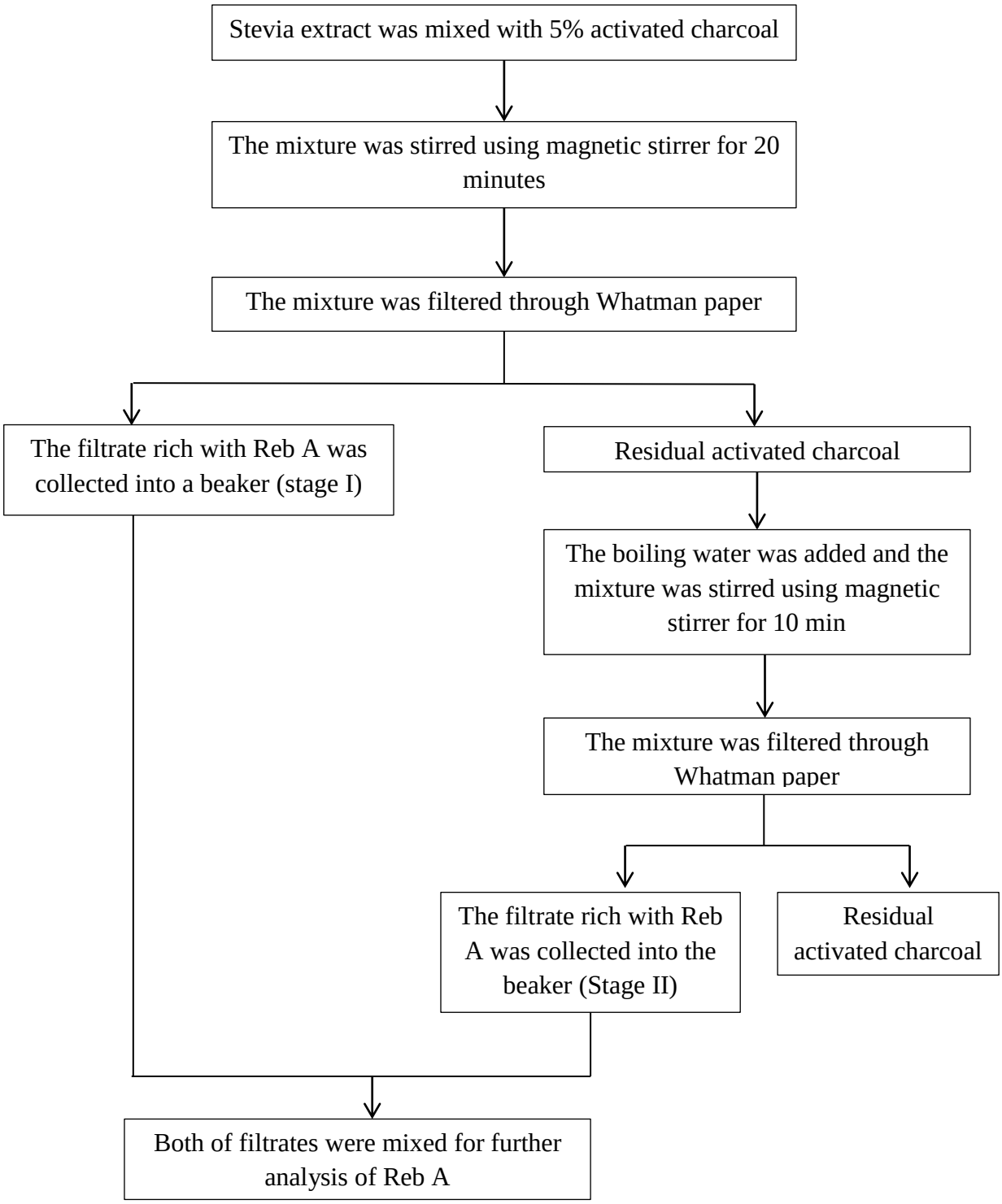


Figure 3.11 Steps involved in purification of Reb A using activated charcoal.

3.5 Analysis

3.5.1 HPLC analysis

Preparation of standard and sample

10mg of stevioside hydrate and Reb A standard was diluted with 20ml of mobile phase to prepare 0.5mg/ml stock solution. Then, it was re-diluted to 0.1 mg/ml by serial dilution to produce five different standard concentrations (0.1mg/ml, 0.2mg/ml, 0.3mg/ml, 0.4mg/ml and 0.5mg/ml). The concentrations of standards can be calculated using the following formula.

$$C1V1 = C2V2 \qquad 3.1$$

Where;

$\mathrm{C1} = \text{the initial concentration}$

$\mathrm{C2} = \text{the final concentration}$

$\mathrm{V1} = \text{the initial volume}$

$\mathrm{V2} = \text{the final volume}$

Afandi (2013), Zllabur (2015) and Carbonell-Capella (2017) analysed the yield of steviol glycosides present in the sample before and after purification using HPLC with C18 column (25 cm × 4.6 mm I.D., 5 µm). The mobile phase was HPLC grade acetonitrile (≥99.9% purity) and distilled water with ratio of 80:20. The pH of the mobile phase was adjusted to 3.0 with phosphoric acid (85% reagent grade). The mobile phase, sample and standard were filtered through 0.45µm Millipore filtration membrane and degassed for 30mins at 27^{o}C to remove any bubbles in the mobile phase, sample and standard.

10μL of sample was introduced into the HPLC column. The flow rate was kept constant at 1mL/min, the detection wavelength was adjusted to 210 nm and the temperature of the column was maintained at 27°C. The mobile phase was pumped through HPLC column until a drift-free baseline was obtained. This process is known as purging which means the process of removing impurities in the HPLC column. The chromatogram of the sample and the standard solution was recorded in 10 minutes. The stevioside and related compounds were determined by comparing the retention time and peak area of the standard. The percentage of steviol glycosides yield was calculated using the following formula (Steviol glycosides, 2007):

$$\% X = \left[\frac{W_s}{W}\right] \times \times \left[\frac{f_x A_x}{A_s}\right] \times 100 \qquad 3.2$$

Where,

W_s is the amount (mg) of stevioside/Reb A in the standard solution

W is the amount (mg) of sample in the sample solution

A_s is the peak area for stevioside/Reb A from the standard solution

A_x is the peak area of X for the sample solution

f_x is the ratio of the formula weight of X to the formula weight of stevioside: 1.00 (stevioside), 0.98 (dulcoside A), 1.20 (rebaudioside A), 1.18 (rebaudioside C), 0.80 (rubusoside), 0.80 (steviolbioside), and 1.00 (rebaudioside B).

3.5.2 Determination of moisture content

The purpose of determination of moisture content is to estimate the temperature and time taken for completely removed the moisture in the leaves using oven drying. Excessive moisture content in the leave will increase the chance of fungal growth. According to the method described by Bucic-Kojic (2007), the moisture content of stevia leaves was determined by drying the stevia leaves in the oven at 60^{o}C to a constant mass. The moisture content was expressed in percentages (%) and calculated by the following formula (Bucic-Kojic, 2007);

$$\%\ Moisture\ content = \frac{M_1 - M_2}{M_1} \times 100 \qquad 3.3$$

Where,

$M_1 =$ a mass of stevia leaves before drying (g)

$M_2 =$ the mass of stevia leaves after drying (g)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this section, the standard chromatogram of Reb A and stevioside were obtained. The relationship between the materials ratio for LLE method and the effects of different percentages of activated charcoal in adsorption method were investigated. The effects of a number of extraction towards the yield of Reb A in both methods were discussed. Both purification methods were compared to determine the best technique that will produce high yield and purity of Reb A.

4.2 Standard curve of stevioside and Reb A

In this study, the yield of Reb A was determined by analytical HPLC. The identification of Reb A in the samples was done by comparing the retention time with the standard solution. Meanwhile, for the quantification of Reb A in the samples, the peak area under the graph was compared with the standard. As can be seen in figure 4.1, the retention time of stevioside in the standard solution is 2.237 min and the retention time of Reb A in the standard was found to be at 2.183 min (Figure 4.2). It was found that the correlation coefficient (r^2) in calibration curves of standard stevioside is 0.99 and 0.98 for standard Reb A, which means that there is a strong relationship between two variables; peak area and amount of compound in the solution.

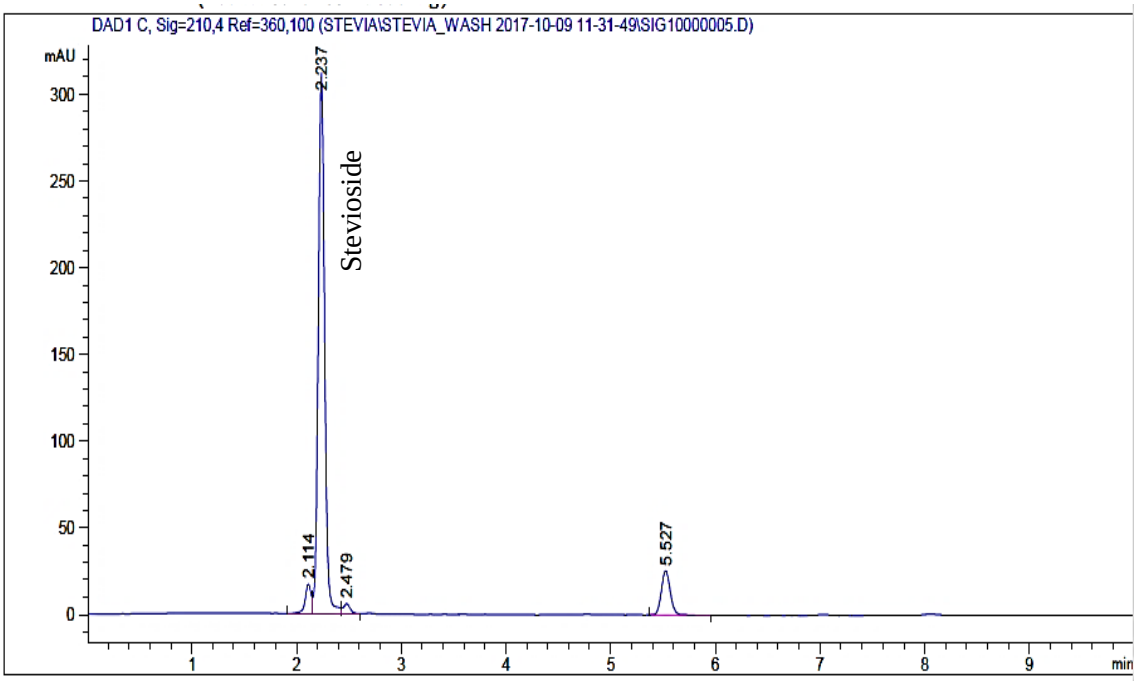


Figure 4.1 HPLC chromatogram of stevioside standard

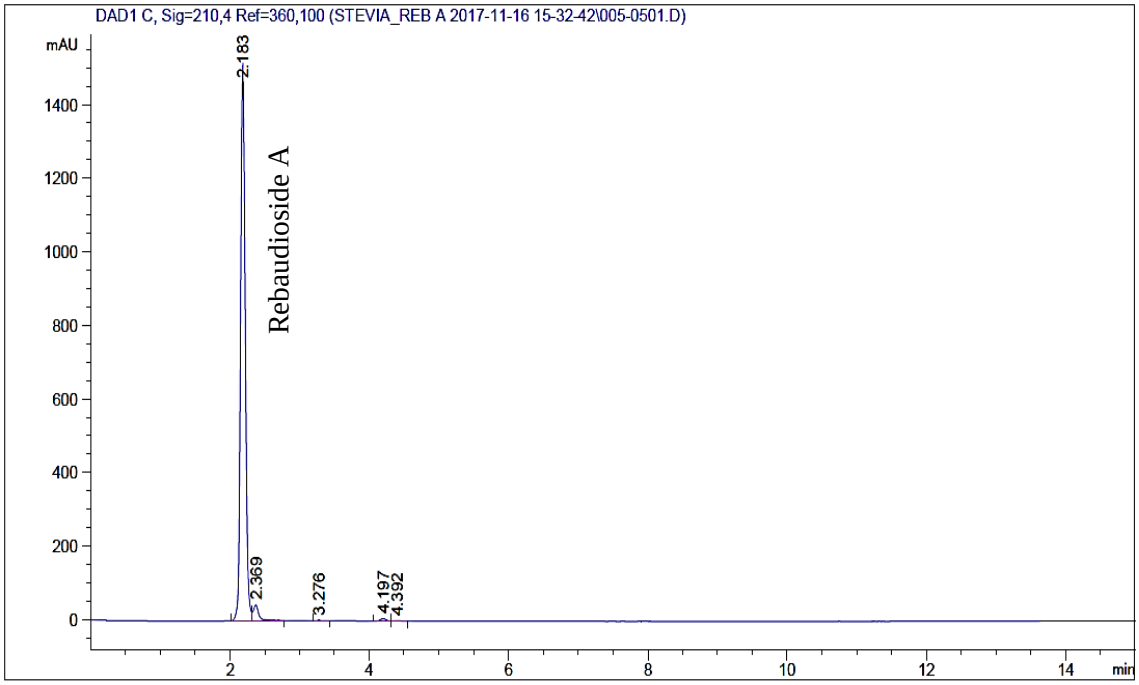


Figure 4.2 HPLC chromatogram of Reb A standard

4.3 Purification of Reb A using LLE method

The effects of the parameters on the yield and purity of Reb A were investigated. The parameters were the ratio of diethyl ether and stevia extract and the number of extraction.

4.3.1 Effects of material ratio

As can be seen in Figure 4.3, the yield of Reb A decreases as the material ratio increases. Diethyl ether is an organic compound belonging to a large group of compounds called ether. The density of diethyl ether is 0.708g/ml and it is immiscible with water (Wiliamson, 2015). Meanwhile, the density of water is 0.999g/ml (Fredlund, 2012). When diethyl ether was mixed with stevia extract, it will form two different layers, where the lower layer is a water layer containing stevioside and some Reb A and the upper layer is a diethyl ether rich with chlorophylls and some of Reb A molecule. The main function of diethyl ether is to remove the green colour of the extract because chlorophyll A is a non-polar molecule, hence, it is more soluble in a non-polar solvent like diethyl ether (Sumanta, 2014).

However, chlorophyll B is polar molecule so it is not dissolved in diethyl ether and it will give a yellow colour to the water layer as shown in Figure 4.4. Reb A sparingly soluble in water, hence, some of Reb A will retain in the water and some will be soluble in diethyl ether. When the ratio of diethyl ether increase, more Reb A was dissolved in diethyl ether, causes decreases in the yield of Reb A. Then, the upper layer (diethyl ether) was discarded and the lower layer was washed with butanol to isolate Reb A from the water into the butanol and the stevioside will remains in the lower layer because stevioside is more soluble in water (Aminha, 2016). However, the present of stevioside in material ratio 2:2 and 3:2 is due to the poor separation of a compound during HPLC analysis. In this study, it was found that the highest yield of Reb A is at 1:2 material ratio.

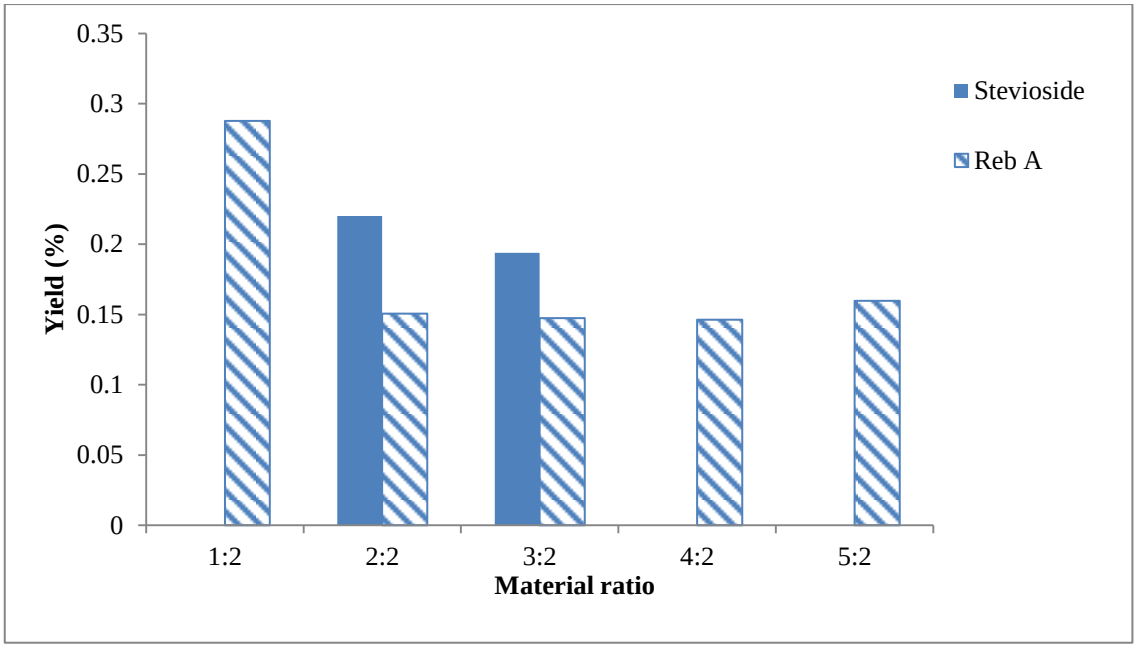


Figure 4.3 Effect of materials ratio towards the yield of Reb A and stevioside

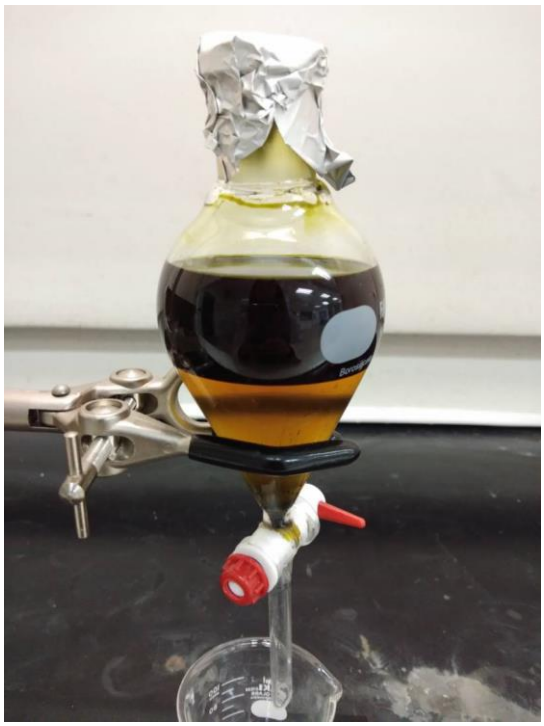


Figure 4.4 Lower layer and upper layer diethyl ether and stevia extract mixture

4.3.2 Effects of number of extraction

Figure 4.5 shows the effect of number of extraction towards the yield of Reb A. The yield of Reb A increases as the number of extraction increases.

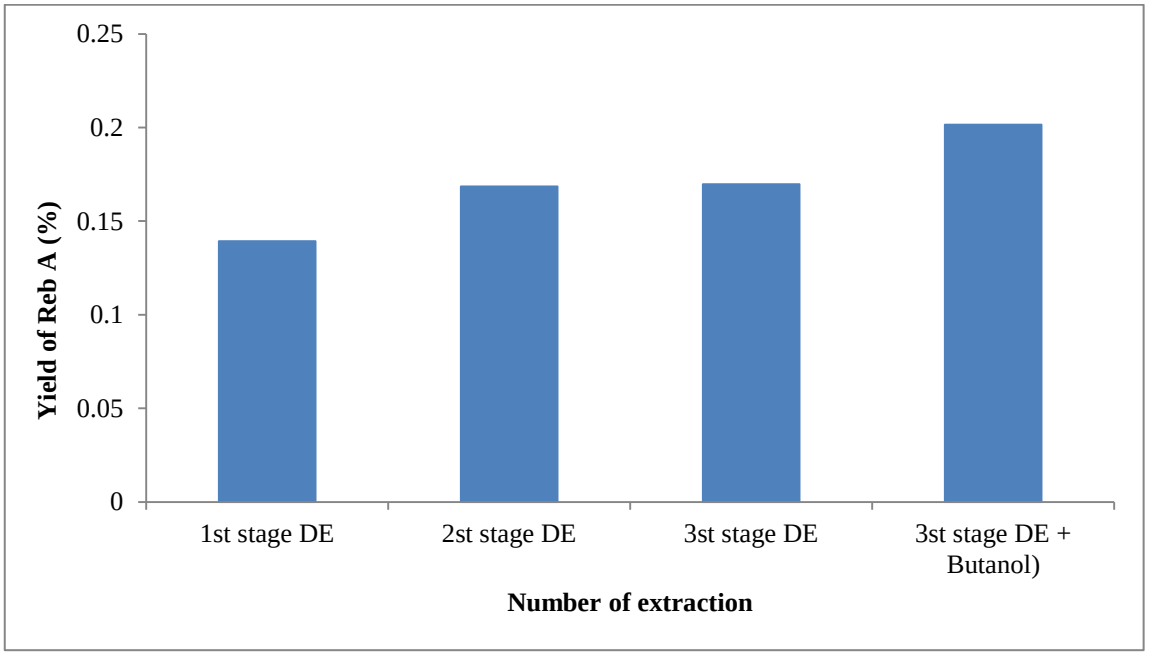


Figure 4.5 Effect of purification stages on the yield of Reb A and stevioside

As stated by Miranda (2015), double or multiple extractions is more efficient to obtain a higher yield of the desired product. Reb A is sparingly soluble in water. As can be seen in Figure 4.5, the yield of Reb A increases when butanol is added because it isolates the whole Reb A from water into the butanol (Aminha, 2016). The extraction needs to be repeated 3 times in order to recover the maximum amount of Reb A.

4.4 Purification of Reb A using adsorption method

In adsorption method using activated charcoal, the manipulated parameters are the percentages of activated charcoal and the number of extraction.

4.4.1 Effect of difference percentage of activated charcoal

The yield of Reb A decreases as the percentages of activated charcoal increases. Figure 4.6 showed the yields of Reb A decreases gradually from 0.10% in 5% of activated charcoal to 0.03% in 10% of activated charcoal. The yields of Reb A was continues to decreases until the equilibrium states were achieved at 15% and 20% of activated charcoal. However, a slightly drop in the yield of Reb A was observed in 25% of activated charcoal but the value is not very significant. As stated by Iqbal (2007), adsorption of adsorbate (in this case the adsorbate is Reb A) on the surface of adsorbent (activated charcoal) increased with the amount of activated charcoal and attained a constant value when equilibrium was established. However, in this study, the yield of Reb A in the filtrate decreases as the percentages of activated charcoal increases because the remaining of Reb A were still trapped in the pore of activated charcoal and not fully been extracted from activated charcoal. This statement explained why the yield of Reb A in the filtrate decreases when the percentages of activated charcoal increases.

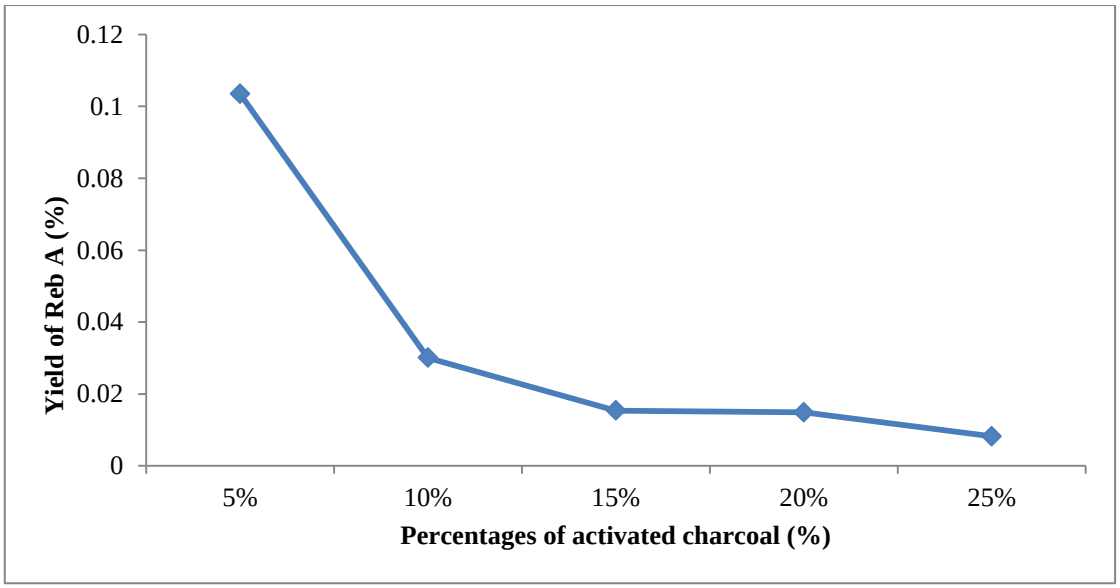


Figure 4.6 Effect of percentage of activated charcoal (%) on the yield of Reb A (%)

Table 4.1 shows the yield of Reb A and stevioside were decreases as the percentages of activated charcoal increased. A completely removal of stevioside from Reb A can be seen in the purification using 20% and 25% of activated charcoal.

Table 4.1 Effect of percentages of activated charcoal (%) on the yields of Reb A and stevioside (%)

Percentages of activated charcoal (%)	Yield of Reb A (%)	Yield of Stevioside (%)
5%	0.1034	2.4234
10%	0.0301	0.1363
15%	0.0154	0.0617
20%	0.0149	0.0000
25%	0.0082	0.0000

Activated charcoal is a structurally homogenous material with a high surface area and has a microporous structure. The basic mechanism of charcoal adsorption is the mass transfer and adsorption of a molecule from a liquid or gas into the solid surface (Figure 4.7). Molecules with very small size or low molecular weight will trap in the micropore of charcoal. Meanwhile, large molecules or high molecular weight molecules will be adsorbed on the surface of activated charcoal (Gawander, 2017). Basically, molecular weight indicates the size of molecules. Reb A (967.021 g/mol) has high molecular weight compared to stevioside (804.88 g/mol) which have low molecular weight (Stevioside, n.d.) (Rebaudioside A, n.d.). Therefore, more stevioside was trapped in the micropores of the activated charcoal. As a result, the yield of stevioside decreases gradually when the percentage of activated charcoal increases compared to Reb A.

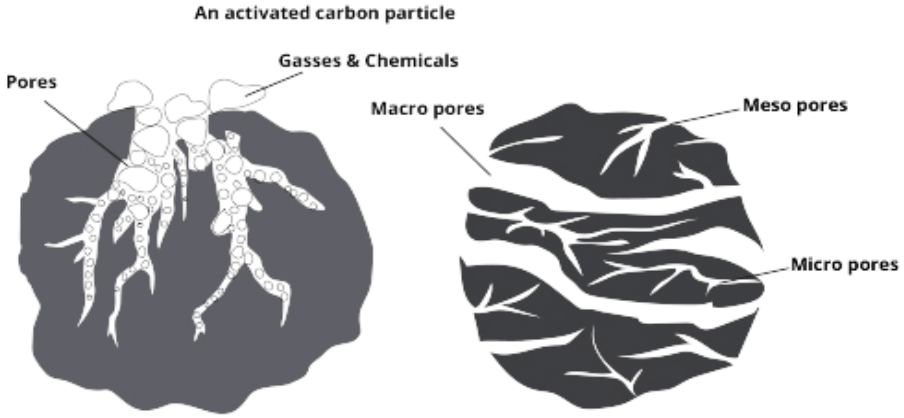
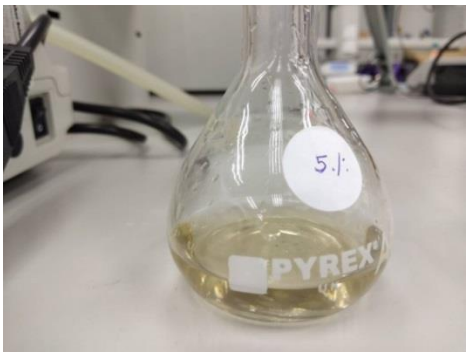
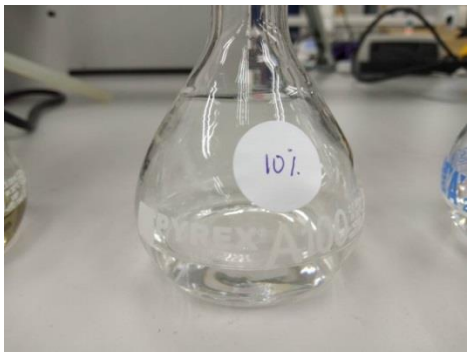


Figure 4.7 Mechanism of adsorption (Source: Gawande, 2017)

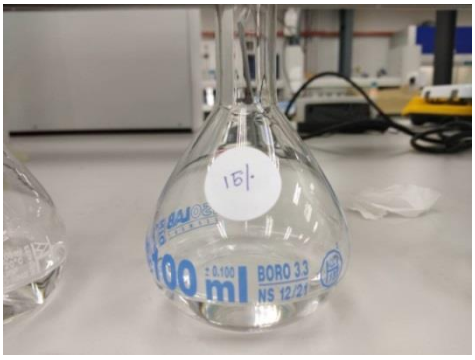
Figure 4.8 below shows the different in colour of filtrate when using different percentages of charcoal.



5% of activated charcoal



10% of activated charcoal



15% of activated charcoal

20% of activated charcoal

25% of activated charcoal

Figure 4.8 Effect of different percentages of charcoal towards the colour of filtrate

4.4.2 Effect of number of extraction

According to (Chen, 2007) when the number of extraction times increases, the yield of the respective bioactive principle may increases. However, in this study, the yield of Reb A was decreased as the number of extraction increased as shown in Figure 4.9. This is due to the differences in the sampling or calculation method to determine the yield of Reb A for each stage of purification. In this study, firstly, the crude stevia extract was mixed with activated charcoal and filtered through filter paper (Stage I). An amount of filtrate (filtrate I) containing Reb A was taken for sampling and test for HPLC analysis. Then, 10ml of boiling water was mixed with residual charcoal from the previous stage and filtered through filter paper (Stage II). The filtrate (filtrate II) was mixed with filtrate I from the first stage of purification and test for HPLC analysis. Obviously, when boiling water was added to recover the remaining Reb A in the residual charcoal and mixed with the previous filtrate, the solution becomes more diluted and that is why the yield of Reb A slightly decreases when the number of purification stages increases.

On the other hand, Afandi (2013) used a cumulative method to determine the yield of Reb A at difference purification stage. For example, the yield of Reb A in the filtrate (filtrate I) during the first stage of purification is 1.63% and the yield of Reb A in the filtrate (filtrate II) during the second stage is 0.47%. Then, the percentage yield of Reb A in the second stage was added with the percentage yield of Reb A in the first stage and become 2.1%. This example explained why the yield of Reb A increased with the stage of purification increased as stated by Afandi (2013).

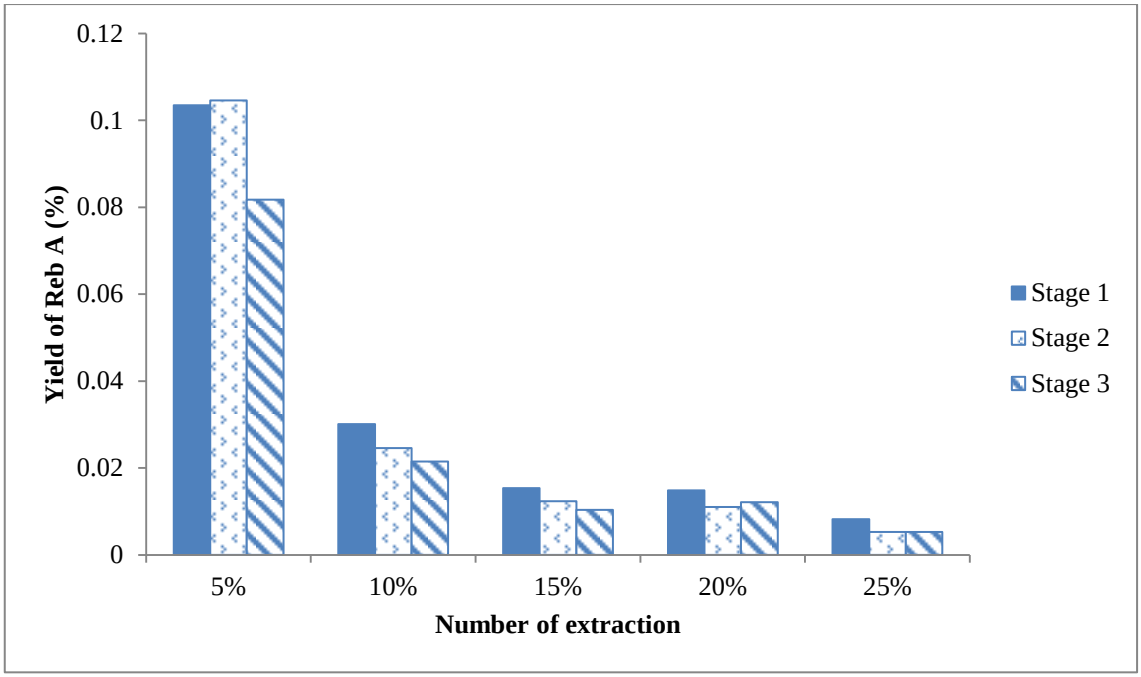


Figure 4.9 Effect of number of extraction on the yield of Reb A (%)

4.5 The moisture content of the dried stevia leaves

Moisture content of stevia leaves was determined by drying 3g of dried stevia leaves in the oven at 60^{o}C to constant mass. The mass of the stevia leaves before drying (W_1) was 3g. The mass of the stevia leaves after drying (W_2) was 2.85g. Hence, the mass loss during drying process was 0.15g. The percent of moisture content was calculated by using equation 3.3:

$$\% \, Moisture = \frac{3g - 2.85g}{3g} \, X \, 100$$

$$= 5\%$$

Thus, the moisture content of dried stevia leaves was 5%.

CHAPTER 5

CONCLUSION

5.1 Conclusion

Selection of suitable method is very important in order to recover high yield of product and to prevent product loss during the purification process. In this present study, two different purification methods which are LLE and adsorption method have been studied. Several parameters were manipulated to study the effect of different parameters towards the yield of Reb A. In LLE method, it was found that the effect material ratio (diethyl ether and stevia extract) was significant on the yield of Reb A. The yield of Reb A continuously decreases when the material ratio rise from 1:2 to 2:4. The number of extraction also plays an important role in the recovery of Reb A. The results from this study showed that when the number of extraction increases, the yield of Reb A increases until it reached an equilibrium state.

Meanwhile, for the adsorption method, percentages of activated charcoal and the number of extraction have been manipulated to study the impacts of these parameters toward the yield of Reb A. It was found that when the percentage of activated charcoal increases, the yield of Reb A decreases. A complete removal of stevioside from Reb A which means the highest purity of Reb A can be obtained using 20% of activated charcoal. However, the number of extraction does not give a big impact on the yield of Reb A. As a conclusion, this study showed that the best purification method to recover high yield and purity of Reb A is LLE method. As can be seen from the analysis, the highest yield of Reb A which is 0.29% was obtained at material ratio 1:2 and the extraction need to be repeated for 3 times in order to recover the maximum amount of Reb A.

5.2 Recommendations

As a recommendations to improve the recovery of Reb A using LLE, other parameters such as the contact time between diethyl ether and stevia extract, the volume of butanol and the contact time with butanol should be studied to determine the effect of these parameters toward the yield and purity of Reb A. Apart from that, in order to completely remove the yellowish colour of the final product, activated charcoal can be used to adsorb remaining chlorophylls in the extract. However, suitable activated charcoal should be used to prevent any losses of Reb A during purification process.

For the adsorption method, difference types and pore size of activated charcoal such as coconut shell charcoal, wood activated charcoal and coal activated charcoal can be used to determine its impact on the yield of Reb A. In addition, the contact time between activated charcoal and stevia extract should be study because contact time also will affect the recovery of Reb A.

In this study, the separation of compount during HPLC analysis was very poor. For the recommendation of future study, the conditions of the HPLC should be optimised before proceeding with sample analysis in order to obtain a good compounds separation. The ratio of mobile phase and the flow rate should be manipulated to obtain good separation of the compounds. Apart from that, needle wash system should be employed to prevent contamination of the current sample with the previous sample. Too much contamination will increases undesirable peak noise and it can give a false result to the user. Moreover, in-line filters and guard columns should be used before the main column. The main function of in-line filters and guard columns is to remove particulate and contaminant which may cause blockage in the HPLC column.

REFERENCES

Abou-arab, A. E., Abou-arab, A. A., & Abu-salem, M. F. (2010). Physico-chemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana bertoni plant, *4*(May), 269–281.

Adari, B. R., Alavala, S., George, S. A., Meshram, H. M., Tiwari, A. K., & Sarma, A. V. S. (2016). Synthesis of rebaudioside-A by enzymatic transglycosylation of stevioside present in the leaves of Stevia rebaudiana Bertoni. *Food Chemistry*, *200*(June), 154–158. https://doi.org/10.1016/j.foodchem.2016.01.033

Afandi, A., Sarijan, S., & Shaha, R. K. (2013). Optimization of rebaudioside a extraxtion from Stevia rebaudiana (Bertoni) and quantification by high performance liquid chromatography analysis. *Journal of Tropical Resources and Sustainable Science*, *1*(1), 62–70.

Ahmed, M. J., & Smith, R. M. (2002). Determination of stevioside by high-performance liquid chromatography with pulsed amperometric detection Short Communication, 170–172.

Aminha, S., Soumya, A. N., Raju, G., Goud, B. M., Irfath, M., & Quadri, S. A. P. (2016). Isolation and Extraction of Artificial Sweetener (Stevia). *World Journal of Pharmaceutical Research*, *3*(8), 481–486.

Bhutia, P. H., & Sharangi, A. B. (2016). IJCST Stevia: Medicinal Miracles and Therapeutic Magic. *International Journal of Crop Science and Technology*, *2*(2), 45–59.

Bucic-Kojic, Ana; Planinic, Mirela; Tomas, Srecko; Bilic, Mate; Velic, D. (2007). Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. *Journal of Food Engineering*, *81*, 236–242. https://doi.org/10.1016/j.jfoodeng.2006.10.027

Carbonell-Capella, J. M., Šic Žlabur, J., Rimac Brnčić, S., Barba, F. J., Grimi, N., Koubaa, M., … Vorobiev, E. (2017). Electrotechnologies, microwaves, and ultrasounds combined with binary mixtures of ethanol and water to extract steviol glycosides and antioxidant compounds from *Stevia rebaudiana* leaves. *Journal of Food Processing and Preservation*, (May 2016), e13179. https://doi.org/10.1111/jfpp.13179

Chen, X. Q., Wu, S. D., Jiang, X. Y., & Chen, Q. Y. (2007). Comparison of Techniques for the Extraction of the Hypotensive Drugs Geniposidic Acid and Geniposide from Eucommia Ulmoides. *Journal of Iran. Chemistry Society*, *4*(2), 205–214.

Chen, Z., Wei, X., Li, J., & Di, D. (2012). Preparative separation of Rebaudiana A from commercialized steviol glycosides by macroporous adsorption resins mixed bed. *Separation and Purification Technology*, *89*, 22–30. https://doi.org/10.1016/j.seppur.2012.01.001

Deshmukh, S., & Kedari, V. (2014). Isolation, purification and characterization of sweetners od Stevia rebaudiana Bertoni for their anticancerous activity against colon cancer. *World Journal of Pharmacy and Pharmaceutical Sciences*, *3*(5), 1394–1410.

Fredlund, D., Hendry, R., & Fredlund, M. (2012). *Unsaturated Soil Mechanics in Engineering Practice*. Canada: John Wiley & Sons.

Gallo, M., Vitulano, M., Andolfi, A., Dellagreca, M., Conte, E., Ciaravolo, M., & Naviglio, D. (2017). Rapid Solid-Liquid Dynamic Extraction (RSLDE): a New Rapid and Greener Method for Extracting Two Steviol Glycosides (Stevioside and Rebaudioside A) from Stevia Leaves. *Plant Foods Hum Nutr*, 141–148. https://doi.org/10.1007/s11130-017-0598-1

Gawande, S. M., & Belwalkar, N. S. (2017). Adsorption and its Isotherm – Theory. *International Journal of Engineering Research*, *ISSN*(66), 312–316. https://doi.org/10.5958/2319-6890.2017.00026.5

Guo, W., Ngo, H., & Li, J. (2012). Bioresource Technology A mini-review on membrane fouling. *Bioresource Technology*, *122*, 27–34. https://doi.org/10.1016/j.biortech.2012.04.089

Heaton, J., & Smith, N. W. (2012). Advantages and Disadvantages of HILIC; a Brief Overview. *Chromatography Today*, (May/June), 44–47.

Huang, X.-Y., Fu, J.-F., & Di, D.-L. (2010). Preparative isolation and purification of steviol glycosides from Stevia rebaudiana Bertoni using high-speed counter-current chromatography. *Separation and Purification Technology*, *71*(2), 220–224. https://doi.org/10.1016/j.seppur.2009.11.025

Idris, S. . M. (2016, November). Unhealthy living makes Malaysia No. 1 in diabetes prevalence in Asean. *Malay Mail Online*.

Iqbal, M. J., & Ashiq, M. N. (2007). Adsorption of dyes from aqueous solutions on activated charcoal. *Journal of Hazardous Materials*, *139*(1), 57–66. https://doi.org/10.1016/j.jhazmat.2006.06.007

Karásek, P., Pól, J., Varad, E., Roth, M., Bene, K., Kotla, P., & Josef, Č. (2007). Comparison of two different solvents employed for pressurised fluid extraction of stevioside from Stevia rebaudiana : methanol versus water, 1847–1857. https://doi.org/10.1007/s00216-007-1404-y

Kim, N., & Kim, D.-K. (2007). E1SPB8_2007_v16n6_1055.pdf. *Food Science and Biotechnology*, *16*(6), 1055–1059.

Kim, N., & Park, I. S. (2001). Purification of saponin compounds in Bupleurum falcatum by solvent partitioning and preparative LC. *Bioscience, Biotechnology, and Biochemistry*, *65*(7), 1648–1651. https://doi.org/10.1271/bbb.65.1648

Larowe, D. E., & Cappellen, P. Van. (2011). Original Submission. *Geochimica Et Cosmochimica Acta*, (January). https://doi.org/10.1016/j.gca.2011.01.020

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Kong, A. H. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, *132*(3), 1121–1132. https://doi.org/10.1016/j.foodchem.2011.11.140

Lv, X. H., Kuang, P. Q., Yuan, Q. P., Liang, H., & Zheng, G. Q. (2014). Preparative Separation of Steviol Glycosides from Stevia rebaudiana Bertoni by Macroporous Resin and Preparative HPLC. *Acta Chromatographica;26 (1): 123-135 Mar 2014*, *26*(1), 123–135. https://doi.org/10.1556/AChrom.26.2014.1.10

M.Roy, G. (1995). *Activated Carbon Applications in the Food and Pharmaceutical Industries*. United State: Technomic Publishing Company, Inc.

Miranda, M., Guzman, D., & Anne, K. (2015). Extraction Volume of NaOH used for titration Original 1 % sample Solution , extracted once Solution extracted twice Weight of Adipic acid in grams Original Solution Solution , extracted once Solution , extracted twice Distribution coefficient Single extra, (2), 4–7.

Mohankumar, C., Jayashree, G. P., Harish, W. M., & Reeja, R. (2011). *245903*. India. Retrieved from http://www.allindianpatents.com/patents/245903-a-process-for-production-of-steviol-from-stevia-species

Pillay, V. (2013). *Modern Medical Toxicology*. (P. Nair, Ed.) (4th ed.). New Delhi: Jaypee Brothers Medical Publishers (P) Ltd.

Pohorecki, R., Bridgwater, J., Molzahn, M., Gani, R., & Gallegos, C. (2010). *Chemical Engineering and Chemical Process Technology* (Volume II). United Kingdom: Eolss Publisher Co.Ltd.

Purkayastha, S., Pugh, G., Lynch, B., Roberts, A., Kwok, D., & Tarka, S. M. (2014). In vitro metabolism of rebaudioside B, D, and M under anaerobic conditions: Comparison with rebaudioside A. *Regulatory Toxicology and Pharmacology*, *68*(2), 259–268. https://doi.org/10.1016/j.yrtph.2013.12.004

Rajab, R., Mohankumar, C., Murugan, K., Harish, M., & Mohanan, P. V. (2009). Purification and toxicity studies of stevioside from Stevia rebaudiana Bertoni. *Toxicology International*, *16*(1), 49–54. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-78650365449&partnerID=40&md5=74a5ba410d06f8e110d68b5e63cdf796

Rao, A. B. (2012). Simple extraction and membrane purification process in isolation of steviosides with improved organoleptic activity. *Advances in Bioscience and Biotechnology*, *3*(August), 327–335. https://doi.org/10.4236/abb.2012.34048

Rao, A. B., Reddy, G. R., Ernala, P., Sridhar, S., & Ravikumar, Y. V. L. (2012). An improvised process of isolation, purification of steviosides from Stevia rebaudiana Bertoni leaves and its biological activity. *International Journal of Food Science and Technology*, *47*(12), 2554–2560. https://doi.org/10.1111/j.1365-2621.2012.03134.x

Rebaudioside A. (n.d.). National Center for Biotechnology Information. Retrieved December 8, 2017, from https://pubchem.ncbi.nlm.nih.gov/compound/6918840

Sigma Aldrich Malaysia. (2017). Retrieved May 3, 2017, from http://www.sigmaaldrich.com/malaysia.html

Steviol glycosides. (2007). *FAO JECFA Monographs*, *4*, 2–5.

Stevioside. (n.d.). National Center for Biotechnology Information. Retrieved December 8, 2017, from https://pubchem.ncbi.nlm.nih.gov/compound/442089

Sumanta, N., Haque, C. I., Nishika, J., & Suprakash, R. (2014). Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents. *Journal of Chemical Sciences*, *4*(9), 63–69.

USP 30. (2007). *Residual Solvents*, 1–10.

Vanneste, J., Sotto, A., Courtin, C. M., Van Craeyveld, V., Bernaerts, K., Van Impe, J., Van Der Bruggen, B. (2011). Application of tailor-made membranes in a multi-stage process for the purification of sweeteners from Stevia rebaudiana. *Journal of Food Engineering*, *103*(3), 285–293. https://doi.org/10.1016/j.jfoodeng.2010.10.026

Wiliamson, K. L., & Masters, K. M. (2015). *Macroscale and Microscale Organic Experiments* (7th editio). United State: Cengage Learning.

Wölwer-Rieck, U. (2012). The Leaves of Stevia rebaudiana (Bertoni), Their Constituents and the Analyses Thereof: A Review, (60), 886–895.

Yadav, A. K., Singh, S., Dhyani, D., & Ahuja, P. S. (2011). A review on the improvement of stevia Stevia rebaudiana (Bertoni). *Canadian Journal of Plant Science, 91(1)*(2011), 1–27. https://doi.org/10.4141/cjps10086

Zllabur, J. S., Voca, S., Dobricevic, N., Brncic, M., Dujmic, F., & Brncic, S. R. (2015). Optimization of ultrasound assisted extraction of functional ingredients from Stevia rebaudiana Bertoni leaves. *International Agrophysics*, *29*(2), 231–237. https://doi.org/10.1515/intag-2015-0017

APPENDICES

1. Examples calculation for different concentrations of standard

<table>
<tr><td colspan="2">Standard stock solution (0.5 mg/ml)

$\frac{10\text{mg}}{20\text{ml}} = 0.5\ \text{mg/ml}$</td></tr>
<tr><td>0.1 mg/ml

$\text{C1V1} = \text{C2V2}$

$\left(\frac{0.5\text{mg}}{\text{ml}}\right)\text{V1} = (\frac{0.1\text{mg}}{\text{ml}})(1.5\text{ml})$

$\text{V1} = 0.3\text{ml of stock solution}$</td><td>0.2 mg/ml

$\text{C1V1} = \text{C2V2}$

$\left(\frac{0.5\text{mg}}{\text{ml}}\right)\text{V1} = (\frac{0.2\text{mg}}{\text{ml}})(1.5\text{ml})$

$\text{V1} = 0.6\text{ml of stock solution}$</td></tr>
<tr><td>0.3 mg/ml

$C1V1 = C2V2$

$\left(\frac{0.5mg}{ml}\right)V1 = (\frac{0.3mg}{ml})(1.5ml)$

$V1 = 0.9ml\ of\ stock\ solution$</td><td>0.4 mg/ml

$C1V1 = C2V2$

$\left(\frac{0.5mg}{ml}\right)V1 = (\frac{0.4mg}{ml})(1.5ml)$

$V1 = 1.2ml\ of\ stock\ solution$</td></tr>
<tr><td colspan="2">0.5 mg/ml

$C1V1 = C2V2$

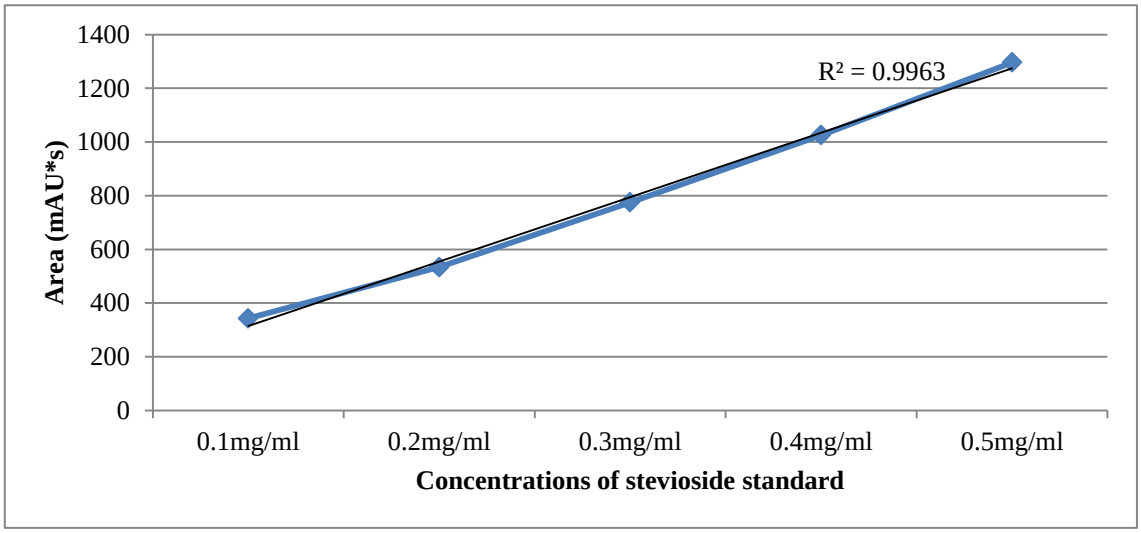
$\left(\frac{0.5mg}{ml}\right)V1 = (\frac{0.5mg}{ml})(1.5ml)$

$V1 = 1.5ml\ of\ stock\ solution$</td></tr>
</table>

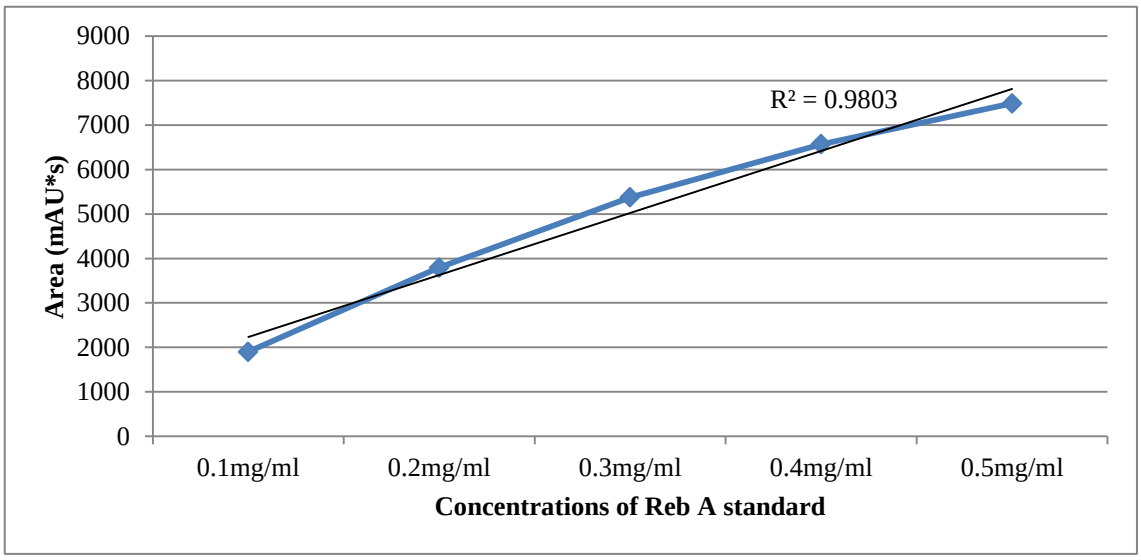
2. Volume of stock solution (ml) and volume of mobile phase (ml) to prepare both standard (stevioside and Reb A)

Concentrations (mg/ml)	Volume of stock solution (ml)	Volume of mobile phase (ml)
0.1mg/ml	0.3	1.2
0.2mg/ml	0.6	0.9
0.3mg/ml	0.9	0.6
0.4mg/ml	1.2	0.3
0.5mg/ml	1.5	0.0

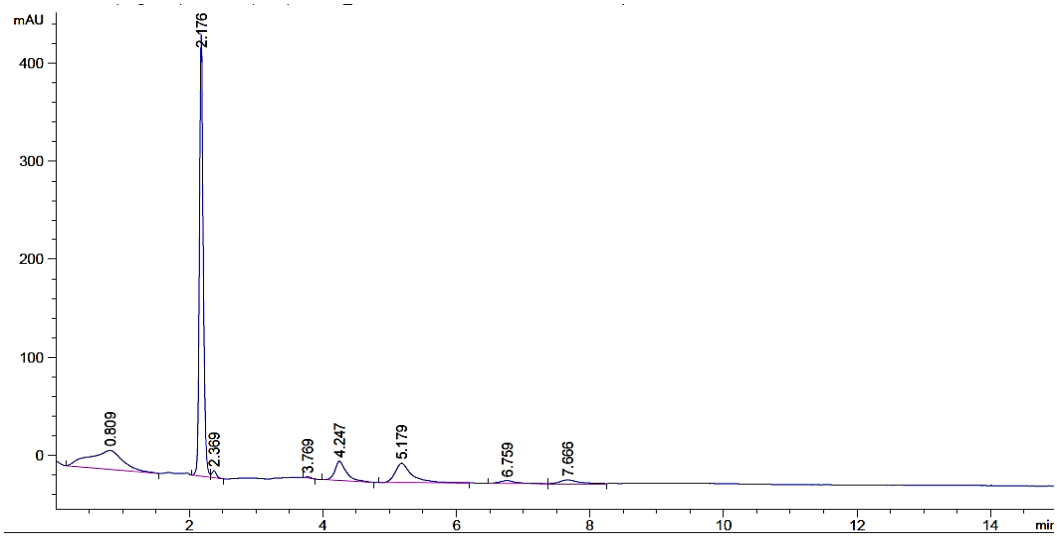
3. Calibration curve of standard stevioside (r^2 = 0.99)



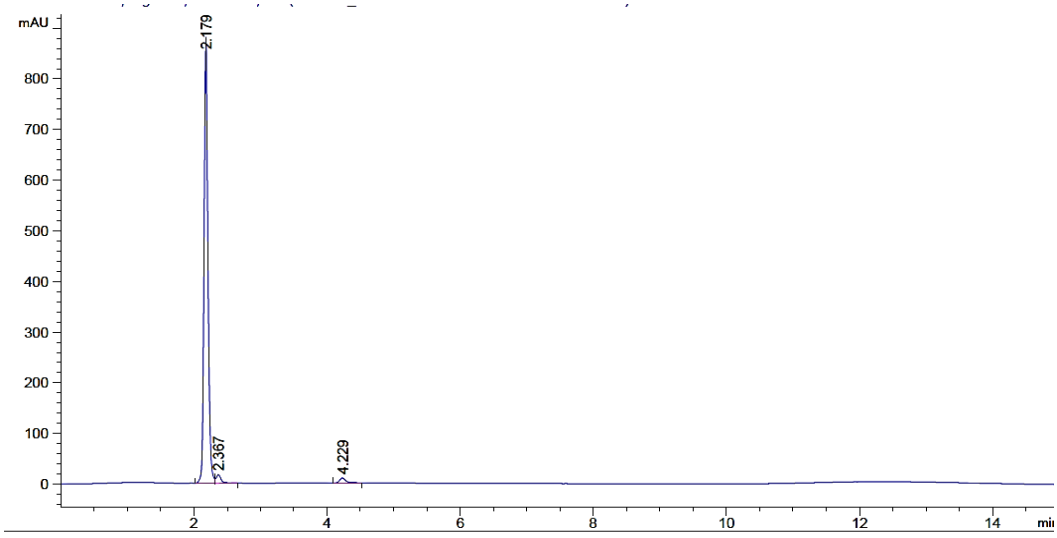
4. Calibration curve of standard Reb A (r^2 = 0.98)



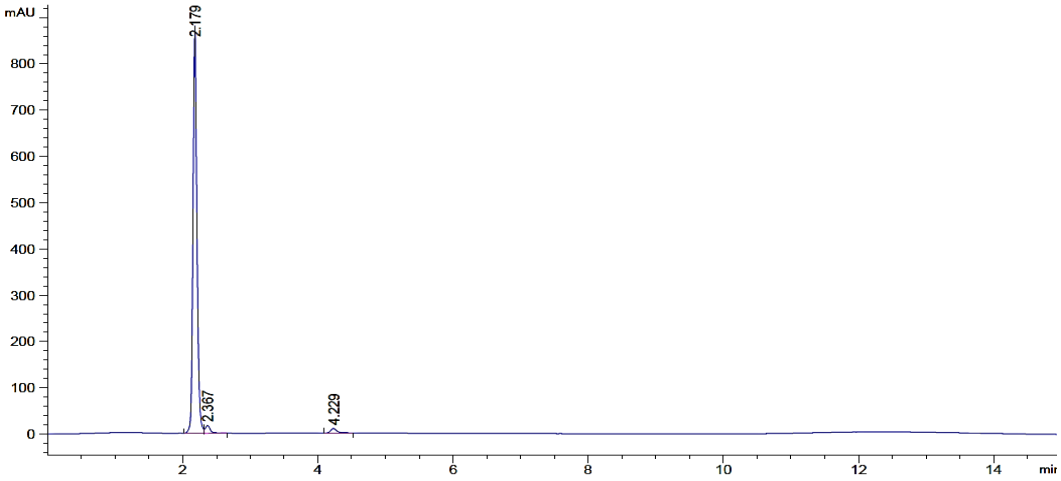
5. Chromatogram of Reb A standard



0.1mg/ml



0.2mg/ml



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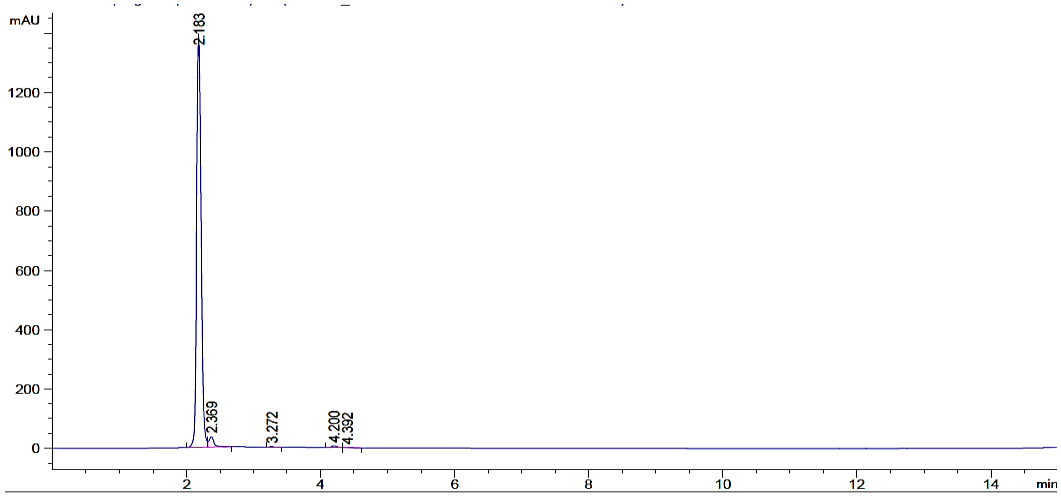
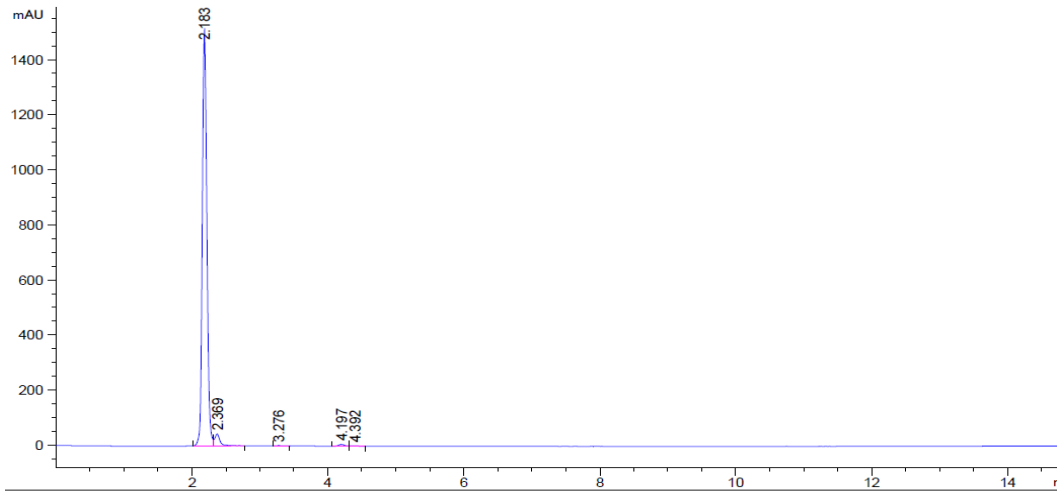
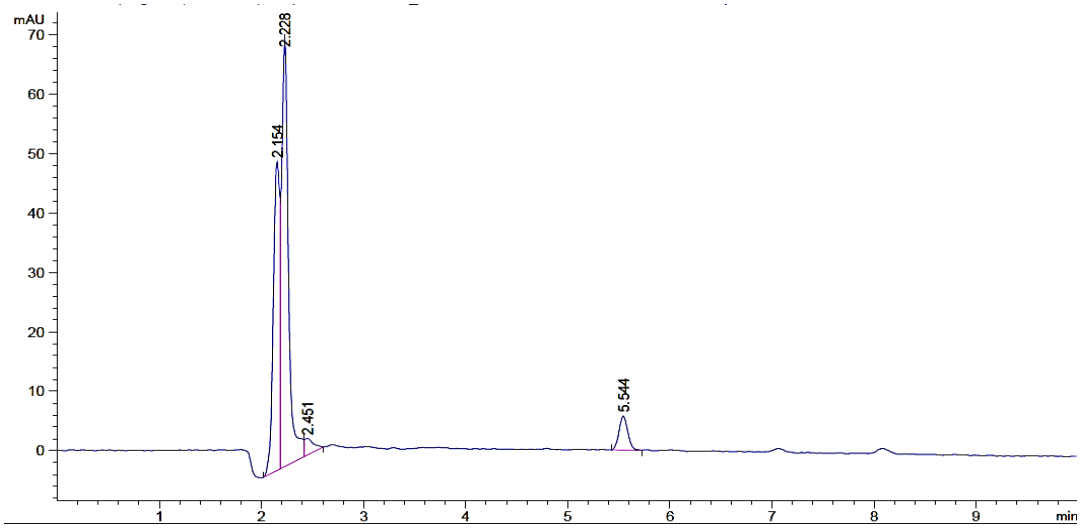


0.4mg/ml

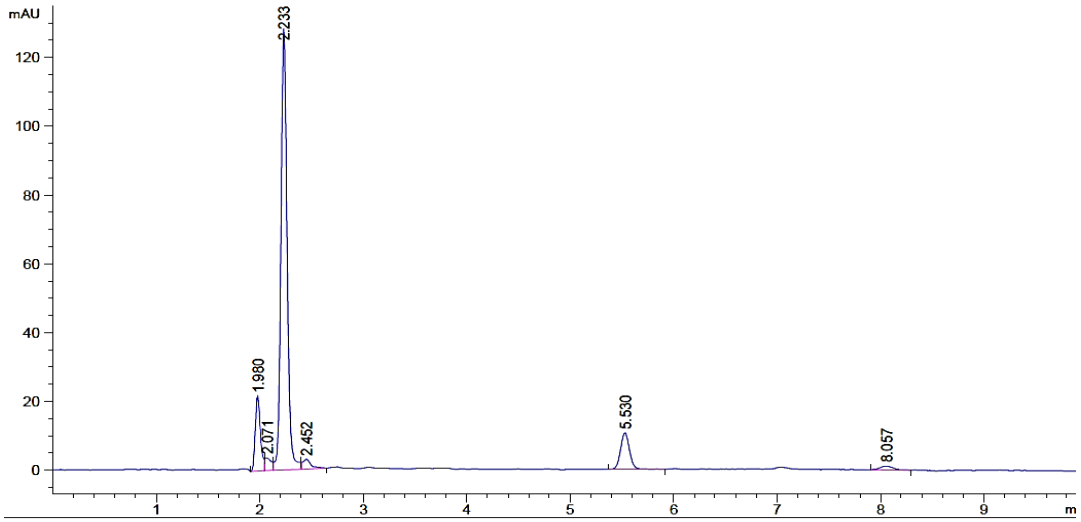


0.5mg/ml

6. Chromatogram of stevioside standard



0.1mg/ml



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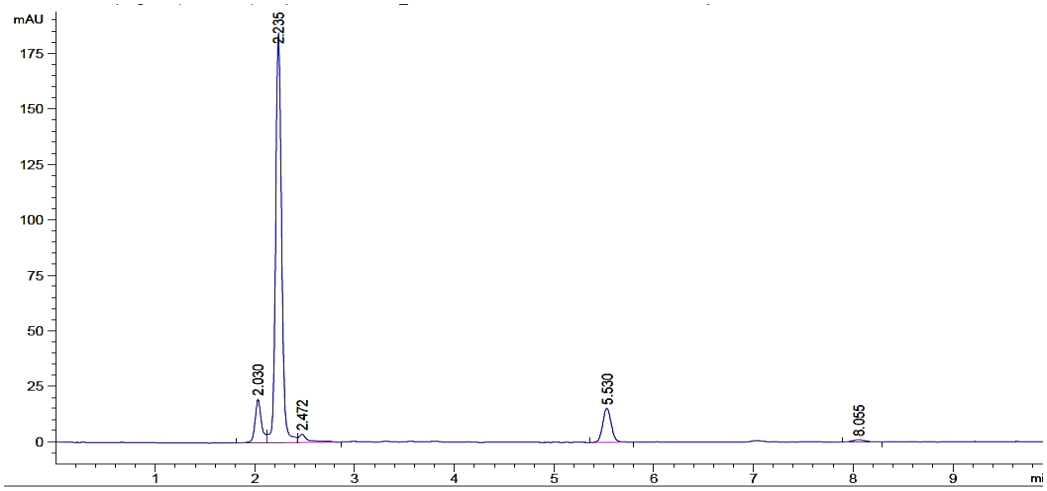

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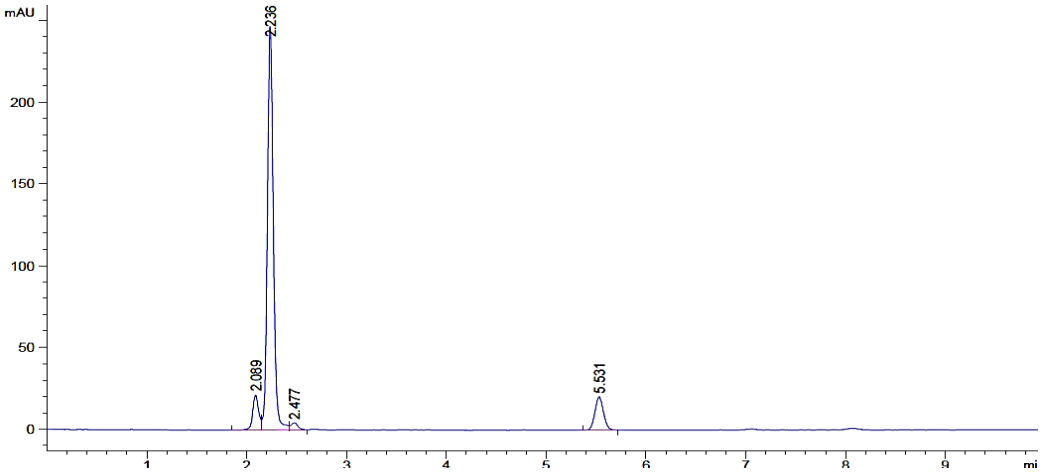

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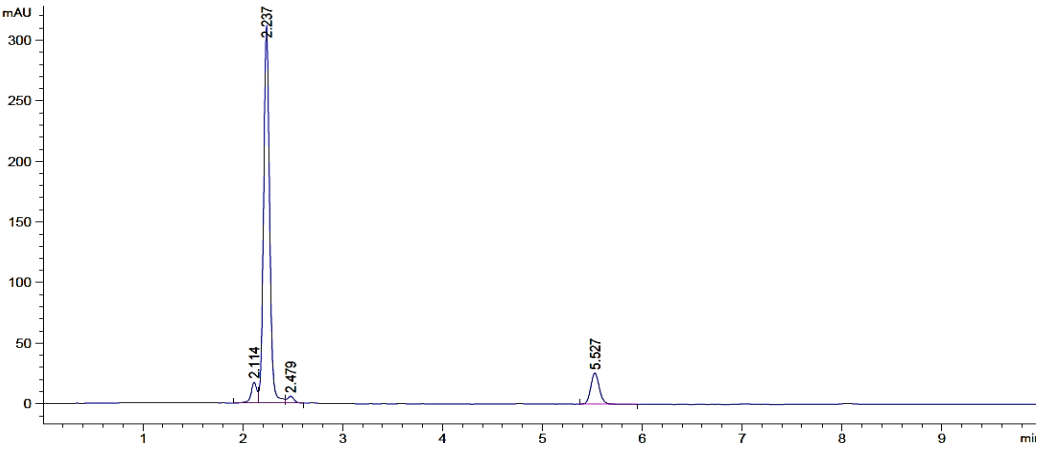

0.5mg/ml