STUDY ON EXTRACTION AND STABILITY OF NATURAL RED COLORANT FROM *HIBISCUS SABDARIFFA L.*

CHAN WILLIAM

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JULY 2014

ABSTRACT

Hibiscus Sabdariffa L. which is also known as Roselle is a tropical shrub that contains anthocyanins, which is responsible for the red colour appearance of the fruit. The major anthocyanins found in Roselle are delphinidin-3-sambubioside and cyanidin-3-sambubioside, while the minor anthocyanin is cyanidin-3-glucoside. Its application in the food industry is wide and includes products such as jam, beverage drink and fruit juice. However, the application of *Hibiscus Sabdariffa L.* as a source of natural food dye has never been studied.

The objective of the study is to use hot water extraction method to extract the anthocyanin from *Hibiscus Sabdariffa L.* and to study the stability of the extract. Hot water extraction method was used to extract anthocyanin. 4g of dried Roselle calyces and 100mL of distilled water was mixed and the extraction was done using shaking water bath. UV-Vis analysis were done on the samples and results show that the optimal temperature and time found for the extraction process is between the range of 50°C - 60°C and 15 minutes. As for the stability of anthocyanin, the addition of food grade stabilizers such as maltodextrin DE15-20 and trehalose proved that it could retard the degradation of anthocyanin with results showing maltodextrin DE15-20 as the better stabilizer. Lastly, all the samples tested with HPLC showed that cyanidin-3-glucoside were present in all the samples.

ABSTRAK

Hibiscus Sabdariffa L. yang juga dikenali sebagai Roselle adalah pokok renek tropika yang mengandungi anthocyanin, yang bertanggungjawab untuk kemunculan warna merah buah. Anthocyanin utama yang terdapat dalam Roselle adalah delphinidin-3-sambubioside dan cyanidin-3-sambubioside, manakala anthocyanin yang lebih kecil adalah cyanidin-3-glucoside. Aplikasinya dalam industri makanan adalah luas dan termasuk produk-produk seperti jem, minuman ringan dan jus buah-buahan. Walau bagaimanapun, penggunaan Hibiscus Sabdariffa L. sebagai sumber pewarna makanan semula jadi tidak pernah dikaji.

Objektif kajian ini adalah untuk menggunakan kaedah pengekstrakan air panas untuk mengeluarkan anthocyanin dari Hibiscus Sabdariffa L. dan untuk mengkaji kestabilan ekstrak. Kaedah pengekstrakan air panas telah digunakan untuk mengeluarkan anthocyanin. 4g buah Roselle yang sudah dikeringkan dan 100ml air suling telah bercampur dan pengekstrakan telah dilakukan dengan menggunakan 'shaking water bath'. Analisis UV-Vis telah dijalankan ke atas sampel dan keputusan menunjukkan bahawa suhu dan masa yang optimum untuk proses pengekstrakan adalah di antara julat 50°C - 60°C dan 15 minit. Bagi kestabilan anthocyanin, penambahan penstabil gred makanan seperti maltodextrin DE15-20 dan trehalose membuktikan bahawa ia boleh melambatkan degradasi anthocyanin dengan keputusan menunjukkan maltodextrin DE15-20 sebagai penstabil yang lebih baik. Akhir sekali, semua sampel yang diuji dengan HPLC menunjukkan bahawa cyanidin-3-glucoside turut hadir dalam semua sampel.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	*absorbance value*
MW	*molecular weight*
D	*dilution factor*
l	*path length in centimeters*

Greek

ε	molar extinction coefficient
µ	micro
n	nano

1 INTRODUCTION

1.1 Motivation and statement of problem

Food dye or food coloring is a type of food additive that is added to food or drinks. It is a form of pigment, dye or substance that imparts color when it is added. Food dye can be found in the form of powder, liquid and gel. Its main function is to provide color to food and drinks. Besides that, it can also be used to offset the color loss of the food due to exposure of light, air, temperature, moisture and storage conditions. It also functions to enhance colors that occur naturally. In the current market, food dye can be divided into two types. They are natural food dye and synthetic food dye.

Natural dyes have been used since the existence of man as mentioned in the study of Kumar & Sinha et. al., (2004). It was used in the wall paintings of the Egyptian pyramids, and other ancient civilizations. However, the invention of 'Mauveine' by William Henry Perkin in 1856 started the trend of using synthetic dyes. This invention created an industry that we all now fondly call as 'fashion' and has been well received back then. It was even expanded to other industry such as food and beverage, paint and textile industry. Various synthetic food dyes were created to meet the demands of the market.

However, in recent times, a growing worldwide concern for food quality and safety has been pushing governments to set new standards (Shahid & Mohammad, 2013). The market pressure and consumer preferences have brought about changes for food manufacturers to increase their interest in finding alternatives to synthetic food dyes and replacing it with natural dyes (Shahid & Mohammad, 2013).

The ever increasing reports of health hazards and toxicity of using synthetic dyes are pushing food industry producers to find an alternative in the form of natural dyes (Santos et. al., 2011). Natural dyes are commonly obtained from the extraction of natural sources such as fruits and plants. For the past decade, extraction of natural dyes has resulted in extraction of beetroot (López et.al., 2009), red cabbage (Chandrasekhar et. al., 2012), acai berry (Tonon et. al., 2010) and many more.

Various methods have been used to extract the color pigments from their respective source of fruits and plants. Methods like exhaust method (Ali et. al., 2009), Soxhlet extraction method (Luque de Castro & Priego-Capote, 2010) and solvent extraction (Abou-arab et.al., 2011) have been used in the extraction of color pigments.

In the current commercial market, the source of red color comes from an insect known as cochineal. Cochineal is defined as a natural organic dyestuff made from the bodies of the female insect *Dactylopius coccus*, which lives on cactus pads in Central and South America (See et al., 2011). In history, cochineal or more known formally as *Dactylopius coccus* has been used as dye for red color since the 16^{th} century (Norrington, 2011). There was not much of a problem with the use of cochineal until recently when people are more conscious about the health issues that is caused by the use of cochineal as the source of red color in their day-to-day food preparation. One of the case is by the famous American coffeehouse chain Starbucks when they introduced a new drink called Strawberry Frappucino that uses cochineal extract for its strawberry base in the year 2012 (Jaslow, 2012). The customers complained that the usage of cochineal poses two problems. The first problem is that it cannot be consumed by vegetarians and secondly, it poses allergic problems.

Therefore, this study is carried out to find a suitable and potential alternative to replace cochineal as the organic dye for red color.

This study focuses on the extraction of Roselle. Roselle or *Hibiscus Sabdariffa L.* is a type of shrub plant that belongs to the Malvaceae family. Its genes belong to the Hibiscus genus. This plant is able to withstand short periods of drought and can be cultivated throughout the tropics and subtropics during hot and rainy seasons (Eslaminejad & Zakaria, 2011).

1.2 Objectives

The following are the objectives of this research:

i. To successfully extract red color from *Hibiscus Sabdariffa L.* using water extraction method.
ii. To study the color stability of the extract from *Hibiscus Sabdariffa L* .

1.3 Scope of this research

The following are the scope of this research:

i) Experimental analysis of anthocyanin extraction using water extraction method whereby the optimal temperature and time for extraction is studied.

ii) Experimental analysis of anthocyanin stability with and without addition of stabilizers for a certain period of time.

iii) Analysis of the extracted anthocyanin by comparing the retention time of the samples with the standard.

To realize the outlined objective, both experiment and data analysis works will be conducted.

1.4 Significant of Study

The result of this study can be further developed for the industrial application of replacing cochineal as source for red color dye. It is hoped that a natural color retention agent could be found to replace the chemicals used to maintain the color of the extract.

2 LITERATURE REVIEW

2.1 Overview

This paper presents the experimental studies of gas-liquid stirred tanks agitated by a pitched paddle impeller operating at an aeration number ranged from 0.005 to 0.024. The gassed and ungassed power draw profile for the pitched paddle impeller was established. The ungassed turbulent power number was found to be around 0.95, which is a typical value for an axial impeller. The power draw decreases quickly from 0.85 to 0.4 during aeration when the aeration number increases from 0.005 to 0.024. It is also possible to correlate the ungassed power number profile according to Smith's (2006) correlation. The mass transfer coefficient of pitched paddle impeller was found to be much higher than those of a conventional Rushton type impeller operating at similar Pg/V.

2.2 Roselle/Hibiscus Sabdariffa L.

Hibiscus Sabdariffa or more commonly known as Roselle in English is a shrub plant that is grown in tropical countries. It belongs to the Malvaceae family of the Hibiscus genus. It is usually cultivated for its leaves, seeds, stem and calyces. It can be planted in a wide range of soil conditions. For domestic cultivation, a relatively infertile soil is sufficient for it to grow. However, for commercial cultivation, the soil needs to be rich in organic materials and nutrients (Tindal HD, 1986).

It takes about six months for a Roselle plant to mature. It is suitable to be grown in tropical and subtropical countries due to the warm and humid climate which will help the cultivation of the Roselle plants. It is not suitable to be cultivated in cold areas as it is susceptible to frost and mist (Morton JF, 1986). Usually the plant is cultivated for its leaves, stem and calcyces. The dried calcyces can be used to prepare tea, syrup, jams, jellies and beverages. (Ansari et. al. , 2013)

In Malaysia, Roselle is known by locals as 'Assam Paya' or 'Assam Susur'. It is locally called by different names in different countries.

Table 2.1: Names of Roselle in different regions (Source: Ansari et al., 2013)

Regions	Vernacular Names
Egypt, Saudi Arabia & Sudan	Karkade
Indonesia	Rosela
Latin America	Jamaica
Gambia	Wonjo
Indian Subcontinent	Meshta
Thailand	Krajeab

In a Roselle plant, the various parts such as the flower, calyces and petals are rich in anthocyanins, a type of chemical component that is found naturally in the plant (Abou-arab et. al. , 2011). Anthocyanin is a type of vacuolar pigment that is water soluble. It normally gives colour such as orange, red, purple and blue to the various plants and fruits (Giusti & Wrolstad, 2003).

2.3 Anthocyanin

Anthocyanin, (from the Greek word, *anthos* = flower and *kianos* = blue) are pigments found in vascular plants(Castañeda-Ovando et. al. , 2009). They are water-soluble, harmless and can be easily incorporated in aqueous media. This makes the usage of anthocyanin more interesting as natural water-soluble colorants. (Pazmino-Duran et.al., 2001). These pigments normally give rise to the color of shiny orange, red, violet, pink and blue colors in the flowers and fruits of some plants.

Another significant of anthocyanin is its antioxidant activity (Castañeda-Ovando et al., 2009). They play a vital role to prevent neuronal and cardiovascular illnesses, cancer and diabetes (Konczak & Zhang, 2004). Recently, there is also a new study on anthocyanin to be a part of a dietary supplement to prevent asthma (Park et. al. , 2007). Besides that, there are also several reports produced that focuses on the effect of anthocyanins in cancer treatment (Lule and Xia, 2005; Nichenametla et. al. , 2006), human nutrition (Stintzing & Carle, 2004) and its biological activity (Kong et. al. , 2003).

Anthocyanidins are the basic structures of anthocyanins. The anthocyanidins consist of an aromatic ring that is bonded to a heterocyclic ring that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring (Konczak & Zhang, 2004).

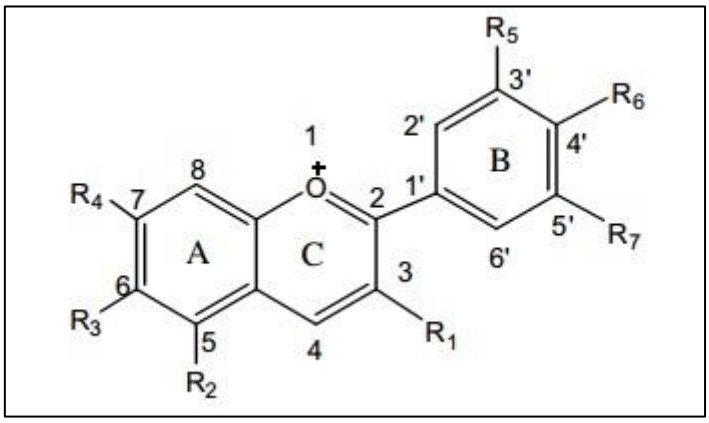


Figure 2.1: General structure of anthocyanin (Source: Castañeda-Ovando et al., 2009)

When anthocyanidins are found in the glycoside form, the structure is known as anthocyanin (Castañeda-Ovando et al., 2009).

In nature, there is a huge variety of anthocyanins available. They are differentiated by:

a) Number of hydroxylated groups
b) The nature and number of bonded sugars to their structure
c) The aliphatic or aromatic carboxylates bonded to the sugar in the molecule
d) The position of the bonds.

Source: (Kong et. al., 2003)

As of now, there are reportedly more than 500 different anthocyanins (Andersen & Jordheim, 2006) and 23 anthocyanidins (Andersen & Jordheim, 2006; Kong et. al., 2003). There are six most common glycoside derivatives of anthocyanidins, which are Pg, Pn, Cy, Mv, Pt and Dp (Clifford, 2000) and the three non-methylated anthocyanidins (Cy, Dp and Pg) are most commonly found in 80% of pigmented leaves, 69% in fruits and 50% in flowers (Dey & Harborne, 1993).

Anthocyanins are highly unstable and are very susceptible to degradation(Giusti & Wrolstad, 2003). It is affected by several factors such as pH, storage temperature, chemical structure, concentration, oxygen, light, solvents, presence of enzymes, flavonoids, proteins and metallic ions (Rein, 2005).

2.4 Anthocyanins in Roselle

The anthocyanins found in Roselle calyces are **delphinidin-3-sambubioside** and **cyanidin-3-sambubioside** (Cissé et al., 2012). It is reported to contain up to 2.5g/100g dry weight of roselle calyces (Cissé et al., 2012). The major anthocyanin found in Roselle calyces are delphinidin-3-sambubioside.

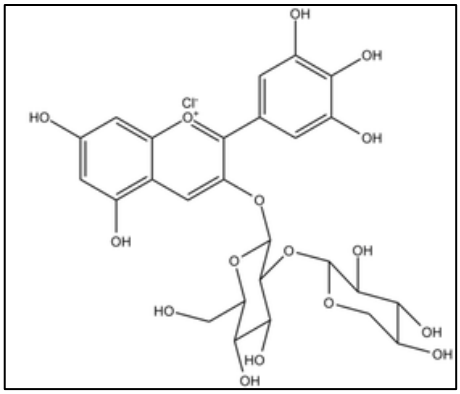


Figure 2.2: Delphinidin-3-sambubioside (Polyphenols, 2013)

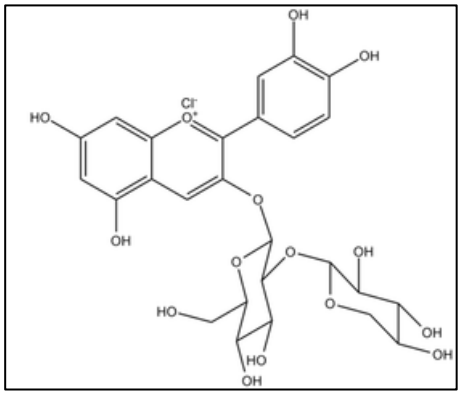


Figure 2.3: Cyanidin-3-sambubioside (Polyphenols, 2013)

The chemical formula of delphinidin-3-sambubioside is $C_{26}H_{29}0_{16}Cl$. Its molecular weight is 632.9 g mol^{-1} while for the cyanidin-3-sambubioside, the chemical formula is $C_{26}H_{29}0_{15}Cl$. The molecular weight for cyanidin-3-sambubioside is 617 g mol^{-1} (Polyphenols, 2013). Both are easily degraded in hydrolysis and hydrogenation at temperatures more than 40°C. These two anthocyanins will make the fruit or plant appear dark red or purple in color (Polyphenols, 2013).

2.5 Rotary Evaporator

Rotary evaporator or commonly termed as ‘rotavap’ is an equipment that is used in the laboratories for the removal of solvents from samples by evaporation. The equipment reduces the volume of the solvent by distributing it as a thin film across the interior of a vessel at elevated temperature and reduced pressure. This type of mechanism promotes rapid removal of excess solvent from the less volatile samples.

Most rotary evaporators have four major components. The components are heat bath, rotor, condenser and solvent trap. The heat bath is usually filled with water and functions to heat the sample. As for the rotor, it is a motor unit that rotates the evaporation flask or vial that contains the sample. The condenser is important as it functions to condense the distilling solvent and usually a coolant is used.

The rotary evaporator normally works in a vacuum condition because by lowering the pressure above a bulk liquid, it will also lower the boiling points of the component liquids in it. It is most conveniently used to remove solvents that are used in research such as n-hexane and ethyl acetate from compounds which are solid at room temperature and pressure.

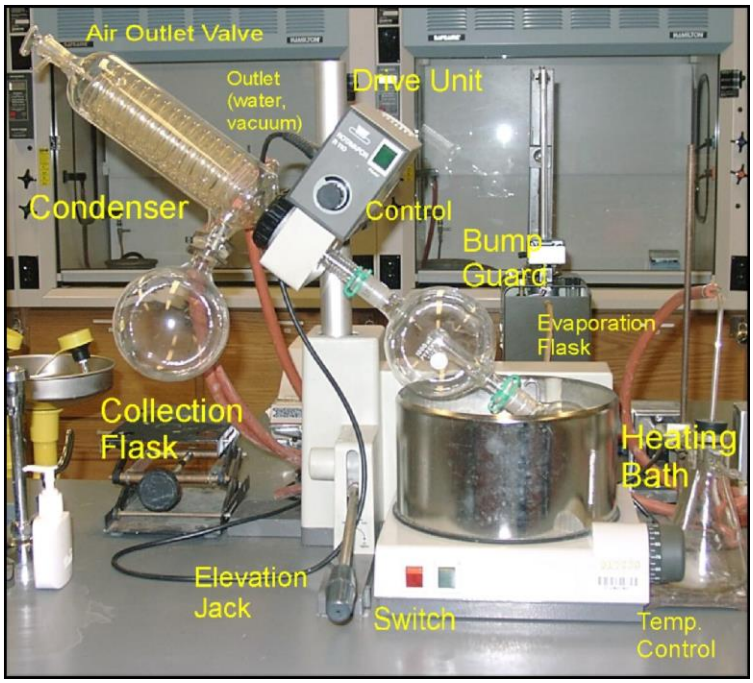


Figure 2.4: Rotary evaporator and its labelled parts

2.6 UV-Vis Spectrometer

UV-Vis spectrometer is laboratory equipment that refers to the absorbance or reflectance spectroscopy in the ultraviolet-visible spectral region. It is usually used to detect light in the visible and adjacent regions of the electromagnetic spectrum. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved.

The theory behind the UV-Vis spectrometer is the transition of electrons from lower energy levels to higher energy levels. When the UV light rays are directed towards the sample, the rays will excite the electrons and cause the electrons to move to higher anti-bonding molecular orbitals. Normally, the more favorable excitation occurs from the highest energy bonding pi-orbital to the lowest antibonding pi-orbital. Therefore, the smaller the energy gap between the highest energy bonding pi-orbital and the lowest antibonding pi-orbital, the more easier for the electrons to be excited. That means the longer the wavelength of light it can absorb. A schematic diagram of the UV-Vis equipment can be seen below:

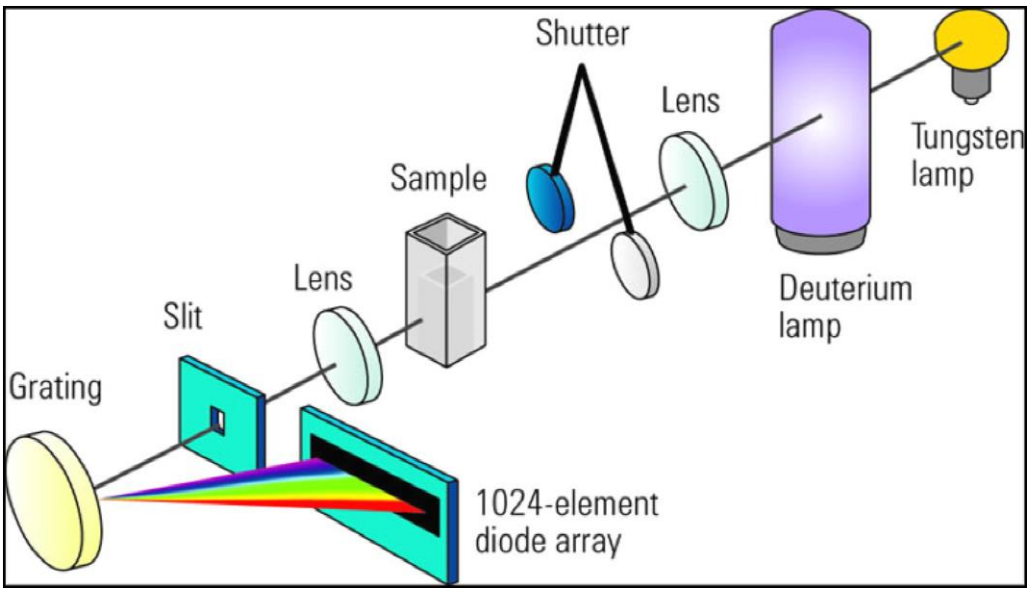


Figure 2.5: Schematic diagram of UV-Vis spectrometer

In laboratory studies, UV-Vis spectrometer is used for the quantitative determination of analytes such as transition metal ions and highly conjugated organic compounds.

2.7 High Performance Liquid Chromatography

High Performance Liquid Chromatography or better known as HPLC is an advanced and improved form of column chromatography. It was originally known as high pressure liquid chromatography as it uses high pressure to generate the flow required for liquid chromatography in packed columns (Waters, 2014).

The HPLC is a separation technique that involves the injection of a small volume of liquid sample into a tube packed with tiny particles, normally 3 to 5 micron (µm) in diameter. These tiny particles are called the stationary phase. The liquid sample are divided into individual components and moves down the packed tube or better known as column with a liquid, which is termed as mobile phase. It is forced through the column by high pressure delivered by a pump. The individual components are separated from one another by the column packing that involves various chemical and physical interactions between their molecules and the packing particles. These separated components are detected at the exit of the column by a detector that measures the amount of the components. Then, the detector sends an output, which is termed as liquid chromatogram.

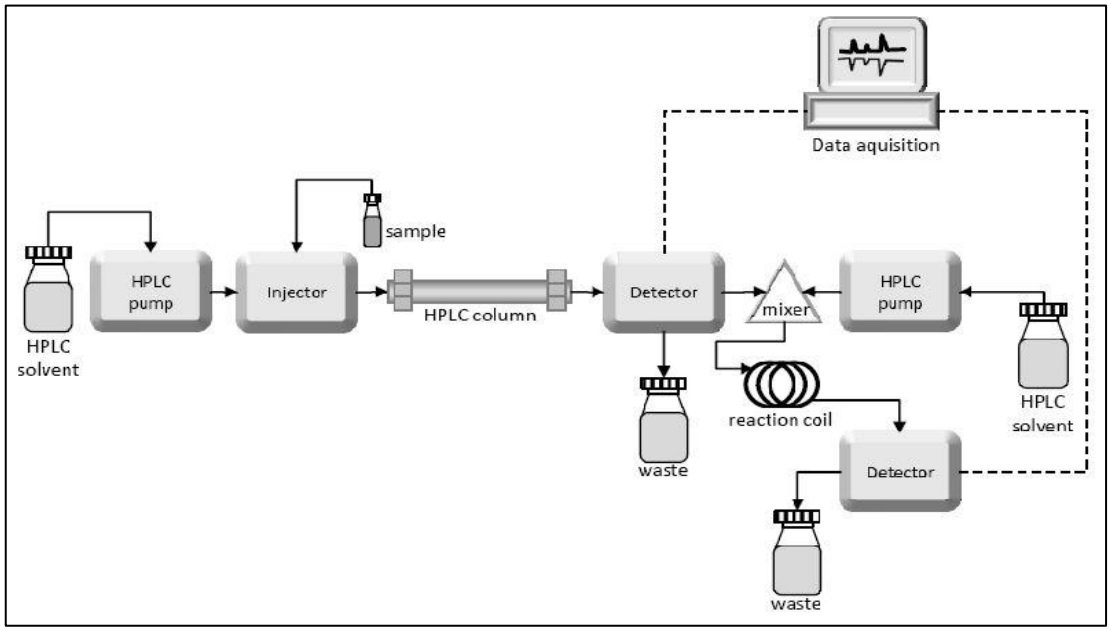


Figure 2.6: Schematic Diagram of High Performance Liquid Chromatography
Source: (INTECH, 2013)

Retention time is the time taken for a particular compound to travel through the column to the detector. The actual measurement of the time is from the time at which the sample is injected to the point where the display shows a maximum peak height for the tested compound. The retention times of each compound are different. The variation due to the retention time depends on a few factors such as:

a) The pressure used.

b) The nature of the stationary phase

c) The exact composition of the solvent

d) The temperature of the column

The usage of HPLC with different columns allows better separation of components of the mixture because it has different surface area for interactions between the stationary phase and the molecules flowing past it. Basically, a column packing with much smaller particles has greater surface area and is much more efficient and vice versa.

2.8 Factors that affect the stability of anthocyanin

The stability of isolated anthocyanin are very weak and very susceptible to degradation (Giusti & Wrolstad, 2003). The stability of anthocyanin are affected factors such as:

a) pH
b) Storage temperature
c) Light

2.8.1 pH and stability of anthocyanin

Anthocyanins are affected by the pH of the solution and can be found in many forms. When the pH of the solution is 1, the flavylium cation is predominant and contributes to the red and purple color. When the pH value is slightly increased to between 2 and 4, the dominant species is the quinodal blue species. This contributes to the blue color of the anthocyanin. When the pH of the solution is at 5 and 6, there are only two species

and it is colourless. The two species are carbinol pseudobase and chalcone. (Castañeda-Ovando et al., 2009)

When the pH values are higher than 7, the anthocyanins are degraded according to the substituent group. A summary of the chemical reaction that occurs in the structure of anthocyanin can be seen in Figure 2.7 below.

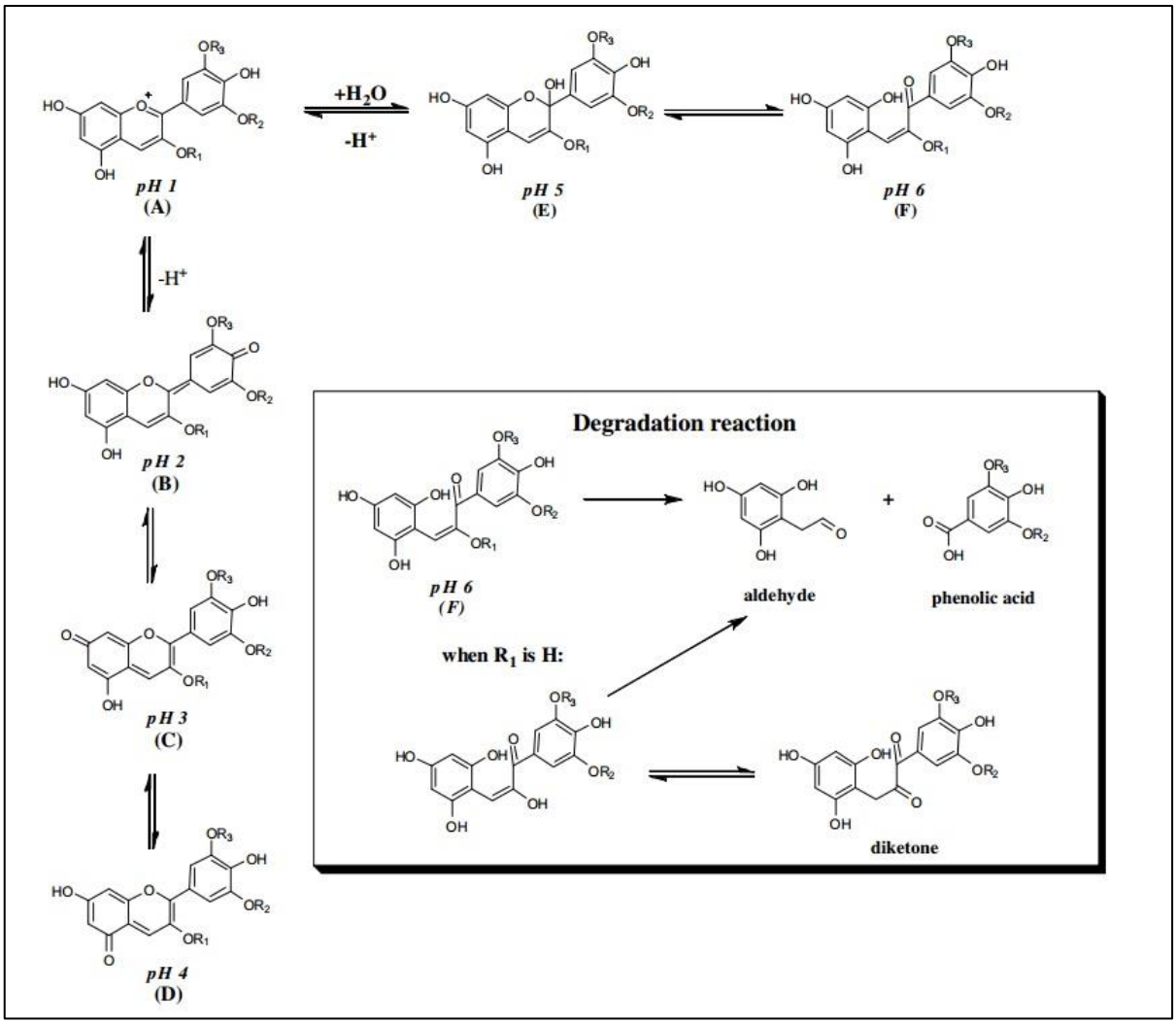


Figure 2.7: Anthocyanin chemical forms depending on pH and degradation reaction for anthocyanins. Where R_1 = H or saccharide, R_2 and R_3 = H or methyl. Source: (Castañeda-Ovando et al., 2009)

2.8.2 Storage temperature and stability of anthocyanin

Based on a study by (Mattila et. al. , 2013), it has been found that the stability of anthocyanin of commercial juices are significantly affected by the storage temperature. In the study, the results showed that at different storage temperatures, the amount of anthocyanins found in the commercial juice differ greatly. Similar study had also been

conducted on the stability of black carrot anthocyanin by (Kırca et. al., 2007). The results of the study showed that degradation of anthocyanins occurs faster at higher temperatures compared to lower temperatures. This has lead us to believe that during the storage of the extracted sample, certain precautionary steps will need to be taken to ensure that the result of our study is not affected.

One of the precautionary steps is to store the extracted sample immediately after extraction at 4°C in the refrigerator.

2.8.3 Light and stability of anthocyanin

The stability of anthocyanins are proven to be affected by light (Cemeroglu et.al., 1994). The degradation pathway is normally modelled as a first-order reaction rate reaction (Cemeroglu et. al., 1994).

From previous studies made on anthocyanin, the effect of light on anthocyanin had reported to degrade the amount of anthocyanin depending on the type of light source used. Based on the study by (Sari et. al., 2012), the result of the study showed that the exposure of anthocyanin to fluorescent light accelerated the degradation of anthocyanin. However, the exposure of UV-B light rays will yield in the accumulation of anthocyanin(Reay & Lancaster, 2001). The effect of UV-B irradiation on the levels of anthocyanin of buckwheat sprouts have also recorded similar findings (Tsurunaga et al., 2013).

The two different findings ultimately lead us to understand more regarding the stability of anthocyanin under the influence of light.

3 MATERIALS AND METHODS

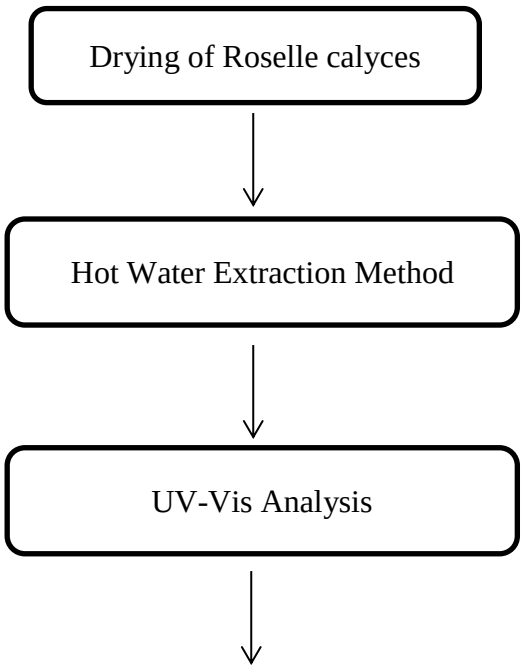
3.1 Overview

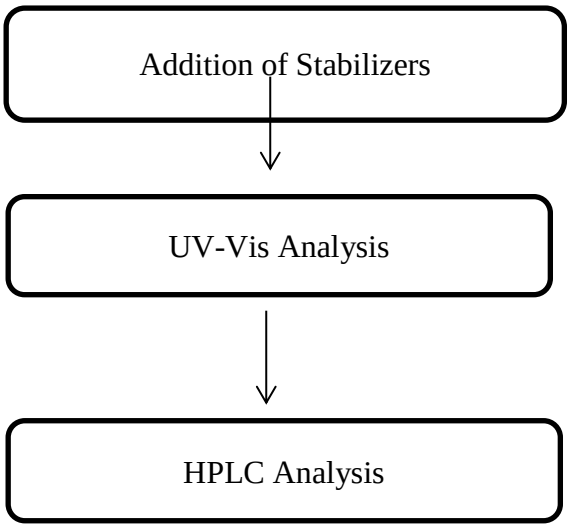
This study comprises of two parts, which is the study of extraction of anthocyanin and the study of stability of the extract. Experimental works were conducted and the results were analysed using UV-Vis spectrometer and HPLC equipment. The experimental work done was to extract anthocyanin using hot water extraction method at temperature of 30, 40, 50, 60 and 70°C and time interval of 5, 10, 15,20, 30 and 40 minutes. As for the stability part, the extraction was conducted with addition of stabilizers such as maltodextrin and trehalose. All the extracts were analysed using UV-Vis spectrophotometer, while for the identification of anthocyanin, the HPLC was used and the extract was compared to the standard, cyanidin-3-glucoside.

3.2 Introduction

This chapter presents on the chemicals, materials and analysis methods used throughout the duration of the study.

3.3 Overall Methodology Flowchart





3.4 Chemicals

The chemicals used in this study were acetonitrile, cyanidin-3-glucoside, maltodextrin and trehalose. The acetonitrile used was HPLC grade and purchased from Merck Malaysia. Maltodexrin DE 15-20 and trehalose used were food grade. The cyanidin-3-glucoside was purchased from Santa Cruz Biotechnology.

3.5 Materials

The main material used in the study was Roselle fruit. The fruit or better known as calyces were purchased from a local farm in Kemaman. The calyces were dried in the oven at 60°C and blended into powder form. The powder form calyces were then stored inside an amber Schott bottle and wrapped with a layer of aluminium foil to prevent any contact with the light.

3.6 Hot Water Extraction Method

The shaking water bath temperature was set accordingly to 30°C. While waiting for the water bath to reach the set temperature, 100mL of distilled water was measured using a measuring cylinder and poured into the 100mL conical flask. Once the temperature of the water bath had reached the set temperature, the conical flask was put into the water bath and wait for the temperature of the water inside the conical flask to be the same as the water bath. Four (4) grams of dried Roselle calyces were mixed with the distilled water. The speed for the shaker was set to 100RPM and time was started. The extraction

time duration was varied for 5, 10, 15, 20, 30 and 40 minutes while the extraction temperature was varied for 30, 40, 50, 60 and 70°C.

After the extraction process had completed, the sample was filtered using a vacuum pump. The filtrate was then further centrifuged to remove any impurities. After that, the sample was taken for UV-Vis analysis

3.7 Addition of Stabilizer to Extract

For the study of the stability of anthocyanin, two types of food grade stabilizers were added to the extract. The stabilizers are maltodextrin DE 15-20 and trehalose. The procedure is similar to the hot water extraction method.

Three (3) grams of stabilizers were added to the mixture of dried Roselle calyces and distilled water and the extraction was carried out at temperatures of 30, 40, 50, 60 and 70°C for the duration time of 5, 10, 15, 20, 30 and 40 minutes. After the extraction process had completed, the sample was filtered using a vacuum pump. The filtrate was then centrifuged and taken for UV-Vis analysis. The filtrate was kept in the vial for 7 days and the readings for the UV-Vis analysis were taken daily.

3.8 UV-Vis spectrophotometer

The UV-Vis spectrophotometer was used to record the absorbance value of the extracts. The absorbance readings can be interpreted as the total anthocyanin content via a formula as stated by (Idham et. al. , 2012). The formula to calculate total anthocyanin content is as shown below:

$$Total\ Anthocyanin\ Content\ \left(\frac{mg}{L}\right) = \frac{A\ X\ MW\ X\ 10^3\ X\ D}{\varepsilon\ X\ l}$$

Where A = absorbance value

MW = molecular weight of cyanidin-3-glucoside (448.8 g/mol)

D = dilution factor (1/25)

l = path length in cm