BATCH EXTRACTION OF CAFFEINE FROM COCOA *MCBC 1*

NURADIBAH BINTI HUSSIN

UNIVERSITI MALAYSIA PAHANG

BATCH EXTRACTION OF CAFFEINE FROM COCOA MCBC 1

NURADIBAH BINTI HUSSIN

A thesis submitted in fulfillment
of the requirements for the award of the Degree of
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"I hereby declare that I have read this thesis and in my opinion this thesis has fulfilled the qualities and requirements for the award of Degree of Bachelor of Chemical Engineering (Biotechnology)"

Signature	:	……
Name of Supervisor	:	DR IR SAID NURDIN
Date	:	……

I declare that this thesis entitled “Batch Extraction of Caffeine from Cocoa *MCBC 1*” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :……

Name : NURADIBAH BINTI HUSSIN

Date : ……

SPECIAL DEDICATION
TO MY FAMILY MEMBERS ESPECIALLY TO MY BELOVED MOTHER,
TINA BIN KAMSAN AND FATHER, HUSSIN BIN SALLEH,
MY SUPERVISOR, DR IR SAID NURDIN, STAFFS OF FKKSA LABORATORY
AND ALL MY FRIENDS

FOR ALL YOUR LOVE AND SUPPORT, THANK YOU VERY MUCH

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I acknowledge my sincere indebtedness and gratitude to my parents for their love, dreams and sacrifice throughout my life. Unfortunately, it is not possible to list all of them in this limited space. I cannot find the appropriate words that could properly describe my appreciation for their devotion, support and faith in my ability to attain my goals.

ABSTRACT

Caffeine is naturally occurring substances found in the leaves seeds of fruits of a group of compounds known as methylxanthines. The most commonly known source of caffeine is coffee and cocoa seeds. However, cocoa seeds have received limited attention as a source for caffeine since it widely used as a source to make a cocoa powder and chocolate. The caffeine extraction from cocoa seeds will be another way to fulfill demand for caffeine. The objectives of this research is to extract the caffeine from Cocoa MCBC I Seeds by the effect of different extraction time, particle size of cocoa seeds powder and ratio of cocoa seeds-solvent towards yields of caffeine by using batch system. This research was divided into five main steps which are the sample preparation, leaching of caffeine, liquid-liquid extraction, drying of caffeine and analysis the crude caffeine using High Performance Liquid Chromatography (HPLC). Extraction yield increase towards the increasing of extraction time. Meanwhile, extraction yield of caffeine increased when using the smallest particle size of cocoa. However, the best ratio of cocoa seeds-solvent to get the highest extraction yield was 1:5. Besides that, either using lower ratio or higher ratio than 1:5, the extraction yield cannot achieve as higher as 1:5 cocoa-solvent ratio. As a conclusion, caffeine can be extracted from cocoa MCBC 1 seeds by using batch system. 60 minutes of extraction time, 400 of the particle size of cocoa seeds powder and 1:5 ratio of cocoa seed-solvent gives highest extraction yield of caffeine.

ABSTRAK

Kafein adalah zat alami yang ditemui dalam daun, biji buah-buahan dari kumpulan sebatian yang dikenali sebagai methylxanthines. Sumber yang biasa bagi kafein adalah kopi dan biji koko. Namun begitu, biji koko kurang mendapat perhatian sebagai sumber kafein kerana banyak digunakan sebagai sumber untuk membuat serbuk koko dan coklat. Ekstrak kafein daripada koko merupakan satu cara lain untuk memenuhi permintaan terhadap kafein. Penyelidikan ini bertujuan untuk mengekstrak kafein daripada biji koko MCBC1 dengan memanipulasi waktu ekstrak, saiz serbuk koko dan nisbah biji koko-pelarut terhadap hasil kafein dengan menggunakan sistem batch. Penyelidikan ini dibahagikan kepada lima bahagian utama iaitu penyediaan sampel, ekstrak kafein dari sampel ke cecair, ekstrak ceceir ke cecair, pengeringan kafein dan analisi kafein menggunakan *High Performance Liquid Chromatography (HPLC)*. Hasil ekstrak meningkat seiring penigkatan masa ekstrak. Namun begitu, hasil ekstrak menurun apabila saiz sampel meningkat. Nisbah biji koko-pelarut yang terbaik yang mendapatkan hasil ekstrak yang tinggi adalah 1:5. Kesimpulannya, kafein boleh dihasilkan dari biji koko MCBC 1 melalui sistem batch. 60 minit masa ekstraksi, 400 dari saiz zarah serbuk biji koko dan nisbah 1:5 biji koko-pelarut memberikan hasil ekstraksi tertinggi kafein.

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LIST OF ABBREVIATIONS/TERMINOLOGY/SYMBOLS

º	- Degree
%	- Percent
µg/ml	- Microgram per milliliter
µm	- Micrometer
C	- Celsius (Temperature)
et al.	- And others
g	- Gram
GC	- Gas Chromatography
HPLC	- High Performance Liquid Chromatography
m	- Meter
MCBC	- Malaysian Cocoa Board Clone
min	- Minute
ml	- Milliliter
mg	- Milligram
M	- Mole
SPE-HPLC	- Solid Phase Extraction- High Performance Liquid Chromatography

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

This introduction gives the idea about the rationale and general understanding of the research. This chapter discovers the subtopic of background of study problem statement research objectives, scope of research and significance of research.

1.1 Background of Study

The quality of the cocoa beans depends on many factors such as the genotype, the agronomic management, the soil factors, the climatic conditions, and the most importantly the post harvest technology (Brunetto et al., 2007). Cocoa based foods are consumed worldwide and have been shown to be very nutritious, containing substantial amounts of amino acids, except methionine and arginine. Vitamins, minerals and fat are also presence a high proportion. Cocoa products contain many physiologically active compounds. The high level of fat contributes to the high gross energy content of the cocoa bean. Despite its high with nutrition value, the presence of caffeine and thebromine alkaloids may limits its potential as a nourishing food (Beckett, 1988).

Caffeine is naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide and is part of a group of compounds known as methylxanthines. The most commonly known sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. Caffeine is pharmacologically active substance and depending on the dose, can be mild central nervous system stimulant. Caffeine does

not accumulate in the body over the course of time and is normally excreted within several hours of consumption (Barone and Roberts, 1996).

Decaffeination is a popular term in present modern world in order to optimize the caffeine contains in various sources. This is simply use of a solvent, which extract caffeine. For this purpose, the currently available solvents are chloroform, methyl chloride, ethyl acetate, super critical carbon dioxide and others (Mumin et al., 2006).

1.2 Problem Statement

Caffeinated products have been consumed by humans since prehistoric times. Today, most of the world's population, regardless of geographical location, gender, age, or culture consumes caffeine daily. With increasing use of caffeine in the beverage and pharmaceutical industries, demand for caffeine continues to increase. Natural sources of caffeine used today include tea leaves and coffee beans. A naturally occurring source of caffeine that has received limited attention is the seed of cocoa tree. This is because nowadays, cocoa are widely used to make cocoa powder and chocolate. The caffeine extraction from cocoa seeds will be another way to fulfill demand for caffeine.

1.3 Objectives

The current research is done to achieve the following objectives:

i) To extract the caffeine from cocoa MCBC I seeds using batch system.

ii) To investigate the effect of extraction time, particle size of sample and cocoa seed-solvent ratio towards extraction yield of caffeine.

1.4 Research Scope

The scopes of this research are:

i) Extraction of caffeine using batch system.

ii) Investigate effect of extraction time, particle size of sample and cocoa seed-solvent ratio towards extraction yield of caffeine.

iii) Analysis of crude caffeine using High Performance Liquid Chromatography (HPLC).

1.5 Significance of Research

This research created another application for cocoa seeds except as a chocolate and cocoa powder. Cocoa also can be use as a raw material for caffeine. It can fulfill demand for caffeine. Extract the caffeine composition in this research is from new breed which is Cocoa *MCBC I.*

CHAPTER 2

LITERATURE REVIEW

2.1 Cocoa

The cocoa tree also known as Theobroma Cocoa is a native of the dense tropical forests of the Amazon where it grows in conditions of semishade, warmth, and high humidity. The genus of Theobroma consists of over twenty species.

Botanically, the term “cocoa” refers to the tree and its fruits. Cocoa describes the bulk commercial dried fermented beans, as well as the powder produced from the beans. The tree can only be cultivated within fairly narrow limits of altitude, latitude and humidity.

The cultivation of 75 percent of the world’s cocoa lies within eight degree of either side of the equator, with exceptions in some areas to about 18 degrees north or south. The optimum growing temperature for cocoa tree is between 18 to 32ºC. (Wood, 1985).

Table 2.1: Chemical Composition of Cocoa Beans

	Knapp and Churchman (1937)		Fincke (1965)		Jensen (1931)		Pearson (1981)			
							Nib, %		Shell, %	
	Nib, %	Shell, %	Nib, %	Shell, %	Nib, %	Shell, %	Max	Min	Max	Min
Water*	2.1	3.8	5	11	3.9	8.1	3.2	2.3	6.6	3.7
Fat (cocoa butter, shell fat)	54.7	3.4	54	3	63.2	3	57	48	5.9	1.7
Ash	2.7	8.1	2.6	6.5	3.1	7.6	4.2	2.6	20.7	7.1
Nitrogen										
Total nitrogen	2.2	2.8	2.1	2.6		2.6	2.5	2.2	3.2	1.7
Protein nitrogen	1.3	2.1								
Theobromine	1.4	1.3	1.2	0.8	1.3		1.3	0.8	0.9	0.2
Protein			11.6	13.5	13.9	15.9				
Caffeine	0.07	0.1	0.2				0.7	0.1	0.3	0.04
Carbohydrates										
Glucose	0.1	0.1	1							
Sucrose	0	0								
Starch	6.1	No true starch	6		6		9	6.5	5.2	3.4
Pectine	4.1	8								
Crude fiber	2.1	18.6	2.6	16.5	2.7	14.8	3.2	2.2	19.2	12.8
Cellulose	1.9	13.7	9							
Pentosans	1.2	7.1	1.5	6	1.4	8				
Mucilage and gums	1.8	9								
Tannins				9						

* Water content can vary according to the degree of drying or roasting.

Source: Bernard, 1999

Cocoa tree grow from seed through three stages of development. The early stages of grow involve the initial growth of the seed. The cocoa initially germinates and pushes the cotyledons about an inch above the surface of the soil and then it produces the first leaves.

The next stage gives the plant further vertical growth until fan branches appear. The number of these sideways growths can vary depending on the variety. The growth of the fan branches forms a jorquette. Further vertical growth depends on chupons, the branches that normally develop on the main trunk and that grow up above the fan branches. The chupons may then form a new jorquette with their own fan branches. The height of the tree is typically between eight and ten meters (Dand., 1999).

Figure 2.1: Fermented and Roasted Cocoa

The most visible use of cocoa is in candies and drinks. Its solids form are ground into powder and its oil is extracted and converted into cocoa butter, these two ingredients can be mixed with sugar, milk, flour and a variety of other common goods to create chocolate bars, cakes and other sweets. In addition, raw cocoa beans are sometimes eaten for their flavonoids to improved cardiovascular health (Eric et al., 2006).

Cocoa butter is used for pharmaceuticals. It is used for encapsulating certain drugs because it can be stored sadely and dissolved readily in the body. Naturally resists rancidity compound in cocoa makes it ideal for products such as cosmetics and soap. A study by Young and Jewell shown that topical application of vitamin E in cocoa can be used to reduce stretch marks and scar removal.

Table 2.2: Composition of Cocoa versus Chocolate (in %)

	Cocoa Beans	Cocoa Butter	Dark Chocolate
Proteins	18.0	6.4	6.0
Lipids	56.0	54.0	27.0
Carbohydrates	13.5	28.0	54.0
Water	3.0	2.0	1.0
Theobromine	1.45	1.1	0.5
C1.1affeine	0.05	0.5	0.07
Theophylline	-	-	0.001

*This average values are subject to considerable variations
Source: Hesse, 2002

2.2 Caffeine

Caffeine is the world's most popular drug, easily surpassing nicotine and alcohol. Caffeine is the only addictive psychoactive substance that has overcome resistance and disapproval around the world that it is freely available almost everywhere, sold without license and even added to beverages intended for children (Bennet and Bonnie, 2001).

Caffeine (1,3,7-trimethylxanthine, guaranine) is a plant-derived alkaloid. Caffeine, theophylline, theobromine and paraxanthine are usually easily to detect in toxicological samples due to dietary exposure to caffeine. The average cup of coffee or tea in United State is reported to contain between 40 and 150 mg of caffeine

(Baselt, 2004) although special coffees may contain much higher doses of caffeine (McCusker, 2003).

Caffeine is an alkaloid of the methylxanthine family. In its pure state, it is an intensely bitter white powder. Its chemical formula is $C_8H_{10}N_4O_2$, its systematic name is 1, 3, 7-trimethylxanthine (Arnaud, 1987) and its chemical formula is shown below.

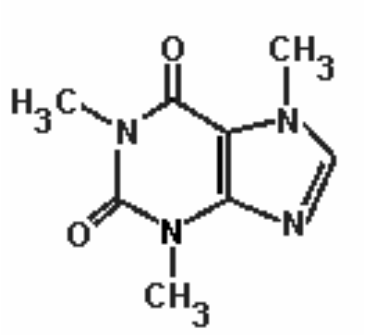

Figure 2.2: Structure of Caffeine

Pure caffeine occurs as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19g, melting point is 236 0C, point at which caffeine sublimes is 1780C at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mm Hg at 178 0C, solubility in water is 2.17%, vapor density 6.7 (Clementz and Dailey, 1988).

Methylxanthine or caffeine contents in many food and beverages have been determined, including coffee, tea, carbonated beverages, some chocolate products, caffeinated water and chewing gum (Hoch, 1998). Most of the research has focused on the caffeine content in coffee, tea, and cola beverages.

However, less data exist on the methylxanthine contents in chocolate foods and beverages. Caffeine and theobromine contents have been reported for some chocolate products including commercial hot chocolate, bakery products, chocolate milk, and cocoa powder. However, methylxanthine concentration data in cocoa are

presented as an average of several unspecified brands or varieties and do not provide detailed information on specific products (Austin et al., 2001).

Austin et al., 2001 investigated that the methyxanthine contents in the chocolate products (cereal and toaster pastry) were lower than other chocolate dessert products such as pudding and cookies. However, the amount of caffeine in all the product tested was quite low as compared with coffee, tea, and cola products.

Caffeine is among the drugs that have been deliberately used for stimulants effects by athletes. It is listed as a banned drug by the International Olympic Committee because of its effects upon performance. Caffeine can be used wisely or it can be used foolishly like any other drug. People who take excessive amount of caffeine through their consumption of tea and coffee can experience various unpleasant side effects as a result. Usually they fail to recognize these as toxic effects. Caffeine can cause headaches, insomnia, dizziness, trembling, diarrhea, breathlessness and anxiety symptoms. It also has various toxic effects on the heart. These can include palpitations, rapid pulse or irregular pulse rate, and when consumed in large amounts, it can lead to increased blood pressure (Gossop, 2007).

Since it acts as stimulants of the central nervous, muscle and circular system, this compound has become a valuable agent for human body. Although caffeine has been extensively consumed in pharmaceuticals, soft beverages, etc, it is creates health risk in children, pregnant women and some patient. Therefore, obtaining of caffeine and decaffeination of food become highly important. It can be synthesized from chloroacetic acid or uric acid (Metin and Hacer, 2004., 2009).

Table 2.3: Caffeine Content of Food Products as Reported by Various Sources.

Product	Volume or weight*	Caffeine content (mg) Range	Average	Reference
Roasted and ground coffee	150 ml	64–124	83	Burg (1975)
(percolated)†	150 ml	—	74	Gilbert *et al.* (1976)
	‡	37–128	82	Stavric *et al.* (1988)
Instant coffee	150 ml	40–108	59	Burg (1975)
	150 ml	—	66	Gilbert *et al.* (1976)
	150 ml	—	66	US FDA (1980)
	‡	21–117	71	Stavric *et al.* (1988)
Roasted and ground coffee	150 ml	2–5	3	Burg (1975)
(decaffeinated)	150 ml	—	2	Gilbert *et al.* (1976)
Instant coffee	150 ml	2–8	3	Burg (1975)
(decaffeinated)	‡	2–6	4	Stavric *et al.* (1988)
Roasted and ground coffee	150 ml	—	112	Gilbert *et al.* (1976)
(drip)	‡	37–148	84	Stavric *et al.* (1988)
All coffee (except decaffeinated)	150 ml	29–176	—	Gilbert *et al.* (1976)
Tea	150 ml	8–91	27	Gilbert *et al.* (1976)
	150 ml	—	24	Stavric *et al.* (1988)
Bagged tea	150 ml	—		Burg (1975)
	150 ml	28–44	—	US FDA (1980)
	150 ml	—	30	Wheeler (Thomas J. Lipton Inc., 1989, personal communication)
*Leaf tea	150 ml	30–48	41	Burg (1975)
	150 ml	—	30	Wheeler (Thomas J. Lipton Inc., 1989, personal communication)
Instant tea	150 ml	24–31	28	Burg (1975)
	150 ml	—	20	Wheeler (Thomas J. Lipton Inc., 1989, personal communication)
Cocoa—African	150 ml	—	6	Burg (1975)
—South American	150 ml	—	42	Burg (1975)
Cocoa	150 ml	—	5	US FDA (1980)
	150 ml	2–7	4	Zoumas *et al.* (1980)
Chocolate bar	28 g	—	20	Gilbert *et al.* (1976)
Milk chocolate	28 g	—	6	US FDA (1980)
	28 g	1–15	6	Zoumas *et al.* (1980)
Sweet chocolate	28 g	5–35	20	Zoumas *et al.* (1980)
Chocolate milk	240 ml	2–7	5	Zoumas *et al.* (1980)
Baking chocolate	28 g	—	35	US FDA (1980)
	28 g	18–118	60	Zoumas *et al.* (1980)
Chocolate candy	28 g	1.5–6	—	Tarka (Hershey Foods, 1989, personal communication)
Soft drinks				
Regular colas	180 ml	15–24	—	NSDA (1993, unpublished data)
Caffeine-free colas	180 ml	0	—	NSDA (1993, unpublished data)
Diet colas	180 ml	13–29	—	NSDA (1993, unpublished data)
Caffeine-free diet colas	180 ml	0	—	NSDA (1993, unpublished data)
Speciality products	180 ml	0–35	—	NSDA (1993, unpublished data)
Others	180 ml	0	—	NSDA (1993, unpublished data)

*All volumes and weights reported by original sources have been converted to metric units for consistency.
†The US FDA cites a range of 75 to 155 mg caffeine per cup (150 ml or 5 fl. oz) of coffee, noting that percolated coffee is in the lower and drip coffee in the upper part of this range.
‡The volumes of coffee per serving prepared at home in this study ranged from 25 ml to 330 ml with a mean of 224 ml (about 7.5 fl. oz). Volumes of commercially prepared coffee ranged from 30 ml to 205 ml with a mean of 171 ml.

Sources: Barone and Roberts, 1996

2.3 Decaffeination

The extraction of caffeine from coffee beans is one of the first Chemical Engineering process. The patent of this process exist since 1905 (Bichel, 1979). The extraction of caffeine from coffee is a solid-liquid process, where the coffee is a transferred from the coffee beans solid matrix to the bulk solvent. The beans are not spherical and the caffeine concentration is time depending during process in both phases (Espinoza-Perez et. al., 2007).

Several investigations were report for extraction by supercritical carbon dioxide from guarana (Mehr et al., 1996; Saldana et al., 2002), coffee beans (Peker et al., 1992), green tea (Chang et al., 2000, Park et al., 2007).

Also, Saldana et al., 2002 investigate that methylxanthines were extracted from guarana seeds, mate leaves and cocoa seeds using supercritical carbon dioxide and ethanol. Furthermore the extraction of tea polyphenols and tea caffeine from green tea leaves were investigates by the microwave-assisted extraction method.

The present experimental study was carried out on the stalk and fiber wastes of Turkish black tea factories by supercritical carbon dioxide extraction. The nature of the raw materials makes leaching of caffeine from tea stalk and fiber wastes economic and commercially feasible. They have no economic value other than being used as very low grade fuel. The purpose of this study was to investigate the influence of operational conditions on extraction yield. The yield was compared with the yield of chloroform extraction in the specified conditions which had been reported in a previous study (Metin and Hacer, 2004).

During the testing in Shufen et. al., 1990, it was found that only 1 ml of 1 M sodium hydroxide solution was needed to regulate the pH between 12.5 - 12.7 for pure sample solution. However, when it was done according to the procedure, over five times of 1 M sodium hydroxide solution used to give the same pH. The reason

may be that the presence of sodium hydrogen carbonate and sodium hydroxide creates a buffereing effect, making control of the pH easier.

The extraction of methylxanthines has been performed using liquid extraction solvents such as dimethyl chloride, chloroform and water (Hulbert et al., 1998, Caudle et. al., 2001). However, chemical solvent need several time for extraction complete. Although water an excellent solvent, is highly non selective and its use may result in the removal of other valuable component from the extracted product, which gradually leads to deterioration of the analytical column (Saldana et al., 2002).

Kim et. al., 2008 said that acetone, methanol, ethanol and acetonitrile as extraction solvents were used to obtain caffeine-free green tea. Also, mixture of methanol and water was used to extract catechin from green tea. The use of the organic solvents in not appropriate because residual organic solvent have potential adverse effect on human health even though effective decaffeination can be achieved using the organic solvents. Therefore, non-toxic and effective decaffeination alternatives such as supercritical fluid extraction using carbon dioxide as a solvent have been explored in recent years. The advantage on using supercritical carbon dioxide extraction ($SCCO_2$) have been well documented by Mansoori et al., 1988, Park et al., 1987, and Martinelli et al., 1991.

2.4 Analysis of Caffeine

HPLC has recently used to identify and quantify simultaneously methylxanthine and polyphenols levels in cocoa and chocolate products (Blauch and Tarka, 1983; Kim et al., 1983; Kreiser and Martin, 1980). Most of the research carried out involved the analysis of commercial cocoa, chocolate liquors, different type of chocolate and cocoa beverages (Timbi et al., 1978).

Pura, 2001 proposed the use of Seppak cartridge for the purification of cocoa extract before injection onto a HPLC reverse-phase column. In this way, the

interfering cocoa pigments are effectively removed, therefore increasing the column life.

Mumin et al., 2006 have developed a HPLC method for the determination of caffeine which was using HPLC instead of using UV-Visible Spectrophotometer. HPLC method is choosing to determine the caffeine because HPLC is the most widely used qualitative and quantitative determination and separation method.

The method is popular because it is non-destructive and can be applied to thermally labile compounds (unlike GC). It is also a very sensitive technique since it incorporates a wide choice of detection methods.

With the use of post-column derivation methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific fields.

Espinoza-Perez et. al., 2007 investigate that the evolution of caffeine concentration was measured both in extract and refined phase by using gas chromatography.

CHAPTER 3

METHOD AND METHODOLOGY

This method of extraction of caffeine was using batch system. This method of this study is based on the study of which the title is Determination and Characterization of Caffeine in Tea, Coffee and Soft Drinks by Solid Phase Extraction and High Performance Liquid Chromatography (SPE - HPLC) (Mumin et al., 2006) and UV Spectrophotometric Determination of Theobromine and Caffeine in Cocoa Beans (Li et al, 1990).

3.1 Material

The green *MCBC1* cocoa seeds were bought from Malaysian Cocoa Board research station in Jengka, Pahang.

3.2 Chemicals

There are list of the chemicals was used in order to done this research:

- Ethanol
- Solid sodium hydrogen carbonate
- 1M sodium hydroxide
- 10% lead ethanoate solution

- Solid anhydrous sodium sulfate
- Distilled water

3.3 Apparatus

There are list of the apparatus was used in order to done this research:

- Beaker (50ml, 100ml, 500ml)
- Separatory funnel (500ml).
- Thermometer
- Measuring cylinder (50ml, 250ml)
- Retort stand
- Stopwatch
- Filter paper
- Syringe
- Conical flask 500ml
- Tray
- Spatula
- Weighing boat
- Volumetric flask (10ml, 100ml)

3.4 Equipments

There are list of the equipments was used in order to done this research:

- Hot plate
- Oven
- Siever
- pH meter
- Water bath

- Sonicator
- Blender
- Rotary Evaporator
- High Performance Liquid Chromatography (HPLC)

3.5 Flow Chart of Methodology

Flow chart of methodology is used as a guideline for the experiment. As illustrated in Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4 and Figure 3.5, the process begins with the drying stage and end with analysis by using HPLC.

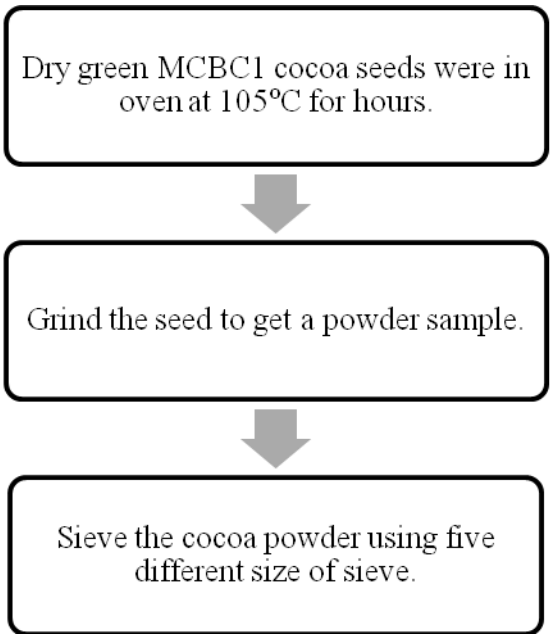

Figure 3.1: Flow of Method for Sample Preparation.

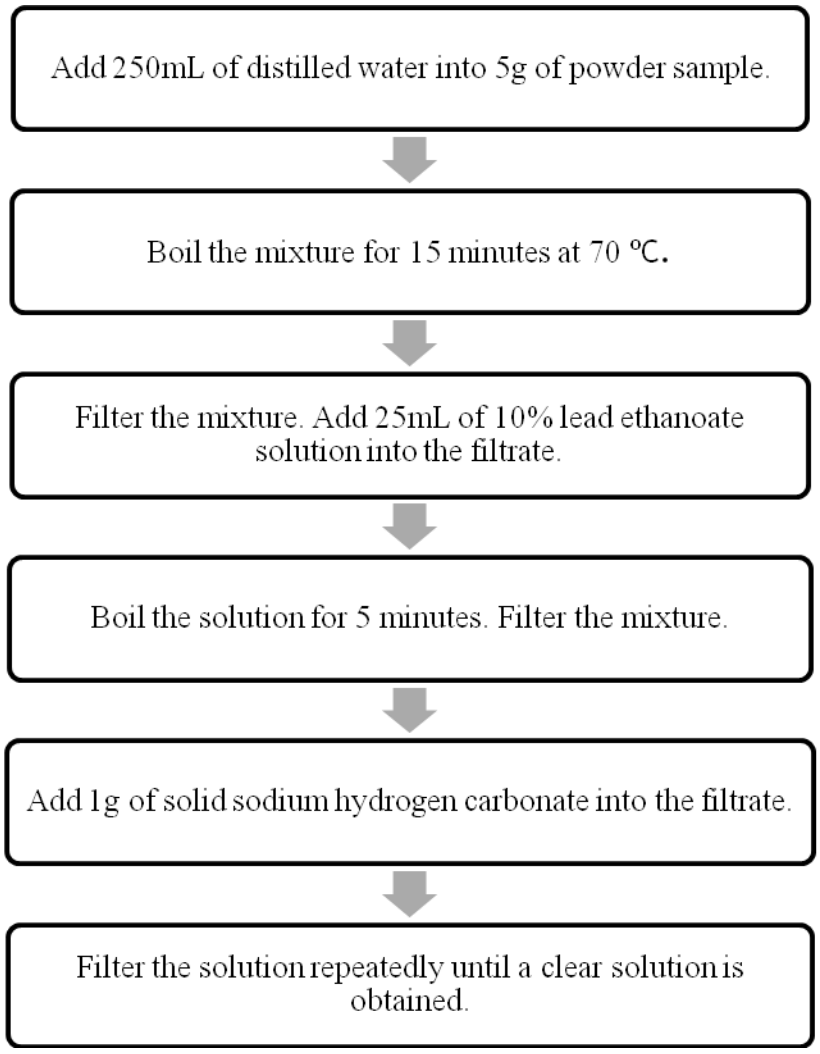

Figure 3.2: Flow of Method for Leaching of Caffeine.

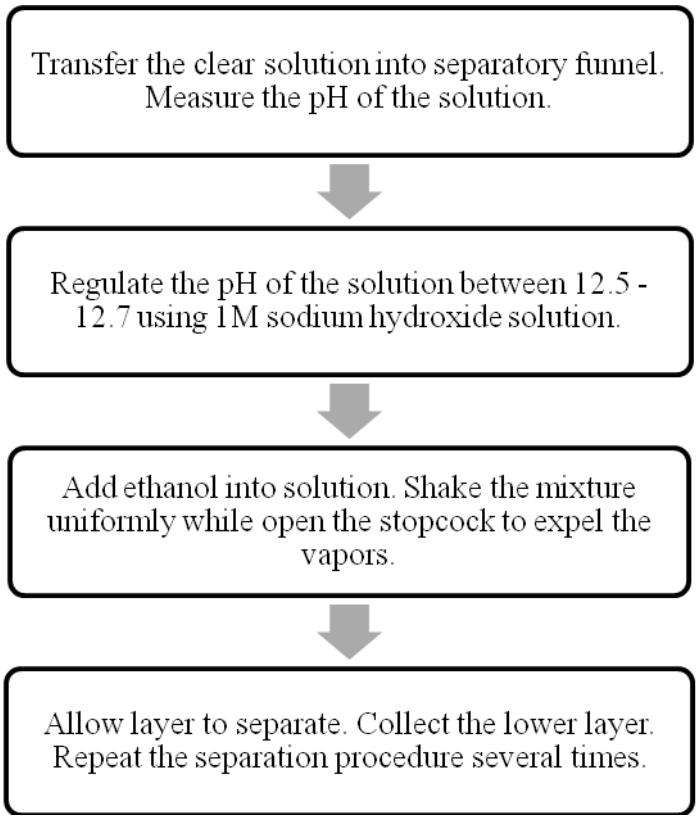

Figure 3.3: Flow of Method for Liquid-liquid Extraction of Caffeine.

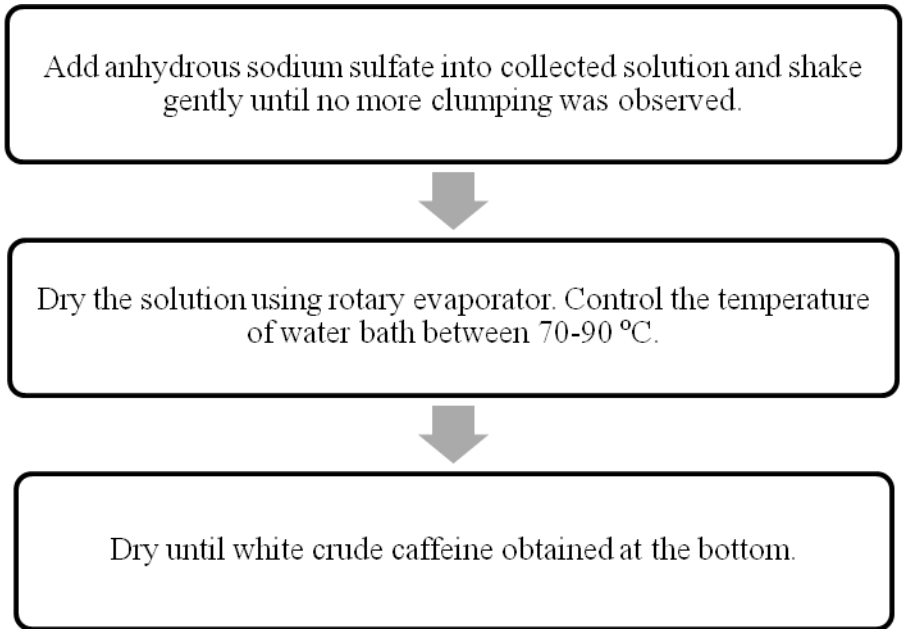

Figure 3.4: Flow of Method for Drying of Caffeine.

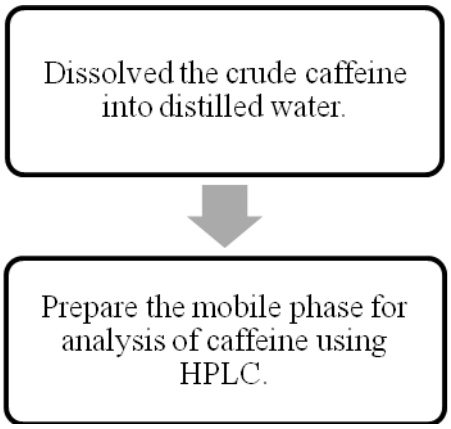

Figure 3.5: Flow of Method for Analysis of Caffeine.

3.6 Preparation of Sample

The green *MCBC1* cocoa seeds were bought from Malaysian Cocoa Board research station in Jengka, Pahang. 5g of the green seeds were weighed and dried in oven at 105^{o}C for hours, to remove the moisture in the seeds. Then, the seeds were grinded to get a powdered sample.

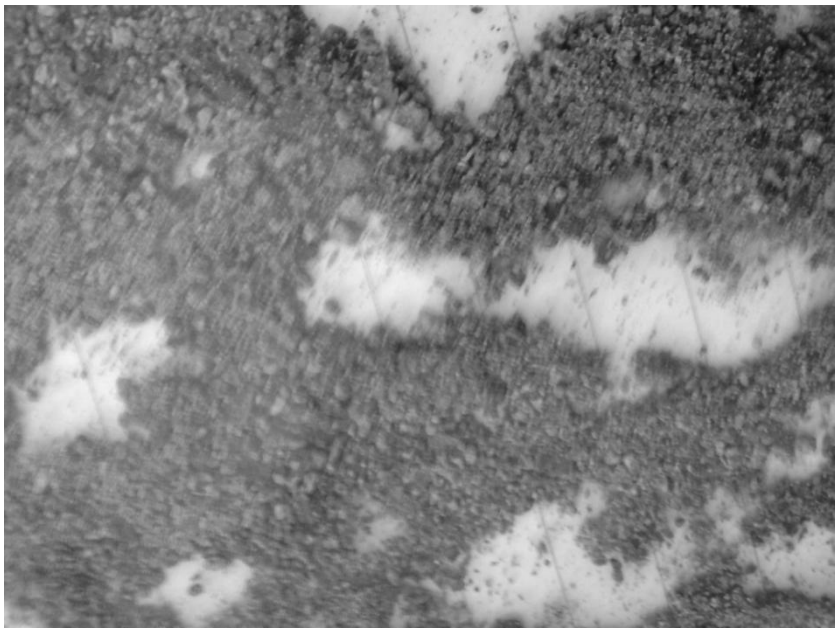

Figure 3.6: Cocoa after Drying and Grind.

3.7 Leaching of Caffeine

The 5g of the prepared powdered sample of *MCBC I* cocoa seeds were putted in a 500ml beaker and subsequently 250ml of distilled water was added into the beaker. The mixture was boiled for 15 minutes at 70^{o}C. Then, the mixture was filtered using Buchner Funnel. The filtrate was collected and 25ml of 10% lead ethanoate solution was added to the filtrate.

The purpose of adding 10% lead ethanoate solution is to convert tannins and other acids into anions (base) that will not be soluble in water and ethanol. This also helps to avoid an emulsion.

The solution was boiled for 5 minutes. The lead ethanoate will form a precipitate, and this precipitate was removed by filtering it in a funnel. Next, 1g of solid sodium hydrogen carbonate was added to the filtrate in order to clear the filtrate by removing the Pb^{2+} ions in solution in a form of white precipitate of $PbCO_3$. Then, the solution was filtered again repeatedly, until a clear solution was obtained.

3.8 Liquid-liquid Extraction

The clear solution obtained was transferred into a 500ml separatory funnel. The pH of the solution was measured using pH meter. If the pH of the solution is not between 12.5 and 12.7, 1M sodium hydroxide solution was added until the pH of the solution regulate between 12.5 and 12.7.

The purpose of the addition of sodium hydroxide is to maintain the anions in the solution, so that tannins and other acids do not soluble in water and ethanol.

Then, the caffeine in the solution was extracted with ethanol. The mixture was shaking uniformly while the stopcock was opened to expel the vapors. The layers were allowed to separate and the lower layer (ethanol with caffeine) was

collected into a 100ml beaker, and the separation procedure was repeated several times to collect into the beaker.

3.9 Drying of Caffeine

Anhydrous sodium sulfate was then added into the collected solution containing the extracts. The anhydrous sodium sulfate would act to remove any water and water-soluble salts that were retained in the ethanol or accidentally transferred during decantation of solution. The ethanol was appeared a bit cloudy, because the anhydrous sodium sulfate was clumped when water present. The anhydrous sodium sulfate was added and shaken gently until no more clumping was observed.

Then, the caffeine in the solution was dried using rotary evaporator, and the temperature of the water bath was controlled low enough between 70-90^{o}C to avoid caffeine decomposition. After few hours, white crude caffeine was obtained at the bottom.

Figure 3.7: Rotary Evaporator

3.10 Analysis of Caffeine

The crude caffeine obtained was analyzed using High Performances Liquid Chromatography (HPLC). These tests proved the presence of caffeine.

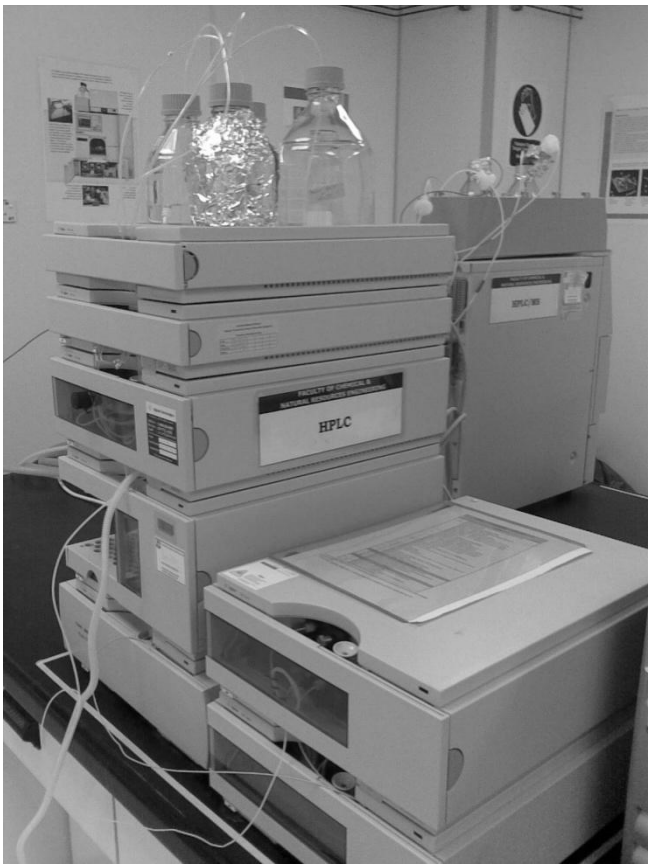

Figure 3.8: HPLC used to analyze the Caffeine.

3.11 Preparation of Chemical and Standard Caffeine, mobile Phase and Vial for HPLC

This method is based on book in title Official Methods of Analysis of the Association of Official Analytical Chemists 15th Edition.

3.11.1 Reagents

The reagents used for analysis are:

1. Glacial acetic acid.
2. Caffeine standard.
3. Methanol (HPLC grade).
4. Sample (crude caffeine).

3.11.2 Apparatus

1. Analytical column	:	ZORBAX Eclipse XDB C18
2. Detector	:	Agilent Photodiode Array Detector (DAD)
3. Wavelength	:	280 nm
4. Flowrate	:	1 ml/min
5. Injection volume	:	20 µl
6. Membrane Filter	:	5 Standard, 15 Sample
7. Syringe	:	10 Unit (0.45µm)
8. Vial (agilent)	:	20 Unit
9. Sonicator	:	For gas degassing
10. Funnel		
11. Volumetric Flask	:	100ml, 1 Unit
12. Volumetric Flask	:	10ml, 5 Unit
13. Micropipette	:	10 - 100µl
14. Filtration Kits		

3.11.3 Method

3.11.3.1 Preparation of Caffeine Standard Solution

3.11.3.1.1 Preparation of Stock Solution from Standard Caffeine

100mg of caffeine standard was weighed in weighing board. The caffeine was dissolved with distilled water and transferred it to a 100ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

3.11.3.1.2 Preparation of Standard Solution for Analysis

Caffeine standard solution was prepared with five different concentrations.

1. Standard 2.5µg/ml: 0.0025ml or 25µl of stock solution was dissolved with distilled water in 10 ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

2. Standard 5.0µg/ml: 0.005ml or 50µl of stock solution was dissolved with distilled water in 10 ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

3. Standard 10.0µg/ml: 0.010ml or 100µl of stock solution was dissolved with distilled water in 10 ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

4. Standard 15.0µg/ml: 0.015ml or 150µl of stock solution was dissolved with distilled water in 10 ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

5. Standard 20.0µg/ml: 0.020ml or 200µl of stock solution was dissolved with distilled water in 10 ml volumetric flask. The solution in volumetric flask was added with distilled water until the graduated level. The solution was shaken until dilute.

3.11.3.2 Preparation of Mobile Phase (Mix water and glacial acetic acid 800ml)

The water and glacial acetic acid was mixed at the ratio of 79:1. 790ml distilled water was used to dissolve 10ml glacial acetic acid. Filtration kits and vacuum pump system was assembled before using it to filter mobile phase mixture. Then, the flask containing filtrate was transferred into ultrasonicator. Sonication process was performed for 10 minutes. After 10 minutes, the solvent was carefully poured into solvent bottle that is connected to HPLC.

3.11.3.3 Preparation of Mobile Phase (Methanol HPLC Grade)

700ml of pure Methanol HPLC grade was poured into a beaker. Filtration kits and vacuum pump system was assembled before using it to filter mobile phase. Then, the flask containing filtrate was transferred into ultrasonicator. Sonication process was performed for 10 minutes. After 10 minutes, the solvent was carefully poured into solvent bottle that is connected to HPLC.

3.11.3.4 Preparation of Sample

Crude caffeine was dissolved with distilled water before sonicate for 10 minutes until no bubble is observed. After that, the sample was continued sonicate for another 10 minutes. 5ml of sample was injected into vial through a syringe filter (0.45μm) for further analysis in HPLC.

CHAPTER 4

RESULT AND DISCUSSION

In this chapter, all the data that get from the experiment were compute into the tables, graph and figures of the data analysis.

4.1 Result Generated from HPLC

All the result for standard solution and samples generated from HPLC are constructing in table form in Appendix A.

4.2 Determination of Caffeine Concentration and Extraction Yield in Sample

Graph for standard curve of caffeine was plotted using data from Appendix A to determine the concentration of caffeine for each sample.

The caffeine concentrations in the sample determine from the standard curve in Appendix B or the linear equation was calculated using Eq. (4.1).

$$\text{Caffeine concentration in the sample } (\mu g/ml) = \frac{DF \times C}{M} \qquad (4.1)$$

Where *DF* is dilution factor of sample, *M* is volume of sample, *C* is concentration of sample solution (µg/ml).

The extraction yield of caffeine for each sample is calculated using Eq (4.2).

$$\text{Extraction yield of caffeine (\%)} = \frac{Mass\ final}{Mass\ initial} \times 100\% \quad (4.2)$$

The concentration and extraction yield of caffeine for each sample is shown in Appendix C. The graph on effect of each parameter was drawn in Figure 4.1, Figure 4.2 and Figure 4.3 to show the relationship on extraction time, particle size of cocoa powder and cocoa seed-solvent ratio toward extraction yield of caffeine.

4.3 Effect Parameters towards Extraction Yield

The graph on effect of each parameter was drawn in Figure 4.1, Figure 4.2 and Figure 4.3 to show the relationship on extraction time, particle size of cocoa powder and cocoa seed-solvent ratio toward extraction yield of caffeine.

4.3.1 Effect of Extraction Time

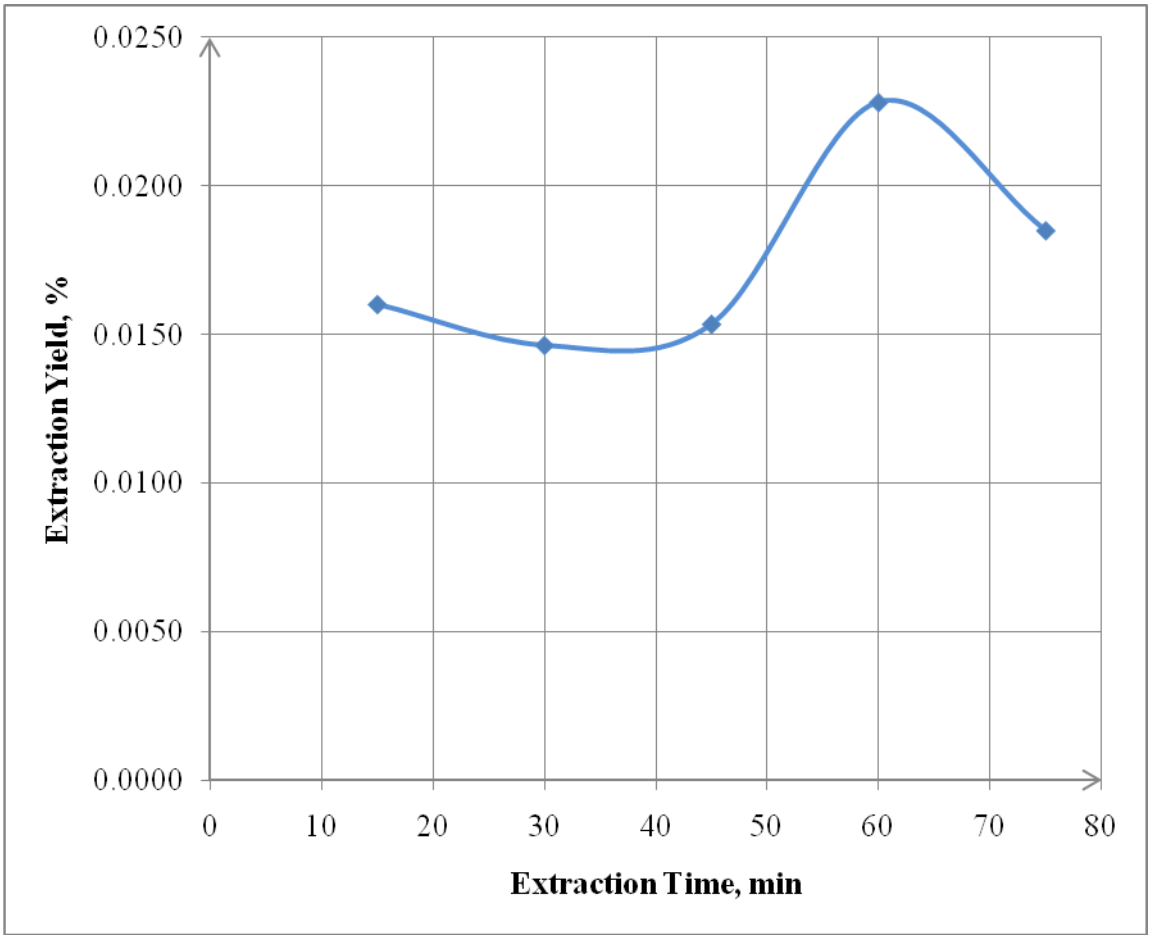

Figure 4.1: Graph of Extraction Yield against Extraction Time.

The relationship between the yield of caffeine and extraction time is shown in Figure 4.1. The yields increased with increasing time. The longer the extraction time, the longer time cocoa spend in the solvent. Thus the rate of diffusion of caffeine into solvent increased.

The percent of extraction was slightly increased with increasing of extraction time (Hameed, et al., 2003). Metin and Hacer, 2004 discovered that the yield increased rapidly with increasing in time. At longer durations at the boiling temperature, a noticeable decomposition of caffeine was observed.

However, the increasing in yield after 60 minutes becomes less. This is because the solvent becomes saturated with caffeine decrease further diffusion of caffeine into solvent. Therefore, extraction time of 60 minutes was a reasonable choice.

4.3.2 Effect of Particle Size of Cocoa Powder

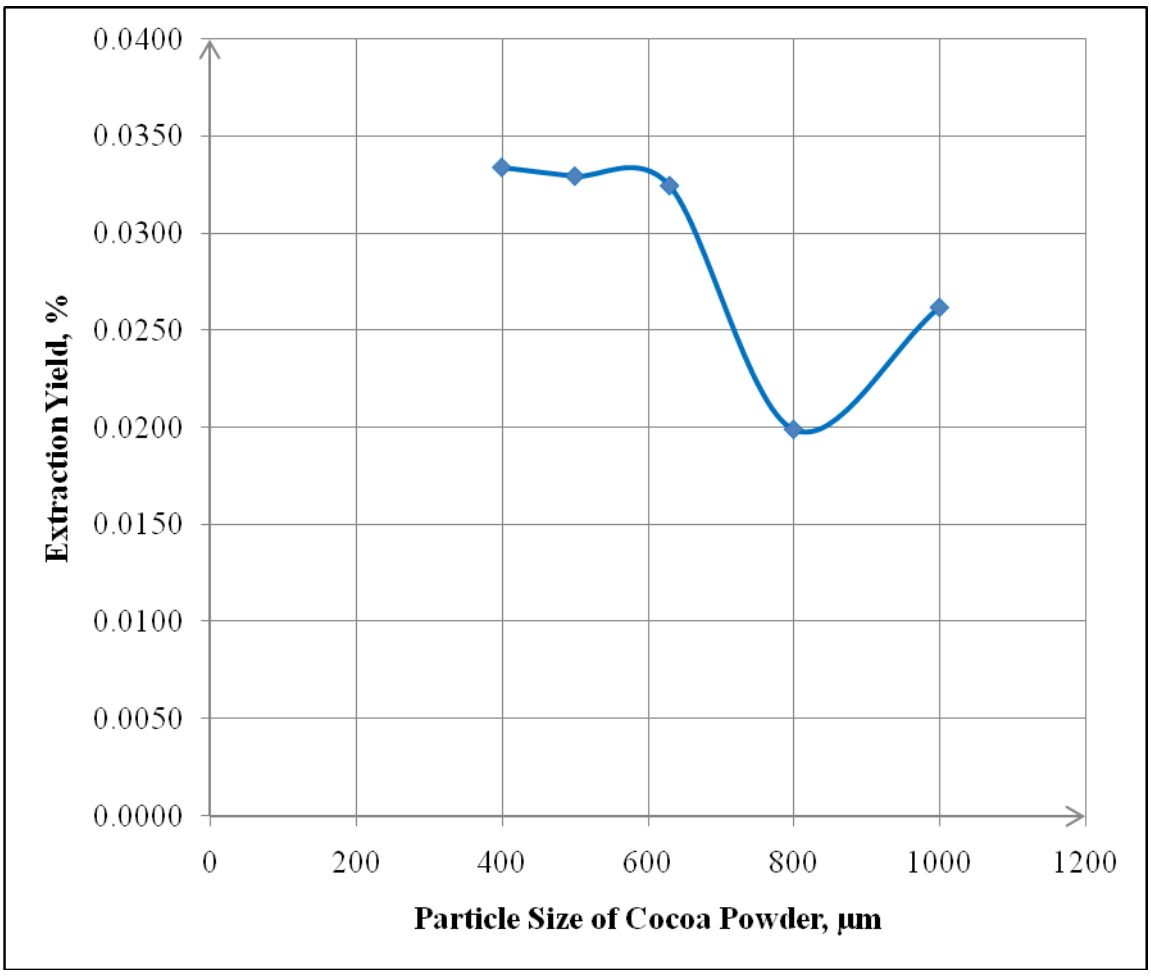

Figure 4.2: Graph of Extraction Yield against Particle Size of Cocoa Powder.

It is known that particle size of cocoa powder affected extraction yield of caffeine. In order to see how those particle sizes affect the yield, experiments were ran using five difference particle size of cocoa powder which are 400, 500, 630, 800 and 1000µm.

As shown in Figure 4.2, extraction yield of caffeine decreased with increasing particle size of cocoa powder as a result of the hindrance of transportation of soluble agents from cocoa to solvent due to the decrease in specific surface area (Metin and Hacer, 2009). The cocoa bean samples should be ground as finely as possible. Li et al., 1990 investigated that 18% error will arise if the sample size is 0.5-2.0 mm compared with less than 0.5 mm. At small particle size, the surface area of the particles becomes large. The diffusion of caffeine from inside of cocoa to surface and to solvent becomes easier. Thus, extraction yield of caffeine increases as particle size decreases.

4.3.3 Effect of Cocoa Seed-Solvent Ratio

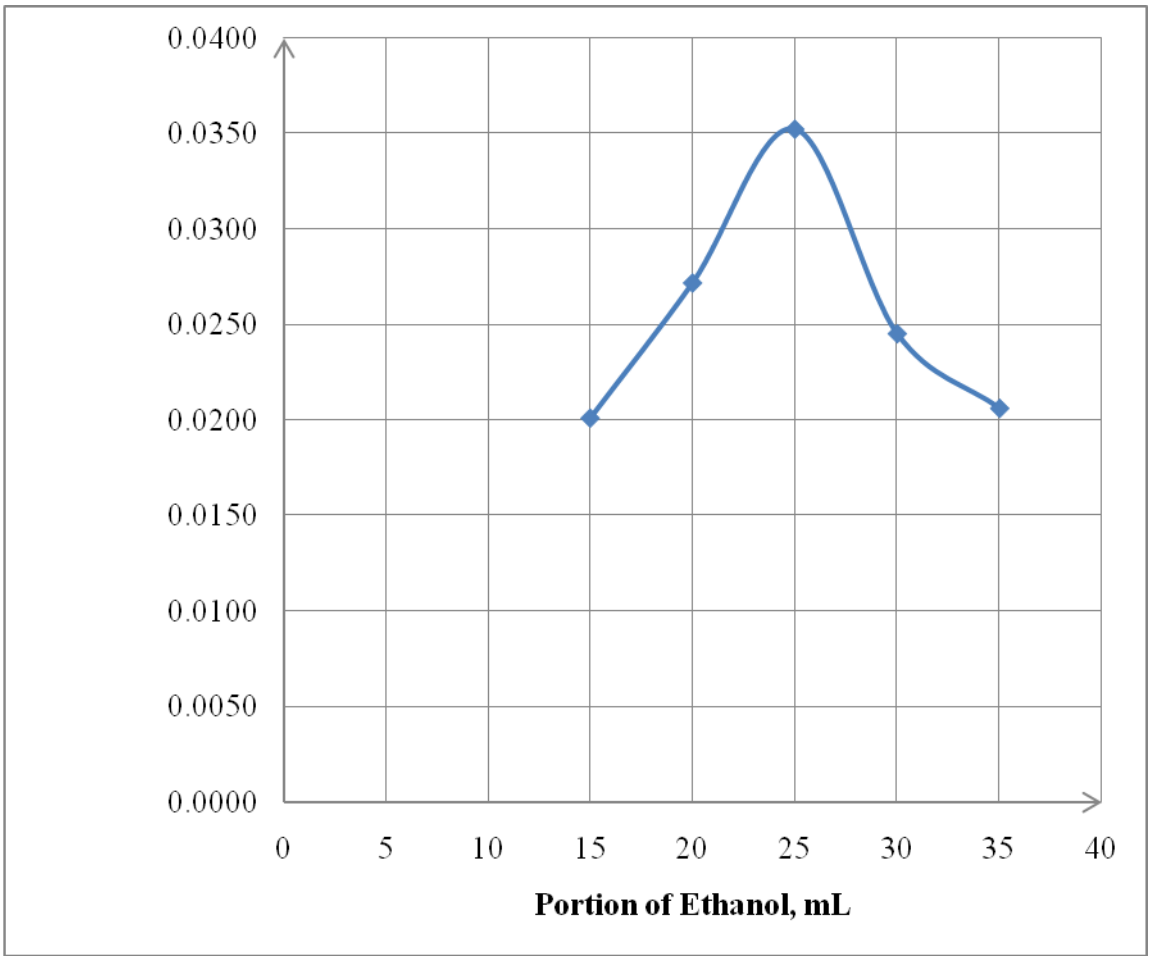

Figure 4.3: Graph of Extraction Yield against Cocoa Seeds-Solvent Ratio.

To investigate the effect of cocoa seed-solvent ratio, the extraction time of 15 minutes, and the particle size of 400µm were kept constant. As can be seen from Figure 4.3, the extraction yield of caffeine increased with increasing of cocoa seed-solvent ratio.

The percent of extraction was increased with the increasing of solvent/feed ratio. Obviously, the percent of extraction increases gradually when the solvent/feed ratio increase until 0.5 and reaches a constant value at ratio greater than 0.5 (Hameed et al., 2003).

However, the caffeine yield increased up until reaches 25ml portion of ethanol (1:5) and then decrease for further increase in portion of ethanol. It is evident from Figure 4.3 that the best ratio of cocoa seed-solvent to get the highest yield of caffeine was 1:5. Besides that, either using lower ratio or higher ratio than 1:5, the yield of caffeine cannot achieve as higher as 1:5 cocoa-solvent ratio.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The extraction yields of caffeine were affected by different extraction time, particle size of cocoa seeds powder and ratio of cocoa seeds-solvent. Caffeine can be extracted from cocoa MCBC 1 seeds by using batch system.

The extraction yield of caffeine increased towards the increasing of extraction time. Meanwhile for the particle size, extraction yield of caffeine increased when the particle size of cocoa decreased. However, the best ratio of cocoa seeds-solvent to get the highest extraction yield was 1:5. Besides that, either using lower ratio or higher ratio than 1:5, the extraction yield cannot achieve as higher as 1:5 cocoa-solvent ratio.

The result showed 60 minutes of extraction time, 400 of the particle size of cocoa seeds powder and 1:5 ratio of cocoa seed-solvent gives highest extraction yield of caffeine.

5.2 Recommendation

In Malaysia, there are several types of cocoa. It is recommended to do research on other types of cocoa seeds to get the cocoa seeds that give higher extraction yield of caffeine. Furthermore, this research is recommended to use other parameters such as different type of solvent use to extract the caffeine, temperature for leaching process and others to obtain the best condition to maximize extraction yield of caffeine. Using different method for the same purpose such as soxhlet extraction and heat reflux extraction other than batch system can be recommended to improve the extraction yield of caffeine. Analysis of caffeine also can be tried using other equipment other than using HPLC. For example, using UV-Visible Spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS)

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APPENDIX A

Result Generated from HPLC

A-1 Standard Solution

Concentration of Standard (µg/ml)	Peak Area (mV.s)
2.5	800.83673
5.0	1179.96204
10.0	2010.83008
15.0	2792.40552
20.0	3615.73071

A-2 Extraction Time

Extraction Time (min)	Peak Area (mV.s)
15	26171.5
30	23952.9
45	25080.4
60	37129.9
75	30176.7

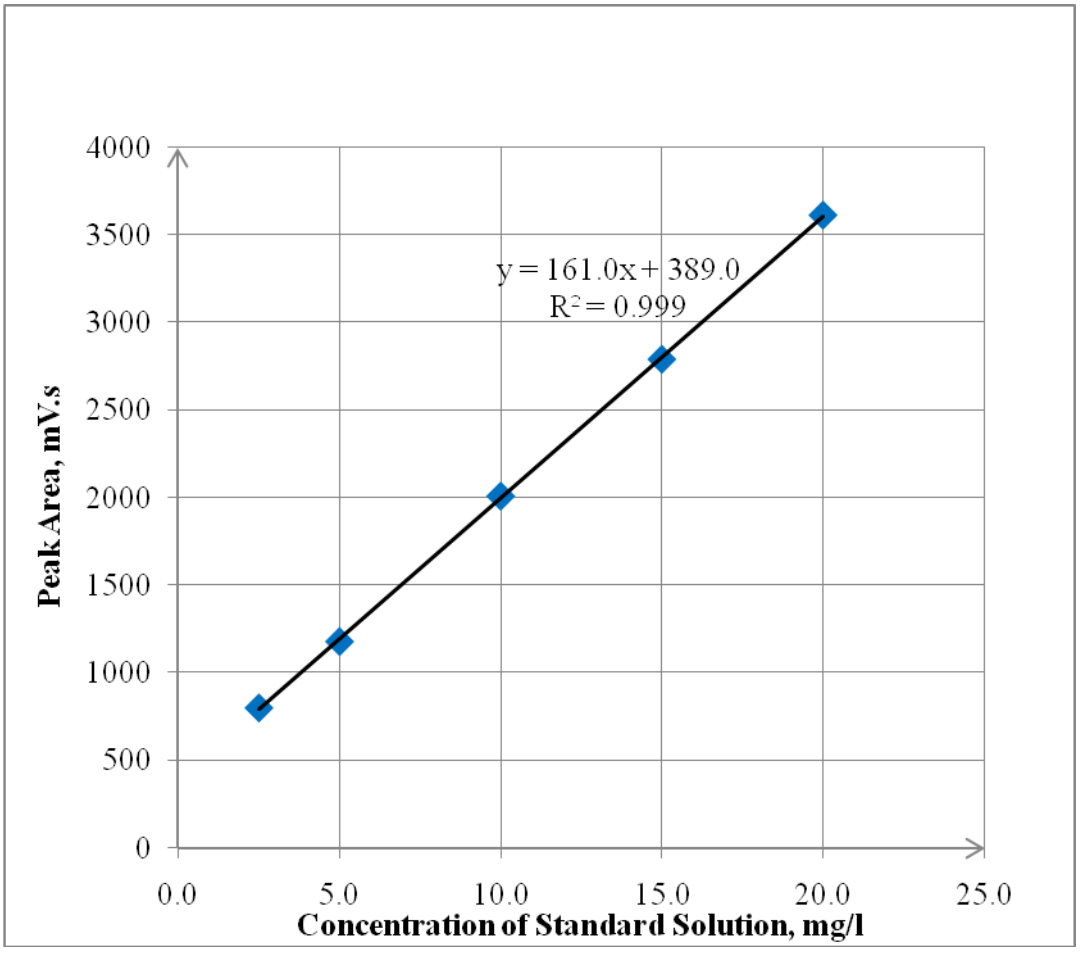
A-3 Particle Size of Cocoa Powder

Particle size of Cocoa Powder (µm)	Peak Area (mV.s)
400	27291.8
500	26929.3
630	26534.7
800	16413.9
1000	21480.3

A-4 Cocoa Seed-Solvent Ratio

Cocoa Seed-Solvent Ratio	Peak Area (mV.s)
1:3	11184.4
1:4	14983.5
1:5	19306.2
1:6	13557.5
1:7	11458.6

APPENDIX B
Standard Curve of Caffeine



APPENDIX C

The concentration and extraction yield of caffeine for each sample

C-1 Effect of Extraction Time to Extraction Yield of Caffeine

Extraction Time (min)	Concentration of caffeine (µg/ml)	Extraction Yield of Caffeine (%)
15	160.14	0.0160
30	146.36	0.0146
45	153.50	0.0154
60	228.20	0.0228
75	185.02	0.0185

C-2 Effect of Particle Size of Cocoa Powder to Extraction Yield of Caffeine

Particle size of Cocoa Powder (µm)	Concentration of caffeine (µg/ml)	Extraction Yield of Caffeine (%)
400	167.098	0.0334
500	164.847	0.0330
630	162.396	0.0325
800	99.534	0.0199
1000	131.002	0.0262

C-3 Effect of Cocoa Seed-Solvent Ratio to Extraction Yield of Caffeine

Cocoa Seed-Solvent Ratio	Concentration of caffeine (µg/ml)	Extraction Yield of Caffeine (%)
1:3	67.052	0.0201
1:4	90.650	0.0272
1:5	117.498	0.0352
1:6	81.792	0.0245
1:7	68.755	0.0206