BATCH EXTRACTION OF 1,3,7-TRYMETHYLXANTHINE FROM *KKM22* COCOA SEEDS

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BATCH EXTRACTION OF 1,3,7-TRYMETHYLXANTHINE FROM *KKM22* COCOA SEEDS

NORHAMIZAH BINTI JOHARI

A report submitted in partial fulfillment of the

requirements for the award of the degree of

Bachelor of Chemical Engineering

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Dedicated to My Beloved Family

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ABSTRACT

1,3,7- trymethylxanthine (caffeine) is a naturally occurring substance found in cocoa seeds. The purpose of this research is to extract caffeine from *Klon Koko Malaysia (KKM)22*, to investigate the effect of particle size, solvent/feed ratio and extraction time, and also to determine the kinetic model for extraction of 1,3,7-trymethylxantine. The sample was prepared by grinding and sieving, followed by solid-liquid extraction using water by heat reflux extracting technique, liquid-liquid extraction with ethyl acetate, drying of caffeine by rotary evaporator, and finally analysis of the caffeine yield. The analysis of the caffeine yield was done using UV/Vis Spectrophotometric method. The caffeine yield was highest at sample particle size of 315 μm (0.22 % w/w caffeine or 2.1724 mg/g cocoa), solvent/feed ratio of 1:1 (0.22 % w/w caffeine or 2,2102 mg/g cocoa), and extraction time of 90 minutes (0.23 % w/w caffeine or 2.288 mg/g cocoa). The best conditions for the highest yield of caffeine from *KKM22* were 315 μm of sample particle size, 1:1 of solvent/feed ratio, and 90 minutes of extraction time.

ABSTRAK

1,3,7-trymethylxanthine (kafein) adalah sebuah zat yang dijumpai secara semulajadi dalam biji koko. Objektif kajian ini adalah untuk mengekstrak kafein dari *Klon Koko Malaysia (KKM) 22*, untuk mengkaji pengaruh saiz zarah koko, nisbah pelarut/sampel, dan masa ekstraksi terhadap hasil kafein dan mengkaji kinetik untuk mengekstrak kafein. Persiapan sampel dilakukan dengan mengisar dan menapis, diikuti oleh ekstraksi pepejal-cair menggunakan air panas dengan teknik ekstraksi refluks, diikuti ekstraksi cair-cair dengan pelarut etil asetat, pengeringan kafein dengan rotary evaporator, dan akhirnya analisis hasil kafein. Analisis hasil kafein dilakukan dengan menggunakan kaedah spektrofotometri UV/Vis. Hasil kafein yang tertinggi diperolehi pada saiz zarah sampel 315 μm (0.22 % w/w kafein atau 2.1724 mg/g koko), nisbah pelarut/sampel 1:1 (0.22 % w/w kafein atau 2.2102 mg/g koko), dan masa ekstraksi 90 minit (0.23 % w/w kafein atau 2.288 mg/g koko). Keadaan terbaik untuk mendapatkan hasil tertinggi kafein dari *KKM22* adalah pada saiz zarah sampel 315 μm, nisbah pelarut/sampel 1:1, dan masa ekstraksi 90 minit.

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LIST OF ABBREVATIONS

HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
KKM22	*Klon Koko Malaysia 22*
SPE	Solid Phase Extraction
UV	Ultraviolet
UV/Vis	Ultraviolet/Visible

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Malaysia was recognized as the largest cocoa producer and ranked 11th in the lost of cocoa cultivating countries, worldwide since a decade ago. Commonly, the alkaloid contents such as caffeine, theobromine, and theophylline in cocoa are extracted before the cocoa is processed, and the alkaloids, especially caffeine are discarded without use (Varma and Nurdin,2011). According to previous research, caffeine has its own benefits such as it is used for pharmaceutical and therapeutic purposes. Since Malaysia as a large producer of cocoa, it can be used to extract caffeine and process the caffeine for benefits without discarding it. Therefore, in order to get a high value of caffeine yield, an efficient and economic method of extraction of caffeine from cocoa is needed. Furthermore, an effective and low cost of solvent is also needed in order to get better extraction caffeine and will increase the profit from caffeine sales.

1,3,7-trimethylxanthine is an alkaloid of the methylxanthine family, thus it is known as Caffeine. Caffeine is an intensely bitter white powder in its pure state. Its IUPAC name is 1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione, with chemical formula $C_8H_{10}N_4O_2$ (Arnaud, M. J., 1987). The structure of caffeine is shown in Figure 1, below (Mumin et. al., 2006).

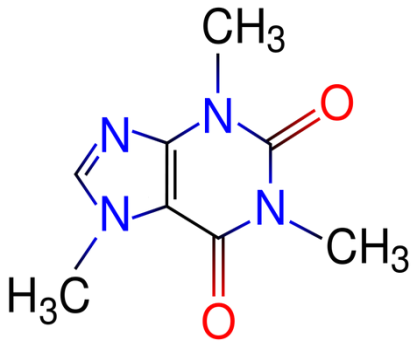


Figure 1.1: The Structure of 1,3,7-trymethylxanthine

Caffeine is one of the most widely consumed molecules in the world, and has been for centuries. It has remained popular because of its many positive effects on the body, which include stimulating the central nervous system, and making one more alert and giving one a boost of energy. Caffeine is most commonly consumed in the form of coffee, tea, or chocolate. (Anonym., 2010). Caffeine is the most widely consumed psychoactive substance and can be a mild central nervous system stimulant. It does not accumulate in the body over a period of time and is normally excreted within several hours of consumption (Barone, 1996).

Cocoa tree is an evergreen tree in the family *Sterculiaceae*, genus *Theobroma*, and species *cacao*, which flourish well in a narrow belt of 10^{o} of either sides of the Equator. Climatically, since cocoa tree grow well in humid tropical climates with regular rains and a short dry season, means that Malaysia is very suitable for cocoa growing. There are three broad types of cocoa; *Forastero, Crillo, and Trinitario* which is a hybrid of *Forastero* and *Crillo* (Anonym, 2010). Cocoa is an important flavouring ingredient in preparation of beverages, confectionary, ice-cream, bakery products, etc. The stimulant action of cocoa based products is due to the presence of alkaloids, theobromine, and caffeine in them. About 90% of the total composition of cocoa is Theobromine, while the remaining is caffeine (Franzke et. al, 1969).

One analysis of the chemical composition of cocoa beans after fermentation and drying is shown in Table 1.1, below:

Table 1.1: Chemical composition of cocoa beans

Contents	Nib % Maximum	Shell % Maximum
Water	3.2	6.6
Fat (cocoa butter, shell fat)	57	5.9
Ash	4.2	20.7
Total nitrogen	2.5	3.2
Theobromine	1.3	0.9
Caffeine	0.7	0.3
Starch	9	5.2
Crude fibre	3.2	19.2

Source: (Minifie, 1989)

This indication of the chemical composition of cocoa beans can vary depending on the type of bean, the quality of the fermentation and drying, and the subsequent processing of the bean (Minifie, 1989).

(Anonym., 2010) stated that caffeine can be extracted from cocoa by various methods, such as water extraction, supercritical carbon dioxide extraction, and organic solvent extraction. Solvents such as chloroform, methyl chloride, ethanol, and ethyl acetate are commonly used for the solvent extraction of caffeine.

Figure 1.2: Heat Reflux Extractor

KKM 22 cocoa is one of local breed of cocoa, which was cloned by *Malaysian Agricultural Research and Development Institute (MARDI).* The composition of caffeine in this cocoa seeds is yet to be analyzed. Therefore, this research may benefit *MARDI* in analyzing their breed.

1.2 PROBLEM STATEMENT

Since a decade ago, Malaysia was recognised as the largest cocoa producer, and Malaysia is ranked 11th in the list of cocoa cultivating countries, worldwide (Anonym, 2005). Commonly, the alkaloid contents (caffeine, theobromine, and theophylline) in cocoa are extracted before the cocoa is processed, and the alkaloids, especially caffeine are discarded without use. Actually, according to some research, caffeine has its own benefits, for example it is used for pharmaceutical purposes. The caffeine has increasing demand in the world, but we are just not making use of the source we has.

Malaysia as a large producer of cocoa, can extract the caffeine in the cocoa, and process the caffeine for benefits without discarding it. Therefore, an efficient and economic method of extraction of caffeine from cocoa is needed to get a high yield of caffeine. Moreover, an effective and low cost solvent is also needed for better extraction of caffeine. This will increase the profit from the sales of caffeine. Thus, this research will find the best solvent for the high extraction yield, and also will optimize the parameters that affect the extraction yield of caffeine.

1.3 RESEARCH OBJECTIVE

To extract 1,3,7-trymethylxanthine from KKM22 cocoa seeds using heat reflux extraction method by the variation of particle size, solvent/feed ratio, and extraction time.

1.4 SCOPE OF RESEARCH

The scope for this research includes;

1) Extraction of 1,3,7-trymethylxanthine from *KKM22* cocoa seeds by using batch extraction method,
2) Evaluating the selected parameters which are particle size, solvent/feed ratio and extraction time,
3) Analyzing the caffeine yield by using UV/Visible Spectrophotometer,
4) Indentifying the effect on caffeine yield by particle size, solvent/feed ratio and extraction time, and
5) Kinetic model approval of caffeine yield.

CHAPTER 2

LITERATURE REVIEW

2.1 COCOA

Cocoa was domesticated by the Mayas and Aztecs thousands of years ago. Cocoa has travelled along the trade routes used by the Mayas, Aztecs, and also the Pipil-Nicaraoseven before the Spanish conquest (Sophie and Michael, 1996). In the year 1525, Criollo types of cocoa have spreaded to Central America, and to a large number of Caribbean islands, including Trinidad, and Jamaica. Then, cocoa was introduced into Central America, particularly Costa Rica, by the Spanish people. Around the year 1750, the French people planted cocoa in Martinique and Haiti, and the Portuguese people planted cocoa in Belem and Bahia, using Lower Amazon (Forastero) type of cocoa (Varma and Nurdin, 2011).

Pittier (1933) stated that in the 18th Century, between Criollo and Forastero types of cocoa was hybridized and Trinitario types of cocoa was founded .According to Pound (1945), the two populations could have met and hybridised on the islands of the Orinoco delta, including Trinidad and the Orinoco valley. In the year 1727, the „Blast", which is a cyclone or an epidemic has destroyed the Criollo plantations in Trinidad. Then, the cocoa plantations were reconstituted using Trinitario seeds from the Orinoco valley. This Trinitario hybrid of cocoa was produced by open pollination. Their superiority in agronomic terms and better resistance to diseases and pests has favoured their use in Trinidad as a replacement for Criollo types of cocoa (Cheesman, 1944).

In the 16th century, cocoa was introduced into Asia and the Pacific. In 1560, the Dutch introduced the Venezuelan Criollo trees into Java, Indonesia. Meanwhile, in the year 1614, the Spanish introduced Criollo types of cocoa into the Philippines from Mexico.

Cocoa was taken by the British to Madras, India from the island of Amboina in the year 1798, and it was introduced into Sri Lanka from Trinidad at about the same time. From Sri Lanka, cocoa was transferred to Singapore and Fiji in year 1880, Samoa in year 1883, Queensland in year 1886, and Bombay in year 1887 (Young, 1994).

In Malaysia, the first cocoa was planted in Malacca in the year 1778. Subsequently, the cocoa planting was started in area at Serdang Agriculture Station and Silam Agriculture Research Center, Sabah. The earliest cocoa commercialization was started between the years 1853 to 1959 where Amelonado cocoa types were first planted at Jerangau, Terengganu. The planted area was about 403 hectarages. Cocoa trial was further undertaken at Serdang, Cheras, Kuala Lipis and Temerloh between the years 1936 to 1940. However, cocoa was only actively planted after the World War II, whereby cocoa officially came to Quoin Hill, Tawau, Sabah in the year 1960. From then on, cocoa has become an important commodity in Malaysian economy (Varma and Nurdin, 2011).

2.1.1 Scientific Classification of Cocoa

Cocoa tree is originated from the Kingdom *Plantae*, Subkingdom *Tracheobionta*, Division *Magnoliophyta*, Class *Magnoliopsida*, Subclass *Dilleniidae*, Order *Malvales*, Family *Sterculiaceae*, Genus *Theobroma L.*, and Species *Theobroma cacao L.* (Anonym., 2010).

2.1.2 Characteristics of Cocoa Tree

The cocoa tree is a tropical plant that grows in hot, rainy climates. The cultivation of cocoa is concentrated on a narrow band of no more than 20 degrees north or south of the Equator. Cocoa trees need rainfall between 1,150 and 2,500 millimeters per year without hot dry winds and drought, and an even temperature between 21°C and 32°C for ideal growth (Anonym., 2010).

Anonym. (2010) stated that the cocoa tree is usually a small tree of 4 to 8 meters tall. Its stem is straight, the wood is light and white, and the bark is thin, and brownish in colour. The leavesof the cocoa tree are alternate, entire, unlobed, 10 to 40 centimeters long, and 5 to 20 centimeters broad. The leaves are poisonous and inedible as they are filled with

a creamy and milky liquid, which tastes spicy and unpleasant.Cocoa trees begin to bear fruit when they are 3 to 4 years old.The cocoa fruit (pods) can reach up to 15 to 25 centimeters in length, 8 to 10 centimeters in wide, and weighs about 500 grams when ripe. Each pod contains about 20 to 40 seeds, which are known as cocoa beans after drying and fermentation. The seeds are in reddish-brown colour externally and are covered by a white, sweet pulp. Each cocoa tree will yield 20 to 30 pods per year and the peak times for harvesting are around the months April and September in Malaysia (Varma and Nurdin, 2011).

2.1.3 Types of Cocoa

There are three broad types of cocoa Forastero and Crillo plus Trinitario which is a hybrid of Forastero and Crillo. Within these types are several varieties, Forastero, which now forms the greater part of all cocoa grown, is hardy and vigorous producing beans with the strongest flavour. Amelonado is the Forastero variety most widely grown in West Africa and Brazil. It has a smooth yellow pod with 30 or more pale to deep purple beans (Anonym., 2010).

Crillo with its mild or weak chocolate flavour is grown in Indonesia, Central and South America. Crillo trees are not as hardy and they produce softer pods which are red in colour, containing 20-30 white, ivory or very pale purple beans. Trinitario plants are not found in the wild as they are cultivated hybrids of the other two types. Trinitario cocoa trees are grown mainly in the Caribbean area but also in Cameroon and Papua New Guinea. The mostly hard pods are variable in colour and they contain 30 or more beans of variable colour but white beans are rare (Anonym., 2010).

2.2 1,3,7-TRYMETHYLXANTHINE

2.2.1 Properties

1,3,7-trymethylxnathine commonly known as caffeine. The pure caffeine was first isolated by a German chemist Friedrich Ferdinand Runge in year 1819 (Weinberg et. al, 2001). The nitrogen atoms in the structure of caffeine are all planar (in sp^2 orbital hybridization), resulting in the aromatic characteristics of caffeine. Caffeine is a readily available by-

product of decaffeination, and it is not usually synthesized (Anonym, 2001). But if desired, caffeine can be synthesized from dimethylurea and malonic acid (Wilson et. al, 2004).

Pure caffeine occurs as odourless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19g/gmol, melting point is 236^{o}C, point at which caffeine sublimes is 178^{o}C, at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapour pressure is 760 mm Hg at 178^{o}C, solubility in water is 2.17g per 100ml water at 25^{o}C, and vapour density is 6.7 (Clementz, 1988).

2.2.2 Applications

Caffeine is one of the few plant products with which the general public is readily familiar, because of its occurrence in beverages such as coffee and tea, as well as various soft drinks. A growing belief that the ingestion of caffeine can have adverse effects on health has resulted in an increased demand for decaffeinated beverages (Mazzafera, P. *et al*, 1991).

Caffeine is the world's most widely consumed psychoactive substance, by which the global consumption of caffeine has been estimated at 120,000 tonnes per year (Anonym, 1997). Caffeine can be a mild central nervous system stimulant, depending on its dose. Caffeine does not accumulate in the body over the course of time and is normally excreted within several hours of consumption. Consumption of caffeine in large amounts, and especially over extended periods of time, can lead to a condition known as caffeinism (Mackay et. al, 1989). Caffeinism usually combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, tremulousness, muscle twitching (hyperreflexia), insomnia, headaches, respiratory alkalosis, and heart palpitations (Leson et. al, 1988). It also increases the production of stomach acid, thus high usage over time can lead to peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease (Anonym, 2009).

Caffeine is a central nervous system and metabolic stimulant (Nehlig et. al, 1992), and it is used both recreationally and medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs. Caffeine and other methylxanthine derivatives are also used on newborns to treat apnea (suspension of

external breathing) and treat irregular heartbeats. Caffeine also stimulates the central nervous system first at the higher levels, resulting in increased alertness and wakefulness, faster and clearer flow of thought, increased focus, and better general body coordination, and later at the spinal cord level at higher doses (Bolton, Ph.D., Sanford, 1981).

2.2.3 Disadvantages

Mackay and Rollins (1989) stated that the consumption of caffeine in large amount, and especially over extended periods of time, can lead to a condition known as caffeinism. Accoring to (Leson *et al.,* 1988), caffeinism usually combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, tremulousness, muscle twitching (hyperreflexia), insomnia, headaches, respiratory alkalosis, and heart palpitations. It also increases the production of stomach acid, thus high usage over time can lead to peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease (Anonym., 2009). Caffeine also stimulates the stomach to pour out large amounts of acid. This in turn leads to burning in the pits of the stomach and aggravates peptic ulcers of the stomach and duodenum. It also may induce benign (non cancerous) breast diseases and may worsen premenstrual symptoms in women who overuse it. Caffeine crosses the placenta and enters the fetal circulation and its use at a pharmacological level has been associated with low birth weight. Excessive consumption during lactation may cause irritability and wakefulness in a breast- fed baby (Eva, 1988).

2.3 EXTRACTION OF 1,3,7-TRYMETHYLXANTHINE

Decaffeination is a popular term in present modern world to optimize the caffeine contents 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 etc (Varma and Nurdin, 2011).

The industrial decaffeination process has evolved over the years. Initially, direct contact methods used chloroform ($CHCl_3$), and more recently methylene chloride (CH_2Cl_2), as the solvent to repeatedly rinse the green (unroasted) cocoa beans that had been softened by steam. Once sufficient caffeine had been removed, the beans would be roasted. Since these organic solvents have a high vapour pressure and low boiling point,

any solvent remaining in the beans is removed during roasting. This method has several brown characteristics. Both of these solvents are carcinogenic and have several human health concerns with methylene chloride having the lesser overall hazard. Chlorinated hydrocarbon waste has significant environmental impacts and is costly to dispose. Roasting also does not guarantee full removal of the solvent, although solvent levels are rarely detectable. Although these solvents have its disadvantages, they are still used because they are not water-soluble, have a low boiling point, and remove caffeine without removing significant amounts of other compounds, leaving the majority of the flavour unaltered (Kirmer, 1988).

Recently the direct contact process has been greened significantly using supercritical CO_2. The green cocoa beans are steam softened with water and then supercritical CO_2 is used to extract the caffeine. Once the system is returned to room temperature and pressure the cocoa beans and separated caffeine are now solvent free as CO_2 returns to the gas phase. Then the CO_2 can be captured and reused. This method has all the advantages of the above technique without the environmental and human health risks (Murray, 1995).

Indirect contact methods have also been developed to decaffeinate cocoa. The green cocoa beans are soaked (steeped) in almost boiling water until the caffeine is removed from the bean. The cocoa solution is then treated with ethyl acetate (a natural ester) which has moderate human health hazards but is not carcinogenic. Ethyl acetate solvates caffeine more effectively than water and extracts the caffeine. The remaining ethyl acetate is removed from the cocoa solution by steaming. The cocoa solution is then combined with the beans which reabsorb the cocoa oils as they are dried. 2-Propanol is also used as extraction solvent rather than ethyl acetate as it is less hazardous to human health (Hampp, 1996).

2.3.1 Types of Solvent

The isolation of caffeine from cocoa is known as decaffeination, which is done by using a solvent that extract the caffeine. The common solvents used for this purpose are chloroform, methyl chloride, ethyl acetate, super critical carbon dioxide, etc. Methylene chloride is also used to extract caffeine from cocoa, and it is highly effective, but

methylene chloride is potentially dangerous under certain circumstances. It can cause faintness, dizziness, and headache if inhaled at high concentrations (Kirmer, D. A., 1988). Ethyl acetate is another compound used to extract caffeine from cocoa. It removes caffeine from cocoa effectively, and it extracts other chemical components from the cocoa as well. Ethyl acetate is much less hazardous to health and environment compared to chlorinated solvents (Johnson, G. D,1988). Water is also is an excellent solvent of methylxanthine but it is highly nonselective. Its use may result in the removal of the other valuable components from the extracted product, which gradually leads to deterioration of the analytical column (Saldana *et al*., 2002).

2.3.2 Methods of Extraction of 1,3,7-trymethyl xanthine

Ramli N. *et al*. (2001) has analyzed the total polyphenols, epicatechin, catechin, theobromine and caffeine contents in Commercial cocoa and chocolate products such as cocoa powder, cocoa beans, cocoa liquor and chocolate using High Performance Liquid Chromatography (HPLC). The methylxanthines were identified and quantified using Bondapak column and mobile phase of methanol:water:acetic acid at ratio 20:79:1. 32 samples of chocolate products were analyzed, and the levels of caffein and theobromine were 0.62-1.14 mg/g and 0.026-0.153 mg/g, respectively. The chocolate coating made from fat substitute had theobromine and caffeine levels ranged from 0.36-0.70 mg/g and 0.027-0.061 mg/g, respectively. The mean theobromine and caffeine levels in local chocolates respectively were 0.72 mg/g and 0.04mg/g in milk chocolate, and 0.85 mg/g and 0.06 mg/g in dark chocolate. In imported chocolates, the mean theobromine and caffeine levels respectively were 1.05 mg/g and 0.12 mg/g in dark chocolate, 0.76 mg/g and 0.04 mg/g in milk chocolate, and 0.74 mg/g and 0.03 mg/g in white chocolate. The imported chocolates have higher level of theobromine and caffeine compared with the local chocolates.

In a research done by Hu, Q. H. *et al*. (1997), caffeine was extracted from tea using ethanol solvent, by heat reflux extraction. A 50% ethanol in water was refluxed at 85^{o}C, for 45 minutes. The extract was then filtered through a filter paper, and the filtered solution was centrifuged for 10 minutes, at a speed of 4000rpm. The supernatant was then analyzed to determine the caffeine composition.

Hu, Q. H. *et al*. (1997) has also done a research of extracting caffeine from tea using ultrasonic extraction method. 50% ethanol in water was used as solvent to extract the caffeine from tea, and the solution was sonicated for 90 minutes in an ultrasonic bath (frequency 50Hz, power 250W) at 20-40^{o}C. Then the extract was filtered, and the filtered solution was centrifuged for 10 minutes, at a speed of 4000rpm. The supernatant collected was the analyzed to know the caffeine composition.

Mumin M. A. *et al*. (2006) has done a research on determination and characterization of caffeine in tea, coffee, and soft drinks by Solid Phase Extraction (SPE) and High Performance Liquid Chromatography (HPLC). Caffeine which is a mild addicting drug was isolated, purified and characterized from tea (black and green) and coffee. The isolation of caffeine was done by liquid-liquid extraction using chloroform as the extracting solvent. Four steps of extraction were carried out such as leaching, dye removal, liquid extraction and recrystallization. Toluene and petroleum ether were the solvent used for recrystallization. The crude caffeine was purified by SPE method. For the characterization of pure caffeine by HPLC, 50mM KH_2PO_4 (pH=2), acetonitrile, and methanol at ratio 40:8:2 was used as solvent as well as mobile phase at ratio. The amount of caffeine in various soft drinks (Cola) that commercially available in Bangladesh were also determined by HPLC method.

Li S. *et. al* (1990) have developed a method for the determination of theobromine and caffeine in cocoa beans using UV spectrophotometer. They have presented a rapid, simple and accurate method for individually determining theobromine and caffeine in cocoa beans. Caffeine alone was completely extracted into chloroform from an aqueous solution at a pH between 12.5 and 12.7, and analyzed by UV spectrophotometer at 275.9nm. For the remaining theobromine in the aqueous solution, a wavelength of 272.7nm was used. A result with relative standard deviation of about 0.65% was obtained.

Abourashed E. A. *et. al* (2004) have done HPTLC determination of caffeine in stimulant herbal products and power drinks. They analyzed the caffeine content in selected herbal products and energy drinks available in the Saudi market by HPTLC–UV densitometric. Pre-coated HPTLC silica gel plates (20 cm × 10 cm), and a solvent system consisted of ethyl acetate–methanol (85:15, v/v), and caffeine were used for the analysis, at

275 nm. The levels of caffeine in the herbal products and the energy drinks were 4.76–13.29% (w/w) and 0.011–0.032% (w/v), respectively.

In a study done by Wanyika *et al.* (2010), the levels of caffeine in certain coffee (nescafe, africafe, dormans) and tea (chai mara moja, kericho gold, sasini, finlays premium) brands were determined using high performance liquid chromatography (HPLC) and UV/Vis Spectrophotometric methods. The levels of caffeine in all the tea and coffee brands were found to be within the documented range. Generally, higher concentration of caffeine in all the samples were realized with the UV/Vis Spectrophotometric method compared to HPLC method. This indicates that acidified water was a better caffeine extractor than pure water. The results showed that the levels of caffeine obtained by UV/Vis Spectrophotometric method were much higher than those obtained by HPLC method. This shows that acidified water is a more efficient extractor of caffeine.

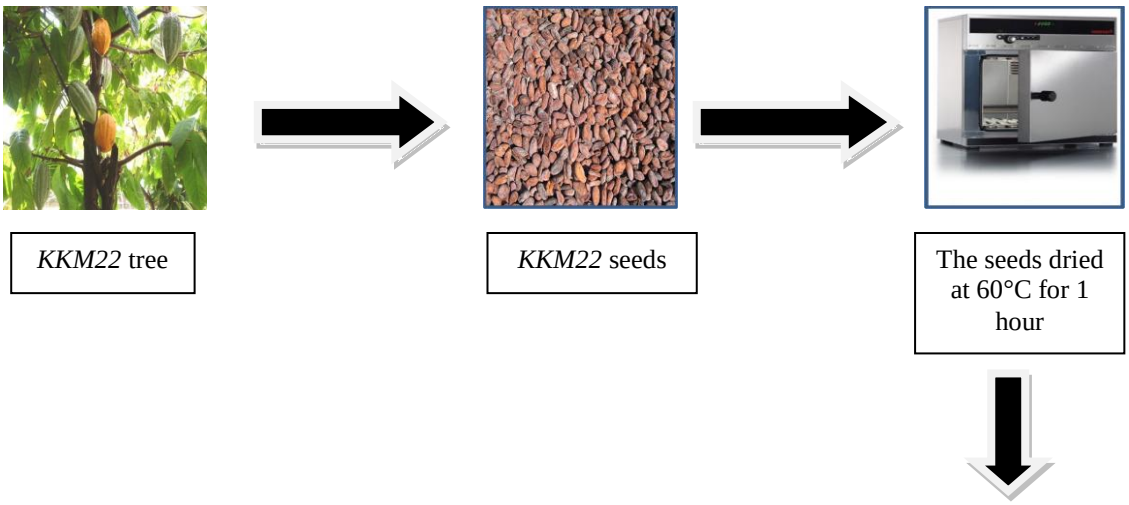
CHAPTER 3

METHODOLOGY

3.1 MATERIALS

The raw material for this research which is *KKM22* cocoa seeds were bought from Malaysian Cocoa Board research station in Jengka, Pahang. Three parameters were set up in order to investigate its effect on the caffeine yield. The apparatus and equipments used in this research were beaker, heater, vacuum filter, separatory funnel, pH meter, rotary evaporator, electronic balance, oven, heat reflux extractor, Buchner funnel, and siever. The chemicals and reagents used in this research were distilled water, 10% lead ethanoate solution, ethyl acetate, solid sodium hydrogen carbonate, 1M sodium hydroxide solution, and anhydrous sodium sulphate.

3.2 FLOWCHART



5 g of powdered seeds were boiled with 250mL distilled water, in heat reflux extractor for 15, 30, 45, 60, and 90 minutes.

The powdered seeds were sieved into 5 different particle sizes (2000, 1000, 800, 630, 315 µm).

The dried seeds were blended.

Solvent extraction was done using ethyl acetate at solvent/feed ratio of 1:5, 1:4, 1:3, 1:2, and 1:1. The lower layer that contains caffeine was collected.

Rotary evaporator was used to evaporate ethyl acetate at 78^{o}C.

The yield of caffeine in the solution was measured using UV/Vis Spectrophotometer.

3.3 METHODS

3.3.1 Preparation of Sample

50 grams of the *KKM 22* cocoa seeds were weighed and dried in the oven at 60°C for 2 hours to remove the moisture in the seeds. Then, the seeds were blended to get powdered sample. Next, the powdered sample was sieved into 5 different particle sizes which are 2000, 1000, 800, 630 and 315 µm.

3.3.2 Preparation of Solutions

10% (w/v) lead acetate solution was prepared by adding 10 grams of anhydrous lead acetate into 100 mL of distilled water. 1M sodium hydroxide solution was prepared by adding 4 grams of anhydrous sodium hydroxide into 100 mL of distilled water.

3.3.3 Solid-Liquid Extraction of 1,3,7-trymethylxanthine

5g from the prepared powdered sample of *KKM 22* cocoa seeds was put in a 500ml beaker and 250ml of distilled water was added into the beaker subsequently. The mixture was boiled in a heat reflux extractor at 70^{o}C for 15 minutes. Water bath was used in the heat reflux extractor. Then, the mixture was filtered by using Buchner Funnel. Next, the filtrate was collected and added with 25ml of 10% lead acetate solution.

The 10% lead acetate solution was to convert tannins and other acids into anions (base) that insoluble in water and ethyl acetate. This also can avoid an emulsion occur during the research. The solution was boiled for 5 minutes. The precipitate that formed during heating process was removed by filtering it in vacuum filter. Next, 1 gram 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 repeatedly and a clear solution was obtained.

3.3.4 Liquid-Liquid Extraction of 1,3,7-trymethylxanthine

The clear solution obtained was transferred into a 500ml separatory funnel. The pH of the solution will be measured using pH meter. If the pH of the solution is not between 12.5 and 12.7, about 5.5ml of 1M sodium hydroxide solution will be added until the pH of the solution regulate between 12.5 and 12.7. The addition of sodium hydroxide is to maintain the basicity of the solution, so that tannins and other acids do not soluble in water and chloroform. At basic condition, water polarity is increase and the caffeine in least polar form will be more readily solvated in ethyl acetate compared to water. After that, the solution was extracted with 5 different solvent/feed ratio which are 1:1, 1:2, 1:3, 1:4 and 1:5. Then, the mixture was shaked uniformly while the stopcock is opened to expel

vapours. The layers were allowed to separate and the lower layer (ethyl acetate) was collected into a 100ml beaker.

Ethyl acetate was used as the solvent in this research. Based on the Material Safety Data Sheet (MSDS), ethyl acetate is a highly flammable liquid which is moderately hazardous. The MSDS for ethyl acetate is shown in Appendix B.

3.3.5 Separation of 1,3,7-trymethylxanthine

The collected solution from liquid-liquid extraction process was added with anhydrous sodium sulphate. Anhydrous sodium sulphate was used to remove any water and water-soluble salts which retained in the ethyl acetate or accidentally transferred during dcantation of solution. When the anhydrous sodium sulphate was added, the solution was appeared a bit cloudy. This is because anhydrous sodium sulphate will be clumped in the present of water. The solution was shake gently so that no more clumping is observed. Then, the caffeine solution containing ethyl acetate was evaporated by using rotary evaporator at operating temperature of 78°C. The temperature must be maintained to avoid decomposition. After 1 hour, the saturated solution of caffeine was obtained.

3.3.6 Analysis of 1,3,7-trymethylxanthine

The saturated caffeine solution obtained was analyzed using UV/Visible Spectrophotometer. A standard calibration curve of absorbance versus caffeine concentration at wavelength of 275.9 nm was prepared. From UV/Visible Spectrophotometric method, the absorbance for all samples was determined using the same wavelength which is 275.9 nm. From the standard calibration curve, the caffeine concentrations for all samples were obtained and the data were recorded.

CHAPTER 4

RESULT AND DISCUSSIONS

4.1 STANDARD CURVE OF CAFFEINE

The absorbance value for each standard concentration of caffeine is shown at Appendix C (Table C.1). The standard curve for caffeine concentrations was drew using the data from Table C.1. Figure 4.1 show the standard curve obtained.

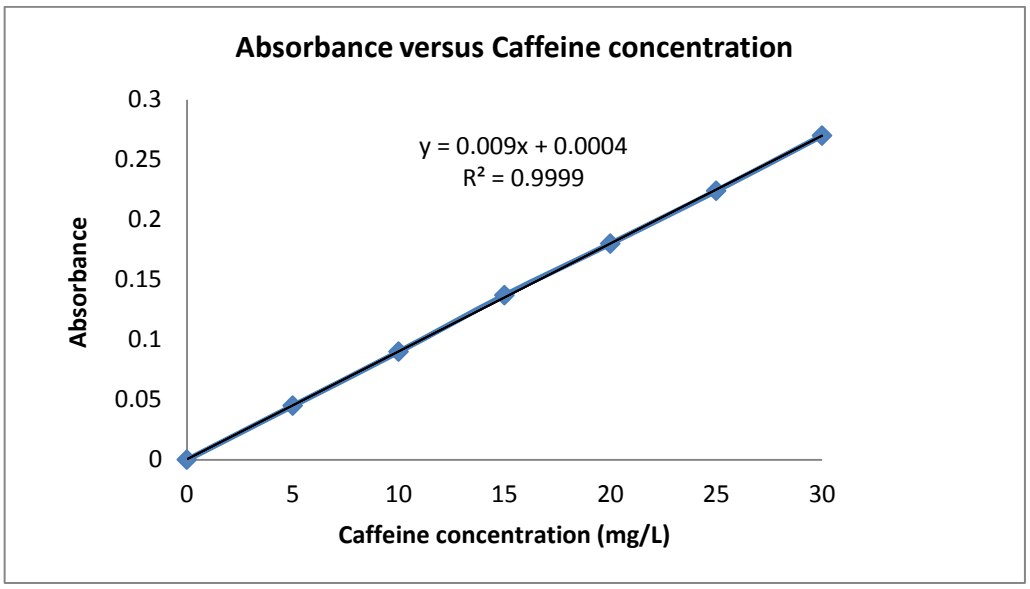


Figure 4.1: Standard Calibration Curve of Caffeine

4.2 THE EFFECT OF *KKM22* PARTICLE SIZE ON THE CAFFEINE YIELD

The effect of *KKM22* particle sizes on the percentage of caffeine yield was investigated and the data obtained was recorded as shown in Appendix C (Table C.2).

The absorbance value for every sample that obtained for all 5 different *KKM22* particle sizes at wavelength of 275.9 nm was read. Then, the standard calibration curve for caffeine concentration was used in order to get the concentration of caffeine for each particle size. Since the absorbance value obtained from the analysis were higher than the absorbance value from the standard curve, the linear equation for the curve was used to calculate the caffeine concentrations. From the caffeine concentration value, the amount of caffeine in 1 gram of *KKM22* powdered sample was calculated. Since 5 grams of *KKM22* was added into 100 mL of water, the concentration of the *KKM22* is 50 g/L. The amount of caffeine was determined by dividing the caffeine concentration (mg/L) by the concentration of *KKM22* solution (g/L). The percentage of caffeine yield was calculated by converting the unit of the amount of caffeine to mg per mg sample and then multiplied with 100%.

Based on the data shown in Table C.2, a graph of caffeine yield against *KKM22* particle size was plotted. The following Figure 4.2 showed the graph.

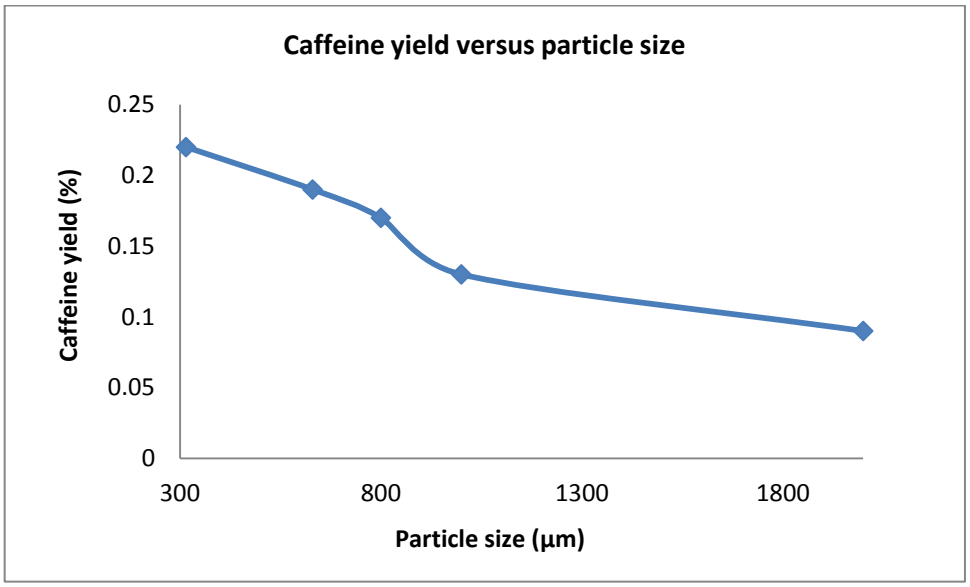


Figure 4.2: Percentage of Caffeine Yield for Different *KKM22* particle size

Figure 4.2 showed that the caffeine yield is inversely proportional to the *KKM22* particle size. As the *KKM22* article size increases, the percentage of caffeine yield is decreases. This is because, the surface area of the particle will become larger when the particle size is small and vice versa. The length of diffusion path for caffeine also becomes shorter when the particle size of *KKM22* is small. Thus, caffeine particles can easily diffuse out to the surface from inside of cocoa and easily extracted by solvent. Therefore, the highest percentage of caffeine yield was obtained at particle size of 315 μm (0.22 % of caffeine or 2.1724 mg/g of cocoa) in the range of particle size between 315 and 2000 μm.

From another research done by Li *et al.* (1990), the highest amount of caffeine also was found at the smallest particle size and the average caffeine yield of 2.316 mg/g sample was obtained for the cocoa beans tested. Thus, it is indicating that this research is feasible.

4.3 THE EFFECT OF SOLVENT/FEED RATIO ON THE CAFFEINE YIELD

The effect of solvent/feed ratio on the percentage of caffeine yield was investigated and the data obtained is shown in Appendix C (Table C.3).

The absorbance value for all 5 different solvent/feed ratios at wavelength of 275.9 nm was read. The value of caffeine concentration (mg/L), amount of caffeine (mg per g sample), and the percentage of caffeine yield were calculated similarly as the effect of particle size data shown in Table 4.2 calculation.

Solvent/feed ratio of 1:1 showed that the amount of solvent is equal to amount of feed, whereby for 100 mL of feed needs 100 mL of solvent. For the solvent/feed ratio of 1:3, the feed need is 3 times the solvent, whereby for 100 mL of feed needs 33.3 mL of solvent. It is similarly for the solvent/feed ratio of 1:5, the feed need is 4 times the solvent, whereby for 100 mL of feed needs 20 mL of solvent. The solvent used in this research is ethyl acetate.

Based on the data shown in Table C.3, a graph of caffeine yield against solvent/feed ratio was plotted. The following Figure 4.3 showed the graph.

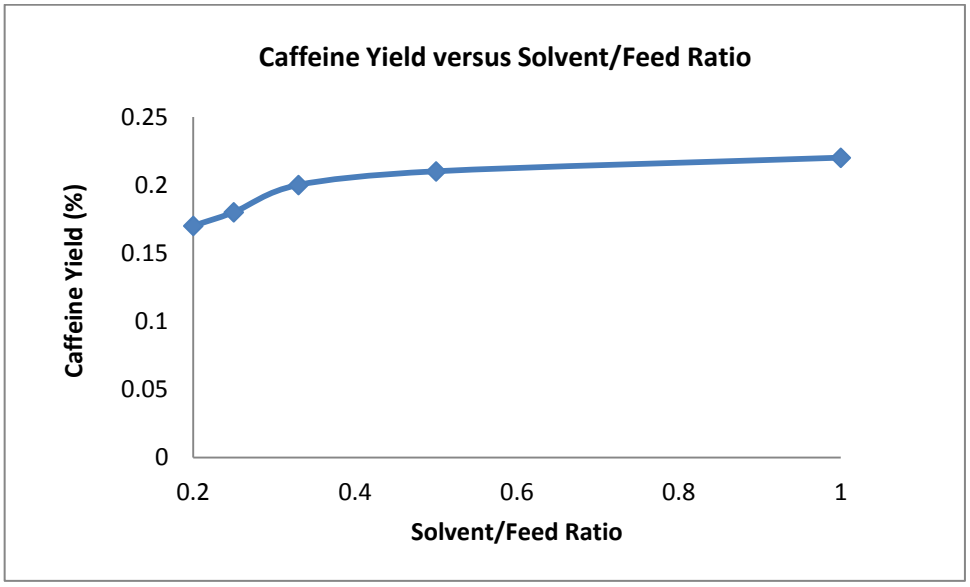


Figure 4.3: Percentage of caffeine yield for different solvent/feed ratio

Figure 4.3 showed that the percentage of caffeine yield is directly proportional to the solvent/feed ratio. As the solvent/feed ratio increased, the percentage of caffeine yield also increases. This is because the volume of solvent per volume feed will become higher at higher solvent/feed ratio. When the volume of (solvent) ethyl acetate is higher in the solution, the contact between the solvent and solute (caffeine) will become frequent and more caffeine will be extracted from the solution. Thus, the highest percentage of caffeine yield was obtained at 1:1 solvent/feed ratio with 0.22% of caffeine (2.2102 mg/g of cocoa).

Theoretically, the high amount of caffeine is extracted at high amount of solvent but economically, it is not relevant since high cost is required when high amount of solvent used.

In order to reduce the cost, it is reasonable to use an optimum of solvent/feed ration that is between 1:5 and 1:1.

The result obtained for *KKM22* shows the similar result as stated by Hameed *et al.* (2003), that the percent of extraction increases as the solvent/feed ratio increases.

4.4 THE EFFECT OF EXTRACTION TIME ON THE CAFFEINE YIELD

The effect of extraction time on the percentage of caffeine yield was investigated and the data obtained is shown in Appendix C (Table C.4).

The absorbance value for all 5 different extraction time at wavelength of 275.9 nm was read. The value of caffeine concentration (mg/L), amount of caffeine (mg per g sample), and the percentage of caffeine yield were calculated similarly as the effect of particle size data shown in Table 4.2 calculation.

Water was used as solvent for solid-liquid extraction of caffeine from *KKM22*. The solid-liquid extraction was done for 5 different extraction times which are 15, 30, 45, 60, and 90 minutes.

Based on the data shown in Table C.4, a graph of caffeine yield against solvent/feed ratio was plotted. The following Figure 4.4 showed the graph.

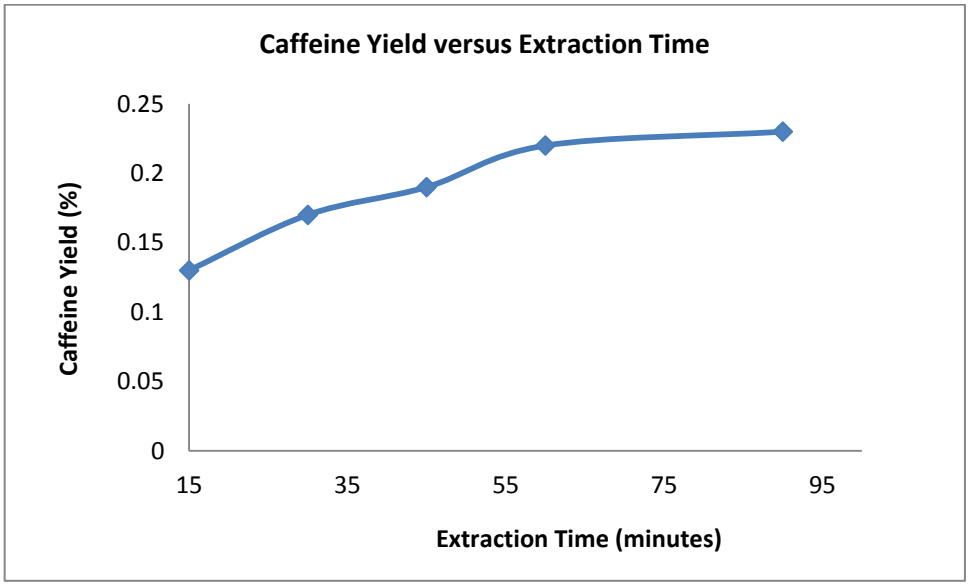


Figure 4.4: Percentage of caffeine yield for different extraction time

Figure 4.4 showed that the percentage of caffeine yield is directly proportional to the extraction time. As the extraction time increases, the percentage of caffeine yield increases. This is because, the longer the extraction time, the longer the particles of KKM22 spend time in the solvent. The diffusion of caffeine from inside of cocoa to the

surface of cocoa and the solvent takes place longer time. Thus, the highest percentage of caffeine yield was obtained at 90 minutes with 0.23% (2.288 mg/g of cocoa).

The result obtained for *KKM22* shows the similar result as stated by Hameed *et al.* (2003), that the percent of extraction increases as the extraction time increases.

From Figure 4.4, the caffeine yield increase slower when it reaches at 60 minutes. This is because the saturated solvent with caffeine decreases further diffusion of caffeine into the solvent. Time is an essential factor in the industrial production because the more the production, the more time can be saved. But, in extraction process an optimum should be applied so that high caffeine yield is obtained and long time is not consumed.

4.5 KINETIC MODEL FOR EXTRACTION OF CAFFEINE

Application of the steady-state model (Spiro & Jago,1982) leads to the first-order rate equation**:**

$$\boldsymbol{ln\left(\frac{C_{\infty}}{C_{\infty}-C}\right)=k_{obs}t}$$

where C is the concentration of the extracted caffeine in the solution at time t and C_{∞} is its concentration at the equilibrium ($t = \infty$**).**

From the caffeine concentration obtained for various extraction times, as showed in Appendix C (Table C.5), the curve for kinetic model was plotted by using the first-order Spiro's equation. The graph obtained is shown as following Figure 4.5.

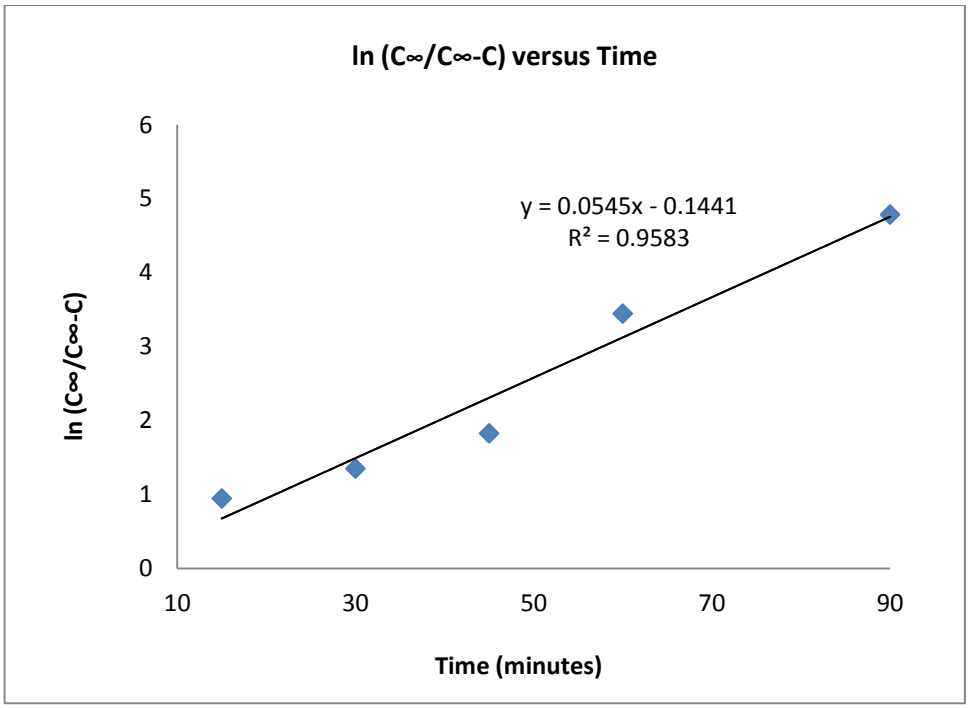


Figure 4.5: Kinetic Model for extraction of caffeine in different extraction time

The curve for kinetic model obtained from this research showed that it is obeyed the first-order of (Spiro & Jago, 1982). The rate constant, *k* value obtained from this research was determined as the gradient from Figure 4.5 which is 0.0545 min^{-1}.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The caffeine has bees resulted from *KKM22* by using batch extraction method, effectively. The effectivity of caffeine yield is influenced by particle sizes, solvent/feed ratio and extraction time. The highest caffeine yield was obtained at *KKM22* of sample particle of 315 µm (0.22%, 2.1724 mg/g of cocoa), solvent/feed ratio of 1:1 (0.22%, 2.2102 mg/g of cocoa), and extraction time of 90 minutes (0.23%, 2.288 mg/g of cocoa).

This research is an important implementation for the potential of extracting caffeine from *KKM22*. At the same time, to get decaffeinated cocoa at low cost and high efficiency. It is recommended to repeat this research by using different solvents for liquid-liquid extraction also should be tried, such as supercritical carbon dioxide, hexane, *etc*. Different solid-liquid extraction method such as soxhlet extraction, which is a continuous method should be tried to compare the performance of the method. The analysis of caffeine yield should be tried using high performance liquid chromatography (HPLC) to compare which one is the most effective to analyze caffeine.

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Appendix A: *KKM22* Cocoa Tree

Appendix B: Material Safety Data Sheet of Ethyl Acetate

ETHYL ACETATE

1. Product Identification

Synonyms: Acetic acid ethyl ester; Acetic ether; Acetoxyethane; Ethyl Acetic Ester; Ethyl ethanoate

CAS No.: 141-78-6

Molecular Weight: 88

Chemical Formula: $CH_3COOC_2H_5$

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2. Hazards Identification

Health Rating: 2 - Moderate (Life)

Flammability Rating: 3 - Severe (Flammable)

Reactivity Rating: 1 - Slight Contact Rating: 2 – Moderate

Lab Protective Equip: GOGGLES & SHIELD; LAB COAT & APRON; VENT HOOD; PROPER GLOVES; CLASS B EXTINGUISHER Storage Color Code: Red (Flammable)

Potential Health Effects

Inhalation:

Inhalation can cause severe irritation of mucous membranes and upper respiratory tract. Symptoms may include burning sensation, coughing, wheezing, laryngitis, shortness of breath, headache, nausea and vomiting. High concentrations may cause lung damage. An irritant to the nose, throat, and upper respiratory tract. Exposure to high concentrations have a narcotic effect and may cause liver and kidney damage.

Ingestion:

Causes irritation to the gastrointestinal tract. Symptoms may include nausea, vomiting and diarrhea.

Skin Contact:

Causes irritation to skin. Symptoms include redness, itching, and pain. Repeated or prolonged contact with the skin has a defatting effect and may cause dryness, cracking, and possibly dermatitis.

Eye Contact:

Causes irritation, redness, and pain.

Chronic Exposure:

Chronic overexposure may cause anemia with leukocytosis (transient increase in the white blood cell count) and damage to the liver and kidneys.

Aggravation of Pre-existing Conditions:

Persons with pre-existing skin disorders or eye problems, or impaired liver, kidney or respiratory function may be more susceptible to the effects of the substance.

---

3. First Aid Measures

Inhalation:

Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion:

Give large amounts of water to drink. Never give anything by mouth to an unconscious person. Get medical attention.

Skin Contact:

Immediately flush skin with plenty of soap and water for at least 15 minutes. Remove contaminated clothing and shoes. Get medical attention. Wash clothing before reuse. Thoroughly clean shoes before reuse.

Eye Contact:

Immediately flush eyes with plenty of water for at least 15 minutes, lifting lower and upper eyelids occasionally. Get medical attention immediately.

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4. Fire Fighting Measures

Fire:

Flash point: -4°C (25°F) CC

Autoignition temperature: 426°C (799°F)

Flammable limits in air % by volume: lel: 2.0; uel: 11.5

Flammable Liquid and Vapor! Contact with strong oxidizers may cause fire.

Explosion:

Above flash point, vapor-air mixtures are explosive within flammable limits noted above. Vapors can flow along surfaces to distant ignition source and flash back. Sealed containers may rupture when heated. Sensitive to static discharge.

Fire Extinguishing Media:

Water spray, dry chemical, alcohol foam, or carbon dioxide. Water may be ineffective. Water spray may be used to keep fire exposed containers cool.

Special Information:

In the event of a fire, wear full protective clothing and NIOSH-approved self-contained breathing apparatus with full facepiece operated in the pressure demand or other positive pressure mode. Water may be used to flush spills away from exposures and to dilute spills to non-flammable mixtures. Vapors can flow along surfaces to distant ignition source and flash back.

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5. Handling and Storage

Protect against physical damage. Store in a cool, dry well-ventilated location, away from any area where the fire hazard may be acute. Outside or detached storage is preferred. Separate from incompatibles. Containers should be bonded and grounded for transfers to avoid static sparks. Storage and use areas should be No Smoking areas. Use non-sparking type tools and equipment, including explosion proof ventilation. Containers of this material may be hazardous when empty since they retain product residues (vapors, liquid); observe all warnings and precautions listed for the product.

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6. Exposure Controls/Personal Protection

Airborne Exposure Limits:

-OSHA Permissible Exposure Limit (PEL): 400 ppm (TWA)

-ACGIH Threshold Limit Value (TLV): 400 ppm (TWA), A4 - Not classifiable as a human carcinogen.

Ventilation System:

A system of local and/or general exhaust is recommended to keep employee exposures below the Airborne Exposure Limits. Local exhaust ventilation is generally preferred because it can control the emissions of the contaminant at its source, preventing dispersion of it into the general work area.

Personal Respirators (NIOSH Approved):

If the exposure limit is exceeded and engineering controls are not feasible, a full facepiece respirator with organic vapor cartridge may be worn up to 50 times the exposure limit or the maximum use concentration specified by the appropriate regulatory agency or respirator supplier, whichever is lowest. For emergencies or instances where the exposure levels are not known, use a full-facepiece positive-pressure, air-supplied respirator. WARNING: Air purifying respirators do not protect workers in oxygen-deficient atmospheres.

Skin Protection:

Wear impervious protective clothing, including boots, gloves, lab coat, apron or coveralls, as appropriate, to prevent skin contact.

Eye Protection:

Use chemical safety goggles and/or a full face shield where splashing is possible. Maintain eye wash fountain and quick-drench facilities in work area.

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7. Physical and Chemical Properties

Appearance:

Clear liquid.

Odor:

Fruity odor.

Solubility:

1 ml/10ml water @ 25°C

Gravity:

0.902 @ 20°C/4°C

pH:

No information found.

% Volatiles by volume @ 21°C (70°F):

100

Boiling Point:

77°C (171°F)

Melting Point:

-83°C (-117°F) **V**

Vapor Density (Air=1):

3.0

Vapor Pressure (mm Hg):

76 @ 20°C (68°F)

Evaporation Rate (BuAc=1):

6

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8. Stability and Reactivity

Stability:

Stable under ordinary conditions of use and storage. Heat will contribute to instability. Slowly decomposed by moisture.

Hazardous Decomposition Products:

Carbon dioxide and carbon monoxide may form when heated to decomposition.

Hazardous Polymerization:

Will not occur.

Incompatibilities:

Avoid heat, flame and other sources of ignition. Contact with nitrates, strong oxidizers, strong alkalis, or strong acids may cause fire and explosions. Will attack some forms of plastic, rubber, and coatings.

Conditions to Avoid:

No information found.

Appendix C: Result Data

Table C.1: Absorbance for Standard Concentration of Caffeine

Caffeine Concentration (mg/L)	Absorbance
0	0.000
5	0.045
10	0.090
15	0.137
20	0.180
25	0.224
30	0.270

Table C.2: Percentage of Caffeine Yield for Different *KKM22* Particle Sizes

Particle Size (μm)	Absorbance	Caffeine Concentration (mg/L)	Amount of Caffeine (mg per g of sample)	Caffeine Yield (%)
2000	0.401	44.5111	0.8902	0.09
1000	0.590	65.5111	1.3102	0.13
800	0.772	85.7333	1.7147	0.17
630	0.852	94.6222	1.8924	0.19
315	0.978	108.6222	2.1724	0.22

Table C.3: Percentage of Caffeine Yield for Different Solvent/Feed Ratio

Solvent/Feed Ratio	Absorbance	Caffeine Concentration (mg/L)	Amount of Caffeine (mg per g of sample)	Caffeine Yield (%)
1:5	0.781	86.7333	1.7347	0.17
1:4	0.813	90.2889	1.8058	0.18
1:3	0.911	101.1778	2.0236	0.20
1:2	0.958	106.4000	2.1280	0.21
1:1	0.995	110.5111	2.2102	0.22

Table C.4: Percentage of Caffeine Yield for Different Extraction Time

Extraction Time (minutes)	Absorbance	Caffeine Concentration (mg/L)	Amount of Caffeine (mg per g of sample)	Caffeine Yield (%)
15	0.603	66.9556	1.3391	0.13
30	0.745	82.7333	1.6547	0.17
45	0.853	94.7333	1.8947	0.19
60	0.995	110.5111	2.2102	0.22
90	1.03	114.4000	2.2880	0.23

Table C.5: Kinetic Model for Extraction of Caffeine

Extraction Time (minutes)	$\ln (C_\infty / C_\infty - C)$
15	0.9492
30	1.3502
45	1.8266
60	3.4474
90	4.7880