INVESTIGATION OF CONDITIONS FOR ASSAM GREEN TEA EXTRACTION AND SEPARATION

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MASTER OF SCIENCE

IN FOOD TECHNOLOGY

MAE FAH LUANG UNIVERSITY

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THESIS SUBMITTED TO
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THIS THESIS HAS BEEN APPROVED

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ABSTRACT

Green tea is becoming an attractive material for research due to increased human preference and health concerns. Many efforts have been made to find an effective way to produce green tea extracts with high contents of catechins (main active ingredient in tea). Therefore, this study investigates the optimum conditions for assam green tea extraction, the kinetics of its compounds during extraction (both in laboratory and pilot plant scale) and also the fractionation of green tea extract compounds. With varying tea-water ratio (1:5, 1:10, 1:20 and 1:30) and pH (4, 5, 6 and 7), extraction in tea-water ratio 1:20 at pH 5 was found to be the optimum condition by providing the maximum amount of total polyphenols. In order to increase the extraction efficiency, a two step extraction procedure was necessary to be conducted.

Extractions for the kinetics study in the laboratory scale were performed (using the optimum condition from the previous study) at five different temperatures (50^0C, 60^0C, 70^0C, 80^0C and 90^0C) for 1, 3, 5, 10, 20, 40 and 80 minutes. The content of total polyphenols, tannin, total amino acids, caffeine and individual catechins were increased rapidly in 10 minute intervals, and then approached the equilibrium value. The relationships between those concentrations and times were found to fit the first order kinetic equations. Extraction at 90^0C was found to be the

optimum condition to obtain green tea extract with the highest equilibrium value for the extraction of total polyphenols, caffeine, individual catechins and total catechins. The maximum rate constant for extraction of total polyphenols, total amino acids, EGCG and ECG were all reached at 90^0C. Moreover, the optimum rate constant for tannin extraction was reached at a temperature of 50^0C, while for extraction of caffeine, EGC, EC, GC, C, G and total catechins were reached at a temperature of 70^0C.

Scaling up the kinetic study to pilot plant scale is important to set up an efficient process for green tea extraction at an industrial scale. The extractor model unit designed by Wongsuwan (2008) was used in this experiment. Extractions were performed with tea water ratio 1:28, pH 5 at a flow rate of 1 kg/s. Similar to the kinetic study in the laboratory scale, the content of total polyphenols, tannin, theanine, caffeine, individual catechins and total catechins were increased very fast in the first 10 minutes. The relationships between those concentrations and times were found to fit the first order of kinetic equations. By providing the maximum of equilibrium value, the optimum temperature for extraction of all the analyzed parameters was 90^0C. Furthermore, the highest rate constant for extraction of total polyphenols, tannin, caffeine, EGCG, EC, GCG and total catechins were reached in extraction at 90^0C, while for extraction of the remaining compounds were achieved at 70^0C.

In fractionation of green tea extract, various resins, including polyamide 6, amberlite XAD7HP, poly (dimmer acid-co-alkyl polyamine) and nylon 6 were used as a stationary phase. It was found that polyamide 6 exhibited the better ability to separate caffeine from polyphenols compounds when water, 50% of ethanol and 75% of ethanol were used as eluting agents. By using the regeneration treatment, polyamide 6 still has ability for caffeine separation after four uses. However it was observed that this ability was decreasing.

Keywords : Assam green tea / Green tea extraction / Kinetic of green tea compounds during extraction / Fractionation of green tea extract

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Tea is a well-known beverage due to its mildly stimulating and refreshing effect, sensory properties, low retail price, and potential health benefits. There are a wide variety of compounds in tea including polyphenols (mainly catechins), polysaccharides, amino acids, chlorophyll and other pigments, caffeine and other alkaloids, vitamins and minerals (Chen *et al.,* 2002; Sharma *et al.,* 2005; Uzunalic *et al.,* 2006). Among varieties of tea, assam tea has been reported to have a higher content of catechins (Wilson, 1999).

Recently, green tea is gaining popularity due to increased preference and health concerns. Numerous epidemiological and pharmacological studies demonstrated that due to the presence of catechins, green tea possesses potent antioxidant and antimicrobial activities, anticarcinogenic and antimutagenic activities, and other clinically relevant potentials (Yoshida *et al.,* 1999; Chang *et al.,* 2000; Wang *et al.,* 2000; Vasisht *et al.,* 2003; Row & Jin, 2006; Uzunalic *et al.,* 2006). Applications of green tea have an important role in the food industry and in medicine for daily use. Its antioxidant activity has been proven to be higher than butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and DL-α-tocopherol, and its toxicity is lower than those (Baptista *et al.,* 1999; Pan *et al.,* 2003; Sharma *et al.,* 2005).

To receive the therapeutic amount for health benefits from catechins, consumers need to consume green tea in sufficient quantity. Producing new tea products such as green tea extract, which provide catechins in high doses could be an alternative effort to overcome this problem. Generally, green tea extract processing consists of extraction, filtration, concentration, separation and drying.

In recent years, many attempts have been made to find practical ways to produce green tea extract with high contents of catechins. Green tea polyphenols, mainly catechins, constituting up to 25% on a dry weight basis are water soluble and can be easily extracted by water. Extraction conditions such as temperature, time, pH, ratio of water to tea and a number of extraction steps varyingly affect the extraction yield, the efficiency and the quality of the extract (Yoshida *et al.,* 1999; Sharma *et al.,* 2005; Uzunalic *et al.,* 2006).

Catechins play an important role in green tea medicinal properties. These compounds are supposed to consist of high amounts to improve the quality of the green tea extract. In the extraction process, other compounds or impurities are normally extracted together with catechins. Therefore, it is necessary to continue with a separation or fractionation process to purify the catechins. There are several methods for separation, including solvent separation, supercritical carbon dioxide, membrane separation, hot water treatment, and by adsorption method.

Using chloroform or methylene chloride, caffeine and related impurities can be separated from tea extracts. However, this is not widely accepted because of their toxicity. Decaffeination of tea using supercritical carbon dioxide is effective but needs expensive instruments, while decaffeination with hot water treatment and membrane separation method do not specifically separate caffeine from other impurities (Row & Jin, 2006; Liang *et al.,* 2007). Several studies have been published about the application of adsorbent to isolate polyphenol compounds from plants containing high levels of polyphenols (Lee, 2004). Basically, adsorption is recognized as an inexpensive and safe separation method. However, optimum adsorbent and eluting agents to fractionate green tea compounds are under investigation.

Therefore, the investigation of optimum conditions for assam green tea extraction, the kinetic study of its compounds during the extraction process, and the fractionation of the desirable compounds (mainly catechins) are still required in order to obtain green tea extract with highly pure catechins compound. Scaling up the study to pilot plant scale is necessary to set up an efficient process for isolating and purifying the desired compounds of green tea at an industrial scale.

1.2 Objective

1.2.1 To study the optimum conditions for extracting assam green tea.

1.2.2 To study the optimum conditions for separating assam green tea extract.

1.3 Expected outcome

This research provides information about the optimum conditions for green tea (*Camellia sinensis* var. *assamica*) extraction, the kinetics of green tea compounds during the extraction process, and also the procedures of green tea extract fractionation.

1.4 Research Scope

1.4.1 Green tea (*Camellia sinensis* var. *assamica*) and green tea extract (spray dried) were used as food models.

1.4.2 The extraction conditions studied in the laboratory scale were the pH of water and tea-water ratio, the number of extraction steps, and the time and temperature of extraction.

1.4.3 Kinetic studies (both in laboratory and pilot plant scale) were conducted to investigate the effect of time and temperatures during extraction process.

1.4.4 Fractionation of green tea extract compounds was observed. The fractionation conditions studied were types of resin (polyamide 6, amberlite XAD7HP, poly (dimmer acid co-alkyl polyamine) and nylon 6), eluting agent (water and water-ethanol mixture), and height of column packed bed.

CHAPTER 2

LITERATURE REVIEW

2.1 Origin and varieties of tea

The tea plant originates from the north of China. It was discovered accidentally by the Chinese emperor Shen Nung around 2737 B.C. From this site, it spread to the southern parts of China and other countries, such as India, Burma, Thailand, Laos and Vietnam. It spread into many tropical and subtropical countries. In China, tea was used mainly as a medicinal drink, and later it became a common beverage, now famous in other parts of the world (Baptista *et al.,* 1999; Bezbaruah, 1999; Caballero *et al.,* 2003; Vasisht *et al.,* 2003; Shahidi & Naczk, 2004; Uzunalic *et al.,* 2006).

Generally, the tea plant (*Camellia sinensis*) was divided into three varieties, var. *sinensis*, var. *assamica*, and var. *cambod*. Morphological characteristics of the three varieties of tea have been found to be related to the quality and yield of tea in different tea growing countries (Bezbaruah, 1999; Caballero *et al.,* 2003; Vasisht *et al.,* 2003).

2.1.1 *Camellia sinensis* var. *sinensis*

This variety of tea grows slowly into a big shrub (about 1 to 3 m high), a dwarf tree with numerous virgate stems arising from the base of the bush near the ground level and giving rise to a dome shaped bush when fully grown. Leaves are erect, small, narrow, and are dark green with a smooth, characteristically thick and mat surface. Flowers are born singly or in pairs in the cataphillary leaf axils (Bezbaruah, 1999). The flowering shoot, pistil and fruit of *Camellia sinensis* var. *sinensis* is shown in Figure 2.1.

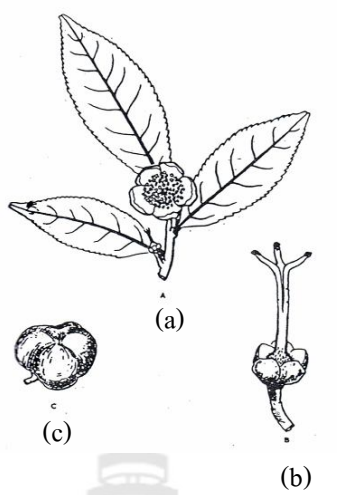


Figure 2.1 Flowering shoot (a), pistil (b) and fruit (c) of *Camellia sinensis* var. *sinensis*

Source: Bezbaruah (1999)

2.1.2 *Camellia sinensis* var. *assamica*

This variety of tea grows faster than does the sinensis variety, about 10 to 15 m high with a vertical branch system and a distinct trunk. Leaves are typically dependant, thin, and glossy with more or less acuminate apex and distinct marginal veins. Flowers from the cataphylary leaf axils are single or in pairs. This variety is less resistant to cold and also yields higher than var. *sinensis* does (Bezbaruah, 1999). The flowering shoot and pistil of *Camellia sinensis* var. *assamica* is shown in Figure 2.2.

2.1.3 *Camellia sinensis* var. *cambod*

The Cambod or southern variety of tea is a fastigate tree with more or less equally developed ascending main stems. It reaches a height of about 6 to 8 m. Its leaves are more or less erect, glossy, yellowish-green when young and light green when mature, often turning coppery-yellow or pinkish-red in autumn (Bezbaruah, 1999). The flowering shoot and pistil of *Camellia sinensis* var. *cambod* is shown in Figure 2.3.

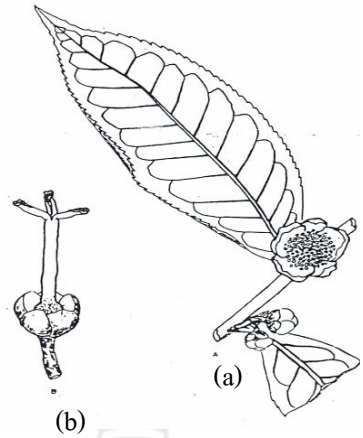


Figure 2.2 Flowering shoot (a) and pistil (b) of *Camellia sinensis* var. *assamica*

Source: Bezbaruah (1999)

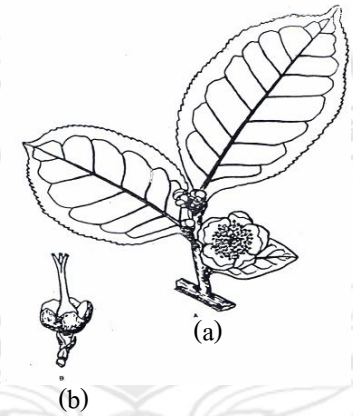


Figure 2.3 Flowering shoot (a), pistil (b) of *Camellia sinensis* var. *cambod*

Source: Bezbaruah (1999)

During their migration from the center of origin, the tea varieties might have hybridized not only with the related *Camellias* indigenous to the south-east Asian region, but also possibly with other genera, resulting in new cultivars (Bezbaruah, 1999).

2.2 Classification of tea product

Based on the extent of enzymatic reactions in the manufacturing process of tea, a tea product is classified into non-fermented green tea (20-22% of world tea consumption), fully fermented black tea (73-78% of world tea consumption), and semi-fermented oolong tea (2-3% world tea consumption). These types of tea products differ in their chemical contents (Halder *et al.,* 1998; Yamanishi, 1999; Krishnan & Maru, 2006). Figure 2.4 shows a picture of green tea, oolong tea, and black tea. Figure 2.5 shows the processing method of green tea, oolong tea and black tea.

(a) (b) (c)

Figure 2.4 Green tea (a), oolong tea (b), black tea (c)

Source: Anonymous (2009a)

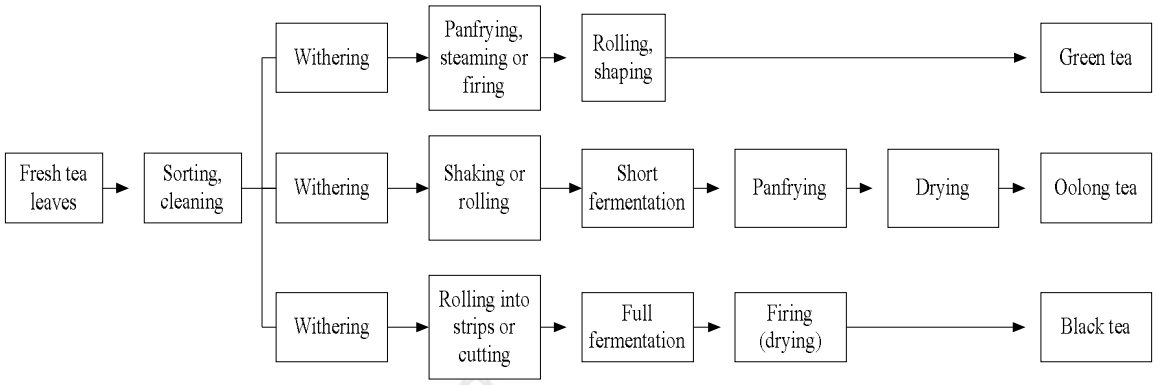


Figure 2.5 Tea product processing chart

Source: Anonymous (2007)

2.2.1. Green tea

Green tea, an unfermented tea, is widely consumed and imported mainly from China and Japan. Recently, world production of green tea is increasing to meet demand such as the manufacturing of instant tea (Pintauro, 1977; Peterson *et al.,* 2005).

Green tea is produced by inactivation of polyphenol oxidase. Parching, roasting, dry heating, and steaming are some techniques used to inactivate polyphenol oxidase (Vasisht *et al.,* 2003). This enzyme is responsible for oxidizing catechins to produce theaflavin and thearubigin, pigments that contribute to the colour and taste of black tea (Sharma *et al.,* 2005). Enzyme deactivation should be done immediately after plucking the tea shoots to prevent oxidation and polymerization of primary polyphenols (Vasisht *et al.,* 2003; Shahidi & Naczk, 2004).

Primary polyphenols are located in the vacuoles of the cells, and they are kept separated from the enzyme in the chloroplast. If the polyphenols and the enzyme come in contact with each other, oxidation and polymerization of polyphenols will occur to form complex compounds (Vasisht *et al.,* 2003).

The amount of polyphenols in green tea is higher than those in oolong and black tea, thus making green tea a considerable interest. Oxidation, hydrolysis, polymerization and transformation are the main chemical reactions that occur during green tea processing (Peterson *et al.,* 2005; Uzunalic *et al.,* 2006; Kim *et al.,* 2007). Generally, the amount of polyphenols content

in green tea after processing will decrease by about 15%. This change plays an important role in forming the characteristic of green tea taste and flavour (Xu & Chen, 2002).

2.2.2 Oolong tea

Oolong tea, semifermented tea, is produced by partial fermentation of tea leaves by endogenous enzymes (polyphenols oxidase) (Peterson *et al.,* 2005). Oolong tea has characteristics between green tea and black tea. It has the colour of black tea and the flavour of green tea (Wilson, 1999). The tea leaves are partially fermented immediately after plucking for about half the time of black tea's fermentation. Due to this fermentation process, the phenolic content of oolong tea is 70% oxidized (Caballero *et al.,* 2003; Vasisht *et al.,* 2003; Shahidi & Naczk, 2004).

2.2.3. Black tea

Black tea, a fully fermented tea, is the most popular among the three types of tea and dominates the market economically (Peterson *et al.,* 2005). The fermentation process should be done immediately after plucking. This process results in oxidation and polymerization of polyphenols. These processes form more complex condensed molecules (theaflavins and thearubigins) that are responsible for the color, strength, briskness, taste, aroma and pungency of black tea. Its infusion has a bright red or copper color, astringent taste and characteristic aroma (Vasisht *et al.,* 2003; Krishnan & Maru, 2006).

Fermentation is the most crucial step in black tea manufacturing. This process usually occurs at room temperature in the presence of oxygen and sufficient humidity. The degree and the rate of oxidation and polymerization depends on the distribution, composition and content of polyphenols in the fresh tea flush, activity of the enzyme, degree of tissue and cellular disruption, and the temperature and oxygen content of the tea leaves (Halder *et al.,* 1998; Baptista *et al.,* 1999; Obanda *et al.,* 2004; Shahidi & Naczk, 2004). Polyphenols oxidase, an important enzyme in black tea fermentation, works best at 28.3°C, and the reaction rate gets slower with an increase or decrease in temperature. Fermentation occurs faster in fresh leaves than in withered leaves (Vasisht *et al.,* 2003).

2.3 Chemical composition of tea leaves

The tea flush is generally referred to as the apical shoots, which consist of the terminal bud and two adjacent leaves. Overall composition of the tea flush is listed in Table 2.1. The composition of tea flush in this table is classified into two compounds including soluble and insoluble (or slightly soluble) in water. The composition of the tea flush may vary with the variety of tea, genetic characters, age of the leaf, its geographical origin, environmental conditions as well as agronomic situations (Baptista *et al.,* 1999; Shahidi & Naczk, 2004; Uzunalic *et al.,* 2006).

2.3.1 Phenolic compounds

A phenolic compounds are chemical compounds that contain at least one aromatic ring (C6) having one or more hydroxyl group. Many phenolics are derivatives formed by condensation or addition reactions. Most of these compounds are derived from three different biogenetic routes: (1) the shikimate/arogenate pathway (shikimic acid pathway), (2) the acetate/malonate pathway (malonic acid pathway), and (3) the acetate/mevalonate pathway (mevalonic acid pathway). Among the biogenetic pathways, the shikimate/arogenate and the acetate/malonate pathways are the most important ways in biosynthesis of the plant phenolics (Dey & Harborne, 1997). Figure 2.6 shows the illustration of the primary metabolic pathways in plants including phenolic compounds.

Polyphenolic (polyphenols) compounds in tea range from 20-35% of dry weight. The major compounds of tea polyphenols are flavanols (flavan-3-ols) that comprise of up to 90% of total polyphenols, flavonols (quercetin, kaempferol and their glycosides), flavones (vitexin, isovitexin), phenolic acids and depsides (gallic acids, cholorgenic acids, and theogallin) (Sarma, 1999). Flavanols mainly consist of catechins such as (-) epicatechin gallate (ECG), (-) epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG), (-) epicatechin (EC), (+) catechin (C) and their derivatives (Chen *et al.,* 2002; Pan, 2003; Shahidi & Naczk, 2004; Peterson *et al.,* 2005).

Table 2.1. Composition of fresh tea flush

Components	Dry Weight (%)
Soluble in water	
Flavanol	18-32
(-) – EGCG	9-14
(-) – EGC	4-7
(-) – ECG	2-4
(-) – EC	1-3
(-) – GC	1-2
(-) – C	0.5-1
Minor catechins	0.4-1
Flavonol glucosides	3-4
Proanthocyanidins	2-3
Caffeine	3-4
Amino acids	2-4
Carbohydrates	3-5
Organic acids	0.5-2
Saponins	0.04-0.07
Pigments	0.5-0.8
Vitamins	0.6-1
Soluble minerals	2-4
Insoluble or slightly soluble in water	
Cellulose	6-8
Lignin	4-6
Polysaccaharides	4-10
Lipids	2-4
Insoluble pigments	0.5
Insoluble minerals	1.5-3
Volatiles	0.01-0.02

Source: Chen *et al* (2002)

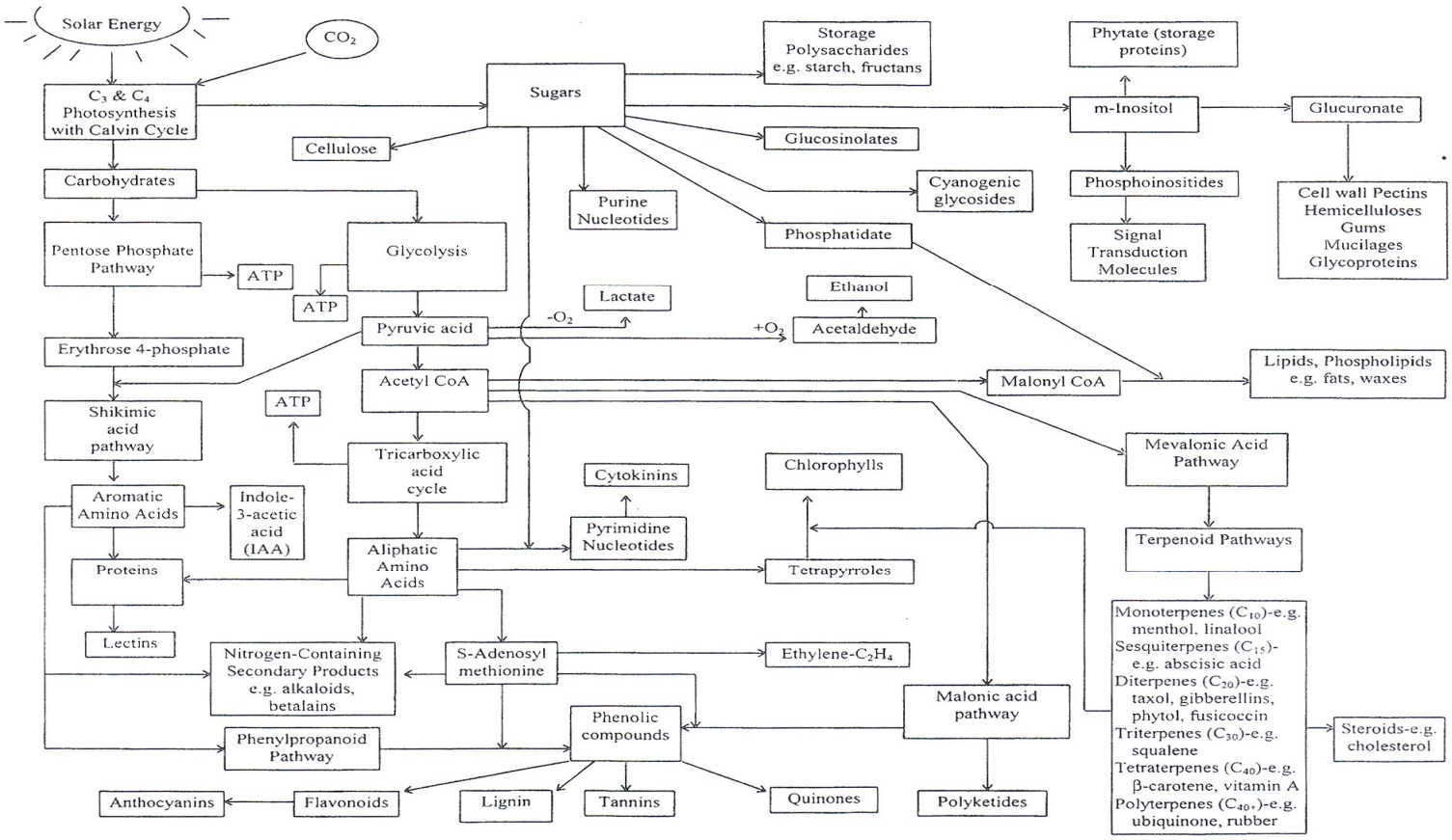


Figure 2.6 Primary metabolic pathways in plants

Source: Kaufman *et al* (1999)

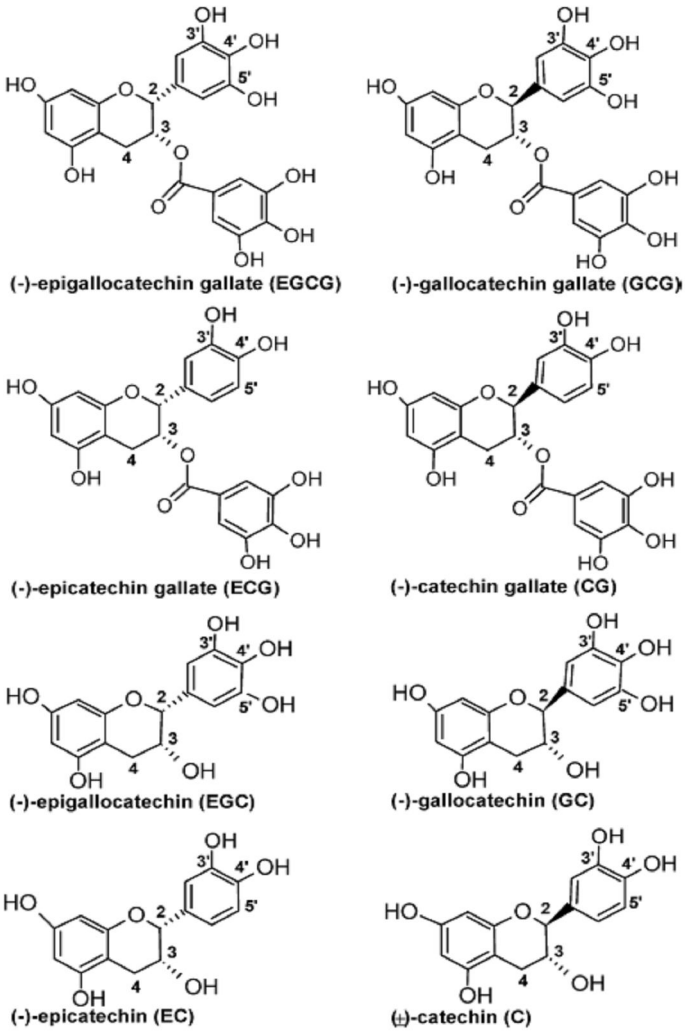


Figure 2.7 Chemical structure of green tea catechins

Source: Wang *et al* (2008)

Normally, catechins contents in tea flush are higher in the summer than those obtained in the spring (Chen *et al.,* 2002). In addition, during the manufacturing of tea and tea products, tea catechins can undergo many chemical reactions including epimerization, which will convert the tea catechins to their corresponding isomers (Belitz & Grosch, 1999; Wang & Helliwell, 2000; Pan, 2003; Shahidi & Naczk, 2004; Peterson *et al.,* 2005).

Tea catechins are divided into four primary compounds (EC, ECG, EGC and EGCG) and four secondary compounds (C, CG, GC and GCG) (Wang & Helliwell, 2000; Row & Jin, 2006; Uzunalic *et al.,* 2006). EGCG is the highest catechin found in green tea followed by EGC, ECG and EC, respectively. Other catechins including (+) gallocatechin (GC), (-) gallocatechin gallate (GCG), (-) catechin gallate (CG) and (+) catechin (C) are presented in minor quantities as the epimers of the primary catechins (Yoshida *et al.,* 1999; Caballero *et al.,* 2003; Su *et al.,* 2003; Vasisht *et al.,* 2003; Lee *et al.,* 2006). Figure 2.7 shows the chemical structure of catechins in green tea.

Catechins in tea contribute to bitter characteristics and astringent tastes of tea, while the brothy and sweet taste come from amino acids such as theanine, glutamic acid and arginine. EC and EGC produce bitterness with a sweet after taste, while their gallates (ECG and EGCG) produce strong bitterness with astringency (Yamanishi, 1999; Yoshida *et al.,* 1999; Su *et al.,* 2003).

2.3.2 Tannin

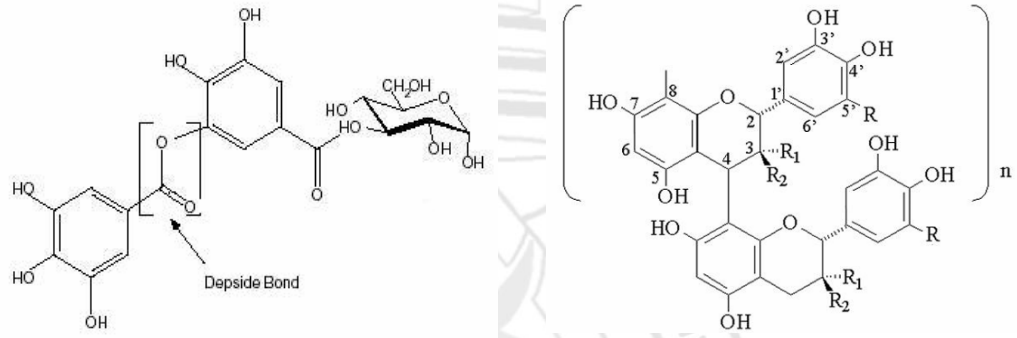
Tannins are phenolic compounds that can precipitate proteins from aqueous solutions. They consist of oligomeric and polymeric constituents. They can form complexes with certain types of polysaccharides, nucleic acids and alkaloids. Because of this property, they can produce turbidity upon steeping the tea leaves (Dey & Harborne, 1997; Chung *et al.,* 1998; Caballero *et al.,* 2003; Shahidi & Naczk, 2004). Tannins are water soluble with the exception of some high molecular weight structures (Boadi & Neufeld, 2001).

Due to its ability to interact with other molecules, tannins may have anti-nutritional effects, including a decrease in food palatability, and a decrease in fiber and protein digestibility (Shahidi & Naczk, 2004). Tannin-amino acids complexes may inhibit the activity of enzymes such as trypsin and lipase, while the complexes with protein may interfere with the utilization of proteins, and also decrease the phenolic antioxidant activity (Shahidi & Naczk, 2004).

Based on the chemical structures, tannins are divided into hydrolysable tannin and condensed tannin (proanthocyanidin). The hydrolysable tannins are molecules with a polyol (commonly D-glucose) as a central core. The hydroxyl groups of this polyol are partially or totally esterified with phenolic compounds like gallotannin and ellagitannin. Meanwhile, condensed tannins are oligomer and polymers of flavonoids, specifically flavan-3-ols, they are

also called proanthocyanidins because of their behavior to give anthocyanidins in the presence of acids (Dey & Harborne, 1997; Chung *et al.,* 1998; Cannas, 2001; Shahidi & Naczk, 2004). Figure 2.8 shows the chemical structure of gallotannin and proanthocyanidin.

Hydrolysable tannins can be hydrolyzed by mild acids or mild bases, and also by hot water or enzymes to yield carbohydrate and phenolic acids. Condensed tannins are not hydrolyzed under physiological digestive conditions, but upon severe acid or alkaline treatment, yielding less soluble polymeric phlobaphanes or monomeric flavonoids such as catechin or epicatechin (Dey & Harborne, 1997; Chung *et al.,* 1998; Cannas, 2001; Shahidi & Naczk, 2004).



Gallotannin Proanthocyanidin

Figure 2.8 The chemical structure of gallotannin and proanthocyanidin

Source: Anonymous (2003a)

2.3.3 Caffeine

The presence of caffeine in tea was first observed in 1820. Pure caffeine is white and has a bitter taste. Since the late 1980's, caffeine has been determined by HPLC and much data on the content of caffeine in fresh tea leaves and tea brews have been reported (Yamanishi, 1999; Caballero *et al.,* 2003; Vasisht *et al.,* 2003). The amount of caffeine in tea leaves varies from 2-5%, while in tea infusion, it ranges from 2- 4%. Commercially, caffeine is produced from tea leaves and tea wastes. Caffeine contributes to the brisk and creamy properties of tea infusion,

which result from the complexity of caffeine with polyphenols (Chen *et al.,* 2002; Caballero *et al.,* 2003; Vasisht *et al.,* 2003).

During tea processing, caffeine content is not significantly reduced, although it may decrease during the firing process. It is soluble in hot water with a bitter taste. Caffeine content in tea flush is higher in spring and gradually decreases with the growth of leaves. Its content in the first and second leaves are higher than that in the mature leaves (Chen *et al.,* 2002). Caffeine is one of the most widely consumed substances in the world. In moderate doses, caffeine is generally considered to have the effect of a mild stimulant, which is helpful in relieving minor fatigue and boredom with a small risk of harmful effects (Caballero *et al.,* 2003). The chemical structure of caffeine is shown in Figure 2.9.

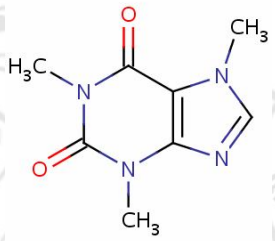


Figure 2.9 Chemical structure of caffeine

Source: William & Markley (2007)

2.3.4 Amino acids

Amino acids contribute make up about 4% of the tea leaves. The major amino acids present in the tea leaves include theanine and glutamic acid, whereas aspartic acid and arginine are the minor amino acids. Free amino acids are the key quality compounds of tea, and they are present in higher amounts in higher grade teas. These compounds play an important role in tea taste, specifically a brothy taste called “umami” (Horie *et al.,* 1997; Yamanishi, 1999; Chen *et al.,* 2002; Vasisht *et al.,* 2003).

Theanine, the most abundant amino acid in tea leaves, is a unique amino acid, produced by tea plants and other certain species of *Camellia* (Vasisht *et al.,* 2003). Its amount is

about half of the total amino acids in the leaves. The metabolism rate of theanine in tea leaves is slow, but it is rapidly transported from the root to the leaf, which leads to its accumulation in the leaves (Horie *et al.,* 1997; Chen *et al.,* 2002).

Some proteins in tea leaves are hydrolyzed into free amino acids due to high temperatures and the moist environment during tea processing. Therefore, the amount of amino acids in made tea is higher than that of the fresh leaves. At the same time, amino acids will change into volatile substances (Xu & Chen, 2002). The chemical structure of L-theanine is shown in Figure 2.10.

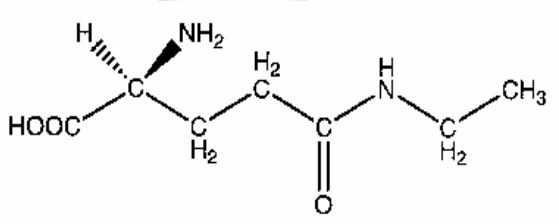


Figure 2.10 Chemical structure of L-theanine

Source: Viklund (2008)

Recently, theanine has been attracting many considerable interests in physiological and pharmacological applications. It has been reported that theanine can promote relaxation, reduce blood pressure, inhibit caffeine's negative effects, have neuroprotection activity, anti-obesity activity, and improve anti-tumor activity. Because of its favorable taste and beneficial health effects, the demand for theanine is increasing, not only as a taste enhancing (food additive) substance, but also as a supplement for human health (Li *et al.,* 2007).

2.4 Tea extracts

Due to its compositions (of mainly catechins), tea and its products possess therapeutic and medicinal properties. Tea extract is one of many new tea product developments that have captured a significant portion of the market share. Tea extract is a liquid derived from the brewing or extraction process. This liquid is further concentrated and dried to form a powdered or granular product (Hampton, 1999).

In the last 10 years, tea extract have been applied in several areas such as in dyes, deodorants, sterilizers and in medical agents. In the pharmacological area, tea extract has some properties such as antiseptic, anti-inflammatory, anti-allergic effects, anti-dental caries. It is currently also under investigation for possibilities for skin care products and for chemo-preventive agent for cancer and cardiovascular diseases (Takeo, 1999).

Due to its potential properties, various types of tea extract powders are produced in the world. Raw material for tea extract powder is mainly the low grade teas. Therefore, their utilization plays an important role in increasing the economic efficiency of the tea industry. Generally, the method consists of extraction with hot water or aqueous ethanol, concentration, separation and drying (as shown in Figure 2.11). Tea extract powder made by this process has about 25-30% of polyphenols and around 20% of catechins. Separation can be added in the processing of tea extracts to purify polyphenols, which are mainly catechins in tea extract (Takeo, 1999).

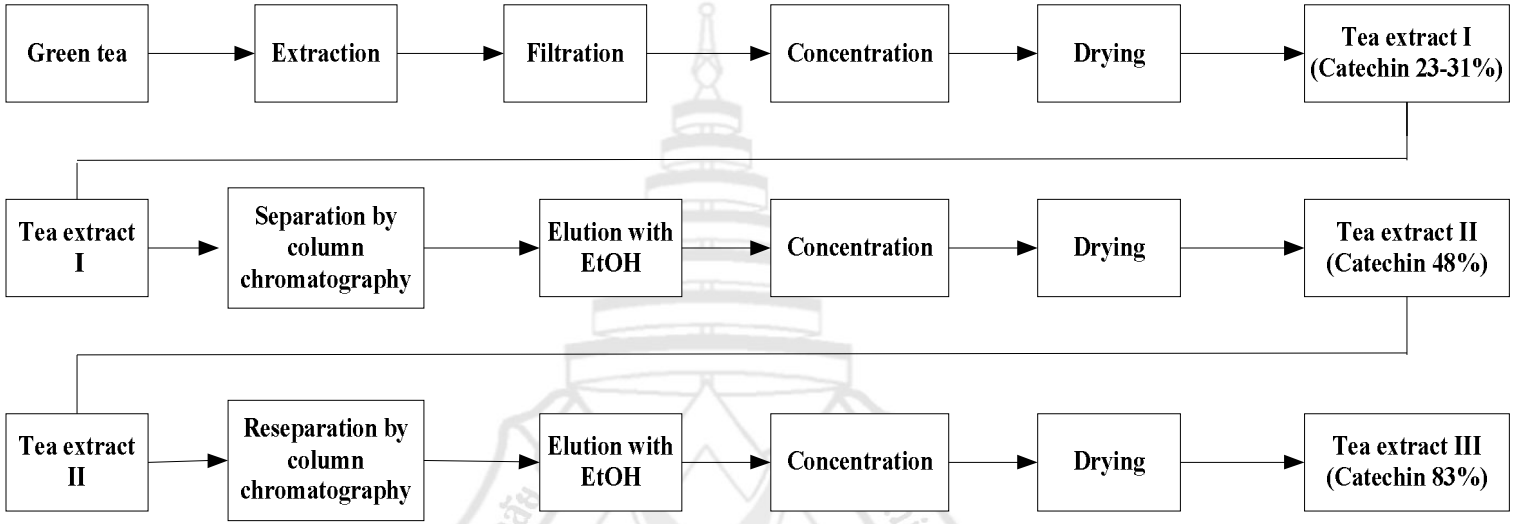


Figure 2.11 Tea extract processing chart

Source: Takeo (1999)

2.5 Phenolic compounds extraction from tea

The phenolic compounds are located in the vacuoles of the cells. The process to release them from the cell structure through rupturing the plant tissues and through the diffusion process are called extraction. Extraction procedures depends on the type of food, phenolic compounds, and the analytical procedure to be used. At present, there are three extraction techniques used: (1) solvent extraction, (2) supercrtitical fluid extraction, and (3) pressurized liquid extraction (Escribano-Bailon & Santos-Buelga, 2003; Lee, 2004).

2.5.1 Solvent extraction

In this process, a solvent is used to extract the soluble compound through diffusion from the solid matrix (plant tissues) as shown in Figure 2.12. Basically, this process consists of the initial stage and the diffusion stage. The initial stage is the process where the solvent gets into the plant tissue and the cell becomes swollen. The sorption of solvent is caused by osmotic forces, both by capillarity and by solvation of the ion in the cells. In this stage, a certain amount of polyphenols from the damaged cells are extracted directly. Meanwhile, the soluble components are dissolved at the same time. In some cases, the insoluble compounds can be extracted by the hydrolysis process. The diffusion stage may occur in two steps; an internal step within the solid phase and external step through the outer layers surrounding the solid matrix (Escribano-Bailon & Santos-Buelga, 2003).

A dried sample is prefered to the fresh one because the enzyme activity in the fresh material can degrade the phenolic compounds. Heat drying and lyophilization are the most common methods used in the drying process. Before extraction, the sample should be prepared with a certain treatment. The most common techniques in sample preparation are almost always to crush, mill, and macerate or grind the food to increase the sample surface area in order to allow better contact with the extracting solvent (Lee, 2004).

Generally, phenolic compounds are extracted by means of alcohol-water mixtures. During most of the extraction process, a mixture of ethanol (80%) and water (20%) are used. Besides ethanol, methanol can be used to extract the soluble phenolic compounds (Caballero *et al.,* 2003). To obtain satisfactory results, it is necessary to extract the sample several times or reflux for 1 h. Polar flavonoids occurring in the form of glycosides, or bound to some other polar

acyl group, (e.g. organic acid) can sometimes be extracted with hot water but usually, organic solvents are necessary to complete the extraction (Lee, 2004).

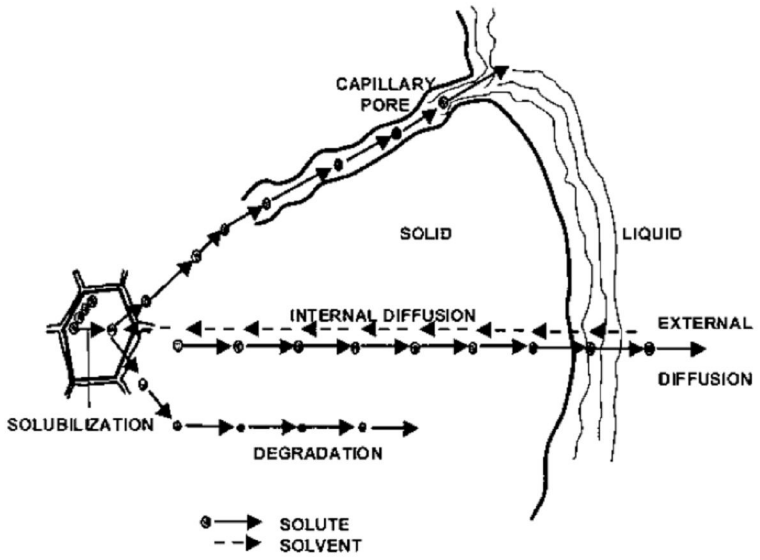


Figure 2.12 Schemes of the main steps in solvent extraction of solid particle

Source: Aguilera (2003)

As in any extraction procedure of sensitive compounds, considerable precautions must be taken to prevent isomerization and oxidation of phenolic acids (Lee, 2004). Polyphenols oxidases are the enzymes that catalyze the oxidation of phenolic compounds into quinines together with subsequent nonenzymatic rapid polymerization. To prevent these enzymatic activities, samples may need to be heated to a temperature of more than 90°C for a few minutes and the pH level needs to be lowered below 4.0 (Caballero *et al.,* 2003).

Besides the conventional methods of maceration/stirring by heat, solvent extraction can be done by ultrasound-assisted extraction, microwave-assisted extraction, or the combination of those three methods. The process of ultrasound-assisted extraction is faster and more complete than conventional methods. This process allows better contact between the solid sample and the solvent because of cell rupturing. The time of extraction depends on sample preparation. Some

complete extractions may be accomplished in 30 minutes. This extraction can also be performed using soxhlet apparatus, thus combining percolation and immersion techniques (Escribano-Bailon & Santos-Buelga, 2003).

The microwave-assisted method (MAE) is a new technique combining microwave and conventional method. Some studies show that MAE needs less time, less solvent, and has a higher extraction rate than conventional methods. Moreover, it is a simple and cheap procedure, so that it can be applied to more materials due to its smaller polarity limitation of extractant (Escribano-Bailon & Santos-Buelga, 2003).

2.5.2 Supercritical fluid extraction

Super critical fluid extraction was developed in the 1960s. Recently, its application has become wider to polyphenol extraction from plant sources. When gas is compressed at a high pressure, it is converted into a liquid. This new form has many properties that offer very attractive extraction characteristics, such as a favorable surface tension, diffusity, viscosity, and other physical properties. These properties improve the extraction rate (Mukhopadyay, 2000). Furthermore, it can reduce any possible degradation process including oxidation and epimerization that may occur during extraction, because it needs less time than conventional method and can also be performed with the absence of oxygen and light (Escribano-Bailon & Santos-Buelga, 2003).

Recently, a continuous extraction process using supercritical fluid extraction (SFE) for phenolics has been evaluated. This method has been reported to successfully extract different classes of compounds from plant materials. Moreover, carbon dioxide is the most common supercritical solvent used in SFE. By varying the CO_2 density, it is possible to selectively extract many different fractions from foods. The limiting property of CO_2 extraction is that it is only capable of dissolving non-polar organic based solute (Lee, 2004).

SFE consists of two steps. In the first step, the phenolic compounds are extracted from the solid matrix after being dissolved in supercritical CO_2. In the second step, the phenolic compounds are trapped in liquid (i.e. methanol) immediately in a process called the trapping step. Many studies have been conducted to optimize phenolic compounds extraction using the SFE method. The parameters needed are CO_2 density, CO_2 flow rate, modifier type, modifier

percentage, extraction temperature and time, trap temperature, trap solid phase, trap rinse solvent, and trap rinse solvent flow rate (Escribano-Bailon & Santos-Buelga, 2003).

2.5.3 Pressurized liquid extraction (PLE)

Recently, this method has been introduced to extract phenolic compounds from plant sources. High temperature and high pressure are used in this technique to accelerate the extraction rate. High pressure allows better contact between the liquid phase and the solid phase (sample), while high temperatures rupture the phenolic matrix bonds. Furthermore, hot solvent can breaks down the cells due to coagulation of the lipoprotein that result in increased permeability of the cell wall. Through the reheating process, the volume of the internal liquid phases increases, leading to increased pressure, which in turn results in centrifugal circulation of the solution through the pores (Escribano-Bailon & Santos-Buelga, 2003).

PLE also offers the capability of conducting extraction in an inert atmosphere and in the absence of light. Since the phenolic compounds are sensitive to oxygen and light, PLE is a suitable method to extract these compounds (Escribano-Bailon & Santos-Buelga, 2003). When using methanol as a solvent in PLE method, the phenolic compounds stabilizes at a range of 40^0C - 150^0C. It has been determined that at a temperature of about 100^0C, the maximum degree of phenolic compound degradation is 10% (Palma *et al.,* 2001).

2.6 Factors affecting the tea extraction process

2.6.1 Temperature and time of extraction

The time and temperature of extraction obviously shows a synergistic effect on polyphenols content. There are many reports about the effect of time and temperature either as individual factors or combinations of both factors on extraction yields and efficiency process. Generally, tea extraction process uses high temperature to achieve high yields and recover flavor components. High temperature increases the extraction rate since heat renders the permeability of the cell wall, improves solubility and the diffusion coefficient of compound to be extracted, and decreases the viscosity of the solvent (Ekayanake *et al.,* 1995; Escribano-Bailon & Santos-Buelga, 2003; Uzunalic *et al.,* 2006).

During green tea extraction with water, there was a sharp rise in the catechins extraction, mainly of EGCG. As the temperature increased from 40^0C to 98^0C, the maximum extraction temperature was achieved at 98^0C in 15 min. EGCG extraction in water for 5, 10 and 15 minutes showed that the amount of EGCG obtained in 5 minutes did not improve further as the extraction time was increased to 15 minutes (Vasisht *et al.,* 2003).

The time of the extraction process affects the quality of the obtained extract. Major catechins extraction reached a maximum level after 20 min at 80°C and 10 min at 95^0C. It then decreases over time. It was found that catechins content increased after a certain time, then remained constant and tended to decrease with the prolonged time. Catechins compounds have a tendency to be degraded at high temperature for a long time period of extraction. In order to prevent catechin degradation during extraction, it is advisable to do extraction at lower temperatures (60^0C or 80°C) for a long time of extraction or higher temperature (95^0C) for a short time of extraction (Uzunalic *et al.,* 2006).

Chemical reactions such as oxidation and epimerization of catechin compounds may occur during the extraction process. Time and temperature in extraction should be carefully controlled to prevent these reactions. As the heating temperature increases, the amount of catechins, such as EGCG, EGC, EC and ECG decreases but the isomers of these catechins, such as GCG, GC, C and CG increases. These results indicate that catechin epimerization takes place under heating conditions (Shahidi & Naczk, 2004; Uzunalic *et al.,* 2006; Kim *et al.,* 2007). In green tea extraction at 100^0C, GCG as the epimer of EGCG was detected. At the same temperature, after 20 min, most of the catechin compounds were epimerized (Row & Jin, 2006). Heating at 100^0C for 3 hours leads to green tea degradation by as much as 25% (Su *et al.,* 2003).

2.6.2 pH of medium

The medium pH determines the degree of solubility of the soluble compounds. The degree of solubility affects the solubilization of phenolic compounds. For example, polyphenols solubilization can be increased by performing the extraction process at low pH with an addition of erythorbic acid, ascorbic acids, or citric acid (Ekayanake *et al.,* 1995; Escribano-Bailon & Santos-Buelga, 2003). Green tea catechins stability is pH dependent. These compounds are very unstable at a pH value higher than 7 and will degrade in a few minutes, but it remains very stable at a pH less than 4 for at least 18 hours (Shahidi & Naczk, 2004).

The mechanism of catechin breakdown under alkaline conditions are still unknown. However it has been demonstrated that at pH level more than 7, catechins tend to form semiquinone free radicals. Addition of ascorbic acids significantly improves the stability of green tea catechins at pH more than 7. Ascorbic acids might protect catechins by recycling the catechin free radical forms or slowing down their oxidation by removing oxygen from the solution (Shahidi & Naczk, 2004).

Monitoring of the EGCG stability as a mixture of polyphenols in tea infusion is studied a using buffer with a different pH. The result indicates that there is a slight degradation of EGCG (around 3%) at pH 4 or below during the first 24 hours. This process is faster in alkaline condition and 65% of the EGCG degrades during the first 48 hours at pH 8. In water extract with pH 5.5, 43% degradation is observed after 48 hours (Vasisht *et al.,* 2003).

Among the four major green tea catechins, EGCG and EGC are the most unstable, while EC and ECG are relatively stable. EGC is almost degraded completely when incubated for 3 hours at pH 7.4, while EGCG is destroyed completely at the end of the 6 hours incubation. Under the same incubation condition, after 3 hours, ECG and EC decrease by 20% and 5% respectively (Su *et al.,* 2003).

2.6.3 Tea-water ratio

The ratio of tea-water affects the quality of the obtained extract. For example, tea concentration may affect the extraction efficiency of green tea catechins. By increasing the amount of solvent, extraction efficiencies and yields of major catechins increase (Yoshida *et al.,* 1999; Pan *et al.,* 2003; Uzunalic *et al.,* 2006).

Various tea-water ratios at 98^0C are used to extract EGCG. Among the ratios (1:10, 1:25, 1:50, 1:100, 1:150, 1:200 and 1:400), the ratio 1:25 to 1:200 give the best result. A tea-water ratio of 1:50 is found to be the most appropriate to extract EGCG (Vasisht *et al.,* 2003).

2.6.4 Number of extraction steps

As a tradition in Japan and some other Southeast Asia countries, people are accustomed to making tea infusions three or four times with the same tea leaves. This ceremony is popularly known as the tea ceremony. The second infusion from this ceremony shows higher levels of catechins especially EGCG, EGC and methyl xanthines, and its contents decreases in the next infusion gradually (Sharma *et al.,* 2005). Application of tea ceremony procedure in the

extraction process could increase the extraction efficiency and the quantity of major catechins in the obtained extract. This method is called a multi-step extraction procedure (Uzunalic *et al.,* 2003).

Bazinet, *et al* (2007) reported that solutions obtained after each extraction step were very different in catechins compositions. In the first extraction step solution, EGC represented 78.9% of the total catechin, and its concentration decreased to 39.5% in the second extraction step solution. However, EGCG content increased in the second extraction step. Its content increased three times, while EGC content decreased approximately by one-third. These results emphasize the fact that it is possible to obtain green tea extract rich in EGCG by brewing the tea in a two-step extraction. This study was conducted based on their previous research. The authors revealed that the best combination for EGC and EC extraction was 50°C over 20-40 minutes, while for EGCG, GCG and ECG extraction was 90°C over 80 minutes.

2.7 Evaporation process

The concentration process is an operation used to concentrate some solute that dissolved in solution. An amount of solvent is separated to produce a concentrated and viscous solution. Different from drying, the concentration process resulted in a flowing liquid instead of a solid (Billet, 1989).

With an effective extraction process, tea extract can be produced to contain about 5% soluble tea matter and 95% water. The next step mainly removes water. It is important to again consider time and temperature. Normally, the process of water removal should be done in the shortest time and at the lowest temperature to prevent degradation that may occur during the process. However, if the temperature used is low, a long time would be needed to drive off the water and produce the concentrated product. There are three main methods used to separate water from tea to produce concentrated extracts: (1) evaporation of water by application of heat, (2) freeze concentration where extract is partly frozen and the ice formed and separated, and (3) membrane technology where water passes through special membranes leaving tea solids behind (Hampton, 1999).

Basically, evaporation is performed to separate liquid mixtures by the application of heat. Some solids dissolved in a liquid can be separated simply by heat to expel the solvent, merely because its vapor pressure is negligible compared to that of the solvent at the evaporation temperature. If a liquid with high temperature is flashed to a pressure lower than that at the boiling point, the solvent will evaporate. This principle is exploited in practice for evaporating a mixture, which has heat sensitive compounds (Billet, 1989).

There are some factors to be considered in evaporation (Glover, 2004) such as:

2.7.1 Heat sensitivity. Many foods, chemicals, pharmaceuticals and other materials are heat sensitive and need a low heating temperature, short time period or the combination of each. These can be achieved by minimising the volume of the product, minimising evaporation time, and performing the process at lower pressure.

2.7.2 Foaming. Vaporization may result in product foaming. The foam can be unstable, and break easily or stable foam, which is hard to break and tends to fill the entire evaporator system.

2.7.3 Solid. As the solid concentration increases, the properties of the concentrate may change.

2.7.4 Viscosity. Increasing the viscosity of the concentrate can reduce the overall heat transfer coefficient.

2.8 Fractionation process

Fractionation is generally used to purify the crude extracts. Fractionation necessarily varies according to the type of food matrix to be analyzed. It is a critical part of a method to remove the potential interfering components. Phenolic compounds fractionation or purification process includes the liquid-liquid partitioning with a nonmiscible solvent. It also includes an adsorption method with sephadex LH-20, polyamide, amberlite XAD-2, nylon, polyamine, cellulose, preparative HPLC and another suitable adsorbent. Finally it also has a solid-phase extraction, which uses commercially available disposable cartridges. Some studies have been

published about using another method to fractionate or purify phenolic compounds, such as membrane separation and supercritical carbon dioxide (Lee, 2004).

2.8.1 Adsorption method

1. Principle of the adsorption method

Adsorption process is used to separate amounts of solute in mixtures, while certain components are adsorbed or accumulated selectively on the surface of the adsorbent. Commonly, the small particles of adsorbents are placed in a fixed bed and certain fluids flow continuously through the bed until the adsorbent is nearly saturated and the desired separation can no longer be achieved (McCabe *et al.,* 2005; Smith, 2005).

Mostly, adsorbents are highly porous materials, and so the adsorption process occurs on the surface of the pores or at specific sites inside in its particles. Separation is achieved due to the differences of molecular weight, shape, or polarity. Therefore, some molecules are more strongly attached on the surface than others, or because the pores are too small to allow the larger molecules to be attached (McCabe *et al.,* 2005). Generally, an adsorbent can hold some molecules strongly enough to reach complete separation of that component from the mixture (Smith, 2005).

Adsorption has several advantages compared to other methods. The adsorption method can be applied when distillation is hard or impossible to separate compounds that have similar boiling points. It can separate a high loading of solute and work well for dilute systems. Besides, it usually requires low energy for operation (McCabe *et al.,* 2005). Due to these advantages, the adsorption method is widely applied in chemical process industries such as in the gas-drying process, the removal of undesired products, the gas separation, the purification, and so on. However, the adsorption process has some drawbacks. Each adsorbent bed must be regenerated after each process, thus requiring more columns in the series. The regeneration treatment results in some losses of adsorbent material, and it takes a long time (Smith, 2005).

There are some factors affecting the capability of the adsorption method (Jindaratsamee, 2006) such as:

1) Bed height. If the fixed bed is short, the break point time is small. Moreover, if the height of the bed is shorter than that of the mass-transfer zone, the molecules are not adsorbed.

2) Inlet concentration (feed concentration).

3) Volumetric flow rate. When the volumetric flow rate is higher, the period that the mixture is inside the bed is reduced. Therefore, the efficiency of adsorption is also reduced.

4) Type of adsorbents. Effective adsorbents should be able to adsorb a lot of desired molecules.

5) Flow direction.

6) Pressure and temperature. Both temperature and pressure are very important for column operation.

2. Elution development in the adsoprtion method

Elution development is best described as a series of adsorption-extraction processes, which are continuous from the time the sample was injected into the system until the solute is eluted out from the adsorbent. Solute molecules will only leave the stationary phase when their kinetic energy is equal to or greater than the potential energy of their association with the stationary phase (Scott, 1995).

Elution development can be modified in a number of ways. In some chromatographic system, such as in liquid and gas chromatography, the column temperature can be raised progressively during the elution process. As the temperature is raised, an increasing number of the solute molecules in the stationary phase randomly acquire sufficient energy to leave the stationary phase (Scott, 1995). The other alternative is to use a gradient elution, where the composition of the mobile phase is continuously changed during the elution development. In gradient elution, the concentration of the stronger eluting solvent, or solvents in the mobile phase is increased continuously during development. This results in increasing the probability of interaction with the solutes. If the solute molecules interact more frequently with the stronger eluting solute, they will be held more strongly in the mobile phase and thus be eluted more rapidly (Scott, 1995). In most adsorption systems, there is an appreciable interaction between the compounds and stationary phase. This interaction can be conceptualized as a partition. As long as the interaction is relatively weak, elution can be accomplished under isocratic conditions. However, if the interaction is strong, gradient elution may be required (Swadesh, 1997).

3. Application of the adsorption method on polyphenols separation

Many types of adsorbents have been applied to fractionate polyphenol compounds. Very useful applications of amberlite XAD-2 have been developed for flavonoids from grape seed extracts and fruit jams. The flavonoid compounds remain in the XAD-2 resin column while sugars, pectins, and other polar compounds are eluted with the aqueous solvent. The flavonoid fractions can then be eluted with MeOH after washing the column with water (Lee, 2004). For fruit products such as orange, grape and lemon, ionic adsorbents (polyamide, polyvinylpyrrolidone, sephadex and nylon) are used to isolate and purify the various phenolic compounds (Lee, 2004).

For tea extracts, a two step separation process is necessary, involving open-column chromatography on sephadex LH-20 to retain the various phenolic compounds, while the sugars and other polar compounds are eluted with the aqueous solvent followed by further fractionation of the flavonoids by polymeric adsorbents on an XAD-7 column. Aqueous methanol is often used as the eluent for phenolics from column chromatographs, but selective elution for flavonol and theaflavin can be accomplished by varying the concentration of acetone from 5% to 10 % in ethanol (Lee, 2004).

The effectiveness of adsorption of tea polyphenol and caffeine with polyamide resin has been studied. The ability of polyamide to adsorb tea polyphenol is stronger than caffeine. Hydrogen bond plays a very important role in this process. Tea polyphenols and caffeine are separated successfully with polyamide resin, and the obtained fractions comprise more than 96% of tea polyphenol and 80% of EGCC with less than 2.8% of caffeine (Tang *et al.,* 2003).

2.8.2 Solid-phase purification method

A solid-phase extraction (SPE) using small disposable cartridges is another efficient sample separation method. Since the introduction of disposable SPE cartridges (small packed chromatography columns) with HPLC packing, SPE has become the preferred method for purifying crude extracts. The full range of silica-based polar and nonpolar stationary phases in small cartridges in a flat matrix is commercially available. The SPE on C18-bonded phase is widely used for isolating phenolics, replacing the use of PVPP or sephadex for purification steps (Lee, 2004).

Solid-phase extraction separation procedures for phenolics have been widely applied for various food products such as kiwi juice, pineapple juice, berries and berry jams, apples and pears, green tea, wine, olive and olive oils, and soy products (Lee, 2004). These methods can also further provide the fractionation of grape phenolics into acidic and neutral groups, select extraction of phenolic compound, and fractionate of the grape catechins and oligomeric proanthocynidins. Separation of free phenolic acids can also be achieved using an anion-exchange cartridge (Escribano-Bailon & Santos-Buelga, 2003).

2.9. Freeze drying

Basically, drying is a process of thermally removing moisture to yield a solid product. Industrial dryers have a difference in type and designs depending on the principal method of heat transfer employed. Mostly, heat is transferred to the surface of the wet solid and then to the interior. However, in dielectric, radio frequency, microwave and freeze drying, energy is supplied to generate heat internally within the solid and then flows to the exterior surfaces (Mujumdar & Menon, 1995).

A large variety of drying equipment is currently available from manufacturers. Several types of drying equipment are vacuum dryers, fluidized-bed dryers, bands (belt dryer), spray dryers, flash dryers, fluid-bed dryers, and miscellaneous dryers. There are also special drying techniques such as infrared drying, freeze drying and microwave drying (Land, 1991).

Freeze drying has been used for many years in many applications, but mainly in the food and pharmaceutical industries. This process has been developed to overcome the loss of heat sensitive compounds, which may degrade during conventional drying operations (Barbosa-Canovas & Vega-Mercado, 1996). Recently, this method has many other uses including the stabilization of living materials such as microbial cultures, preserving of whole animal specimens, and drying plasma and blood products, antibiotics and other biological material (Anonymous, 2004).

Freeze dried products can be stored for an unlimited period of time, and do not need refrigeration. They retain most of the physical, chemical, biological properties of their initial

state. The removal of water or other solvents in freeze drying is called sublimation. Sublimation occurs when a frozen liquid is converted to its gaseous state without passing through the liquid phase (Barbosa-Canovas & Vega-Mercado, 1996; Anonymous, 2004).

The freeze drying process consists of three main steps: prefreezing, primary drying and secondary drying. Prefreezing is used to reduce the freeze drying time cycle. Before freeze drying, the material should be adequately frozen. Once the material is frozen, the freezing conditions are maintained by a refrigeration system until the product is ready for drying. The prefreezing method and the final temperature of the frozen product can affect the effectiveness of freeze drying (Barbosa-Canovas & Vega-Mercado, 1996; Anonymous, 2004).

The primary drying step involves ice sublimation under vacuum conditions. When the energy for the latent heat is transferred, ice is sublimated, resulting in a dry, structurally intact product. The ice sublimation rate depends on the difference in vapor pressure of the product compared to the vapor pressure in the ice collector (Barbosa-Canovas & Vega-Mercado, 1996; Anonymous, 2004). After the primary drying step is complete, and all the ice has sublimated, bound moisture is still present in the product. At this time the heating rate must decrease in order to maintain the temperature of the product ranging from 30^{0}C to 50°C. The secondary drying step is aimed to desorb the moisture from the internal surface within the dried product (Barbosa-Canovas & Vega-Mercado, 1996; Anonymous, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw materials

1. Assam green tea (*Camellia sinensis* var. *assamica*) from Chiang Rai Province, Thailand. The green tea was ground and sieved to pass completely through a sieve of aperture 500 μm (ISO 1572:1975). The ground green tea was packed in vacuum packaging and in an airtight container. It was stored at room temperature in a cool and dry place.

2. Green tea extract powder and residual green tea extract powder were provided by the Thailand Research Fund, Thailand.

3.1.2 Chemicals

1. Chemical for processing

1) Amberlite XAD7HP (analytical reagent, Sigma, France)
2) Citric acid (analytical grade, Fischer, Germany)
3) Ethanol (commercial grade, Sophorn laboratory, Thailand)
4) Nylon 6 (analytical reagent, Fluka, Switzerland)
5) Polyamide 6 (analytical reagent, Fluka, Germany)
6) Poly (dimmer acid-co-alkyl polyamine) (pro analysis, Aldrich, USA)

2. Chemicals for analysis

1) Acetonitrile (HPLC grade, Merck, Germany)
2) Acetone (analytical reagent, Merck, Germany)
3) Caffeine anhydrous (analytical reagent, Sigma, Germany)
4) Folin ciocalteu (analytical reagent, BDH, England)
5) Gallic acid (analytical reagent, Sigma, Germany)

6) Hydrochloric acid (analytical reagent, JT.Baker, England)

7) Lead (II) acetate trihydrate (analytical reagent, Carlo Erba, Italy)

8) L-Theanine (analytical reagent, Sigma, Germany)

9) Methanol (analytical reagent, Carlo Erba, Italy)

10) Nynhydrin (analytical reagent, Univar Ajax Fine Chem, Australia)

11) Phosporic acid (HPLC grade, Merck, Germany)

12) Polyvynilpyrrolidone (analytical reagent, Sigma, USA)

13) Potassium dihydrogen phosphate (analytical reagent, Univar Ajax Fine Chem, Australia)

14) Standard substances: EC, EGC, ECG, EGCG, CG, GC, GCG, caffeine (HPLC grade, Merck, Germany)

15) Sodium carbonate (analytical reagent, Univar Ajax Fine Chem, Australia)

16) Sodium hydroxide (analytical reagent, Carlo Erba, Italy)

17) Sodium phosphate (analytical reagent, Univar Ajax Fine Chem, Australia)

18) Sulfuric acid (H_2SO_4) (analytical reagent, Carlo Erba, Italy)

19) Tannic acid (analytical reagent, Carlo Erba, Italy)

20) Trifluoroacetic acid (HPLC grade, Merck, Germany)

3.1.3 Equipment and apparatus

1. Processing equipment

1) Tea extractor model unit for kinetic study in the pilot plant scale (designed by Wongsuwan, 2008)

2) Analytical balance BT 224 s (Sartorius, Thailand)

3) Hot plate SLR (Schott instrument GMBH, Germany)

4) pH-meter pH510 (Eutech instrument, People's Republic of China)

5) Freeze dryer (Heto Drywinner, France)

6) Rotary evaporator (Eyela, Japan)

7) Waterbath WB22 (Memmert, Germany)

2. Analysis instruments and glasswares

1) Hot air oven UNB (Memmert, Germany)

2) High Performance Liquid Chromatography (Alltech, USA and Agilent technology, USA)

3) Microwave moisture analyzer (Sartorius, Thailand)

4) Spectrophotometer UV-9200 (Ray-Leigh, People's Republic of China)

5) TLC set (TLC aluminium sheet and UV lamp)

6) Vacuum pump and filtering flask

7) Glasswares

3.2 Methods

3.2.1 Chemicals analysis

1. Chemicals analysis of raw material and green tea extract

Analysis of moisture content, total polyphenols, tannin, caffeine, total amino acids, individual catechins (EGCG, EGC, ECG, EC, GCG, GC, CG, C and G) and theanine of raw material were measured to identify whether the raw materials were suitable for the research and to compare it with the obtained extracts. The detail of the analysis method is shown in Appendix A.

1) Moisture content (gravimetric method) based on ISO 7513:1990

2) Total polyphenols content (colorimetric method) based on ISO 14502-1:2005

3) Tannin content (gravimetric method) based on FAO/IAEA: 2000

4) Total amino acids content (colorimetric method) based on Yoa *et al*: 2006

5) Caffeine content (colorimetric method) based on Yoa *et al*: 2006

6) Individual catechins (EGCG, EC, ECG, EGC,CG, GC and GCG), caffeine and theanine (HPLC method)

2. Chemicals analysis of freeze dried extract

Moisture content for freeze dried fractions was measured by using a microwave moisture analyzer, while analysis of caffeine, theanine and individual catechins for the fraction was found by using the same method as described in 3.2.1.1.

3.2.2 Study the optimum condition of extraction in the laboratory scale

1. Effect of pH and tea-water ratio

As described in Chapter 2, tea-water ratio and pH affect the extraction of green tea compounds. In order to obtain the high quality of green tea extract, these factors were optimized using fixed time and temperature. In this study, tea water-ratio were varied to 1:5, 1:10, 1:20, 1:30, while the pH of water was adjusted to 4, 5, 6 and 7 with the addition of citric acid.

1) Green tea extraction

Green tea was extracted with a certain ratio as described above. Extraction was performed at 95^0C for 10 minutes in a thermostated waterbath (time and temperature used according to Uzunalic *et al.,* 2006).

2) Chemicals analysis

The aqueous extract was separated from solid green tea residue to get the filtrate. Total polyphenols content, tannin content, caffeine content and total amino acids content of the filtrate were then analyzed (using the colorimetric procedure as described in 3.2.1.1).

3) Statistical analysis

All data from the experiment were subjected to analysis of variance (ANOVA). Mean different analysis was conducted by Duncan's multiple-range test in order to obtain the optimum of treatment combinations. Analysis was performed by using Statistical Package for the Social Sciences (SPSS 16.0 for windows, SPSS Inc, Chicago, IL) (Kirkpatrick & Feeney, 2003). The optimum combination (tea-water ratio and pH) was used to study the effect of multi-step extraction.

2. Effects of multi-step extraction

As described in Chapter 2, the number of extraction steps affects the quality of the extracts obtained as well as increases efficiency. This study was conducted in order to observe the number of steps required for extracting all of the green tea compounds and improve the extraction efficiency. In this study, the extraction was performed in four steps.

1) Green tea extraction

The extraction was performed using the best tea-water ratio and pH as noted from the previous study. The green tea extraction procedure was similar to the procedure described in 3.2.2.1. The residue from the first extraction step was extracted again under the same conditions. This extraction was repeated four times.

2) Chemicals analysis

The four subsequent extracts from this study were then analyzed for their total polyphenols content, tannin content, caffeine content and total amino acids content (using the procedure as described in 3.2.1.1.

3) Determination of percent yield

Percent yield shows the ability of the process to extract all of the compounds of green tea in high amounts. The percent yield is calculated by comparing the amounts of a compound in a raw material and its obtained extract by using the following formula:

$$\% \text{ yield} = \frac{\%\text{w/w of compound in green tea extract}}{\%\text{w/w of compound in raw material}} \text{x100 \%} \quad (3.1)$$

4) Statistical analysis

All data from the experiment was subjected to analysis of variance (ANOVA) as described in 3.2.2.1. The mean different analysis was conducted by Duncan's multiple-range test in order to decide the number of steps considered necessary to achieve the objective of this study.

3.2.3 Kinetic study in the laboratory scale

Time and temperature affects the quality of the obtained extract from green tea. In this study, time and temperature of extraction were varied. Green tea was extracted at five different temperatures (50°C, 60°C, 70°C, 80°C and 90°C) for 1, 3, 5, 10, 20, 40 and 80 minutes.

1. Green tea extraction

The extraction was performed using the best tea-water ratio and pH from 3.2.2.1. This process was conducted in varying time and temperatures as described above. During the extraction process, a stable temperature was maintained.

2. Chemicals analysis

The aqueous extract was separated from the solid green tea residue to get the filtrate. The total polyphenols content, tannin content, caffeine content, total amino acids content

and individual catechins (EGCG, EGC, ECG, EC, GCG, GC, CG, C and G) content were analyzed (using the procedure as described in 3.2.1.1).

3. Determination of rate constant

The reaction rate constant and reaction order were measured by using Spiro's steady state kinetic model in the following equation. The graph of ln (C_∞/ (C_∞-C)) versus time was plotted with a slope as a reaction rate constant (Jaganyi and Price, 1999).

$$\ln\left(\frac{c_\infty}{c_\infty - c}\right) = k_{\text{obs}}t + a \qquad (3.2)$$

Where:

C : the concentration value at time t (% w/w of dried basis)

C_∞ : its concentration value when the equilibrium is reached (% w/w of dried basis)

k_{obs} : an observed first order rate constant (minute^{-1})

t : extraction time (minute)

a : the intercept

3.2.4 Kinetic study in pilot plant scale

It is necessary for scaling up the laboratory process to a pilot plant scale so that the laboratory results will be more applicable. The time and temperature of green tea extraction in pilot plant were varied. In this study, green tea was extracted at temperatures of 70°C, 80°C and 90°C for 1, 3, 5, 10, 20, 30, 40, 60, 80 and 120 minutes.

1. Green tea extraction

In this experiment, green tea was extracted using an extractor model unit designed by Jiraborn Wongsuwan (2008) as shown in Figure 3.1. The detail of this extractor unit was shown in Appendix D. The extraction was performed by using the optimum pH from 3.2.2.1 with a tea-water ratio 1:28 (250 grams of green tea with 7 liters of water) and at a flow rate of 1kg/s. The tea-water ratio and flow rate used in this study had been adjusted to the extractor unit specification and capacity.

2. Chemical analysis

The obtained extracts were then analyzed for total polyphenols content, tannin content, theanine content, caffeine content and individual catechins (EGCG, EGC, ECG, EC, GCG, GC and CG) content using the procedure as described in 3.2.1.1.

3. Determination of rate constant

The rate constant and reaction order were calculated using the same formula as described in 3.2.3.

Figure 3.1 Tea extractor model unit

Source: Wongsuwan (2008)

3.2.5 Fractionation of green tea extract

1. Preliminary study

1) Selection of eluting agent

The aim of this study was to determine the best eluting agent to separate green tea compounds in the column chromatography. This study was conducted by using a TLC aluminium sheet with silica gel as the adsorbent (Figure 3.2).

a) Sample preparation

Green tea extract powder, pure caffeine and pure catechins (EGCG, ECG, EGC, EC and C) were used as the material for developing in TLC. A small amount of each material was dissolved in a small amount of acetone forming a concentrated solution.

b) TLC development

This step was performed by making a small spot of all materials on the base line of the TLC sheet and then putting the sheet in the TLC tank containing the solvent. Water-ethanol mixtures in various ratios (0%, 25%, 50%, 75% and 95% ethanol in water) were used as solvents in the TLC development. The TLC development was completed after the solvent reached about 0.5 cm from the upper side of the sheet. The TLC sheet was then visualized under a UV light. The eluting agents that could separate green tea extract compounds properly were then used as the eluting agents in the study.

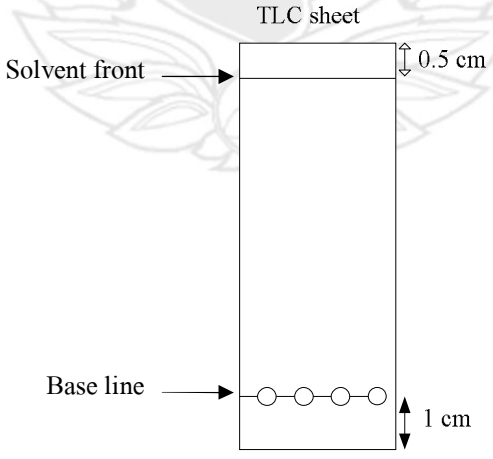


Figure 3.2 TLC Aluminium Sheet

c) Determination of retention factor (Rf)

The position of any solute spot in TLC is characterized by the retention factor (Rf). It was calculated by the following formula (Scott, 1995):

$$Rf = \frac{\text{distance between the center of spot and the base line (cm)}}{\text{distance of the solvent front from the base line (cm)}} \quad (3.3)$$

2) Effect of resin type and its packed bed height

This study was performed to determine the optimum resin and packed bed height for total polyphenols and caffeine separation. Various resins including polyamide 6, amberlite XAD 7HP, Poly (dimmer acid-co-alkyl polyamine) and nylon 6 were used as the stationary phase. Each resin was packed into two different size columns (20g for each column). The difference on column size affects the difference of packed bed height.

a) Column preparation

It was necessary to check the stopper to ensure it worked properly. Cotton was put on the bottom side of the column. Each resin was mixed with a sufficient amount of distilled water and then filled into the column (shown in Figure 3.3).

b) Sample preparation

Green tea extract solution was used as a sample in this study. Green tea extraction was conducted using the optimum condition from 3.2.2.1. Green tea was extracted using water at pH 5 (adjusted with citric acid) at a tea-water ratio of 1:20. The obtained green tea extract solution (10 ml) then evaporated using a rotary evaporator. This process was performed to concentrate the solution of green tea extract.

c) Fractionation procedure

The sample (evaporated green tea extract solution) was loaded into the column gradually. It was important to make sure that the entire sample was already applied through the column. The next step was to elute the column with the selected mobile phase (water and water-ethanol mixture) from 3.2.5.1.1). Fractions were collected for every 15ml. These fractions were picked randomly and then analyzed for the contents of total polyphenols and caffeine (using procedure as described in 3.2.1.1.).

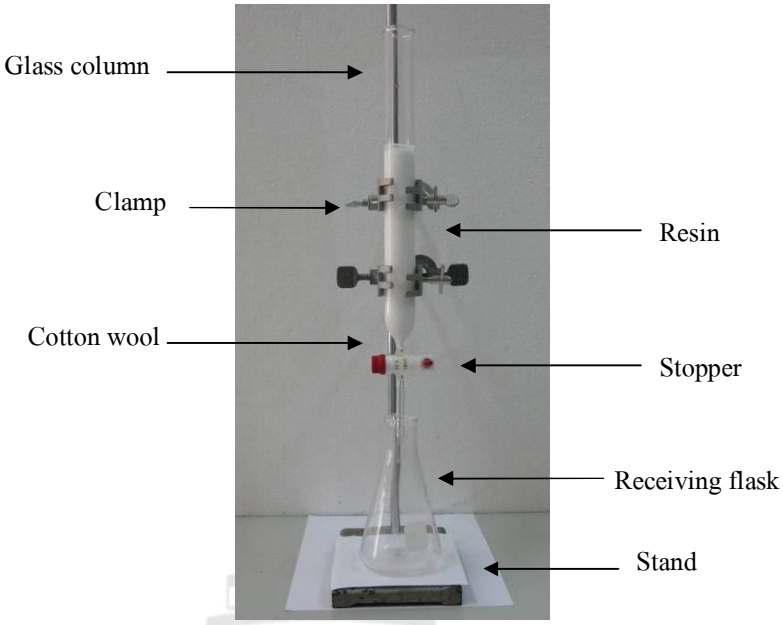


Figure 3.3 Column chromatography

3) Effect of elution system (isocratic and gradient) of water-ethanol mixture

The optimum resin and its packed bed height for total polyphenols and caffeine separation from a previous study were used to study the effect of an elution system of a water-ethanol mixture. Same with the previous study, water was used as the first mobile phase to elute the caffeine compound, while the content of total polyphenols was eluted by a water-ethanol mixture. In an isocratic system, 50% of ethanol was used directly after water elution, while in a gradient system, the ethanol concentration was increased gradually, starting with 10% ethanol. The procedure of column preparation, sample preparation, fractionation procedure and chemical analysis used in this study was described in 3.2.5.1.2).

2. Fractionation of green tea extract powder

1) Fractionation of residual green tea extract

The best resin and its packed bed height as well as the elution system from 3.2.5.1 was then used to fractionate residual green tea extract powder. This material was used in

this study to increase the concentration of each compound that was consisted in the sample loaded in the column chromatography. Before using it as the sample, the content of caffeine, theanine and individual catechins of green tea extract powder was analyzed (using the procedure as described in 3.2.1.1.

a) Fractionation procedure

The residual green tea extract powder (5g) was dissolved in water and then subjected gradually into the column. This process used the same procedure of column preparation in 3.2.5.1.2). In this study, fractions were collected every 5 ml in glass vials. These fractions were picked randomly and then analyzed for the content of total polyphenols, caffeine, theanine as well as individual catechins (using the procedure as described in 3.2.1.1). All of the fractions were combined and then divided into three main groups based on the caffeine concentration. These three main fractions were then analyzed with the same analysis and procedure.

b) Determination of percent of eluted compounds

$$\% \text{ eluted (w/w)} = \frac{\text{weight of a compound in fraction}}{\text{weight of a compound in raw material}} \text{x} 100\,\% \quad (3.4)$$

2) Fractionation of green tea extract

This study was performed with the same procedure in 3.2.5.2.1). Green tea extract powder was used as the sample instead of residual green tea extract due to its better quality. Based on the trend described from 3.2.5.2.1), the fractions were collected every 50 ml. All of the fractions were then divided into three main groups based on caffeine content. The chemical analysis and determination of eluted compounds percentage conducted in this study is same as described in 3.2.5.2.1).

a) Freeze drying

The three main fractions were then dried using a freeze dryer. Two of the combined fractions eluted by a water-ethanol mixture were evaporated until only half of the volume remained to remove the ethanol before they were subjected into the freeze drying process. The freeze drying process was conducted at temperatures less than -40^0C (condenser) and 20^0C

(the shelf) for about 20 hours. The moisture content, tannin content, caffeine content, theanine content and individual catechins content of the resulted dried fractions were then analyzed.

b) Determination of percent yield

Percent yield shows the ability of the fractionation process to purify the compounds of green tea in high amounts. The yield determined for individual catechins, theanine and caffeine. It is calculated by comparing the amount of a compound in raw material and obtained freeze dried extract by using the following formula:

$$\% \text{ yield} = \frac{\%\text{w/w of compound in freeze dried extract}}{\%\text{w/w of compound in raw material}} \text{x} 100\,\% \qquad (3.5)$$

3) Regeneration treatment of polyamide 6 used in green tea extract fractionation

To increase the amount of freeze dried extract as well as to improve the efficiency of the fractionation process, the fractionation of green tea extract was repeated four times using the same resin from previous fractionation processes. The resin from the fractionation process in 3.2.5.2.2) was subjected into regeneration treatment (the regeneration procedure is shown in Appendix E) and then was used to fractionate green tea compounds using the same sample, procedure (fractionation and freeze drying), and chemical analysis.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Chemical compositions of raw material

Table 4.1 shows chemical compositions of green tea used as raw materials in this study (laboratory and pilot plant scale) and also chemical compositions of green tea as studied from the literature. The chemical compositions were different to each other. There are many factors affecting the difference of green tea chemical compositions, such as season of harvesting, leaf age, the manufacturing process, and so on (Baptista *et al.,* 1999; Shahidi & Naczk, 2004; Uzunalic *et al.,* 2006).

Commonly, the moisture content of green tea is below 6%, and the moisture content of the green tea sample in this study were in the range of that value (2.378 and 4.951%). The content of total polyphenols, tannins, total amino acids and caffeine of green tea samples both for study in laboratory and pilot plant scale were 20.710 and 18.709%, 10.604 and 9.153%, 6.012 and 6.036%, 4.612 and 1.636% of dry weight, respectively. Compared to the references, these values were in the range of the common values from green tea compounds. Phenolics compounds in green tea ranges from 11% to 20%, while the amount of caffeine and total amino acids in green tea varied from 1% to 4% and 2% to 6% of dry weight, respectively.

Table 4.1 Chemical compositions of green tea

Chemical properties	% w/w Dry basis		
	Raw material for laboratory scale study	Raw material for pilot scale study	Green tea (from literatures)****
Moisture content	2.378 ± 0.054	4.951 ± 0.141	< 6[(c)]
Total polyphenols content	20.710 ± 0.569	18.709 ± 1.285	11-20[(b,d)]
EGCG	0.676 ± 0.027**	2.579 ± 0.660**	3-10[(b,e,f)]
EGC	4.590 ± 0.438**	2.366 ± 0.208**	1-3.5[(b,e,f)]
ECG	2.646 ± 0.069**	nd	1-2.5[(b,e,f)]
EC	2.613 ± 0.257**	4.680 ± 0.588**	0.4-1[(b,e,f)]
GCG	nd*	nd	0.2-1.5[(e,f)]
GC	3.493 ± 0.240**	1.119 ± 0.136**	0.17-0.24[(e)]
CG	nd	nd	0.02-0.03[(e)]
C	2.122 ± 0.552**	-	0-0.08[(e,f)]
G	1.245 ± 0.070**	-	0.12-0.4[(g)]
Total of catechins	17.386 ± 0.902***	10.744 ± 1.592***	10-18[(b,d,e,f)]
Tannin content	10.604 ± 0.471	9.153 ± 0.293	2.5-20[(a,d)]
Total Amino acids content	6.012 ± 0.086	6.036 ± 0.097	2-6[(b)]
Theanine	-	6.717 ± 2.447**	1-2.5[(b)]
Caffeine content	4.612 ± 0.404**	1.636 ± 0.142**	1-4[(b,d)]

* not detected, ** HPLC analysis, *** Sum of individual catechins

**** Source: Savolainen, 1991[(a)]; Saijo, 1999[(b)]; Wilson, 1999[(c)]; Chen *et al.,* 2002[(d)]; Wang *et al.,* 2003[(e)]; Peterson *et al.,* 2005[(f)]; Sharma *et al.,* 2005[(g)]

According to the literature, tannin content in green tea has a wide range (2.5-20%). This content presumably depends on the analysis method. Due to its complexity, several methods have been developed for tannin quantification. Some of them resulting in tannin underestimation, while the others resulting in overestimation. Generally, tannin quantification can be divided into: (1) colorimetric assay, such as folin-ciocalteu, vanillin-HCl, butanol-HCl, rhodanine, and Wilson

and Hagerman assay, (2) gravimetric assay, such as the gravimetric method with ytterbium and PVP/PVPP, and the gravimetric method based on the detergent system, (3) protein precipitation assay, such as radial diffusion assay, and (4) mixed assay, which combines some previous methods (Cannas, 2001). The tannin analysis used in this study was gravimetric method with PVPP. PVPP irreversibly binds tannin in the sample. With this method, tannin was calculated as the difference between total polyphenols before and after PVPP additions (Cannas, 2001; FAO/IAEA, 2000).

Individual catechins were also determined. The content of EGCG, EGC, ECG, EC, GC, C and G of the sample for the laboratory scale study were 0.676, 4.590, 2.646, 2.613, 3.493, 2.122 and 1.245%, respectively. Meanwhile the content of EGCG, EGC, EC, and GC of the sample for the pilot plant scale study were 2.579, 2.366, 4.680 and 1.119%, respectively. The total catechins (sum from individual catechins) of the sample for the laboratory and pilot plant scale study were 17.386 and 10.744%. These values were in the range of total catechins obtained from the literature (10-18%).

4.2 Study of the optimum condition of extraction in the laboratory scale

4.2.1 Effect of pH and tea-water ratio

The extraction was performed in a waterbath at 95^0C for 10 minutes. The temperature and time used was based on the best condition for green tea extraction from Uzunalic *et al* (2006). The authors stated that the maximum extraction efficiency of catechins was obtained at 80°C for 20 minutes and at 95^0C for 10 minutes. Degradation of catechins were discovered when the extraction was conducted at high temperature and long extraction time.

This study investigated the effect of pH and tea-water ratio on total polyphenols, tannin, total amino acids and on the caffeine contents from the green tea extraction process. The optimum extraction conditions in this study were considered to be the extraction conditions that provide the maximum total polyphenols content and the minimum tannin content. All of the parameters (total polyphenols, tannin, total amino acids, and caffeine content) during extraction were analyzed by the colorimetric method. From the results, it can be concluded that all of the

parameters were significantly affected ($p \leq 0.05$) by pH and tea-water ratio described in the following parts.

1. Effect on total polyphenols (expressed as gallic acids) extraction

Total polyphenols contents extracted from green tea can be seen on Table 4.2. Both factors (tea-water ratio and pH) were affecting the amount of total polyphenols in the extracts. Total polyphenols in the obtained extracts were in the range of 7.114% - 10.837% green tea in dry basis. The highest amount of total polyphenols was obtained by the extraction with tea-water ratio 1:30 at pH 4, while the lowest amount was obtained using a tea-water ratio1:5 at pH 6 and 7.

Table 4.2 Total polyphenols (expressed as gallic acid) contents of green tea extracts extracted with various tea-water ratio and pH

Total polyphenols contents (%w/w dry basis)				
Tea-water ratio	**pH**			
	4	**5**	**6**	**7**
1:5	$8.368 \pm 0.046^{aB*}$	8.408 ± 0.011^{aB}	7.140 ± 0.011^{aA}	7.114 ± 0.011^{aA}
1:10	9.520 ± 0.027^{bC}	9.510 ± 0.037^{bC}	9.071 ± 0.174^{bB}	8.543 ± 0.009^{bA}
1:20	10.820 ± 0.104^{cC}	10.783 ± 0.051^{cC}	10.165 ± 0.009^{dB}	9.457 ± 0.027^{dA}
1:30	10.837 ± 0.027^{cC}	10.805 ± 0.265^{cC}	9.941 ± 0.041^{cB}	8.973 ± 0.060^{cA}

*Different lower-case letters in the same column indicate significant difference ($\alpha = 5$ %) among tea-water ratio, and different capital letters in the same row indicate significant difference ($\alpha = 5$ %) in pH

Increasing tea-water ratio tended to give better results in terms of higher contents of total polyphenols. With varying tea-water ratio (1:5, 1:10, 1:20 and 1:30), ratio 1:20 and 1:30 have a better result than other ratios. The same result was reported by Pan *et al* (2003),

tea water ratio 1:20 was sufficient to reach the high extraction yield. By increasing the ratio of material to solvent, the extraction efficiency and amount of phenolic compounds was increased (Uzunalic *et al.,* 2005), which is consistent with the findings in Vasisht, *et al* (2003).

Since polyphenols are located in the vacuoles of the cells, polyphenols extraction occurrs through rupturing the tea plant tissues and through the diffusion process. In tea-water ratio 1:20 and 1:30, the amount of water is sufficient to get into the tea plant tissues, and to affect the permeability of the cell wall and the membrane. At this stage the polyphenols were extracted into water (Escribano-Bailon & Santos Buelga, 2003; Vasisht *et al.,* 2003).

In the same tea-water ratio treatment, pH 4 and 5 provided higher amounts of total polyphenols than that in pH 6 and 7. Generally, some phenolic compounds are very sensitive to light and oxidation. They are subjected to isomerization and degradation during processing, acids can help to prevent these reactions (Lee, 2004). Moreover, addition of acids can prevent enzymatic oxidation from polyphenol oxidases, which catalyze the oxidation of phenols to quinones with subsequent nonenzymatic rapid polymerization. Because phenolic acids usually occur in conjugated forms and are seldom found in free state, it is necessary to do hydrolysis. Acid hydrolysis has the purpose of rupturing glycosidic linkages to release any bound phenolics (Lee, 2004). Therefore, in this study, decreasing the pH of the extractant (water) tended to increase the polyphenols contents of the obtained extracts.

From Table 4.2, it can be seen that extraction results in tea-water ratio of 1:20 and 1:30 at pH 4 and 5 were not significantly different, therefore it can be concluded that extraction in those conditions were found to be the most appropriate to extract total polyphenols. Moreover, processing in nearly neutral conditions are preferred to acidic conditions because it will have a wider application and can reduce the processing cost, because less citric acid is required. Similar to pH, a tea-water ratio of 1:20 was considered to be more economically efficient than a tea-water ratio of 1:30. Therefore, the optimum treatment combination to obtain the maximum amount of total polyphenols extracted from green tea was an extraction with tea-water ratio of 1:20 at pH 5. With this optimum condition, 10.783% of total polyphenols were extracted from green tea.

2. Effect on tannin extraction

Table 4.3 shows the tannin content of green tea extracts. The two factors observed in this study were significantly affecting the obtained tannin in the extracts. Tannin content in the extracts ranged from 3.317% to 7.000% of green tea in dry basis.

Similar to total polyphenols content, higher tea-water ratio and lower pH provided higher tannin content in the obtained extracts. The highest tannin content was obtained by extraction with a tea-water ratio of 1:30 (at all pH values), while the lowest was obtained by extraction with a tea-water ratio of 1:5 and 1:10 at pH 7.

Table 4.3 Tannin contents of green tea extracts extracted with various tea-water ratio and pH

Tannin contents (% w/w dry basis)				
Tea-water ratio	**pH**			
	4	**5**	**6**	**7**
1:5	$4.989 \pm 0.008^{aB*}$	4.644 ± 0.298^{aB}	4.583 ± 0.257^{aB}	3.888 ± 0.389^{bA}
1:10	5.588 ± 0.077^{bD}	4.671 ± 0.033^{aC}	4.273 ± 0.079^{aB}	3.317 ± 0.094^{aA}
1:20	5.933 ± 0.079^{cB}	4.863 ± 0.245^{aA}	5.099 ± 0.039^{bA}	4.907 ± 0.033^{cA}
1:30	7.000 ± 0.182^{dB}	6.574 ± 0.079^{bA}	6.489 ± 0.235^{cA}	6.449 ± 0.134^{dA}

* Different lower-case letters in the same column indicate significant difference ($\alpha = 5$ %) among tea-water ratio and different capital letters in the same row indicate significant difference ($\alpha = 5$ %) in pH

Tannin is water soluble with the exception of some high molecular weight structures, so it can be extracted in water. Hydrolyzable tannin, a type of tannin that can be hydrolyzed easier than condensed tannin, is extracted more in high temperatures due to the hydrolysis process. Besides high temperature, this compound can be hydrolyzed by acid (Cannas,

2001). Therefore, extraction in acidic conditions (especially at tea-water ratio of 1:5, 1:10 and 1:20) resulted in more tannin in the obtained extracts.

3. Effect on total amino acids (expressed as theanine) extraction

Table 4.4 shows the amount of total amino acids extracted from green tea. Similar to total polyphenols and tannin content, the content of total amino acids in the obtained extracts from green tea were affected by two factors (tea-water ratio and pH). Total amino acids content in green tea were ranging from 3.584% to 4.940% of green tea in dry basis.

Table 4.4 Total amino acids contents (expressed as theanine) of green tea extracts extracted with various tea-water ratio and pH

Total amino acids contents (%w/w dry basis)				
Tea-water ratio	**pH**			
	4	**5**	**6**	**7**
1:5	$4.475 \pm 0.011^{bC*}$	4.582 ± 0.104^{bC}	4.335 ± 0.049^{dB}	4.050 ± 0.090^{cA}
1:10	4.940 ± 0.011^{cD}	4.547 ± 0.044^{bC}	3.971 ± 0.055^{cB}	3.584 ± 0.069^{aA}
1:20	4.471 ± 0.110^{bC}	4.243 ± 0.048^{aB}	3.888 ± 0.019^{bA}	3.895 ± 0.086^{bA}
1:30	3.926 ± 0.171^{aC}	4.050 ± 0.231^{aC}	3.727 ± 0.000^{aAB}	3.631 ± 0.016^{aA}

* Different lower-case letters in the same column indicate significant difference ($\alpha = 5$ %) among tea-water ratio and different capital letters in the same row indicate significant difference ($\alpha = 5$ %) in pH

Tea to water ratio affected the amount of total amino acids in the obtained extract. With varying tea-water ratio (1:5, 1:10, 1:20 and 1:30), 1:10 tended to give higher total amino acids contents than other ratios. Compared to tea-water ratios of 1:20 and 1:30, extraction using lower extractant volume (water) at 1:10 gave higher total amino acids in the obtained extract. The same result was found by Kovacs *et al* (1998). The authors revealed that the relative

compositions of the amino acids were not influenced by the extractant volume. It indicated that amino acids extraction from the samples of plant origin is more advantageous when the extraction was conducted in lower extractant volume.

Moreover, it can be seen that extractions at lower pH tended to increase the amount of total amino acids in the obtained extracts. In the same tea-water ratio, extraction at pH 4 and 5 tended to give higher contents of total amino acids than at pH 6 and 7. Amino acids can be yielded by hydrolyzed proteins at high temperature, moist environment or by acids (Xu & Chen, 2002). Therefore, total amino acids extracted at lower pH are higher than that in higher pH. To get the maximum amount of total amino acids from green tea, extraction can be performed with a ratio of 1:10 at pH 4.

4. Effect on caffeine extraction

The contents of caffeine extracted from green tea can be seen on Table 4.5. As well as other compounds, the contents of caffeine in the obtained extracts of green tea were affected by tea-water ratio and pH. Caffeine extracted from green tea ranged from 2.219% to 4.038% of green tea in dry basis. The maximum amount of caffeine can be obtained by extraction with a tea-water ratio of 1:30 at pH 4. Meanwhile, the lowest caffeine produced by extraction with a tea-water ratio 1:5 at pH 6.

Increasing tea-water ratio tended to increase the amount of caffeine extracted from green tea. Ratio 1:30 had a better result than other ratio to get more caffeine content in the extract. Similar to total polyphenols and tannin contents, the volume of extractant (water) in this ratio was in the sufficient amount to extract caffeine from green tea. In the extraction process, the extractant was used to penetrate into the solid matrix through the pores and capillaries of the sample, to affect the permeability of the cell walls and membranes, and to transport the solute to the exterior of the solid matrix (Aguilera, 2003; Escribano-Bailon & Santos-Buelga, 2003).

The pH of water and tea-water ratio significantly affected the extraction of total polyphenols, tannin, total amino acids and caffeine. Each of these compounds has its own optimum condition of extraction. In general, extraction in lower pH tended to provide higher content of total polyphenols, tannin, total amino acids and caffeine. Presumably, it was due to the increasing of solubility of these compounds in low pH extraction. pH determines the degree of

solubility. Commonly, the degree of solubility of the soluble compounds can be increased by extraction in low pH (Ekayanake *et al.,* 1995; Escribano-Bailon & Santos Buelga, 2003).

Table 4.5 Caffeine content of green tea extracts extracted with various tea-water ratio and pH

Caffeine contents (% w/w Dry Basis)				
Tea-water	**pH**			
ratio	**4**	**5**	**6**	**7**
1:5	$2.522 \pm 0.009^{aC*}$	2.422 ± 0.052^{aB}	2.219 ± 0.005^{aA}	2.479 ± 0.012^{bC}
1:10	2.768 ± 0.025^{bB}	2.369 ± 0.017^{aA}	2.376 ± 0.025^{bA}	2.409 ± 0.026^{aA}
1:20	3.577 ± 0.051^{cC}	3.297 ± 0.051^{bB}	3.303 ± 0.011^{cB}	3.188 ± 0.006^{cA}
1:30	4.038 ± 0.009^{dC}	3.349 ± 0.026^{bB}	2.814 ± 0.026^{dA}	2.848 ± 0.009^{dA}

* Different lower-case letters in the same column indicate significant difference ($\alpha = 5$ %) among tea-water ratio, and different capital letters in the same row indicate significant difference ($\alpha = 5$ %) in pH

By increasing tea-water ratio, the extraction efficiency and yields of green tea compounds were increased (Yoshida *et al.,* 1999; Pan *et al.,* 2003; Uzunalic *et al.,* 2006). By varying tea-water ratio (1:5, 1:10, 1:20 and 1:30), the optimum of total polyphenols extraction was reached in a tea-water ratio of 1:20 and 1:30, while the highest content of tannin and caffeine were obtained from extraction in a tea-water ratio of 1:30. However, the highest total amino acids content were obtained from extraction in a tea-water ratio of 1:10.

Based on the fact that polyphenols are active ingredients in tea and play an important role in tea's health benefits, this study focused on total polyphenols extraction. The optimum condition for total polyphenols extraction (tea-water ratio of 1:20 at pH 5) was applied to investigate the effect of multi-step extraction, described in the following part. By using this condition, the obtained content of total polyphenols, tannin, total amino acids and caffeine were 10.783, 4.863, 4,243 and 3.297% of green tea in dry basis, respectively.

4.2.2 Effect of multi-step extraction

Total polyphenols are the major parts in green tea. Considering the amount of these compounds, their role, and their economical aspects, from section 4.2.1, it can be concluded that extraction with tea:water ratio 1:20 at pH 5 was the optimum condition for extracting total polyphenols from green tea. With this optimum condition, total polyphenols in the obtained extract was 10.783% of green tea in dry basis. Comparing the amount of this compound from the obtained extract and raw material, most total polyphenols were not extracted in a one-step extraction. Due to this reason, multi-step extraction procedures are recommended.

A multi-step extraction procedure was conducted where the residue from the first step was repeatedly extracted again under the same conditions. A higher degree of extraction can be achieved by separating the first extract from the leaf and treating the partially extracted leaf with fresh hot water. Since the fresh water is added, the residual tea solids inside the leaf once more start migrating outwards into the liquids (Hampton, 1999). The extraction process occurs until the new equilibrium is reached. At this time, most of the tea constituents moved out of the leaf to form a weak tea extract. Therefore, with a multi-step extraction, the cumulative amounts of extracted compounds increased, while the extraction yields decreased in each step (Hampton, 1999).

Table 4.6 shows the composition of green tea extracts as well as the cumulative amount of each compound using an optimum extraction conditions. According to significance levels, three steps of extraction were considered to be enough for extracting the total polyphenols contents from green tea. The same trend was found in tannins and caffeine contents. For total amino acids contents, a 4 step extraction was needed to obtain the maximum amount of these compounds. Considering the economical aspects, the third step of extraction was not necessary because it was only able to extract 8.759, 2.282, 5.123 and 0.022% of total polyphenols, tannin, total amino acids and caffeine, respectively. Due to this reason, it can be concluded that the two steps of extraction is enough economically for extracting green tea compounds, especially total polyphenols. With two steps of extraction, the cumulative amount of total polyphenols, tannin, total amino acids and caffeine in the obtained extracts were 14.773, 6.218, 5.350 and 4.252%, respectively, while the percent yield for these compounds were 71.332, 58.638, 88.989 and 92.194%, respectively.

Table 4.6 Compositions of green tea extracts extracted with 4 Steps (Tea-Water Ratio = 1:20, pH = 5)

%w/w Dry basis								
Extraction	**Total polyphenols**		**Tannins**		**Total amino acids**		**Caffeine**	
step	**Amount**	**% yield**	**Amount**	**% yield**	**Amount**	**% yield**	**Amount**	**% yield**
1	$10.783 \pm 0.051^{c*}$	52.066	4.863 ± 0.245^{c}	45.860	4.243 ± 0.048^{d}	70.576	3.297 ± 0.051^{c}	71.487
2	3.990 ± 0.631^{b}	19.266	1.355 ± 0.164^{b}	12.778	1.107 ± 0.011^{c}	18.413	0.955 ± 0.011^{b}	20.707
3	1.814 ± 0.009^{a}	8.759	0.242 ± 0.053^{a}	2.282	0.308 ± 0.004^{b}	5.123	0.001 ± 0.003^{a}	0.022
4	1.302 ± 0.009^{a}	6.287	0.045 ± 0.047^{a}	0.424	0.177 ± 0.005^{a}	2.944	-	-
Cumulative (2-steps)	14.773	71.332	6.218	58.638	5.350	88.989	4.252	92.194

*Different lower-case letters indicate significant difference ($\alpha = 5$ %) among number of extraction step.

4.3 Kinetic study in the laboratory scale

Compounds inside the green tea have different characteristics and different diffusity. The effect of time and temperature become significantly relevant when considering the difference between particle characteristics of green tea compounds (Hampton, 1999). Because the particles move at different rates, time obviously becomes crucial. Temperature is also important because some materials with high molecular weight require additional encouragement in the form of extra heat energy in order to get them to pass through into the surrounding fluid more quickly (Hampton, 1999). The temperature should be high enough to ensure the extraction is economically efficient. Therefore, the kinetic study should be performed by which the results will be used for designing the extraction process.

This study investigated the effect of time and temperature on extraction of total polyphenols, tannin, total amino acids, caffeine and individual catechins including EGCG (epigallocatechin gallate), EGC (epigallocatechin), ECG (epicatechin gallate), EC (epicatechin), GCG (gallocatechin gallate), GC (gallocatechin), CG (catechin gallate), C (catechin) and G (gallic acid). Total polyphenols, tannin and total amino acids contents were analyzed by the colorimetric method, while the other parameters were analyzed using HPLC method.

The kinetic rates of these compounds during green tea extraction were calculated by using the equation described in Chapter 3 (the detail can be seen in Appendix C). The extractions were performed using the optimum conditions from 4.2 (extraction at tea-water ratio 1:20, pH 5) at five different temperatures (50°C, 60°C, 70°C, 80°C and 90°C) for 1, 3, 5, 10, 20, 40 and 80 minutes. The chemical compositions of green tea samples in this study are shown in Table 4.1.

4.3.1 Total polyphenols (expressed as gallic acids)

Total polyphenols contents were expressed in unit of percentage of gallic acids equivalent. The mean values of this contents and standard deviations were plotted in Figure 4.1. The total polyphenols contents of green tea extracted at various times and temperatures ranged from 3.812% to 11.158% of green tea in dry basis. At all temperatures, it tended to increase with the increasing of time and temperatures.

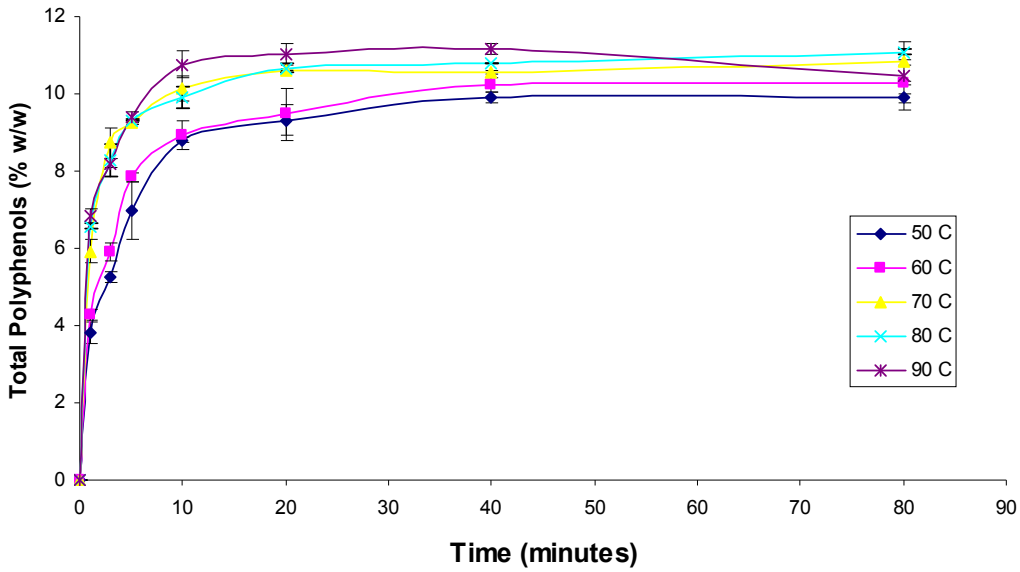


Figure 4.1 Effect of time and temperatures on total polyphenols contents extracted from green tea

The rate constant of total polyphenols extraction at all temperatures were then determined. The total polyphenols contents increased rapidly for the first 10 minutes and then remained stable after that time. Therefore, the rate constant was determined for 10 minute of extraction. The relationships between concentration values with time were found to fit the first order kinetics. Referring to Spiro's steady state kinetic model (Jaganyi and Price, 1998), the rate constant was calculated using the equation (3.2). The observed first order rate constant were obtained from the slope in the graph between ln $(C_\infty / (C_\infty - C)$ versus time. The first order plot for total polyphenols extraction from green tea at various temperatures and times is shown in Figure 4.2. Rate constant (k_{obs}), equilibrium concentration (C_∞), intercept (a) and R square (R^2) for each temperature is listed in Table 4.7.

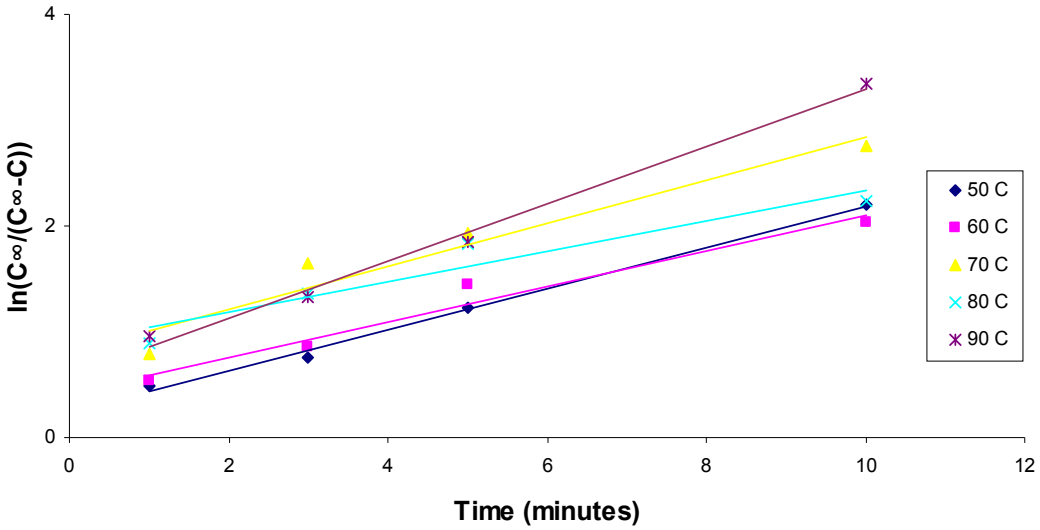


Figure 4.2 First order plot for total polyphenols extraction from green tea at various times and temperatures

Table 4.7. Kinetics and equilibrium data for total polyphenols extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
50	9.901	0.194	0.996	0.243
60	10.281	0.168	0.966	0.418
70	10.825	0.203	0.940	0.813
80	11.089	0.144	0.921	0.901
90	11.158	0.272	0.993	0.581

The highest value of total polyphenols content at 50°C, 60°C, 70°C, 80°C and 90°C were 9.901, 10.281, 10.825, 11.089 and 11.158% of green tea in dry basis, respectively. These values were increased with the increasing of temperature. Total polyphenols content reached a maximum value (11.158%) when green tea was extracted at 90°C for 40 minute. Based on the

concentration, extraction at 90°C was found to be the optimum condition for total polyphenols extraction.

It was observed that, in the first order kinetic, the rate constant was independent to temperatures. The highest rate for total polyphenols extraction was reached in extraction at 90°C. At this temperature, the content of total polyphenols was 6.852% of green tea in dry basis and then increased to 10.767% for 10 minutes. Furthermore, the rate constant of extraction at 80°C was lower than that at 50°C, 60°C and 70°C. In extraction at 80°C, the concentration of total polyphenols in the first minute was 6.575%. It was higher than the initial concentration in extraction at 50°C, 60°C and 70°C. From this point, these initial concentrations were increased when the time of extraction increased. The rate of this increasing concentration of total polyphenols extraction at 80°C was lower than that at 50°C, 60°C, and 70°C.

4.3.2 Tannin

Tannin contents from green tea extracted at various temperatures and times is shown in Figure 4.4. The tannin content was found to vary from 0.822% to 7.283% of green tea in dry basis. The equilibrium value of tannin was increased as the temperature increased. Similar to total polyphenols, concentration of tannin during extraction was obviously increased rapidly at the first 3 and 10 minutes of extraction and then continued to increase slightly.

The rate constant of tannin extraction at each temperature was then measured. Kinetics and equilibrium data for tannin extraction is shown in Table 4.8. Similar to total polyphenols, the rate constant for tannin extraction was independent to temperatures. The maximum rate constant was reached in extraction at 50°C, while the lowest rate constant was observed in extraction at 70°C. Tannin is a multiple structure unit with free phenolic groups. Tannin is composed of a very diverse group of oligomers and polymers. Its molecular weight ranges from 500 to more than 20,000, while total polyphenols is a total phenolic compounds (including free phenolic acids and polymerized forms). These free phenolic acids have a lower molecular weight than the polymerized form (Cannas, 2001). Therefore, compared to total polyphenols, tannin extraction has a higher rate constant.

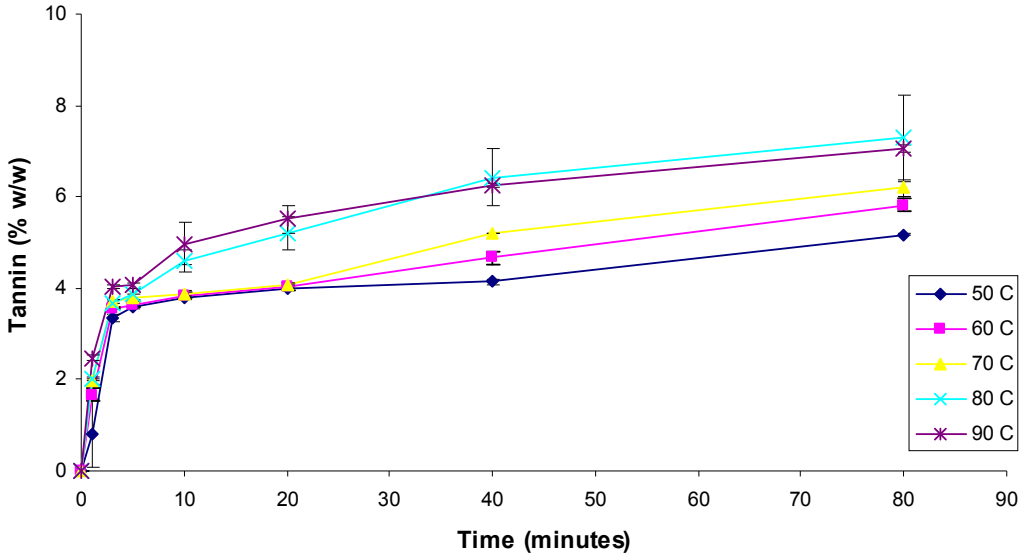


Figure 4.3 Effect of time and temperatures on tannin contents extracted from green tea

Table 4.8. Kinetics and equilibrium data for tannin extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
50	5.169	0.105	0.621	0.429
60	5.824	0.067	0.586	0.518
70	6.191	0.053	0.501	0.562
80	7.283	0.067	0.857	0.375
90	7.051	0.079	0.891	0.464

The equilibrium value of tannin at temperature 50°C, 60°C, 70°C, 80°C and 90°C were 5.169, 5.824, 6.191, 7.283 and 7.051% of green tea in dry basis, respectively. Extraction at 80°C provided the highest equilibrium value for tannin. However, compared to the extraction at 90°C, the equilibrium value of extraction at 80°C was higher. It might be due to tannin hydrolysis

that yields carbohydrates and simple phenols. High temperature catalyzes the reaction (Cannas, 2001; Shahidi & Naczk, 2004). Since total polyphenols including all phenolic compounds (simple phenol and also the polymerized forms), green tea extract obtained from extraction at 90°C have the highest total polyphenols content, but its tannin content was lower than that in extraction at 80°C.

4.3.3 Total amino acids

Total amino acids contents of green tea extract were varied from 0.918% to 4.534% in dry basis. The mean values of total amino acids contents during extraction were plotted in Figure 4.4. Similar to total polyphenols and tannin, the concentrations of total amino acids were increased rapidly for the first 10 minutes of extraction. The concentrations then remained stable after that point and reached the equilibrium value.

The kinetics and equilibrium data for total amino acids extraction at various temperatures are shown in Table 4.9. The equilibrium values of total amino acids during extraction at 50°C, 60°C, 70°C, 80°C, and 90°C were 4.205, 4.281, 4.401, 4.534 and 4.420% of green tea in dry basis, respectively. These values were almost similar. From this data, it can be revealed that temperatures may not influence the equilibrium values of total amino acids.

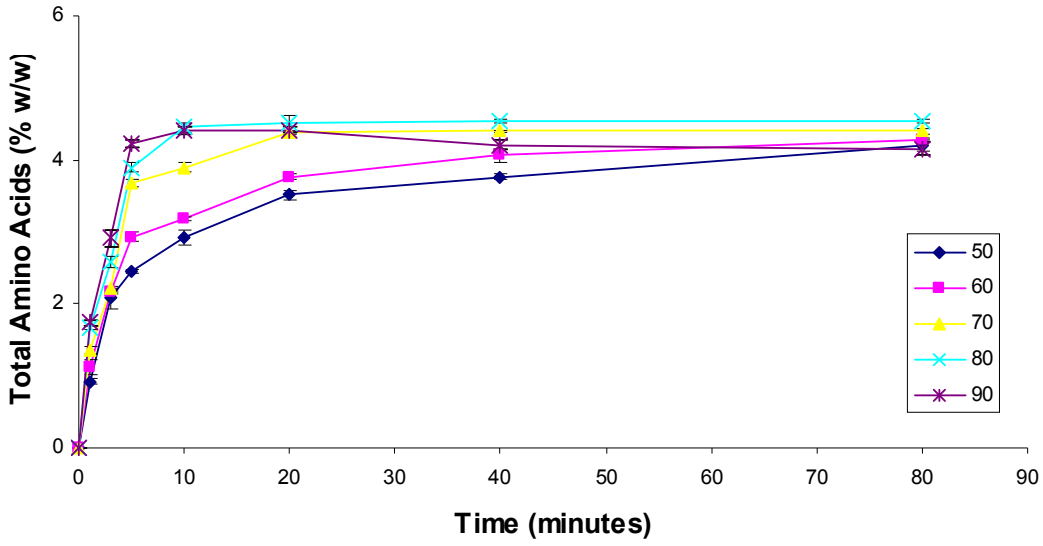


Figure 4.4 Effect of time and temperatures on total amino acids contents extracted from green tea

Table 4.9. Kinetics and equilibrium data for total amino acids extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
50	4.205	0.096	0.897	0.13
60	4.281	0.113	0.852	0.34
70	4.401	0.204	0.850	0.28
80	4.534	0.410	0.987	-0.13
90	4.420	0.702	0.983	-0.51

From Table 4.9, it shows that the rate constants for extraction of total amino acids were increased as the temperature increased. Even though, the equilibrium value for extraction at 80°C was higher than the equilibrium value at 90°C, the rate constant did not show the same trend. The rate constant of total amino acids extraction at 90°C was higher than that at 80°C. It means that the rate was faster for total amino acids concentration to reach the equilibrium value during extraction at 90°C was faster than extraction at 80°C.

4.3.4 Caffeine

Caffeine content in green tea extracts were in the range of 0.621% to 2.396% in dry basis. The caffeine contents during the extractions were plotted in Figure 4.5. At all extraction temperatures, caffeine content increased with time. Similar for total polyphenols, tannin and total amino acids, it was found that the caffeine content rapidly raised for the first 10 minutes and then approached the equilibrium value. According to Labbe, *et al* (2006) the caffeine concentration was influenced to a small extent by temperature and was rapidly made soluble in solution to its final concentration. Moreover, its concentration had reached a maximum in the first few minutes of extraction.

The kinetics and equilibrium data for caffeine extraction is shown in Table 4.10. The equilibrium values of caffeine tended to increase with the increasing of temperature. The lowest caffeine concentration was obtained in extraction at 50°C, while the highest concentration was reached at 90°C. The data shows that the rate constant for caffeine extraction was independent to temperature. Similar to the rate constant of extraction of the previous compounds, the rate

constant for caffeine extraction was independent to temperature. The maximum rate constant for caffeine extraction was reached in extraction at 70°C.

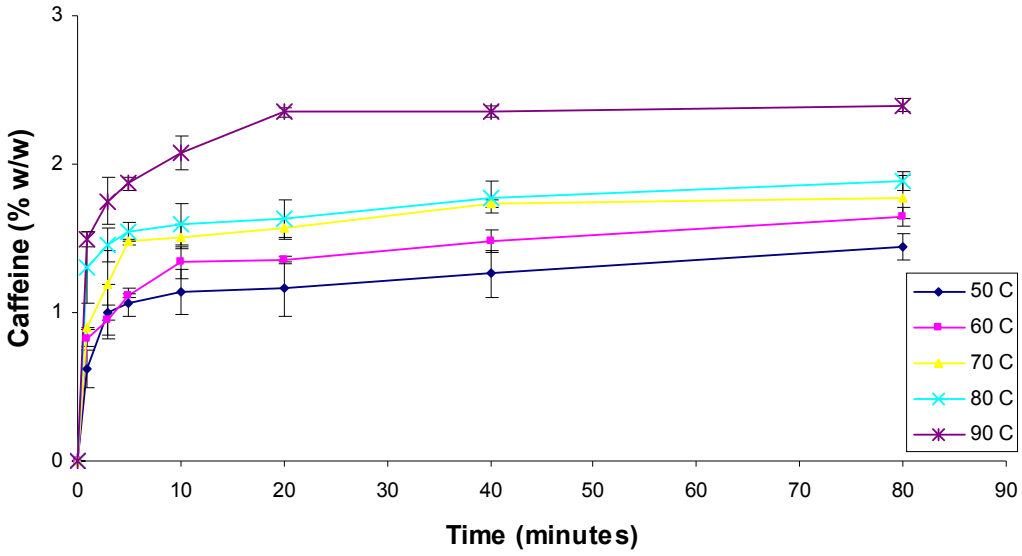


Figure 4.5 Effect of time and temperatures on caffeine contents extracted from green tea

Table 4.10. Kinetics and equilibrium data for caffeine extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
50	1.441	0.098	0.766	0.704
60	1.643	0.111	0.996	0.565
70	1.777	0.130	0.787	0.752
80	1.886	0.073	0.861	1.212
90	2.396	0.112	0.986	0.919

Compared to total polyphenols and tannin, caffeine was extracted in its high amount since the first minute of extraction (the data is shown in Appendix C). The difference in water solubility and molecular weight between caffeine and other compounds might be an important reason (Liang *et al.,* 2007). Even tough it was extracted in high amounts in the first minute, the rate constant for caffeine extraction was lower than that for total polyphenols and tannin extraction. From this case, further experiments involving a broader extraction time (especially before 1 minute) are needed.

The rate constant for caffeine extraction was lower than the rate constant for total amino acid extraction. In total amino acids analysis (colorimetric method), theanine was used as the standard. Molecular weight of theanine (174) is lower than caffeine (194) (Zhu *et al.,* 2004). Compounds with smaller molecular weight could diffuse and dissolve more easily into water (Liang *et al.,* 2007). Therefore, the total amino acids (expressed as theanine) diffused faster than caffeine.

According to the rate constant and equilibrium value, extraction at 90°C was found to be the most appropriate to obtain the maximum total polyphenols and the minimum tannin content. Moreover, the highest rate constant for total amino acids extraction and the highest equilibrium value of caffeine were reached at extraction in this temperature.

4.3.5 Individual catechins

Individual catechins measured in this study were EGCG, EGC, ECG, EC, GCG, GC, C and G. The kinetic rate of these catechins was also determined. The concentrations of all compounds were plotted in Figure 4.6 – 4.7. Similar to other compounds, catechins concentrations tended to increase with the increasing of time and temperature. All of the concentrations tended to increase rapidly in the first 10 minutes, and then became stable, approaching the equilibrium value.

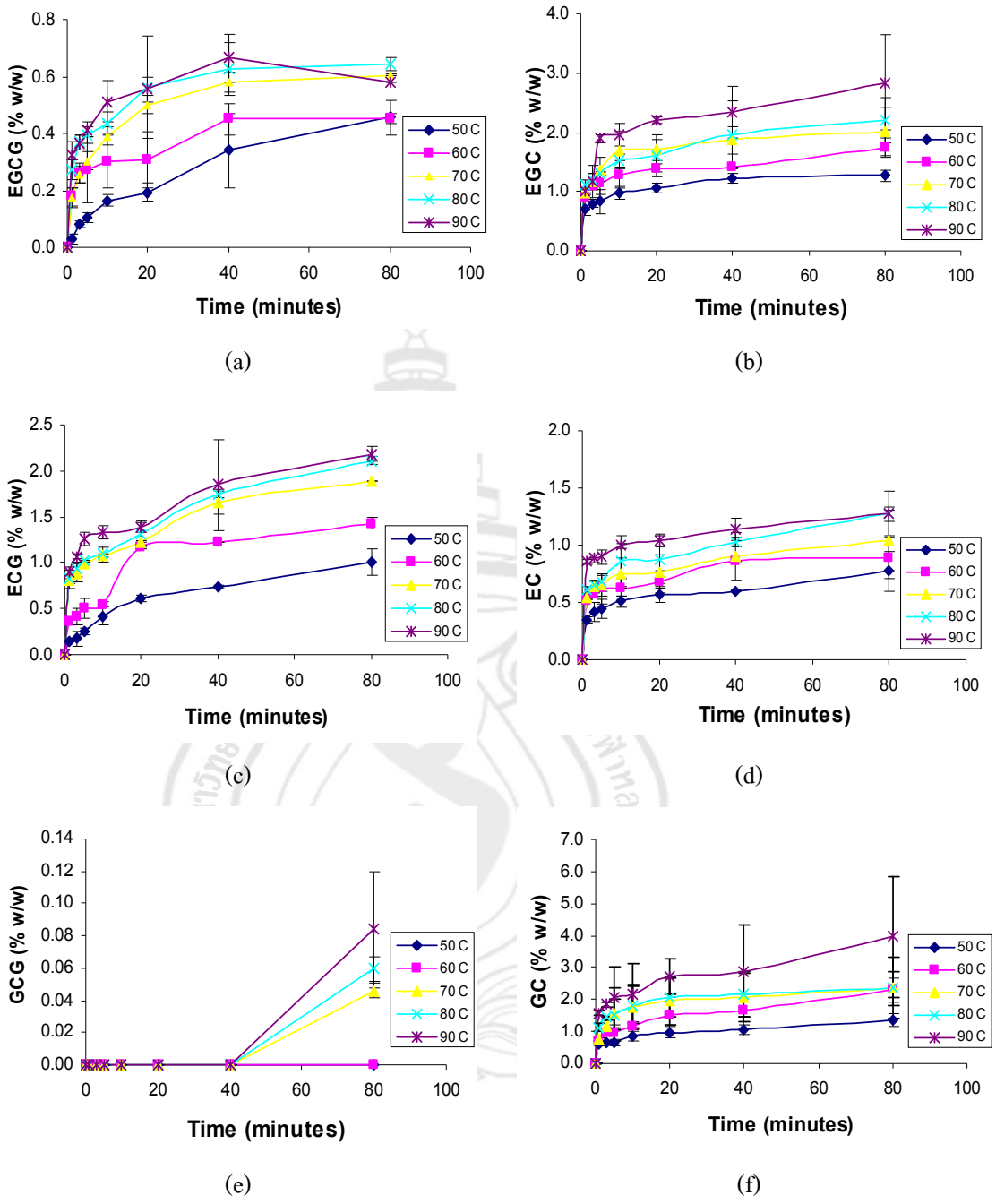


Figure 4.6 Catechins contents extracted from green tea at various times and temperatures. (a) EGCG, (b) EGC, (c) ECG, (d) EC, (e) GCG and (f) GC

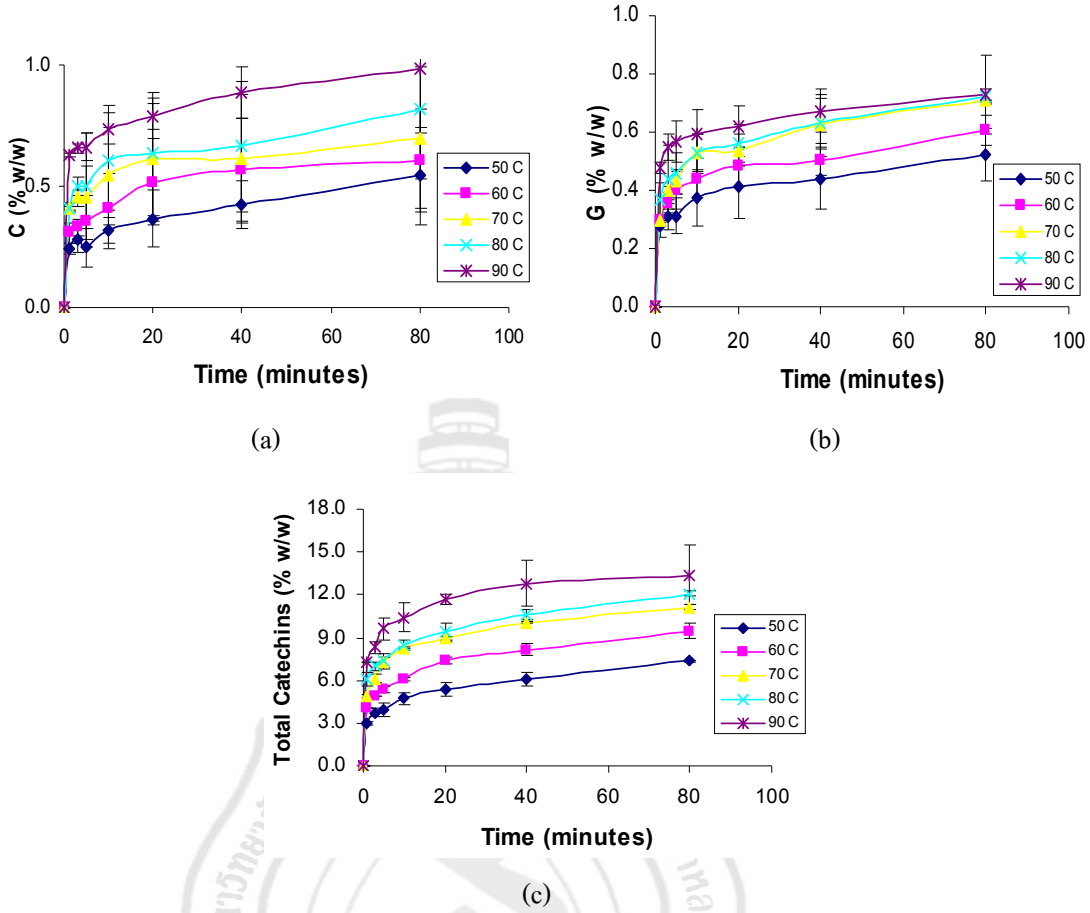


Figure 4.7 Catechins contents extracted from green tea at various times and temperatures. (a) C, (b) G and (c) total catechins

Table 4.11. Kinetics and equilibrium data for major catechins extraction (1 – 10 minutes)

^{0}C		EGCG	EGC	ECG	EC
50	C_∞ (%w/w db)	0.456	1.266	1.008	0.773
	k_{obs} (min^{-1})	0.040	0.070	0.043	0.050
	R^2	0.985	0.989	0.974	0.969
	a	0.05	0.76	0.088	0.587
60	C_∞ (%w/w db)	0.452	1.738	1.426	0.892
	k_{obs} (min^{-1})	0.059	0.066	0.021	0.037
	R^2	0.815	0.968	0.908	0.730
	a	0.56	0.72	0.284	0.902
70	C_∞ (%w/w db)	0.605	2.015	1.891	1.037
	k_{obs} (min^{-1})	0.076	0.131	0.032	0.057
	R^2	0.090	0.995	0.949	0.966
	a	0.30	0.51	0.541	0.722
80	C_∞ (%w/w db)	0.643	2.201	2.113	1.274
	k_{obs} (min^{-1})	0.060	0.055	0.024	0.053
	R^2	0.865	0.992	0.917	0.945
	a	0.59	0.63	0.501	0.558
90	C_∞ (%w/w db)	0.664	2.842	2.185	1.281
	k_{obs} (min^{-1})	0.091	0.087	0.045	0.044
	R^2	0.994	0.778	0.873	0.967
	a	0.55	0.40	0.535	1.036

Table 4.12. Kinetics and equilibrium data for minor and total catechins (1 – 10 minutes)

^{0}C		GC	C	G	Total catechins
50	C_∞ (%w/w db)	1.344	0.544	0.523	7.355
	k_{obs} (min^{-1})	0.047	0.031	0.055	0.055
	R^2	0.924	0.921	0.960	0.982
	a	0.527	0.55	0.695	0.497
60	C_∞ (%w/w db)	2.298	0.605	0.607	9.459
	k_{obs} (min^{-1})	0.034	0.049	0.067	0.051
	R^2	0.913	0.999	0.955	0.969
	a	0.374	0.65	0.662	0.553
70	C_∞ (%w/w db)	2.363	0.700	0.708	11.142
	k_{obs} (min^{-1})	0.107	0.067	0.087	0.084
	R^2	0.923	0.946	0.979	0.968
	a	0.366	0.800	0.506	0.542
80	C_∞ (%w/w db)	2.358	0.820	0.721	12.075
	k_{obs} (min^{-1})	0.082	0.066	0.064	0.054
	R^2	0.951	0.956	0.979	0.981
	a	0.641	0.66	0.684	0.670
90	C_∞ (%w/w db)	3.963	0.987	0.727	13.395
	k_{obs} (min^{-1})	0.031	0.039	0.063	0.080
	R^2	0.886	0.949	0.847	0.940
	a	0.521	0.97	1.122	0.750

Kinetics and equilibrium data for catechins extraction are shown in Table 4.11 and 4.12. According to equilibrium value, the most appropriate temperature for all individual catechins extraction was 90°C. The equilibrium concentration of these compounds reached the

maximum value when green tea was extracted at 90°C. The same result was found by Labbe *et al* (2006). Compared to lower temperature, higher concentrations of catechins compounds were observed in green tea extracted at temperatures of 70, 80 and 90°C. The best temperature and time combination for catechins extraction were at 90°C for 80 minutes. Higher temperature can improve the extraction yield as well as the extraction rate because heat renders the permeability of the green tea cell wall, improving solubility and the diffusion coefficient of the compounds (Ekayanake *et al.,* 1995; Escribano-Bailon & Santos-Buelga, 2003; Uzunalic *et al.,* 2006).

From Figure 4.6, it was found that GCG extracted only at temperature 70°C, 80°C and 90°C. This compound seems to continue to increase after 80 minutes. Since GCG was not detected in raw materials, it indicates that epimerization of EGCG to GCG occurred. Wang & Helliwell (2000) stated that, epimerization between the epi- and non-epistructure for all individual catechin compounds at temperature above 70°C was observed.

From table 4.11 and 4.12, it can be seen that among all individual catechins, the extraction of ECG has the lowest rate constant, while the highest rate constant occurred in EGC extraction. ECG has a molecular weight of 442.4, while EGC is 306.3 (Anonymous, 2007). Therefore, in the interval from 1 to 10 minutes of extraction, EGC diffused and dissolved faster into water than ECG.

4.4 Kinetic study in pilot plant scale

The results from study of the optimum condition of extraction in the laboratory scale (4.3) were applied to investigate the effect of time and temperature extraction as well as the kinetics of green tea compounds during extraction in the pilot plant scale. Since the multi-step extraction procedure was considered necessary, the extractor unit (shown in Figure 3.1) was designed to perform in a continuous system. During the extraction, the extractant (water) was pumped continually through the material (green tea). According to the results from 4.2, the extraction should be conducted at a tea-water ratio of 1:20 and pH 5. However, due to the limitation of the unit, the extractions in this study were performed at a tea-water ratio of 1:28 and pH 5. This condition was based on the capabilities of the extractor unit.

Extraction time for the pilot plant scale was lengthened, because this process involved a high volume of water and green tea. The process was conducted at three different temperatures and the sample was taken at 1, 3, 5, 10, 20, 40, 60, 80 and 120 minutes. When the extraction process began, green tea leaves absorbed water through the cellular structure and started to swell. Not so many extractions occured in the first few seconds. Since the leaf has swollen, the soluble materials inside the leaves had more freedom to move within the fluid and migrate to the edges of the leaf, passing through the cells walls into the liquid. Extraction continued to occur until the concentration of the materials that had been extracted into the surrounding fluid approximately equaled the concentration of the materials left inside the leaf. At this stage, the rate of extraction getting slower and reaches a limit. The concentration at this limit is called an equilibrium concentration. This process was described at Figure 2.12.

Total polyphenols, tannin, theanine, caffeine and individual catechins including EGCG, EGC, ECG, EC, GCG, GC and CG contents during the extraction process in the pilot plant scale were observed. The contents of total polyphenols and tannin were analyzed using colorimetric methods, while the other compounds were analyzed using the HPLC method. The kinetic rates of these compounds during the extraction process were also measured (the detail is shown in Appendix D). The chemical composition of green tea used in this study is shown in Table 4.1. From the table, it can be seen that green tea sample did not consist of ECG, GCG and CG. However, if it compared to the sample used in the previous study, the chemical compositions were different. The difference in geography might be the important reason.

4.4.1 Total polyphenols

Time and temperature affected the total polyphenols extraction. The total polyphenols content extracted at various times and temperature is shown in Figure 4.8. Total polyphenols contents in green tea extracts ranged from 0.707% to 11.180% in dry basis.

The increasing of total polyphenols during the extraction process at three temperatures was observed. It was found that total polyphenols increased with time and increasing temperature. The equilibrium concentration for total polyphenols extracted at 70°C, 80°C and 90°C were 9.998, 11.021 and 11.180% of green tea in dry basis, respectively. Kinetics and equilibrium data for total polyphenols extraction is shown in Table 4.13. The rate constant of total polyphenols extraction increased as temperature increased. The most appropriate

temperature for total polyphenols extraction was 90°C. 11.180% of total polyphenols was reached in extraction at 90°C. The rate constant of extraction at this temperature was 0.048 min^{-1}. The same result was found by Ekwongsupasarn (2005), among three temperatures (70°C, 80°C and 90°C), extraction at 90°C was the optimum temperature, providing the highest equilibrium concentration and rate constant of total polyphenols extraction both in tea product and residue.

Table 4.13. Kinetics and equilibrium data for total polyphenols extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	*a*
70	9.998	0.042	0.677	0.152
80	11.021	0.038	0.814	0.189
90	11.180	0.048	0.970	0.265

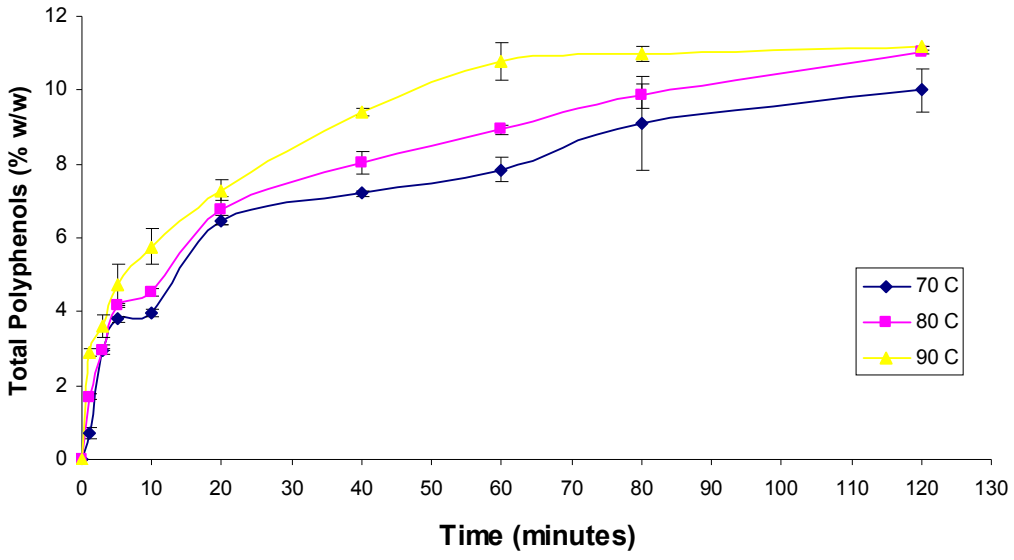


Figure 4.8 Effect of time and temperatures on total polyphenols contents extracted from green tea

4.4.2 Tannin

Tannin content in green tea extracts were in the range of 0.340% - 7.364% of dry basis. The equilibrium value for tannin content in extraction at 70°C, 80°C and 90°C were 6.180, 7.007 and 7.364% of green tea in dry basis, respectively. Tannin contents during extractions were plotted in Figure 4.9. Tannin extraction has a similar trend with total polyphenols extraction. The equilibrium values and rate constants were increased over time.

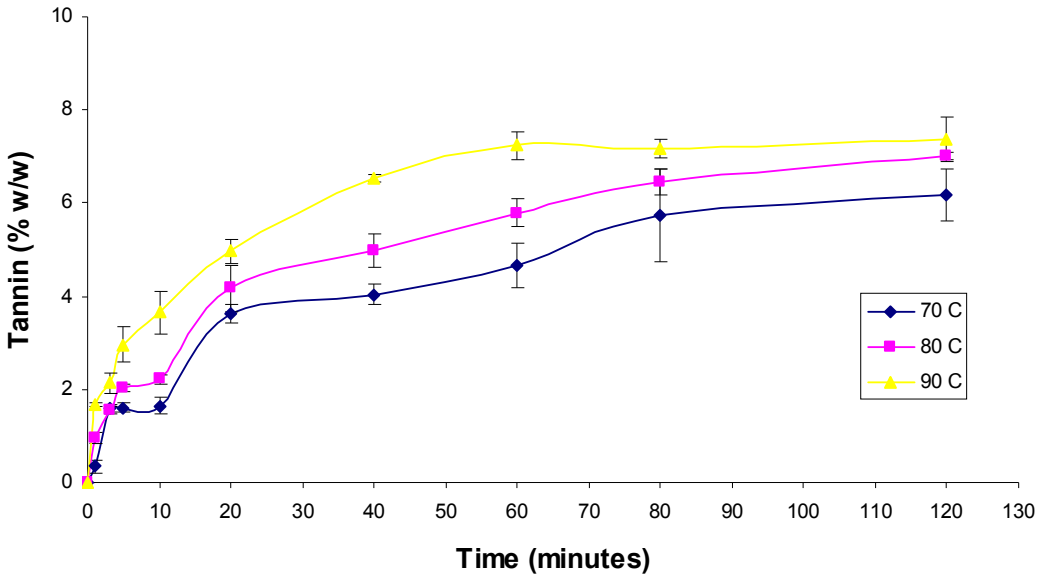


Figure 4.9 Effect of time and temperatures on tannin contents extracted from green tea

Table 4.14. Kinetics and equilibrium data for tannin extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
70	6.180	0.022	0.473	0.137
80	7.007	0.024	0.814	0.166
90	7.364	0.048	0.964	0.220

Kinetics and equilibrium data for tannin extraction is shown in Table 4.14. The highest concentration of tannin was observed in extraction at 90°C. The rate constant at this extraction was 0.048 min^{-1}. While the rate constant for tannin extraction at 70°C and 80°C were 0.022 min^{-1} and 0.024 min^{-1}. Similar to total polyphenols, 90°C was the optimum temperature for tannin extraction. In contrast to the kinetic study in the laboratory scale, the rate constant for total polyphenols and tannin extraction in the pilot plant scale were the same. During 1 to 10 minutes these compounds were extracted in the same rate.

4.4.3 Theanine

Theanine in green tea extracts varied from 0.028% to 2.292% in dry basis. The concentration of theanine during extraction is shown in Figure 4.10. Theanine contents increased as the time increased and then reached a maximum value at 120 minutes. Similar to total polyphenols and tannin content, it was found that theanine contents from extraction at 90°C were higher than extraction at 70°C and 80°C. However, in the end of the extraction process, this content at three temperatures was almost the same.

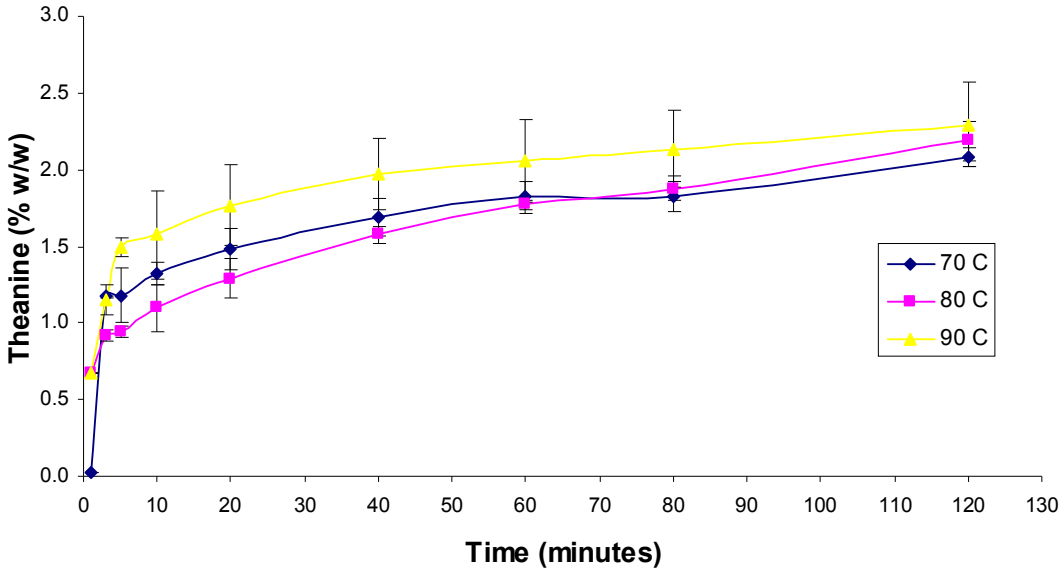


Figure 4.10 Effect of time and temperatures on theanine contents extracted from green tea

Kinetics and equilibrium data for theanine extraction is shown in Table 4.15. The equilibrium values increased as temperature was increased. 70°C was the optimum temperature for theanine extraction. Even though the equilibrium value of theanine content extracted at 80°C and 90°C were higher than at 70°C, the rate constant for these three temperatures did not have the same trend. It was shown that extraction at 70°C needed a shorter time to reach the equilibrium value than at 80°C and 90°C.

Table 4.15. Kinetics and equilibrium data for theanine extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
70	2.087	0.089	0.597	0.246
80	2.189	0.033	0.875	0.388
90	2.292	0.086	0.802	0.410

4.4.4 Caffeine

Caffeine content in green tea extracts were in the range of 0.082% - 0.695% in dry basis. Similar to other compounds, this content increased with the increasing of extraction time. Caffeine content during the extraction process is shown in Figure 4.11. From the figure it can be seen that caffeine contents in extraction at 70°C and 80°C were similar, whereas the caffeine content extracted at 90°C was higher. The same result was found by Wongsuwan (2008). The maximum concentration value reached in extraction at 70°C, 80°C and 90°C were 0.540%, 0.564% and 0.695%, respectively.

Kinetics and equilibrium data for caffeine extraction is shown in Table 4.16. Moreover, it was found that extraction at 90°C was the optimum condition for caffeine concentration by providing the highest equilibrium concentration and rate constant. Similar to extraction in the laboratory scale, due to its molecular weight (as described in 4.3), rate constant for caffeine extraction was lower than that for theanine. The same result was found by Wongsuwan (2008). By using the same extractor unit for the pilot plant scale, extraction for total

amino acids (expressed as theanine) has the highest rate constant compared to total polyphenols, tannin and caffeine.

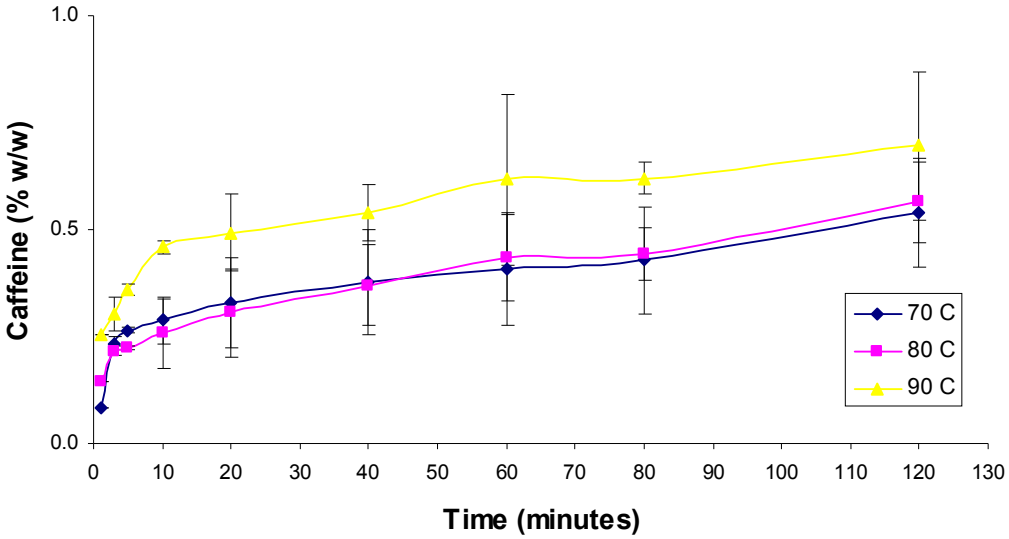


Figure 4.11 Effect of time and temperatures on caffeine contents extracted from green tea

Table 4.16 Kinetics and equilibrium data for caffeine extraction (1 – 10 minutes)

Temperature (^{0}C)	C_∞ (%w/w dry basis)	k_{obs} (min^{-1})	R^2	a
70	0.540	0.057	0.702	0.270
80	0.564	0.030	0.826	0.330
90	0.695	0.071	0.998	0.371

4.4.5 Individual catechins

EGCG, EGC, EC, GCG and GC were the compounds from catechins that were detected in green tea extracts. The concentration of each compound during extraction at various temperatures and times were plotted in Figure 4.12. The total of these compounds was also measured. The content of all compounds tended to increased over time. The content of EGCG, EGC and EC in green tea extracts were in the range of 0.013% - 0.725%, 0.025% - 0.741% and 0.007% - 1.416% of green tea in dry basis, respectively. Meanwhile the content of GCG, GC and total catechins were in the range of 0.000% - 0.055%, 0.034% - 0.745% and 0.079% - 3.660% of green tea in dry basis, respectively.

It was observed that GCG can be extracted after only 10 minutes of extraction at all temperatures studied. The contents of each compound from extraction at 90°C were higher than that at 70°C and 80°C. From Table 4.1, it can be seen that GCG was not detected in green tea. Therefore, it is concluded that epimerization of EGCG to its corresponding epimer (GCG) was took place.

It was found that EGCG contents from extraction at 70°C were slightly higher than its content at 80°C. A similar trend was found for EGC and GCG content. From Figure 4.12. it can be seen that EGC and GCG content from extraction at 70°C were higher than that at 80°C. However, in the end, it reached the same value for EGC content, while GCG content from extraction at 80°C was higher than 70°C. After all, the total catechins content extracted at 90°C were certainly higher than other temperatures. Total catechins content extracted at 70°C and 80°C were almost similar at the first 20 minutes of extraction and then these contents from extraction at 80°C tended to be higher than that at 70°C.

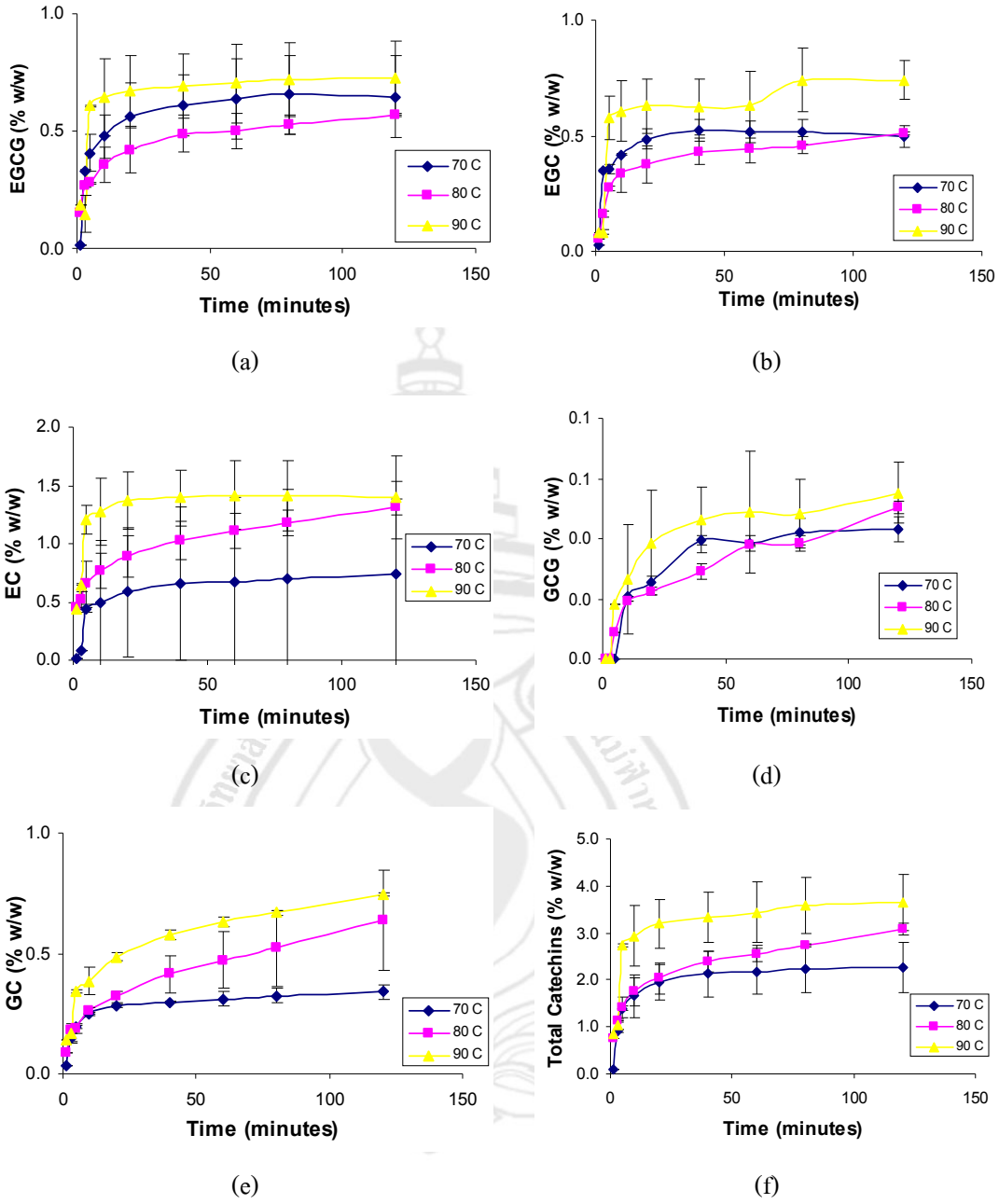


Figure 4.12 Catechins contents extracted from green tea at various times and temperatures. (a) EGCG, (b) EGC, (c) EC, (d) GCG, (e) GC and (f) total catechins

Table 4.17 Kinetics and equilibrium data for EGCG, EGC, EC, GCG, GC and total catechins (1 – 10 minutes)

^{0}C		EGCG	EGC	EC	GCG	GC	Total catechins
70	C_∞ (%w/w db)	0.654	0.525	0.735	0.043	0.341	2.262
	k_{obs} (min^{-1})	0.129	0.182	0.129	0.078	0.124	0.143
	R^2	0.838	0.882	0.826	0.821	0.940	0.910
	a	0.137	-0.128	-0.081	-0.205	0.112	-0.020
80	C_∞ (%w/w db)	0.570	0.508	1.311	0.050	0.637	3.077
	k_{obs} (min^{-1})	0.069	0.105	0.052	0.056	0.039	0.060
	R^2	0.916	0.932	0.963	0.821	0.925	0.972
	a	0.325	0.086	0.383	-0.146	0.154	0.260
90	C_∞ (%w/w db)	0.725	0.741	1.416	0.055	0.745	3.660
	k_{obs} (min^{-1})	0.232	0.173	0.226	0.079	0.061	0.158
	R^2	0.783	0.815	0.852	0.901	0.842	0.824
	a	0.022	0.166	0.213	-0.112	0.164	0.174

The rate for extraction of all compounds were then measured. The kinetics and equilibrium data is shown in Table 4.17. For EGCG, EC, GCG and total catechins extraction, 90°C was found to be the optimum temperature by providing the highest equilibrium value and rate constant. Based on the equilibrium value, 90°C was also the optimum for EGC and GC extraction, but according to the rate constant, 70°C was the optimum temperature for EGC and GC extraction.

Among individual catechins, GC extraction has the lowest rate constant, while extraction of EGCG has the highest rate constant. From this study, it can be concluded that EGCG dissolved and extracted at the highest rate compared to other catechins. The kinetic study in the laboratory and pilot plant scale for catechins extraction has different trends. The difference in extraction system might be an important reason. In the laboratory scale, the extraction was performed in a batch without agitation, while in the pilot plant scale, it was performed

continuously. In a continuous system, the water was pumped continuously through the material, giving higher extraction efficiency.

Based on the theory, through equation (3.2), the line of the first order plot should pass through the origin (axis). However, for the kinetic study of some compounds (both in laboratory and pilot plant scale) a non-zero intercept was found. As has been reported in many other situations, a semi-empirical intercept (*a*) or non-zero intercept was found. Presumably this intercept is a result of a complex infusion process. The intercept is affected by the loss of solubles, the green tea structure and its uptake of water at the beginning of the infusion process. This intercept serves to indicate the quality of the data and deviations from the model employed (Jaganyi and wheeler, 2003).

4.5 Fractionation of green tea extract

4.5.1 Preliminary study

1. Selection of eluting agent (mobile phase)

This study aimed to select the best mobile phase to separate compounds in green tea using the TLC (Thin Layer Chromatography) methods. TLC is a simple and quick method to determine how many compounds are in the mixture and the identity of a compound by comparing its Rf (retention factor) with the Rf of a known compound (Anonymous, 2009b). Compounds in a mixture will be successfully separated if the TLC is developed in a suitable mobile phase.

Figure 4.13 shows the TLC aluminium sheets (normal phase) for green tea samples, caffeine, EGCG, ECG, EGC and EC. Mobile phases used to develop the TLC sheets in picture below, from left to right, were water, 25%, 50%, 75% and 95% of ethanol, respectively. Furthermore, compounds spotted in each TLC sheet, from left to right, were catechin (C), caffeine, green tea extract (three spots), EGCG, ECG, EGC and EC, respectively. The caffeine spots are visible under UV light only.

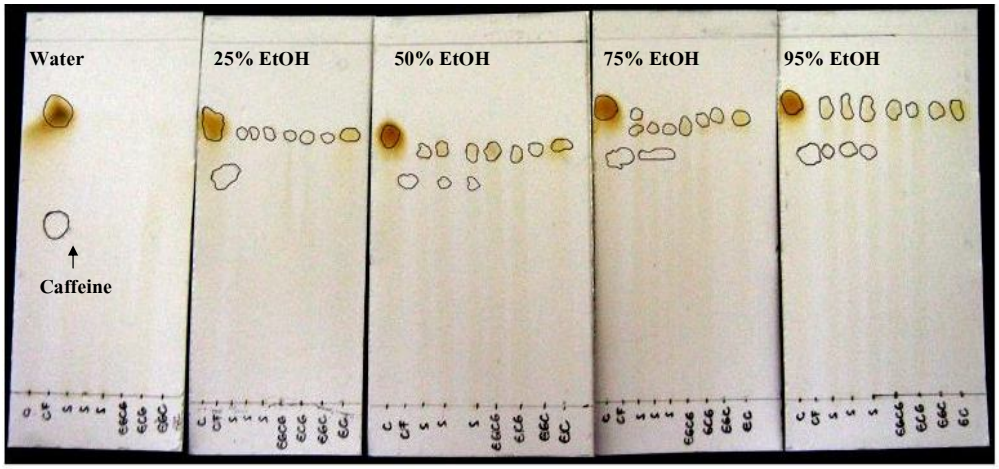


Figure. 4.13 Thin layer chromatography of green tea extract, caffeine and catechins compounds (from left to right: C, caffeine, sample, sample, sample, EGCG, ECG, EGC and EC)

On the TLC sheet that was developed in water, the spot that can be seen was caffeine and catechin. The catechin spot appeared in this TLC sheet because it was spotted in the highest concentration compared to the other samples. Therefore, the catechin spot was the biggest in each TLC sheet. All of the sample spots appeared on the sheets developed in 25% until 95% ethanol. It was observed that the sample spots (green tea extract) were separated into 2 spots in the TLC, developed in 50%, 75% and 95% of ethanol. It means that these mobile phases have an ability to separate compounds from green tea extract. The retention factor of the first spots of green tea extract was similar to catechins spot, while the second spot was similar to caffeine spots. However, the retention factors of these spots were not significantly different between TLC developed in 50%, 75% and 95% of ethanol. In these TLC sheets, it was found that the retention factor of catechins spots were about 0.60 – 0.68 and the retention factor for caffeine was about 0.52 – 0.56.

In silica gel, the dominant interactive forces between adsorbent and samples to be separated are dipole-dipole and hydrogen bond type. Highly polar molecules interact strongly with the polar Si-O bonds of silica gel and tend to stick or adsorbed into fine particles of silica, while weakly polar molecules are held less tightly (Grinberg, 1990; Anonymous, 2009b). Weak

polar molecules commonly tend to move through the silica more rapidly than highly polar molecules. Caffeine (moment dipole = 3.64) is more polar than phenols (moment dipole = 1.7). Therefore, the retention factors of catechins (phenols group) were higher than caffeine (Grinberg, 1990; Anonymous, 2009b).

The polarity of the mobile phase affects the relatives rates of compounds to move through the TLC sheet. The polar mobile phase can more effectively compete with the polar molecules of a mixture (sample) for the polar site on the adsorbent (silica gel) surface and will also better elute the polar compounds. As a consequence, a highly polar mobile phase will elute even highly polar molecules rapidly through the column. If a mobile phase is too polar, the elution becomes too rapid and little or no separation is achieved (Anonymous, 2009b). Therefore, the spots of green tea extract were separated only in TLC that was developed in 50%, 75% and 95% of ethanol due to its lower polarity than 25% of ethanol.

2. Effect of resin type and its packed bed height

As described in Chapter 2, there are many types of adsorbent (resin) that have been applied in fractionation of phenolic compounds. Commonly, the phenolic compounds remain in the adsorbent, while sugars, pectins, alkaloids and other polar compounds are eluted with water and/or aqueous solvent. The phenolic fractions can then be eluted with organic solvents (mainly methanol and ethanol). Based on the results in 4.5.1.1, it was found that water-ethanol mixtures (50%, 75% and 95% of ethanol in water) have the ability to separate compounds of green tea extract using silica gels as the stationary phase.

The main purpose from this section was to determine the suitable resin and the effect of its packed bed height for total polyphenols and caffeine separation. A sample for column chromatography was green tea extract that was extracted using the optimum condition from previous studies. Dried and ground green tea was extracted at a tea-water ratio of 1:20 and pH 5. Every 10 ml of the green tea extract was evaporated and then loaded to each column. The content of total polyphenols and caffeine (from colorimetric method) of green tea extract was used as a sample in this study, shown in Table 4.18.

Table. 4.18 Total polyphenols and caffeine of green tea extract

Total polyphenols (TPP)		Caffeine	
Optical density	**mg/ml**	**Optical density**	**mg/ml**
0.68	6.30	0.35	1.61

Various resins including polyamide 6, amberlite XAD7HP, nylon 6 and poly (dimmer acid-co-alkyl polyamine) were used (shown in Figure 4.14). Each resin was packed into two columns with different diameter sizes (medium and small size columns). The details of packed bed, column size, and sample for each resin is shown in Table 4.19. These two column sizes for each resin were used to compare the caffeine and total polyphenols separation from the obtained fractions. With the same weight of the resin, the different column size affected the difference of packed bed height of the resin in the column as well as the retention factor (Rf) of compounds from the sample.

The mobile phases used in these columns were water (to elute caffeine) and 50% ethanol (to elute total polyphenols). The amount of water used for each column was similar (300 ml). Every 15 ml of fractions were collected from each column. These fractions were randomly picked for total polyphenols and caffeine analysis.

(a) (b) (c) (d)

Figure. 4.14 Physical appearance of polyamide 6 (a), amberlite XAD7HP (b), poly (dimmer acid-co-alkyl polyamine) (c), and nylon 6 (d)

Table 4.19 Type of resin, packed bed height and tea sample used in this study

Resin	Column	Column size		Packed bed		Sample	
		Outside diameter (cm)	Outside diameter (cm)	Weight (g)	Height (cm)	Volume (ml)	Evaporated weight (g)
Polyamide 6	Medium	3.3	25.5	20	16.3	10	1.01
	Small	1.64	70	20	56	10	2.18
Amberlite XAD7HP	Medium	3.30	27	20	5.30	10	0.81
	Small	2.61	37	20	10.70	10	0.82
Poly (dimmer acid-co-alkyl polyamine	Medium	3.16	26	20	8	10	1.19
	Small	1.78	39.20	20	18	10	1.32
Nylon 6	Medium	3.17	26.40	20	5.92	10	0.26
	Small	1.82	38.50	20	14	10	0.27

1) Polyamide 6

The peak profile of total polyphenols and caffeine content from polyamide 6 is shown in Figure 4.15 and 4.16. There were 20 fractions from the polyamide 6 column eluted by water. The first 10 fractions both came from medium and small sized column from this resin were rich in caffeine, while the remaining fractions were rich in polyphenols.

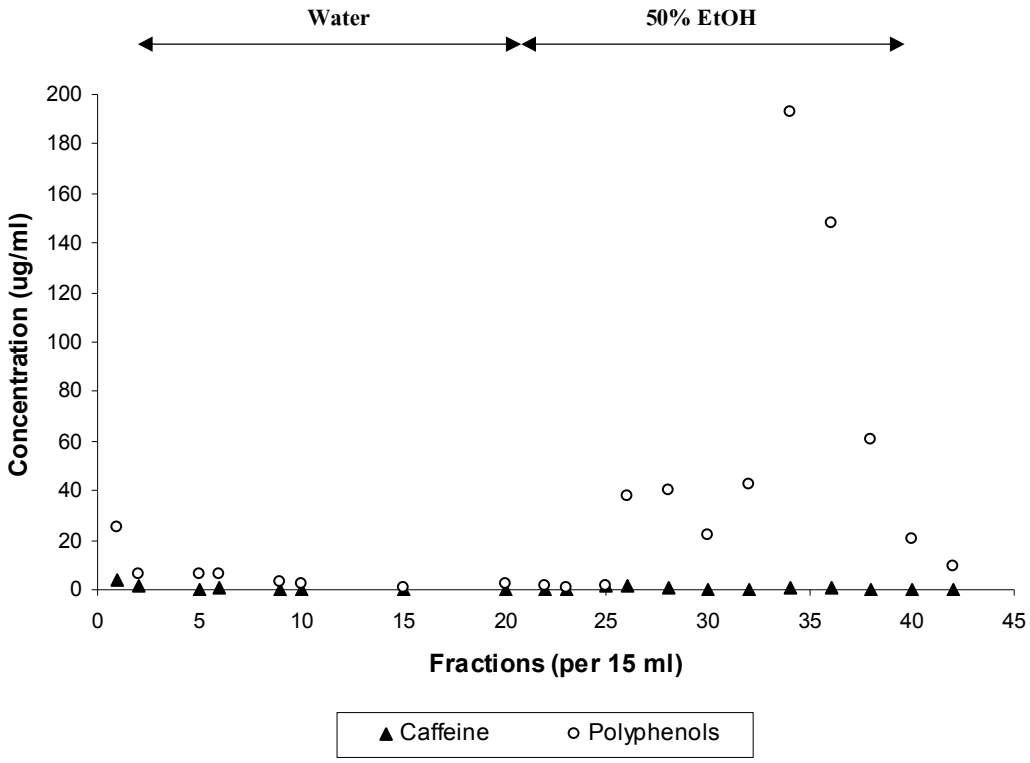


Figure 4.15 Peak profile of total polyphenols and caffeine contents from polyamide 6 with medium sized column

From the figure, it can be seen that most caffeine fractions were eluted by water. The remaining caffeine was eluted by 50% of ethanol. Compared to the first 20 fractions, the caffeine contents in these fractions eluted by 50% ethanol were very low. It was less than 1 μg/ml in each fraction. Therefore, from this result, it can be concluded that polyamide 6 has the ability to separate polyphenols and caffeine. Green tea caffeine and polyphenols can be separated by loading the green tea extract into columns containing polyphenols-adsorbing resin, and separating caffeine with water and desorbing polyphenols with 50-55% of ethanol in water (Bailey *et al*, 2001).

Fractionation through the adsorption method is based on differences in polarity, molecular weight and shape of different molecules from the solution. It leads to differences in affinity for the adsorbent (Silva *et al*, 2006). There are several types of interactions

between compounds and adsorbent, such as ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole and van der Walls forces (Anonymous, 2009b).

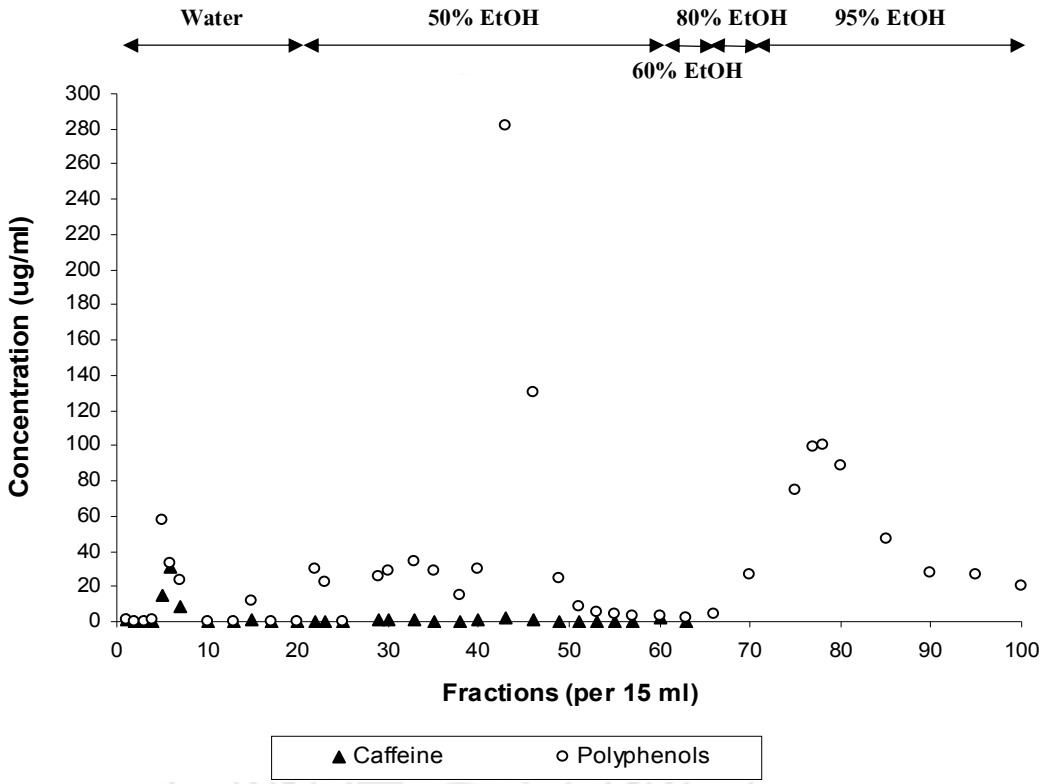


Figure 4.16 Peak profile of total polyphenols and caffeine contents from polyamide 6 with small sized column

Polyamide 6 is a amide-containing polymers characterized by the presence of piperazine amide and/or substituted piperazine amide in the polymer chain (the structure is shown in Figure 4.17). It is a synthetic polymer obtained by various methods (lactame polymerization or polycondensation of fatty acids with aliphatic amines). This resin has been widely used as adsorbents to separate polyphenolic compounds from liquids. Polyphenolic compounds are adsorbed into polyamide 6 particles (Grinberg, 1990; Michos, 1999). Polyamide has a linear molecule in which the -CO-NH- groups are separated by alkyl chains. The molecules

are held together by a three dimesional network of (=NH……OC) hydrogen bonds. Consequently, polyamide can interact with different solutes through the –NH- and –CO- groups. In the adsorption of proton donor molecules, the amide carbonyl group can plays the role of hydrogen acceptor in the hydrogen bond formation with solutes. The amide nitrogen groups play the part of hydrogen donor. It also contributes to the adsorption properties, although to a minor degree (Grinberg, 1990).

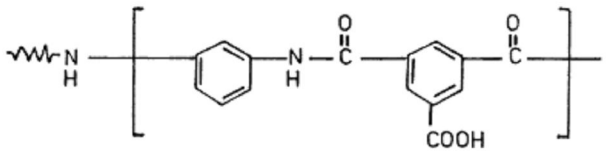


Figure 4.17 Polyamide stucture (linear hydrophilic structure)

Source: Rao *et al* (2003)

Polyamide has better polyphenol-adsoption capacity and better selectivity. It was found that caffeine and polyphenols compounds can be separated with polyamide 6 (Ping li *et al*, 2005). When a very polar eluent is used, the polyamide acts like a non polar stationary phase (Grinberg, 1990). Therefore, adsorption of tea polyphenols with polyamide 6 is stronger than that of caffeine. Hydrogen bond plays an important role in this adsorption (Tang *et al*, 2003). Polyphenols compounds are held more tightly in polyamide than caffeine. Therefore, caffeine can be eluted easily before polyphenols by polar mobile phase (water).

From Figure 4.15 and 4.16, the total polyphenols content can be eluted slightly with water elution. In the first 20 fractions from polyamide 6 column (medium and small sized column), there were peaks of polyphenols in a lower level. However, most polyphenols compounds were eluted by 50%. In the small column, the retention time of polyphenols compounds was slower than that in the medium size column. Ethanol concentration for elution in small size column was increased to elute all polyphenols compounds. More than 100 fractions

were needed in the small sized column to get the entire caffeine and polyphenols eluted, while medium sized column just resulted in 43 fractions.

2) Amberlite XAD7HP

Caffeine and total polyphenols peak profile from amberlite XAD7HP resin is shown in Figure 4.18 and 4.19. Same with polyamide columns, there were 20 fractions collected from water elution. The eluting process was then continued by a water-ethanol mixture. The first 20 fractions from amberlite XAD7HP columns (both medium and small size columns) contained caffeine (less than 1 µg/ml) and polyphenols (not more than 80 µg/ml) in a low level. From the figure, it can be seen that the difference of packed bed height of amberlite XAD7HP did not affect its ability to separate caffeine and polyphenols. Most caffeine and polyphenols compounds were eluted by 50% of ethanol. It is concluded that caffeine and polyphenols compounds were not separated with this resin.

Amberlite XAD7HP is a non ionic polymeric adsorbent. Due to its aliphatic nature, this resin can adsorb non-polar compounds from aqueous systems and also polar compounds from non-polar solvents (Anonymous, 2003b). Several studies have demonstrated that amberlite XAD7HP has an ability to adsorb polyphenols fractions (Silva *et al*, 2006; Yamanaka *et al*, 2007). In this study, polyphenols compounds were adsorbed in amberlite XAD7HP. It was indicated by polyphenols peak that was starting to appear from fraction number 21. This compound was not eluted easily and rapidly with water elution. However, the adsorption of tea polyphenols and caffeine with amberlite was the same. Therefore, these compounds were eluted together with 50% ethanol elution.

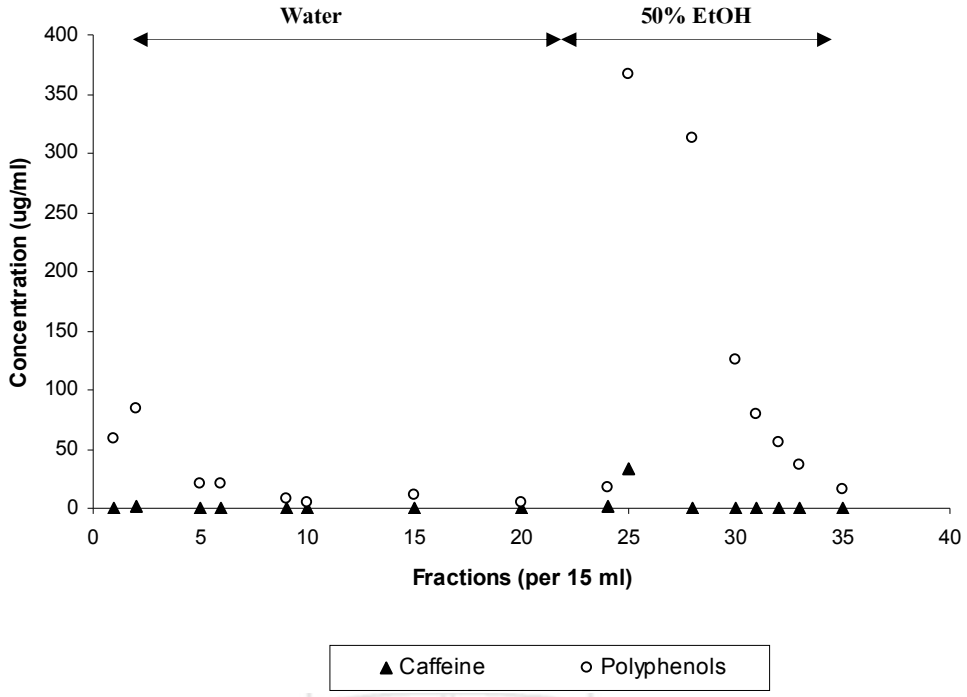


Figure 4.18 Peak profile of total polyphenols and caffeine contents from amberlite XAD7HP with medium sized column

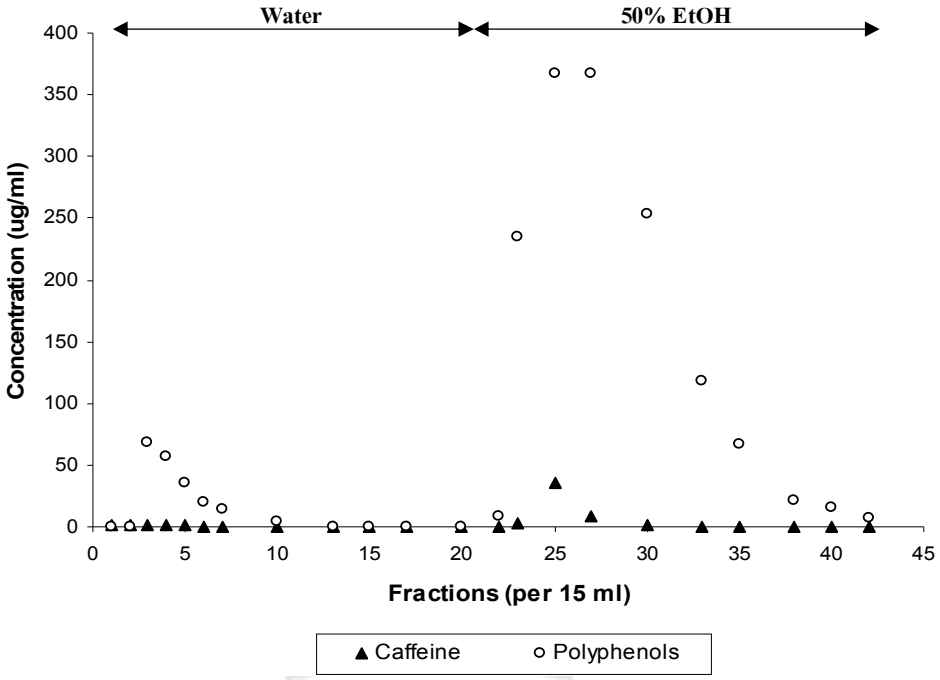


Figure 4.19 Peak profile of total polyphenols and caffeine contents from amberlite XAD7HP with small sized column

3) Poly (dimmer acid-co-alkyl polyamine)

This resin was packed into two columns that had different diameter sizes (shown in Table 4.19). The peak profile of total polyphenols and caffeine contents eluted from this resin is shown in Figure 4.20 and 4.21. Same with the first two resins, the first mobile phase for these two columns was water. From the figure, it was shows that both polyphenols and caffeine compounds were eluted immediately from the column with water elution only. Separation of those compounds did not occurr with this resin. Packed bed height had no effect on the ability of this resin to adsorb and separate polyphenols from caffeine compounds.

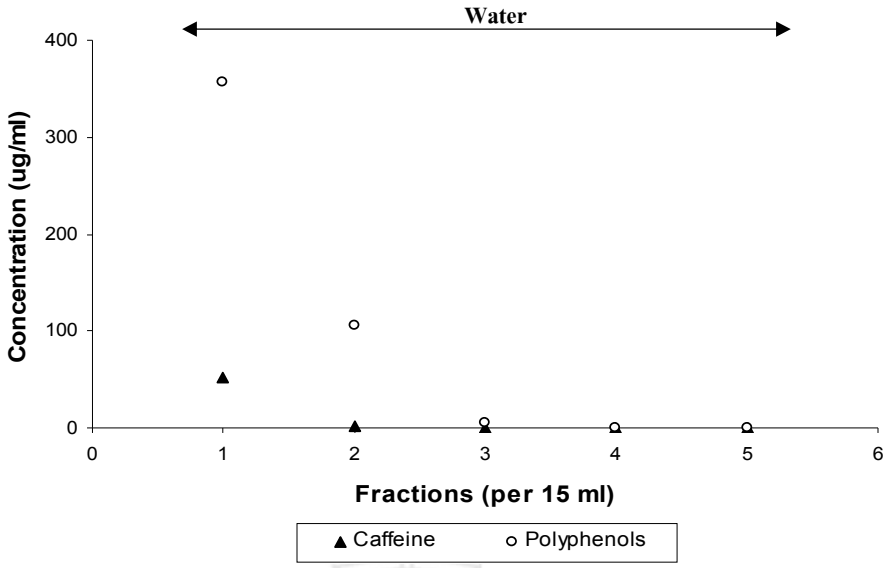


Figure 4.20 Peak profile of total polyphenols and caffeine contents from poly (dimmer acid-co-alkyl polyamine) with medium sized column

This resin (polyamine) is a polyphenolic-adsorbing polymer. Its ability will improve when it is combined/modified with other polymers such as polyamide (Michos, 1999). However, the result of this study shows that poly (dimmer acid-co-alkyl polyamine) could not adsorb polyphenols from green tea extract solution. Presumably, it is because poly (dimmer acid-co-alkyl polyamine) have large particle sizes (as shown in Figure 4.14), which cause the green tea extract compounds to be immediately eluted by water.

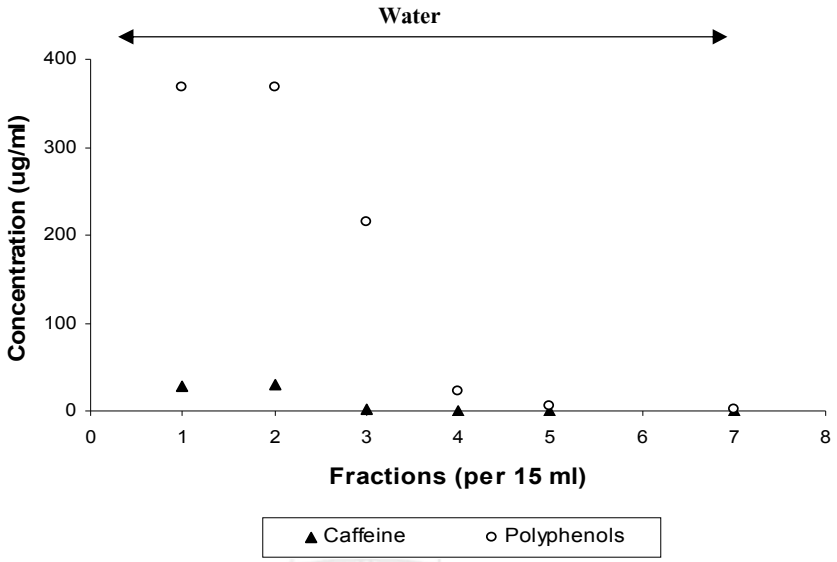


Figure 4.21 Peak profile of total polyphenols and caffeine contents from poly (dimmer acid-co-alkyl polyamine) with small sized column

4) Nylon 6

Same with other resins, this resin was packed into two columns (shown in Table 4.19). The peak profile of total polyphenols and caffeine contents eluted from this resin is shown in Figure 4.22 and 4.23. Similar to poly (dimmer acid-co-alkyl polyamine), the caffeine and polyphenols compounds were eluted immediately with water elution only. Neither adsorption nor separation of caffeine and polyphenols compounds occured with this resin.

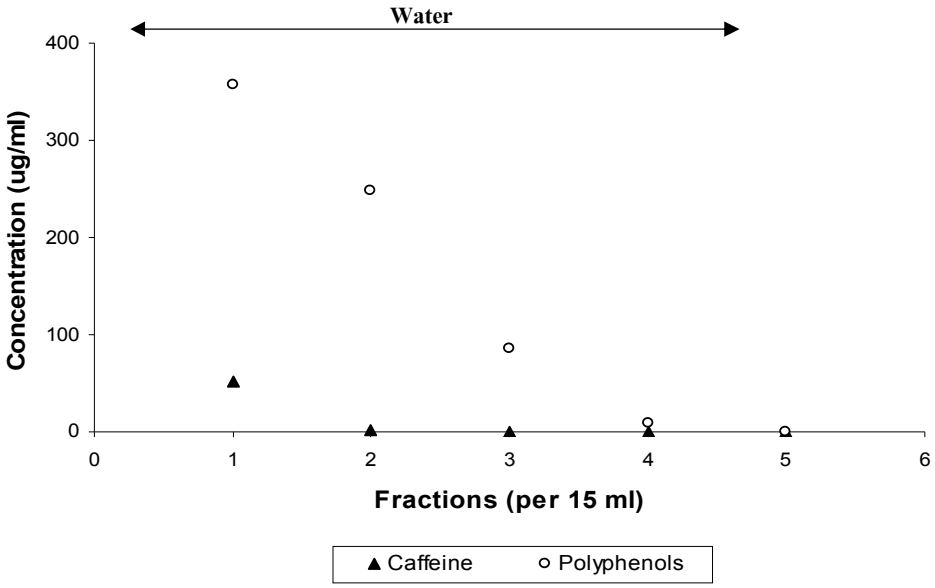


Figure 4.22 Peak profile of total polyphenols and caffeine contents from nylon 6 with medium sized column

Nylon 6 is a commercial aliphatic polyamide. Normally it can be used as an adsorbent to separate polyphenolic compounds from liquids (Michos, 1999). However, in this study, green tea polyphenols were not adsorbed in nylon 6. Similar to poly (dimmer acid-co-alkyl polyamine), it might be due to the large particle of this resin (as shown in Figure 4.14). Therefore, the green tea extract compounds are directly eluted as soon as the column is eluted with water.

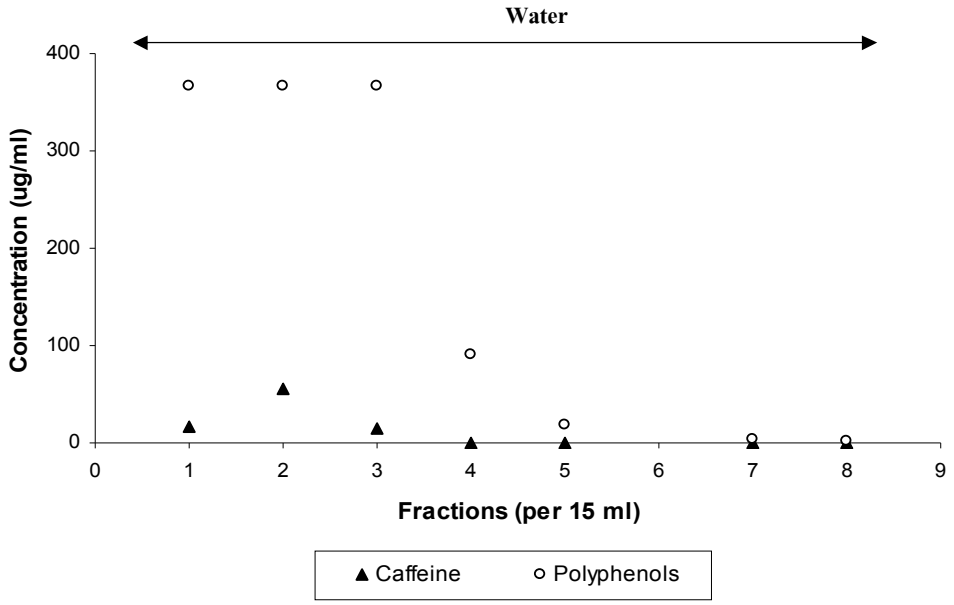


Figure 4.23 Peak profile of total polyphenols and caffeine contents from nylon 6 with small sized column

From all resins, polyamide 6 exhibited the best ability in caffeine and polyphenols separation. The caffeine compound was eluted from the column by water. From the 10 ml of green tea extract that was loaded into the column, caffeine fractions were collected with 300 ml of water. It resulted in 20 fractions. The first 10 fraction were rich in caffeine. Packed bed height of this resin affected the caffeine and polyphenols separation. It was observed that the retention time for polyphenol compounds in a small sized column was longer than that in a medium sized column. Polyphenol eluted by 50% ethanol in both columns, this elution was faster in a medium sized column. Therefore, polyamide 6 and a medium sized column were selected. This condition was then used to study the effect of the elution system of a water-ethanol mixture.

3. Effect of elution system (isocratic and gradient) of water-ethanol mixture

It had been demonstrated that polyamide 6 can be purified polyphenols by water-ethanol mixture elution, both with isocratic or gradient elution systems (Amico *et al*, 2007). In the process of polyphenols separation from green tea, caffeine and other extraneous materials can be separated by eluting the adsorbent with water containing 0-10% of ethanol (Bailey, 2001). Therefore, this study was conducted to determine which conditions were better to separate caffeine completely from polyphenols compounds. Polyamide 6 was packed into 2 medium sized columns. The isocratic water-ethanol mixture was used in one column, while the other one was using a gradient. The first mobile phase for both columns was water to elute the caffeine and the remaining caffeine and polyphenols eluted by water-ethanol. Isocratic elution was elution with 50% of ethanol (directly after water elution), while gradient elution was with a water-ethanol mixture starting from 10% of ethanol. As described in Chapter 2, gradient elution is one developmental way, where the concentration of the mobile phase progressively changed during the elution. In gradient elution, solute molecules from the sample may interact more frequently with the stronger eluting solute and held more strongly in the mobile phase. As a consequence, compounds can be eluted faster (Scott, 1995).

The peak profile of total polyphenols and caffeine from this resin with isocratic and gradient elution can be seen in Figure 4.15 and Figure 4.24, respectively. Water elution was resulting in 20 fractions from both columns. The first 10 fractions were rich in caffeine content. From the figure, it can be seen that most caffeine was eluted by water. This compound continued to be eluted slightly by the water-ethanol mixture but in a low level (not exceed than 1.5 µg/ml). With isocratic elution, fractions consisting of polyphenols started from fraction number 25, while in gradient elution, it started from fraction number 60 (after 50% ethanol was used as the mobile phase). It can be concluded that polyphenols compounds eluted mostly by a water-ethanol mixture start from 50% or 60% of ethanol.

After adsorbed in polyamide resin, green tea polyphenols compounds can be desorbed by water-ethanol mixture elution. The desorption rate of green tea polyphenols tended to be increasing with the increasing ethanol concentration in water-ethanol mixture. The desorption rate of green tea polyphenol with 10-30% of ethanol were ranged from 14.1- 55.2%, while the

desorption rate of green tea polyphenol with 45-95% of ethanol ranged from 83-93.5% (Ping li *et al*, 2005).

Because polyphenols compounds eluted faster in an isocratic elution system than in a gradient system, isocratic elution of a water-ethanol mixture was then selected for use in the following study.

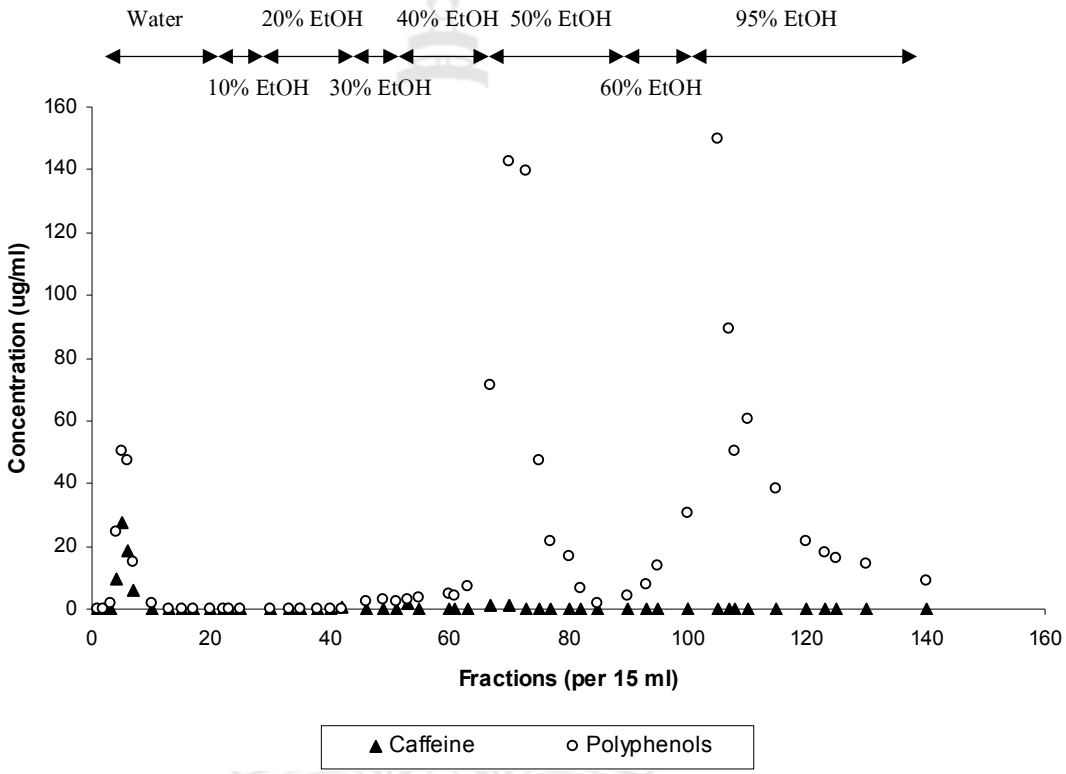


Figure 4.24 Peak profile of total polyphenols and caffeine contents from polyamide 6 with medium sized column and gradient elution

4.5.2 Fractionation of green tea extract

1. Fractionation of residual green tea extract

Residual green tea extract powder used in this study came from a commercial ready-to-drink green tea residue. To study the fractionation of this material, resin polyamide 6 packed in medium sized column with isocratic of water-ethanol mixture was used. Some fractions from this study were subjected to HPLC analysis to observe the order of polyphenols compounds eluted out from the column.

With the same amount of green tea extract used in 4.5.1 (10 ml of green tea extract), most of the catechins compounds from the obtained fractions were not detected by the HPLC instrument. Presumably the fractions were too diluted. Therefore, the residual green tea extract was used in this study in order to increase the concentration of each compound, so that can be detected easily by HPLC. The chemical compositions of residual green tea extract are shown in Table 4.20. The residual green tea extract consisted of 9.152% of theanine, 2.999% of caffeine and 9.713% of total catechins in dry basis.

Table 4.20 Chemical compositions of residual green tea extract

% w/w Dry basis									
		Individual catechins							
Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**	**Total catechin**
9.512	2.999	0.755	1.280	1.071	0.254	1.465	2.859	2.029	9.713

Every 5 ml of fractions were collected from this column. The content of total polyphenols and caffeine in the obtained fractions is shown in Figure 4.25. This column resulted in more than 2 liters of fractions. These fractions were picked randomly and subjected to colorimetric and HPLC analysis. The first mobile phase for this column took 400 ml of water to separate the caffeine. The first caffeine peak appeared in the first 200 ml of fraction, while the

second one appeared in the fraction volume 440-560 ml. It indicates that caffeine was not eluted completely with only water, but also with 50% of ethanol elution. The same result was found in 4.5.1.2 and 4.5.1.3. Most caffeine fractions were eluted by water. However, in the fractions eluted by 50% of ethanol, the caffeine fraction still detected in lower concentration (not more than 1.5 μg/ml). In this study, the caffeine peak in fractions eluted by 50% of ethanol become more obvious because was green tea extract, which is more concentrated than samples used in previous studies (4.5.1.2 and 4.5.1.3).

After water elution, water-ethanol mixtures were then used to elute the polyphenols compounds. 50% of ethanol solution was used directly as a second mobile phase after water elution. During polyphenols elution, the ethanol concentration gradually increased to improve the desorption of polyphenols.

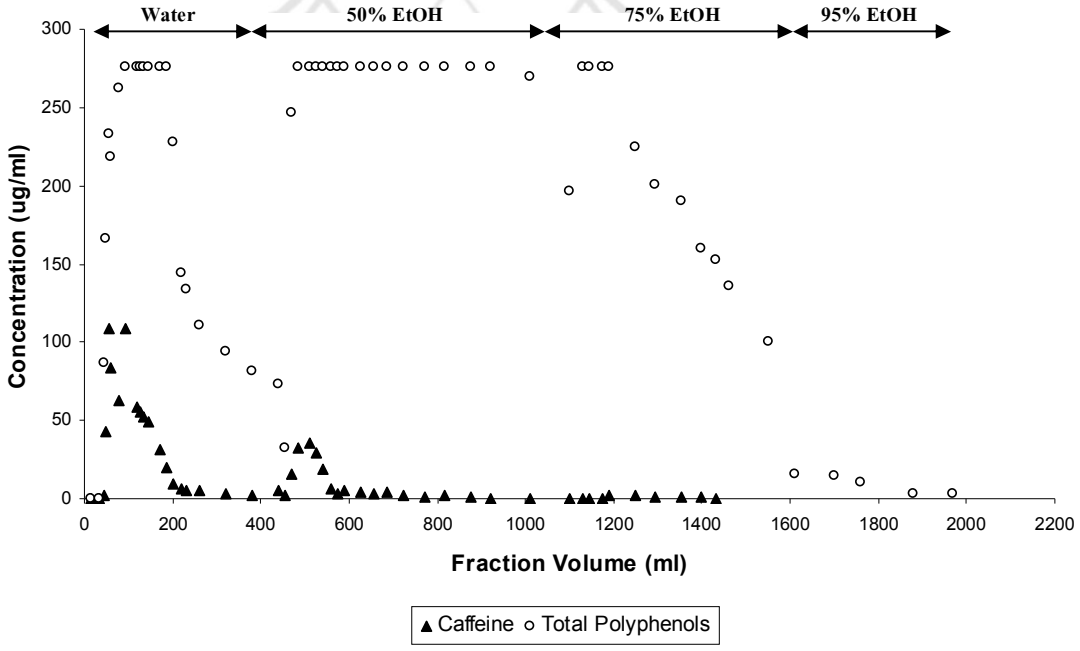


Figure 4.25 Total polyphenols and caffeine contents of residual green tea extract fractions

Table. 4.21 Chemical compositions of fractions from residual green tea extract

Eluting agent	Fraction volume (ml)	% Eluted								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
Water	55-60	2.574	1.323	nd*	nd	nd	nd	nd	0.335	nd
	95-100	2.067	1.322	nd	nd	nd	nd	nd	0.293	nd
	230-235	0.121	0.077	nd	nd	nd	nd	nd	nd	nd
50% EtOH	455-460	0.010	0.019	0.017	0.014	nd	0.078	nd	0.020	nd
	510-515	6.273	2.008	6.889	3.585	0.539	32.839	0.019	3.891	nd
	655-670	1.617	nd	6.572	nd	1.291	nd	2.986	nd	0.631
	920-925	0.442	nd	0.126	nd	nd	nd	1.413	nd	0.331
75% EtOH	1190-1195	0.339	nd	nd	nd	nd	nd	0.179	nd	0.027
95% EtOH	1610-1615	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1880-1885	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd = not detected

Table. 4.22 Chemical compositions of combined fractions from residual green tea extract

Fraction (ml)	Fraction volume (ml)	% Eluted								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
45-190	145	33.426	31.752	nd*	nd	nd	nd	nd	6.273	nd
190-455	265	4.409	0.810	nd	nd	nd	nd	nd	9.458	nd
455-1550	1095	nd	23.922	99.553	35.858	97.943	94.851	91.200	63.248	42.512

*nd = not detected

Ten fractions from this column were then subjected to HPLC analysis and the result is shown in Table 4.21. The results were presented in % eluted (the formula is shown in 3.2.5.2). It was observed that caffeine and GC were eluted by water, while theanine was eluted by water, 50% of ethanol and 75% of ethanol. Moreover, EGC, EC, EGCG and ECG were eluted by 50% of ethanol, while GCG and CG were eluted by 50% and 75% of ethanol. There was no compound detected in fractions eluted by 95% of ethanol. Therefore, elution with 95% of ethanol was not necessary.

According to caffeine content, all of the fractions were combined and then divided into three main fractions. The first main fraction combined from fraction volumes of 45 ml to 190 ml. These fractions were eluted by water and had a caffeine peak. The second main fraction combined volumes of 190 ml to 455 ml. Similar to the fractions from the first main fraction, this fraction was eluted by water and had a low caffeine and polyphenols peak. Meanwhile, the third main fraction combined volumes of 455 ml to 1,095 ml, eluted by 50% and 75% of ethanol. The HPLC analysis results of these three main combined fractions are shown in Table 4.22.

From Table 4.22, it is shown that the first main fraction was rich in theanine and caffeine. Comparing to the amount of the sample, this fraction consisted of 33.426% theanine and 31.752% caffeine. Besides, this fraction also consisted of 6.273% of GC. The second main fraction consisted of theanine, caffeine and GC in the lower concentration than that from the first main fraction. From this result, it was found that 37.835% of theanine, 32.562% of caffeine and 15.731% of GC from the residual green tea extract was eluted by water.

The third main fraction was rich in catechins but still had caffeine in a lower level. However, the theanine compound was not detected in this fraction, presumably due to its low concentration. From this result, it can be concluded that from 5g of residual green tea extract, 23.922% of caffeine, 63.248% of GC, 35.858% of EGC, 94.851% of EC, 99.553% of EGCG, 91.200% of GCG, 97.943% of ECG and 42.512% of CG were eluted by 50% and 75% of ethanol, respectively.

2. Fractionation of green tea extract

The green tea fractionation study was conducted based on the result 4.5.2.1 Green tea extract was used as the sample in this study instead of a residual green tea extract. The chemical compositions of green tea extract are shown in Table 4.23. Theanine and caffeine content were 13.260% and 3.1216% of dry basis, respectively, while the total of individual catechins was 22.037%. Compared to the residual green tea extract in 4.5.2.1, this green tea extract have a better quality due to its compounds that have a higher concentration than those in residual green tea extract.

Table 4.23 Chemical compositions of green tea extract

% w/w Dry basis									
		Individual catechins							
Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**	**Total catechins**
13.260	3.126	2.335	2.520	1.638	4.918	3.002	5.916	1.708	22.037

The colorimetric analysis of total polyphenols content and caffeine content were used to ensure the result from this column similar to the result in 4.5.2.1 Caffeine and polyphenols contents of the fractions is shown in Figure 4.26. Same with the column from 4.5.2.1, the first mobile phase was water and then continued to be 50% and 75% of ethanol. From the previous study, it was found that most caffeine compounds were eluted out from the column by water in the first 200 ml of fractions. For this column, 300 ml of water was used to elute the caffeine content. Similar to the column in section 4.5.2.1, caffeine from this column was not separated completely by water elution. Polyphenols compounds and the remaining caffeine were eluted from the column by 50% of ethanol (700 ml) and 75% of ethanol (650 ml). This column resulted in about 1,650 ml of fractions. The peak profile in Figure 4.26 was almost similar with

the peak profile from the column in 4.5.2.1. It can be concluded that with the same conditions, fractionation of 5g of residual green tea extract and 5g of green tea extract were almost similar.

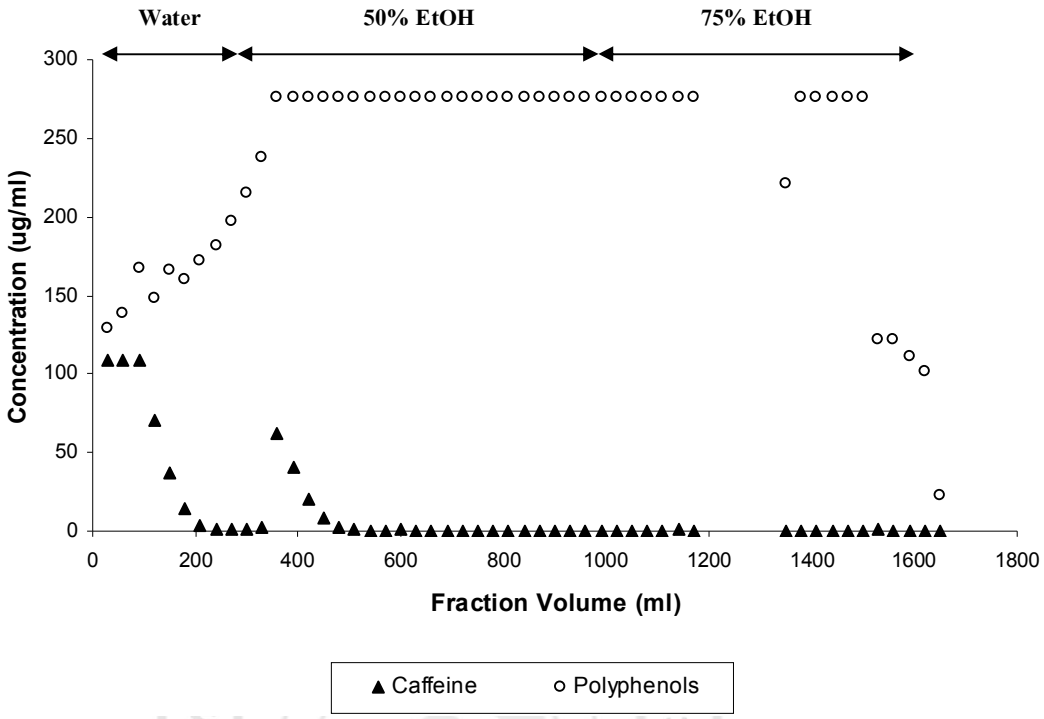


Figure 4.26 Total polyphenols and caffeine of green tea extracts fractions

The column with green tea extract as a sample was once again repeated for the HPLC analysis. Considering the efficiency, fractions were collected for every 50 ml for 50% and 75% of ethanol elution and 300 ml for water elution. This column resulted in 28 of the fractions. Eight of them were picked randomly and subjected to HPLC analysis. The HPLC analysis result is shown in Table 4.24.

Table. 4.24 Chemical compositions of fractions from green tea extract

Eluting agent	Fraction volume (ml)	% Eluted								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
Water	0-300	11.531	58.104	nd	nd	nd	nd	nd	9.482	nd
50% EtOH	400-450	0.271	5.349	nd	21.838	4.436	6.835	nd	7.607	nd
	750-800	4.329	nd	10.163	nd	6.191	nd	11.588	nd	6.350
75% EtOH	1150-1200	0.508	nd	1.833	nd	0.976	nd	4.012	nd	2.371
	1400-1450	0.736	nd	0.066	nd	nd	nd	1.680	nd	nd
	1750-1800	0.721	nd	nd	nd	nd	nd	nd	nd	nd

* nd = not detected

Table. 4.25 Chemical compositions of combined fraction from green tea extract

Fraction (ml)	Volume (ml)	% Eluted								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
300	300	11.531	58.104	nd*	nd	nd	nd	nd	9.482	nd
300-600	300	74.802	3.348	36.247	77.084	37.688	84.583	19.726	59.041	nd
600-1650	1050	22.799	24.924	10.852	nd	11.097	nd	14.908	13.596	21.222

* nd = not detected

All of the obtained fractions were then combined and divided into three different main fractions according to the caffeine content. The first main fraction (300 ml) was the fraction eluted by water. The second main fraction (300 ml) was the fractions eluted by 50% of ethanol, based on the peak profile from Figure 4.26. Besides polyphenols, these fractions also have caffeine in a lower level. Meanwhile, the third main fraction (1,050 ml) was the fractions eluted by 50% and 75% of ethanol. The HPLC result of these three main fractions is shown in Table 4.29.

From the result, 11.531% of theanine, 58.104% of caffeine and 9.482% of GC were eluted from the column by water elution. Theanine was consisted in all fractions. This means that theanine eluted gradually from the column by water and water-ethanol mixtures. After water elution was finished, the remaining caffeine, theanine and catechins were eluted from the column by 50% and 75% of ethanol elution.

The first main fraction was rich in caffeine and also had theanine and GC in low concentration. The second main fraction that was eluted by 50% ethanol consisted of theanine, caffeine, GC, EGC EC, EGCG, GCG and ECG. In addition, the third main fraction consisted of theanine, caffeine, GC, EGCG, GCG, ECG and CG. These three main fractions were evaporated and then subjected to a freeze drying process. The yield (%) of it is shown in Table 4.26 as polyamide 6 (R0).

3. Regeneration treatment of polyamide 6 used in fractionation

The polyamide resin from 4.5.2.2 was regenerated with NaOH solution (the procedure is shown in Appendixe E) and then packed again into the new column. Using the same condition, sample and procedure, three main fractions were collected. Each of them had a volume 300 ml, 300 ml and 1,050 ml, respectively. This process was repeated four times. Each of the three main fractions from four columns was then subjected to evaporation and a freeze drying process. The yield (%) of those dried fractions is shown in Table 4.26 as polyamide 6 (R1), polyamide 6 (R2), polyamide 6 (R3) and polyamide 6 (R4), respectively. It was calculated by comparing the amount of compounds in the freeze dried extract with that in the raw material.

Fractions from polyamide 6 (R0) to polyamide 6 (R4) have the same compounds but are different in concentration. The first main fraction was theanine and caffeine, the second

main fraction was mixed fraction (consisting of individual catechin, theanine including caffeine), while the third main fraction was catechin fraction (consisting of EGCG, ECG and GCG).

Table. 4.26 Yield (%) of freeze dried of green tea extract combined fractions

Compound	Fraction	% Yield of freeze dried fraction				
		(R0)	(R1)	(R2)	(R3)	(R4)
EGCG	I	nd*	nd	nd	nd	nd
	II	41.509	35.182	33.518	32.030	32.849
	III	51.464	45.752	25.007	15.514	11.846
EGC	I	nd	nd	nd	nd	nd
	II	44.161	36.880	34.308	15.143	14.403
	III	nd	nd	nd	nd	nd
ECG	I	nd	nd	nd	nd	nd
	II	24.182	22.362	20.980	21.150	19.363
	III	54.934	54.838	36.168	23.228	17.583
EC	I	nd	nd	nd	nd	nd
	II	47.547	39.668	38.239	20.711	20.895
	III	nd	nd	nd	nd	nd
GCG	I	nd	nd	nd	nd	nd
	II	10.750	10.050	9.868	8.010	6.556
	III	67.954	45.706	33.893	29.344	27.309
GC	I	nd	nd	nd	nd	nd
	II	29.842	27.813	27.458	20.376	19.962
	III	nd	nd	nd	nd	nd

*nd = not detected

Table. 4.26 Yield (%) of freeze dried of green tea extract combined fractions (Cont.)

Compound	Fraction	% Yield of freeze dried fraction				
		(R0)	(R1)	(R2)	(R3)	(R4)
Theanine	I	31.193	25.066	24.687	21.324	19.510
	II	10.768	9.682	9.612	8.811	8.378
	III	nd	nd	nd	nd	nd
Caffeine	I	54.105	42.333	40.172	39.509	26.236
	II	32.422	30.142	25.302	21.510	26.007
	III	nd	nd	nd	nd	nd

*nd = not detected

From polyamide 6 (R0), the second main fraction consisted of 55.53% of total catechins, 10. 656% of theanine and 7.562% of caffeine in dry basis (chemical compositions of freeze dried fractions are shown in Appendix E). Meanwhile, the third main fraction consisted of 28.176% of total catechins (in dry basis) with no caffeine (the chemical compositions of each freeze dried fractions are shown in Appendix E). A different result was found by Tang *et al* (2003). The authors found that green tea polyphenols and caffeine were separated successfully by polyamide resin. The obtained extract consisted of more than 96% of tea polyphenols (with EGCG about 80%) and in caffeine, less than 2%. This difference might be due to the difference in combining and dividing of obtained fractions after fractionation.

Regeneration with NaOH solution was performed to wash the remaining green tea extract compounds inside the resin. In the regeneration treatment, the resin was infiltrated and washed by NaOH solution twice. It was conducted to ensure that the remaining compounds were washed completely from the resin.

With the application of regeneration treatment, the concentration of all compounds in the three main fractions were gradually decreased from polyamide 6 (R0) to polyamide 6 (R4). After five uses, the polyamide resin still had the ability to separate out

caffeine. However, it was also observed that this ability decreased. It might be due to the fact that some compounds still adsorbed inside the resin and so decreased the ability of the polyamide to adsorbed more green tea extract compounds loaded to the column.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

This study investigated the optimal conditions for assam green tea extraction. In general, it was divided into 4 main parts. The first part was to study the optimum condition in laboratory scale. It was an investigation of the effect of pH and tea-water ratio on the green tea extraction. Extraction with a tea-water ratio of 1:20 at pH 5 was found to be the optimum condition for total polyphenols. The content of total polyphenols, tannin, total amino acids and caffeine in green tea extract extracted with this condition were 10.783, 4.863, 4.243 and 3.297% of green tea in dry basis, respectively. Since polyphenols compounds were the main active ingredient in green tea, this condition was then applied to observe the effect of a multi-step extraction. It was found that two step extraction procedure was needed to extract more polyphenols. The cumulative amount of total polyphenols, tannin, total amino acids and caffeine extracted with two step of extractions were 14.773, 6.218, 5.350 and 4.252% of green tea in dry basis, respectively.

This optimum condition was then used in the second part, a kinetic study in the laboratory scale. The extractions were performed in 5 different temperatures for 1, 3, 5, 10, 20, 40 and 80 minutes. During extraction, the concentration of total polyphenols, tannin, total amino acids, caffeine, individual catechins as well as total catechins increased over time. It increased rapidly in the first few minutes and then remained stable, approaching the equilibrium value. The kinetic rates during extractions were also measured.

According to the equilibrium value, 90°C was the optimum temperature in terms of resulting in the highest equilibrium value for extraction of total polyphenols, caffeine, individual catechins (EGCG, EGC, ECG, EC, GC, C and G) and total catechins. In extractions at 90°C, the

equilibrium values of these compounds were 11.158, 2.396, 0.664, 2.842, 2.185, 1.281, 0.08, 3.963, 0.987, 0.727 and 13.395% of green tea in dry basis, respectively. Meanwhile, extraction at 80°C was the optimum temperature for tannin and total amino acids extraction. The equilibrium values of these compounds were 7.283% and 4.534% of green tea in dry basis, respectively.

The optimum rate constant for extraction of total polyphenols, total amino acids, EGCG and ECG were reached in extraction at 90°C. The kinetic rate of these compounds were 0.272, 0.702, 0.091, 0.131 and 0.045 min^{-1}, respectively. However, the optimum rate for extraction of tannin (0.105 min^{-1}) was reached in extraction at 50°C. Meanwhile, the optimum rate for caffeine (0.130 min^{-1}), EGC (0.131 min^{-1}), EC (0.057 min^{-1}), GC (0.107 min^{-1}), C (0.067 min^{-1}), G (0.087 min^{-1}) and total catechins (0.084 min^{-1}) were reached at 70°C.

The third part was the kinetic study of green tea extraction on a pilot plant scale. The extractions were conducted in a tea-water ratio of 1:28 at pH 5. Similar to the kinetic study in the laboratory scale, it was found that the content of total polyphenols, tannin, theanine, caffeine, individual catechins and total catechins were increased in the increasing of extraction times. All of the analyzed parameters in this study (total polyphenols, tannin, theanine, caffeine, EGCG, EGC, EC, GCG, GC and total catechins) reached its highest equilibrium value in extraction at 90°C. The equilibrium of these values were 11.180, 7.364, 2.292, 0.695, 0.725, 0.741, 1.416, 0.055, 0.745 and 3.660% of green tea in dry basis. The highest rate constant for extraction of total polyphenols, tannin, caffeine, EGCG, EC, GCG and total catechins were reached in extraction at 90°C. Whereas for the other compounds (theanine, EGC and GC), the highest rate constants were obtained in extraction at 70°C. The optimum rate constant for extraction of total polyphenols, tannin, theanine, caffeine, EGCG, EGC, EC, GCG, GC and total catechins were 0.048, 0.48, 0.089, 0.071, 0.242, 0.182, 0.226, 0.079, 0.124, and 0.158 min^{-1}, respectively.

The last study was green tea extract fractionation. The main purpose of this study was to separate caffeine and polyphenols compounds. Resin polyamide 6 was the most suitable resin for this purpose. Water, 50% of ethanol and 75% of ethanol in water were used as eluting agents. Water was used to elute the caffeine from the column, while 50% of ethanol and 75% of ethanol were used to elute polyphenols compounds. However, it was observed that caffeine was not completely separated with water elution only.

After the fractionation process, all of the obtained fractions were classified into three main fractions based on the caffeine content. These three main fractions were then freeze dried in order to obtain the fractions in the dried form and analyzed for the content of EGCG, EGC, ECG, EC, GCG, GC, theanine and caffeine. These procedures were repeated four times. After five times, polyamide 6 resin still had an ability to separate out caffeine, however this ability decreased.

5.2 Recommendations

5.2.1 Further study about the kinetic of green tea compounds during extraction process is needed. The extraction process was very rapid in the first few minute. Therefore, sampling in that interval time during extraction should be increased so that the kinetics rate can be more accurately calculated.

5.2.2 Fractionation of green tea extract compounds using poly (dimmer acid-co-alkyl polyamine) and nylon 6 with smaller particle size should be done to investigate its ability in caffeine and polyphenols separation.

5.2.3 Further study about regeneration of polyamide 6 treatments and its life span are needed to investigate the optimum condition to regenerate this resin as well as its mechanism that occurred.

REFERENCES

Amico, V., Chillemi, R., Mangiafico, S., Spatafore, C & Tringali, C. (2007). "Polyphenol-enriched fractions from sicilia grape pomace: HPLC-DAD analysis and antioxidant activity" **Bioresource technology.** 99, 5960-5966.

Aguilera, J.M. (2003). **Solid liquid extraction**. Available from http://143.225.163.184 /_docenti/cavella-silvana/doc/cavella-34-solid-liquid-extraction.pdf (10 January 2009).

Anonymous. (2003a). **MALDI-TOF mass spectrometric analysis of hydrolysable tannins**. Available from http://taninos.tripod.com/hidrolisables.htm (22 March 2009).

Anonymous. (2003b). **Amberlite XAD7HP-industrial grade polymeric adsorbent**. Availlable from http://www.advancedbiosciences.com (20 August 2008).

Anonymous. (2004). **A guide to freeze drying for laboratory**. Available from http://chiron.no/pdf/ Labconco_ guide_to_freeze_drying.pdf (10 October 2008).

Anonymous. (2007). **Natural herbs**. Available from http://www.herbs-tech.com/product/sc_tea catechins.asp (10 November 2007).

Anonymous. (2009a). **Dragon pearl tea**. Available from http://www.dragonpearltea.com (22 March 2009).

Anonymous. (2009b). **Thin layer chromatograpy (TLC) – CU boulder organic chemistry undergraduate courses**. Available from http://www.orgchem.colorado.edu /hndbksupport /TLC/TLC.html (16 April 2009).

Bailey, D.T., Yuhasz, R.L & Zheng, B. (2001). **Method for isolation of caffeine-free catechins from green tea**. Available from http://patft.uspto.gov (22 December 2007).

Baptista, J. A. B., Tavares, J. F. P & Carvalho, R. C. B. (1999). "Comparative study and partial characterization of azorean tea polyphenols" **Food composition and analysis**. 12, 273-287.

Barbosa-Canovas, G & Vega-Mercado, H. (1996). **Dehydration of foods**. New york: Chapman and may.

Bazinet, L., Labbe, D & Tremblay, A. (2007). "Production of green tea EGC and EGCG-enriched fractions by a two step extraction procedure" **Separation and purification technology.** 56, 53-56.

Bezbaruah, H.P. (1999). Origin and history of development of tea. In **Global advances in tea science**. New Delhi: Aravali Books International. pp. 382-392.

Belitz, H. D & Grosch, W. (1999). **Food chemistry**. Berlin: Springer-Verlag.

Billet, R. (1989). **Evaporation technology**. Weinhein: VCH publishers.

Boadi, D.K & Neufeld, R.J. (2001). "Encapsulation of tannase for the hydrolysis of tea tannins" **Enzyme and microbial technology.** 28, 590-595.

Caballero, B., Trugo, L. C & Paul, M. F. (2003). **Encyclopedia of food sciences and nutrition**. 4507-4511. London: Elsevier Science Ltd.

Cannas, A. (2001). **Tannins: Fascinating but Sometimes Dangerous Molecules**. Available from http://www.abc.cornell.edu (23 June 2008).

Chang, C. J., Chiu, K. L., Chen, Y. L & Chang, C. Y. (2000). "Separation of Catechins from Green Tea Using Carbon Dioxide Extraction" **Food Chemistry**. 68, 109-113.

Chen, Z.M., Wang, H.F., You, X.Q & Xu, N. (2002). The Chemistry of Tea Non-Volatiles. In **Tea: Bioactivity and Therapeutic Potential**. London: Taylor and francis. pp. 57-70.

Chung, K.S., Wei, C.I & Johnson, M.G. (1998). "Are tannins a double-edged sword in biology and health?" **Trends in food science and technology**. 9, 168-175.

Dhey, P.M & Harborne, J.B. (1997). **Plant biochemistry**. London: academic press.

Ekayanake, A., Kirksey, S. T & Pultinas, E. P. (1995). **Process making a stable green tea extract and product**. Available from http://patft.uspto.gov (22 December 2007).

Ekayanake, A., Pultinas, E.P., & Edmund, P. (1997). **Tea extract and process**. Available from http://patft.uspto.gov (22 December 2007.

Ekwongsupasarn, P. (2005). **Feasibility study of the use of residue from tea manufacturing process.** Master Thesis in Food Engineering, Gradiuate Scholl, King Mongkut's University of Technology.

Escribano-Bailon, M.T & Santos-Buelga, C. (2003). Polyphenol extraction from foods. In **Methods in polyphenols analysis**. Cambridge: UK.Royal Society of Chemistry. pp. 1-16.

FAO/IAEA. (2000). **Quantification of tannins in tree foliage**. Vienna: FAO/IAEA.

Glover, W.B. (2004). **Selecting evaporators for process application**. Available from http://www.lcicorp.com /evap/docs/CE_evap_selection.pdf (10 October 2008).

Grinberg, N. (1990). **Modern thin layer chromatography**. New York: Marcel Dekker.

Halder, J., Tamuli, P & Bhaduri, A.N. (1998). "Isolation and characterization of polyphenol oxidase from indian tea leaf (*Camellia sinensis*)" **Nutritional biochemistry.** 9, 75-80.

Hampton, M.G. (1999). Manufacture of instant tea. In **Global Advances in Tea Science**. New Delhi: Aravali Books International. pp. 767-784.

Horie, H.; Mukai, T & Kohata, K. (1997). "Simultaneous determination of qualitatively important components in green tea infusions using capillary electrophoresis" **Chromatography.** 758, 352-335.

ISO. (1975). Method 1572 – **Tea: preparation of ground sample of known dry matter content.**

ISO. (1990). Method 7513 – **Determination of moisture content (loss in mass at 103°C).**

ISO. (2005). Method 15402-1 – **Content of total polyphenols in tea.**

Jaganyi, D & Price, R.D. (1999). "Kinetics of tea infusion: the effect of the manufacturing process on the rate of extraction of caffeine" **Food Chemistry**. 64, 27-31.

Jaganyi, D & Wheeler, P.J. (2003). "Rooibos tea: equilibrium and extraction kinetics of aspalathin" **Food chemistry**. 83, 121-126.

Jindaratsamee, P. (2006). **Adsorption of polyphenols by amberlite IRA-67 modified with boronic acids**. Master Thesis in Chemical Engineering, Graduate School, King Mongkut's University of Technology.

Kaufman, P.B., Cseke, L.J., Warber, S., Duke, J.S & Brielmann, L. (2006). **Natural products from plants**. Boca Raton: CRC Press.

Kirkpatrick, L. A & Feeney, B. C. (2003). **A simple guide to SPSS for windows**. Canada: Thomson Learning.

Kim, E.S., Liang, Y.R., Jin, J., Sun, Q.F., Lu, J.L., Du, Y.Y & Lin, C. (2007). "Impact of heating on chemical compositions of green tea liquor" **Food chemistry.** 103, 1263-1267.

Kovacs, A., Ganzler, K & Sarkadi, L.S. (1998). "Microwave-assisted extraction of free amino acids from foods" **Z Lebensm Unters Forsch A.** 207, 26-30.

Krishnan, R., & Maru, G.B. (2006). "Isolation and analysis of polymeric polyphenol fractions from black tea" **Food chemistry**. 94, 331-340.

Labbe, D., Tremblay, A., & Bazinet, L. (2006). "Effect of brewing temperature and duration on green tea catechin solubilization: basis for production of EGC and EGCG-enriched fractions" **Separation and Purification Technology.** 56, 53-56.

Land, C.M.V. (1991). **Industrial drying equipment**. New York: Marcel Dekker.

Lee, H.S. (2004). Phenolic compounds in food. In **Handbook of Food Analysis : Physical Characterization and Nutrient Analysis**. New York: Marcel Dekker, Inc, pp. 657-696.

Lee, N.Y., Jo, C., Sohn, S.H., Kim, J.K & Byun, M.W. (2006). "Effects of gamma irradiation on the biological activity of green tea byproduct extracts and a comparison with green tea leaf extracts" **Journal of food science.** 71, 269-274.

Li, J.; Li, P & Liu, F. (2007). "Production of theanine by *Xerocomus badius* (mushroom) using submerged fermentation" **LWT-Food science and technology**.

Liang, H., Liang, Y., Dong, J., Lu, J., Xu, H & Wang, H. (2007). "Decaffeination of fresh green tea leaf (*Camellia sinensis*) by hot water treatment" **Food chemistry**. 101, 1451-1456.

McCabe, W.L., Smith, J.C. & Harriot, P. (2005). **Unit operations of chemical engineering**. Singapore: McGraw hill.

Michos, D. (1999). **Polyamide compositions for removal of polyphenols from liquids**. Available from http://www.freepatentsonline.com/5856429.html (20 August 2008).

Mujumdar, A.S. & Menon, A.S. (1995). Drying. In **Handbook of industrial drying**. New York: Marcell Dekker, pp. 1-10.

Mukhopadyay, M. (2000). **Natural extracts using supercritical carbon dioxide**. Florida: CRC Press, pp. 15-20.

Obanda, M., Owuor, P.O., Mang'oka, R & Kavoi, M.M. (2004). "Changes in thearubigin fractions and theaflavin levels due to variations in processing conditions and their influence on black tea liquor brightness and total colour" **Food chemistry.** 85:,163-173.

Palma, M., Pineiro, Z & Barosso, C.G. (2002). "In-line pressurized-fluid extraction solid-phase extraction for determining phenolic compounds in grapes" **Chromatography.** 968, 1-6.

Pan, X., Guoguang, N & Huizhou, L. (2003). "Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves" **Chemical engineering and processing.** 42, 129-133.

Peterson, J., Dwyer, J., Bhagwat, S., Haytowitz, D., Holden, J., Eldridge, A.L., Beecher, G & Aladesanmi, J. (2005). "Major flavonoids in dry tea" **Food composition and analysis**. 18, 487-501.

Pintauro, N. D. (1977). **Tea and soluble tea product manufacture**. New Jersey: Noyes data corporation.

Ping Li., Wang, Y., Ma, R. & Zhang, X. (2005). "Separation of tea polyphenols from green tea leaves by a combined CATUFM-adsorption resin process" **Food engineering.** 67, 253-260.

Rao, A.P., Joshi, S.V., Trivedi, J.J., Devmurari, C.V. & Shah, V.J. (2003). "Structure–performance correlation of polyamide thin film composite membranes: effect of coating conditions on film formation" **Membrane science**. 211, 13-24.

Row, K.H. & Jin, Y. (2006). "Recovery of catechin compounds from korean tea by solvent extraction" **Bioresouce technology.** 97, 790-793.

Saijo, R. (199). Japanese green tea-characteristics and manufacture. In **Global advances in tea science**. New Delhi: Aravali Books International LTD. pp. 761-766.

Sarma, S. (1999). Biosynthesis of precursor for liquor and flavor characteristics in black tea. In **Global advances in tea science**. New Delhi: Aravali Books International LTD. pp. 723-732.

Savolainen, H. (1991). "Tannin content of tea and coffee" **Applied of toxicology.** 12, 191-192.

Scott, R.P.W. (1995). **Techniques and practice of chromatography**. New York: Marcell Dekker.

Shahidi, F & Naczk, M. (2004). **Phenolics in foods and nutraceuticals**. Boca Raton: CRC press.

Sharma, V., Gulati, S. & Ravindranath. 2005. "Extractability of tea catechins as a function of manufacture procedure and temperature of infusion" **Food chemistry.** 93, 141-148.

Sharma, V., Gulati, A., Ravindranath, D. & Kumar, V. (2005). "A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC" **Food composition and analysis**. 18, 583-594.

Silva, E.M., Pompeu, D.R., Larondelle, Y. & Roges, H. (2007). "Optimisation of the adsorption of polyphenols from *Inga edulis* leaves on macroporous resins using an experimental design methodology" **Separation and purification technology.** 53, 274-280.

Smith, R. (2005). **Chemical process design and integration**. England: John willey and sons, Ltd.

Su, Y.L.; Leung, L. K.; Huang, Y. & Chen, Z.Y. (2003). "Stability of tea theaflavins and catechins" **Food chemistry.** 83, 189-195.

Swadesh, J. (1997). **HPLC practical and industrial application**. USA : CRC Press.

Takeo, T. (1999). Product development in tea. In **Global advances in tea science**. New Delhi: Aravali Books International. pp. 805-812.

Tang, K. W., Zhou, C.S., Zhong, S.A. & Zhu, J.D. (2003). **Study on adsorption of tea polyphenol and caffeine with polyamide resin**. Available from http://www.ncbi.nlm.nih.gov (22 May 2008).

Uzunalic, A.P., Mojca, S., Zeljko, K., Bernd, W., Frank, O. & Sabine, G. (2006). "Extraction of active ingredients from green tea (*Camellia sinensis*): extraction efficiency of major catechins and caffeine" **Food chemistry.** 96, 597-605.

Vasisht, K., Sharma, P.D., Karan, M., Rakesh, D.D., Vyas, S., Sethi, S. & Manktala, R. (2003). **Study to promote the industrial exploitation of green tea polyphenols in india**. Italy: ICS-UNIDO.

Viklund, A. (2008). **Hypericum's-HBC protocols all natural depression treatment**. Available from http://hypericum.wordpress.com (22 March 2009).

Wang, H. & Helliwell, K. (2000). "Epimerisation of catechins in green tea infusions" **Food chemistry**. 70, 337-344.

Wang, H., Provan, G.J. & Helliwell, K. (2000). "Tea flavonoids: their functions, utilisation and analysis" **Trends in food science and technology.** 11, 152-160.

Wang, H., Provan, G.J. & Helliwell, K. (2003). "HPLC determination of catechins in tea leaves and tea extracts using relative response factors" **Food chemistry.** 81, 307-312.

Wang, R., Zhou, W. & Jiang, X. (2008). "Reaction kinetics of degradation and epimerization of epigallocatechin gallate (EGCG) in aqueous system over a wide temperature range" **Food chemistry** 56, 2694-2701.

Wilson, K. (1999). **Coffee, cocoa and tea**. New York: Oxon CABI.

William, W.M. & Markely, J.L. (2007). **Biological magnetic resources data bank**. Available from http://www.bmrb.wisc.edu/metabolomics/gen_metab_summary_5.php?molName= (10 November 2007).

Wongsuwan, J. (2008). **Improvement of tea extractor and preliminary study of tea powder processing**. Master Thesis in Food Engineering, Graduate School, King Mongkut's University of Technology.

Xu, N & Chen, Z.M. (2002). Green tea, black tea and semi fermented tea. In **Tea: bioactivity and therapeutic potential**. London: Taylor and Francis. pp. 35-42.

Yamanishi, T. (1999). Tea flavour. In **Global advances in tea science**. New Delhi: Aravali Books International. pp. 693-706.

Yamanaka, A., Kouchi, T., Kasai, K., Kato, T., Ishihara, K & Okuda, K. (2007). "Inhibitory effect of cranberry polyphenol on biofilm formation and cysteine proteases of *Porphyromonas gingivalis*" **Periodontal research.** 4, 589-592.

Yoa, L., Liu, X., Jiang, Y., Caffin, N., D'arcy, B., Singanusong, R., Datta, N & Xu, Y. (2006). "Compositional analysis of tea from australian supermarkets" **Food chemistry**. 94, 115-122.

Yoshida, Y., Kiso, M & Goto, T. (1999). "Efficiency of the extraction of catechins from green tea" **Food chemistry.** 67, 429-433.

Zhu, X., Chen, B., Ma, M., Luo, X., Zhang, F., Yao, S., Wan, Z., Yang, D & Hang, H. (2004). "Simultaneous analysis of theanine, chlorogenic acid, purine alkaloids and catechins in tea samples with the help of multi-dimension information of on-line high performance liquid chromatography/electrospray–mass spectrometry" **Pharmaceutical and biomedical analysis**. 34, 695-704**.**

APPENDIX A

CHEMICALS ANALYSIS METHOD

A.1. Moisture content and dry matter content (ISO 1573, 1980)

A.1.1 Measurement :

1. Dry alumunium can in the oven at 103°C ± 2°C for 1 h. cool in the desiccator 30' and weight (Wc)
2. Weight sample in alumunium can 3 g (Wg)
3. Heat in the oven at 103°C ± 2°C for 6 h
4. Cool in the desiccator and weight again
5. Heat again in the oven for 1 h, cool in the desiccator and weight again,

repeat these operation, if necessary, until the difference between two successive weighing doesn't exceed 0.005 g (W_2)

A.1.2 Calculation

$$\% \text{ (W/W) Moisture Content} = \frac{Wg - (W_2 - Wc)}{Wg} x100$$

$$\% \text{ (W/W) Dry Matter Content} = \frac{W_2 x100}{Wg}$$

Wg = weight sample (g)

Wc = weight alumunium can (g)

W_2 = weight sample +alumunium can after dry (g)

A.2. Moisture content and dry matter content (microwave moisture analyzer)

Moisture content and dry matter content analysis using microwave moisture analyzer was used for freeze dried fractions only to reduce the required amount of sample for analysis. These parameters were determined and calculated automatically by the instruments.

Choose the suitable method that available in the instrument to measure the moisture content and dry matter content of freeze dried fractions. Put the sufficient amount of sample (must be in the range of the required weight), the amount of sample depend on the chosen method. The operation procedure based on the type of moisture analyzer that being used. The value of moisture content and dry matter content would be shown in the monitor of instrument after a few seconds.

A.3. Total polyphenols content (ISO 14502, 2005)

A.3.1 Preparation of solutions

1. Folin-Ciocalteu reagent (10% v/v)

Pipette 5 ml of Folin-Ciocalteu reagent into 50 ml volumetric flask and then diluted with distilled water

2. Na_2CO_3 (5% w/v)

Dissolve 37.50 g anhydrous Na_2CO_3 in distilled water into 500 ml volumetric flask

A.3.2 Preparation of standard

1. Gallic acid stock solution 1000 µg/ml

Dissolve 0.1 g of gallic acid in water into 100 ml volumetric flask. Pipette gallic acid stock standard solution for 1, 2, 3, 4 and 5 ml into 100 ml volumetric flask and then diluted to 10, 20, 30, 40 and 50 µg/ml with distilled water

A.3.3. Measurement

1. Dilute extract solution to 10^2. Dilute 1 ml in distilled water into 100 ml volumetric flask
2. Pipette 1 ml gallic acid working standard solution and sample into test tube
3. Pipette 5 ml diluted Folin-Ciocalteu reagent into each test tube
4. Pipette 4 ml Na_2CO_3 into each test tube
5. Incubate at room temperature for 1 h. Measure absorbance at 765 nm using distilled water as blank

A.3.4 Calculation

$$\text{Total polyphenols content (\% w/w) dry basis} = \frac{(Ag - Aint)\, x Vg\, x\, DF}{Sstd\, x\, Wg\, x\, 10000\, x\, DM} x100$$

Where:

Ag : absorbance sample

A int. : intercept value of standard curve

Vg : volume of extract solution (ml)

DF : dilution factor

Sstd : slope of standard curve

Wg : weight of sample (g)

DM : dry matter of sample (%)

A.4. Tannin (FAO/IAEA, 2000)

A.4.1 Reagents

1. Folin Ciocalteu reagent (1 N)

Dilute commercially available Folin-Ciocalteu reagent (2N) with an equal volume of distilled water. Transfer it in a brown bottle and store in a refrigerator (4°C).

2. Sodium carbonate (20%)

Weigh 40 g sodium carbonate (x10 H_2O), dissolve it in about 150 ml distilled water and make up to 200 ml with distilled water.

3. Insoluble polyvinyl pyrrolidone

4. Standard tannic acid solution (0.1 mg/ml)

Dissolve 25 mg tannic acid (TA) in 25 ml distilled water and then dilute 1:10 in distilled water (always use a freshly prepared solution).

A.4.2 Measurement

1. Take suitable aliquots of the tannin-containing extract (initially try 0.02, 0.05 and 0.1 ml) in test tubes, make up the volume to 0.5 ml with distilled water, and add 0.25 ml of the Folin- Ciocalteu reagent and then 1.25 ml of the sodium carbonate solution. Vortex the tubes

2. Record absorbance at 725 nm after 40 min. Calculate the amount of total phenols as tannic acid equivalent from the above calibration curve

A.4.3 Removal of tannin from tannin-containing extract

1. Take 1 ml distilled water add with 1.0 ml of the tannin-containing extract and then 0.1 ml of PVPP suspension (25 mg/ml in distilled water). Vortex it.

2. Keep the tube at 4°C for 15 min, vortex it again, then centrifuge (3000 g for 10 min) and collect the supernatant.

3. Measure the phenolic content of the supernatant as mentioned above (take at least double the volume you used for total polyphenols estimation). Centrifuge again at 10000

rpm for 15 min, collect the supernatant (this supernatant has only simple phenolics other than tannins).

4. Record absorbance at 725 nm after 40 min. Calculate the amount of total phenols (II) as tannic acid equivalent from the above calibration curve, tannin is the difference between total phenols (I) and total phenols (II)

Table A.1 Preparation of calibration curve

Tube	Tannic acid (0.1 mg/ml)	Distilled water (ml)	Folin reagent (ml)	Sodium carbonate solution (ml)	Absorbance at 725 nm	Tannic acid (mg)
Blank	0.00	0.50	0.25	1.25	0.000	0
T1	0.02	0.48	0.25	1.25	0.112	2
T2	0.04	0.46	0.25	1.25	0.218	4
T3	0.06	0.44	0.25	1.25	0.327	6
T4	0.08	0.42	0.25	1.25	0.432	8
T5	0.10	0.40	0.25	1.25	0.538	10

A.4.4 Calculation

$$\text{Total phenol content (\% w/w) dry basis} = \frac{(Ag - Aint) \times Vg \times DF}{Sstd \times Wg \times Va \times 10000 \times DM} \times 100$$

Where:

Ag	: sample absorbance	Wg	: weight of sample
Aint	: intercept from standard curve	S std	: slope from standard curve
Va	: sample volume for measurement	Vg	: volume of solution
DF	: dilution factor	DM	: dry matter of sample (%)

A.5. Total amino acids (Yoa *et al*, 2006)

A.5.1 Reagent

1. Buffer solution. Na_2HPO_4 (23.38 g) is dissolved in distilled water (1000 ml) and KH_2PO_4 (9.08 g) is dissolved in distilled water (1000 ml). A buffer solution consisting of 95% (v/v) of Na_2HPO_4 solution and 5% (v/v) of KH_2PO_4 solution is prepared.

2. Ninhydrin solution. Ninhydrin (2 g) is dissolved in distilled water (50 ml) and $SnCl_2$ (80 mg) is added to the solution diluted to 100 ml with distilled water.

A.5.2 Measurement

1. Tea solution (1 ml), buffer solution (0.5 ml) and ninhydrin solution (0.5 ml) are placed in a 25 ml volumetric flask and the flask is heated in a boiling water bath for 15 min.

2. The flask cooled to room temperature and the solution in the flask is diluted to 25 ml with distilled water.

3. The absorbance of the diluted solution is measured using a UV/Visible spectrophotometer at 570 nm.

A.5.3 Standard curve

1. Theanine is used to prepare the standard curve. Theanine stock solution (1 mg/ml, w/v in distilled water; 5 ml) is diluted to 50 ml with distilled water.

2. Then, 0, 1, 2, 3, 4, or 5 ml of the diluted theanine solution are separately added, each with buffer solution (0.5 ml) and ninhydrin solution (0.5 ml) to different 25 ml volumetric flasks.

3. Each mixture is heated in a boiling water bath for 15 min.

A.5.4 Calculation

Total amino acids = E x Vo x 100/1000/V1/W

= 0.1 E Vo/V1/W

Where:

E : mg of amino acids from the standard curve against the absorbance reading of the spectrophotometer

V0 : the total volume of the tea solution (250 ml)

V1 : the volume used for the measurement (1.0 ml)

W : the dry weight of the tea sample

A.6. Caffeine (Yoa *et al*, 2006)

A.6.1 Reagent

1. Lead acetate solution. $(CH3COO)_2Pb$ (100 g) is dissolved and diluted to 200 ml with distilled water.

2. Hydrochloric acid solution. Hydrochloric acid (36% HCl, specific gravity 1.18, 0.9 ml) is diluted to 1000 ml with distilled water.

3. Sulfuric acid solution. Sulfuric acid (98% H_2SO_4, specific gravity 1.84, 167 ml) is diluted to 1000 ml with distilled water.

A.6.2 Measurement

1. Tea solution (10 ml), hydrochloric acid solution (5 ml) and lead acetate solution (1 ml) are mixed in a 100 ml volumetric flask and diluted to 100 ml with distilled water. The solution is then filtered through Whatman No. 1 qualitative filter paper.

2. The filtrate (25 ml) and sulfuric acid solution (0.3 ml) are placed in a volumetric flask and diluted to 50 ml with distilled water. The solution is filtered using the same type of filter paper.

3. The absorbance of the filtrate is measured using a UV/Visible spectrophotometer at 274 nm.

A.6.3 Standard curve

1. Caffeine stock solution (10 ml, 1 mg/ml, w/v in distilled water) is diluted to 200 ml with distilled water.

2. 0, 10, 20, 30, 40, or 50 ml of the diluted caffeine solution are separately mixed, each with hydrochloric acid solution (4 ml) in a volumetric flask and diluted to 100 ml with distilled water.

3. The measuring steps were repeated

A.6.4 Calculation

$$\text{Caffeine (\%)} = (E/1000) \times Vo \times (100/V1) \times (50/25) /W$$

$$= 0.2\ E\ Vo/V1/W$$

Where:

E : mg of caffeine from the standard curve against the reading of the spectrophotometer

E/1000 : to convert mg into g

V_O : the total volume of the tea solution (250 ml)

V1 : the volume used for the measurement (10 ml)

A.7. Catechins and caffeine analysis by HPLC method

(Tea Institute, Mae Fah Luang University)

A.7.1 Prepare standard solutions

1. Dissolving each standards (0.01 g) with 1 ml of acetonitrile and 0.5 ml EDTA, then adjusted to 10 ml with deionized water. All of standards solution then was mixed and diluted to concentration ranging from 0.2 ppm to 100 ppm with deionized water 1 ppm = 1 mg/L).

2. Filtering 1 ml aliquot of a standard through a syringe filter and collecting it in a vial. Injecting standard solution into HPLC.

A.7.2 Prepare mobile phase

Mobile phase is a 13:87 mixture of mobile phase A and mobile phase B with isocratic elution.

1. Mobile Phase A

Acetonitrile (100%, HPLC grade) filtered with nylon membrane (0.45 µm)

2. Mobile Phase B

Dissolved 1 ml of trifluoroacetic acid (TFA) and 4 ml of EDTA (20 µg/ml) with certain amount of deionized water, then adjusted to 1000 ml with deionized water. Filtered the solution with cellulose membrane (0.45 µm)

A.7.3 Condition of HPLC (Alltech, USA)

1. Column : Platinum EPS C18 100A 3U
2. Column Temperature : 30^0C
3. Separation module : Water 2695
4. Detector : 210 nm (photo diode array 2996)
5. Flow rate : 2 ml/min
6. Injection volume : 10 µl

7. Run time : 12 min

A.8. catechins, caffeine and theanine analysis by HPLC method (Departement of biochemistry, King Mongkut's University of Technology Thonburi)

A.8.1 Prepare standard solutions

1. Dissolving each standards with 10 of acetonitrile in deionized water to concentration 100 ppm and then mixed all of the standard solution

2. Filtering 1 ml aliquot of a standard through a syringe filter and collecting it in a vial. Injecting standard solution into HPLC.

A.8.2 Prepare mobile phase

Mobile phase is a 13:87 mixture of mobile phase A and mobile phase B with isocratic elution

1. Mobile Phase A

Acetonitrile (100%, HPLC grade) filtered with nylon membrane (0.45 µm)

2. Mobile Phase B

Dissolved 1 ml of phosphoric acid with certain amount of deionized water, then adjusted to 1000 ml with deionized water. Filtered the solution with cellulose membrane (0.45 µm)

A.8.3 Condition of HPLC (Agilent Teachnology, USA)

1. Column : Agilent eclipse XDB-C18, 5µm, 4.6 x 150 mm
2. Degasser : G1322A
3. Quat pump : G1331A
4. Detector : 210 nm (photo diode array G1328)
5. Flow rate : 0.5 ml/min
6. Injection volume : 20 µl
7. Run time : 60 min
8. Post time : 10 min

APPENDIX B

STUDY THE OPTIMUM CONDITION OF EXTRACTION IN THE LABORATORY SCALE

B.1 Layout of experimental design

Table B.1 Layout of experimental design

pH	Tea-water ratio											
	1:5			1:10			1:20			1:30		
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
4	Y_{111}	Y_{112}	Y_{113}	Y_{211}	Y_{212}	Y_{213}	Y_{311}	Y_{312}	Y_{313}	Y_{411}	Y_{412}	Y_{413}
5	Y_{121}	Y_{122}	Y_{133}	Y_{221}	Y_{222}	Y_{223}	Y_{321}	Y_{322}	Y_{323}	Y_{421}	Y_{422}	Y_{423}
6	Y_{131}	Y_{132}	Y_{133}	Y_{231}	Y_{232}	Y_{233}	Y_{331}	Y_{332}	Y_{333}	Y_{431}	Y_{432}	Y_{433}
7	Y_{141}	Y_{142}	Y_{143}	Y_{241}	Y_{242}	Y_{243}	Y_{341}	Y_{342}	Y_{343}	Y_{441}	Y_{442}	Y_{443}

B.2 Raw data

Table B.2 Total polyphenols, tannin, total amino acids and caffeine of the green tea extracts extracted from various tea-water ratio and pH (% w/w dry basis)

Condition		Total polyphenols	Tannin	Total amino acids	Caffeine
Tea:water ratio	pH				
1:5	3.82 ± 0.04	8.368 ± 0.046^{b*}	4.989 ± 0.008^{ef}	4.475 ± 0.011^{de}	2.522 ± 0.009^{d}
	4.86 ± 0.08	8.408 ± 0.011^{bc}	4.644 ± 0.298^{d}	4.582 ± 0.104^{e}	2.422 ± 0.052^{c}
	5.86 ± 0.05	7.140 ± 0.011^{a}	4.583 ± 0.257^{d}	4.335 ± 0.049^{cd}	2.219 ± 0.005^{a}
	7.45 ± 0.05	7.114 ± 0.011^{a}	3.888 ± 0.389^{b}	4.050 ± 0.090^{b}	2.479 ± 0.012^{d}
1:10	3.82 ± 0.04	9.520 ± 0.027^{e}	5.588 ± 0.077^{g}	4.940 ± 0.011^{f}	2.768 ± 0.025^{e}
	4.86 ± 0.08	9.510 ± 0.037^{e}	4.671 ± 0.033^{de}	4.547 ± 0.044^{e}	2.369 ± 0.017^{b}
	5.86 ± 0.05	9.071 ± 0.174^{d}	4.273 ± 0.079^{c}	3.971 ± 0.055^{b}	2.376 ± 0.025^{bc}
	7.45 ± 0.05	8.543 ± 0.009^{c}	3.317 ± 0.094^{a}	3.584 ± 0.069^{a}	2.409 ± 0.026^{bc}

Table B.2 Total polyphenols, tannin, total amino acids and caffeine of the green tea extracts extracted from various tea-water ratio and pH (% w/w dry basis) (cont.)

Condition		Total polyphenols	Tannin	Total amino acids	Caffeine
Tea:water ratio	**pH**				
1:20	3.82 ± 0.04	10.820 ± 0.104^{h}	5.933 ± 0.079^{h}	4.471 ± 0.110^{de}	3.577 ± 0.051^{j}
	4.86 ± 0.08	10.783 ± 0.051^{h}	4.863 ± 0.245^{def}	4.243 ± 0.048^{c}	3.297 ± 0.051^{h}
	5.86 ± 0.05	10.165 ± 0.009^{g}	5.099 ± 0.039^{f}	3.888 ± 0.019^{b}	3.303 ± 0.011^{hi}
	7.45 ± 0.05	9.457 ± 0.027^{e}	4.907 ± 0.033^{def}	3.895 ± 0.086^{b}	3.188 ± 0.006^{g}
1:30	3.82 ± 0.04	10.837 ± 0.027^{h}	7.000 ± 0.182^{j}	3.926 ± 0.171^{b}	4.038 ± 0.009^{k}
	4.86 ± 0.08	10.805 ± 0.265^{h}	6.574 ± 0.079^{i}	4.050 ± 0.231^{b}	3.349 ± 0.026^{i}
	5.86 ± 0.05	9.941 ± 0.041^{f}	6.489 ± 0.235^{i}	3.727 ± 0.000^{a}	2.814 ± 0.026^{ef}
	7.45 ± 0.05	8.973 ± 0.060^{d}	6.449 ± 0.134^{i}	3.631 ± 0.016^{a}	2.848 ± 0.009^{f}

* Different lower-case letter in the same column indicate significant difference (α = 5%) among tea-water ratio and pH

APPENDIX C

KINETIC STUDY IN THE LABORATORY SCALE

C.1 Layout of experimental design

Table C.1 Layout of experimental design

Min	Temperature (°C)														
	50			60			70			80			90		
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
1	Y_{111}	Y_{112}	Y_{113}	Y_{211}	Y_{212}	Y_{213}	Y_{311}	Y_{312}	Y_{313}	Y_{411}	Y_{412}	Y_{413}	Y_{511}	Y_{512}	Y_{513}
3	Y_{121}	Y_{122}	Y_{133}	Y_{221}	Y_{222}	Y_{223}	Y_{321}	Y_{322}	Y_{323}	Y_{421}	Y_{422}	Y_{423}	Y_{521}	Y_{522}	Y_{523}
5	Y_{131}	Y_{132}	Y_{133}	Y_{231}	Y_{232}	Y_{233}	Y_{331}	Y_{332}	Y_{333}	Y_{431}	Y_{432}	Y_{433}	Y_{531}	Y_{532}	Y_{533}
10	Y_{141}	Y_{142}	Y_{143}	Y_{241}	Y_{242}	Y_{243}	Y_{341}	Y_{342}	Y_{343}	Y_{441}	Y_{442}	Y_{443}	Y_{541}	Y_{542}	Y_{543}
20	Y_{151}	Y_{152}	Y_{153}	Y_{251}	Y_{252}	Y_{253}	Y_{351}	Y_{352}	Y_{353}	Y_{451}	Y_{452}	Y_{453}	Y_{551}	Y_{552}	Y_{553}
40	Y_{161}	Y_{162}	Y_{163}	Y_{261}	Y_{262}	Y_{263}	Y_{361}	Y_{362}	Y_{363}	Y_{461}	Y_{462}	Y_{463}	Y_{561}	Y_{562}	Y_{563}
80	Y_{171}	Y_{172}	Y_{173}	Y_{271}	Y_{272}	Y_{273}	Y_{371}	Y_{372}	Y_{373}	Y_{471}	Y_{472}	Y_{473}	Y_{571}	Y_{572}	Y_{573}

C.2 Raw data

Table C.2 Total polyphenols, tannin, total amino acids and caffeine content of green tea (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5)

Condition		Total Polyphenols	Tannin	Total amino acids	Caffeine
^{0}C	Min				
50	1	3.812 ± 0.272	0.822 ± 0.733	0.918 ± 0.038	0.621 ± 0.131
	3	5.265 ± 0.150	3.358 ± 0.079	2.083 ± 0.145	1.005 ± 0.186
	5	6.985 ± 0.737	3.574 ± 0.008	2.457 ± 0.029	1.066 ± 0.097
	10	8.802 ± 0.060	3.775 ± 0.013	2.913 ± 0.105	1.142 ± 0.154
	20	9.320 ± 0.398	3.998 ± 0.047	3.514 ± 0.072	1.163 ± 0.183
	40	9.895 ± 0.132	4.134 ± 0.042	3.768 ± 0.048	1.260 ± 0.161
	80	9.901 ± 0.111	5.169 ± 0.027	4.205 ± 0.072	1.441 ± 0.093
60	1	4.277 ± 0.131	1.667 ± 0.147	1.117 ± 0.096	0.826 ± 0.058
	3	5.912 ± 0.228	3.556 ± 0.020	2.166 ± 0.033	0.947 ± 0.098
	5	7.851 ± 0.115	3.644 ± 0.060	2.932 ± 0.061	1.118 ± 0.011
	10	8.939 ± 0.366	3.841 ± 0.060	3.179 ± 0.029	1.337 ± 0.106
	20	9.478 ± 0.666	4.042 ± 0.079	3.768 ± 0.029	1.355 ± 0.021
	40	10.228 ± 0.169	4.666 ± 0.151	4.059 ± 0.083	1.484 ± 0.075
	80	10.281 ± 0.063	5.824 ± 0.146	4.281 ± 0.022	1.643 ± 0.060
70	1	5.917 ± 0.310	1.984 ± 0.079	1.355 ± 0.050	0.895 ± 0.004
	3	8.727 ± 0.397	3.724 ± 0.044	2.207 ± 0.049	1.187 ± 0.234
	5	9.251 ± 0.027	3.806 ± 0.065	3.673 ± 0.048	1.478 ± 0.017
	10	10.133 ± 0.333	3.880 ± 0.069	3.888 ± 0.066	1.509 ± 0.083
	20	10.624 ± 0.009	4.086 ± 0.033	4.370 ± 0.011	1.570 ± 0.069
	40	10.566 ± 0.042	5.199 ± 0.08	4.401 ± 0.019	1.737 ± 0.027
	80	10.825 ± 0.078	6.191 ± 0.166	4.401 ± 0.033	1.777 ± 0.147

Table C.2 Total polyphenols, tannin, total amino acids and caffeine content of green tea (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5) (cont.)

Condition		Total Polyphenols	Tannin	Total amino acids	Caffeine
^{0}C	Min				
80	1	6.575 ± 0.080	2.003 ± 0.014	1.662 ± 0.031	1.301 ± 0.240
	3	8.267 ± 0.416	3.650 ± 0.109	2.578 ± 0.083	1.457 ± 0.116
	5	9.320 ± 0.024	3.876 ± 0.109	3.895 ± 0.072	1.545 ± 0.066
	10	9.906 ± 0.292	4.597 ± 0.223	4.452 ± 0.061	1.595 ± 0.142
	20	10.672 ± 0.129	5.195 ± 0.356	4.509 ± 0.110	1.628 ± 0.131
	40	10.783 ± 0.009	6.431 ± 0.640	4.534 ± 0.033	1.778 ± 0.109
	80	11.089 ± 0.079	7.283 ± 0.935	4.534 ± 0.033	1.886 ± 0.067
90	1	6.852 ± 0.186	2.473 ± 0.074	1.745 ± 0.041	1.489 ± 0.051
	3	8.199 ± 0.109	4.019 ± 0.038	2.913 ± 0.116	1.749 ± 0.159
	5	9.415 ± 0.123	4.055 ± 0.075	4.230 ± 0.050	1.868 ± 0.045
	10	10.767 ± 0.361	4.968 ± 0.465	4.414 ± 0.055	2.077 ± 0.118
	20	11.021 ± 0.279	5.510 ± 0.303	4.420 ± 0.050	2.350 ± 0.034
	40	11.158 ± 0.133	6.243 ± 0.027	4.211 ± 0.069	2.358 ± 0.041
	80	10.461 ± 0.901	7.051 ± 0.069	4.161 ± 0.096	2.396 ± 0.045

Table C.3 The content of major catechins (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5)

Condition		EGCG	EGC	ECG	EC
^{0}C	Min				
50	1	0.031 ± 0.018	0.713 ± 0.119	0.151 ± 0.015	0.348 ± 0.026
	3	0.081 ± 0.010	0.802 ± 0.067	0.174 ± 0.081	0.419 ± 0.088
	5	0.104 ± 0.016	0.835 ± 0.203	0.252 ± 0.041	0.442 ± 0.083
	10	0.163 ± 0.021	0.975 ± 0.113	0.417 ± 0.101	0.509 ± 0.048
	20	0.193 ± 0.031	1.059 ± 0.091	0.613 ± 0.031	0.571 ± 0.068
	40	0.340 ± 0.129	1.236 ± 0.080	0.733 ± 0.020	0.603 ± 0.007
	80	0.456 ± 0.061	1.266 ± 0.093	1.008 ± 0.136	0.773 ± 0.173
60	1	0.178 ± 0.033	0.899 ± 0.006	0.360 ± 0.038	0.514 ± 0.008
	3	0.261 ± 0.034	1.078 ± 0.045	0.410 ± 0.089	0.576 ± 0.034
	5	0.271 ± 0.116	1.139 ± 0.193	0.499 ± 0.104	0.622 ± 0.130
	10	0.302 ± 0.093	1.290 ± 0.236	0.546 ± 0.024	0.627 ± 0.078
	20	0.307 ± 0.098	1.393 ± 0.144	1.173 ± 0.020	0.680 ± 0.100
	40	0.450 ± 0.055	1.410 ± 0.008	1.224 ± 0.004	0.856 ± 0.005
	80	0.452 ± 0.016	1.738 ± 0.171	1.426 ± 0.065	0.892 ± 0.178
70	1	0.173 ± 0.034	0.992 ± 0.048	0.801 ± 0.029	0.547 ± 0.021
	3	0.254 ± 0.030	1.165 ± 0.142	0.888 ± 0.096	0.637 ± 0.048
	5	0.304 ± 0.030	1.372 ± 0.197	0.989 ± 0.021	0.647 ± 0.088
	10	0.390 ± 0.031	1.692 ± 0.309	1.075 ± 0.014	0.753 ± 0.134
	20	0.500 ± 0.033	1.725 ± 0.244	1.228 ± 0.010	0.769 ± 0.144
	40	0.580 ± 0.034	1.872 ± 0.235	1.656 ± 0.134	0.904 ± 0.213
	80	0.605 ± 0.002	2.015 ± 0.406	1.891 ± 0.001	1.037 ± 0.264

Table C.3 The content of major catechins (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5) (Cont.)

Condition		EGCG	EGC	ECG	EC
^{0}C	Min				
80	1	0.270 ± 0.035	1.114 ± 0.070	0.831 ± 0.116	0.608 ± 0.027
	3	0.368 ± 0.027	1.184 ± 0.118	0.929 ± 0.011	0.653 ± 0.038
	5	0.395 ± 0.030	1.307 ± 0.024	1.020 ± 0.075	0.674 ± 0.081
	10	0.436 ± 0.038	1.531 ± 0.109	1.090 ± 0.075	0.858 ± 0.223
	20	0.563 ± 0.179	1.718 ± 0.278	1.315 ± 0.150	0.875 ± 0.208
	40	0.628 ± 0.093	1.959 ± 0.562	1.744 ± 0.037	1.024 ± 0.043
	80	0.643 ± 0.023	2.201 ± 0.389	2.113 ± 0.042	1.274 ± 0.065
90	1	0.323 ± 0.045	1.015 ± 0.042	0.896 ± 0.028	0.855 ± 0.021
	3	0.368 ± 0.028	1.168 ± 0.264	1.067 ± 0.029	0.885 ± 0.034
	5	0.414 ± 0.028	1.895 ± 0.078	1.252 ± 0.072	0.900 ± 0.065
	10	0.512 ± 0.072	1.970 ± 0.188	1.332 ± 0.076	0.995 ± 0.025
	20	0.555 ± 0.044	2.201 ± 0.050	1.388 ± 0.050	1.047 ± 0.057
	40	0.664 ± 0.086	2.334 ± 0.440	1.848 ± 0.491	1.139 ± 0.091
	80	0.580 ± 0.002	2.842 ± 0.798	2.185 ± 0.078	1.281 ± 0.191

Table C.4 The content of minor catechins and total catechins (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5)

Condition		GCG	GC	C	G	Total Catechins
^{0}C	Min					
50	1	nd *	0.618 ± 0.077	0.242 ± 0.022	0.279 ± 0.039	3.003 ± 0.137
	3	nd	0.651 ± 0.069	0.277 ± 0.048	0.308 ± 0.047	3.716 ± 0.304
	5	nd	0.669 ± 0.101	0.248 ± 0.078	0.311 ± 0.060	3.927 ± 0.459
	10	nd	0.863 ± 0.179	0.322 ± 0.082	0.375 ± 0.099	4.765 ± 0.415
	20	nd	0.973 ± 0.167	0.366 ± 0.115	0.410 ± 0.108	5.347 ± 0.458
	40	nd	1.069 ± 0.155	0.422 ± 0.098	0.438 ± 0.103	6.101 ± 0.450
	80	nd	1.344 ± 0.195	0.544 ± 0.200	0.523 ± 0.090	7.355 ± 0.046
60	1	nd	0.701 ± 0.013	0.307 ± 0.043	0.300 ± 0.007	4.084 ± 0.015
	3	nd	0.943 ± 0.070	0.331 ± 0.035	0.353 ± 0.014	4.898 ± 0.031
	5	nd	0.972 ± 0.257	0.359 ± 0.106	0.400 ± 0.064	5.379 ± 0.209
	10	nd	1.156 ± 0.155	0.412 ± 0.144	0.441 ± 0.068	6.112 ± 0.115
	20	nd	1.533 ± 0.556	0.517 ± 0.178	0.482 ± 0.069	7.440 ± 0.248
	40	nd	1.644 ± 0.528	0.567 ± 0.212	0.504 ±0.056	8.139 ± 0.433
	80	nd	2.298 ± 0.364	0.605 ± 0.210	0.607 ± 0.051	9.459 ± 0.531
70	1	nd	0.775 ± 0.055	0.413 ± 0.022	0.297 ± 0.002	4.894 ± 0.137
	3	nd	1.137 ± 0.243	0.452 ± 0.034	0.401 ± 0.009	6.121 ± 0.258
	5	nd	1.559 ± 0.445	0.453 ± 0.130	0.430 ± 0.040	7.233 ± 0.385
	10	nd	1.759 ± 0.656	0.545 ± 0.200	0.527 ± 0.067	8.250 ± 0.057
	20	nd	1.965 ± 0.762	0.613 ± 0.254	0.537 ± 0.056	8.907 ± 0.101
	40	nd	2.062 ± 0.761	0.613 ± 0.267	0.625 ± 0.106	10.047 ± 0.046
	80	0.046 ± 0.005	2.363 ± 0.969	0.700 ± 0.289	0.708 ± 0.005	11.142 ± 0.179

Table C.4 The content of minor catechins and total catechins (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:20, pH 5) (cont.)

Condition		GCG	GC	C	G	Total Catechins
^{0}C	Min					
80	1	nd	1.127 ± 0.138	0.410 ± 0.006	0.367 ± 0.055	6.029 ± 0.473
	3	nd	1.477 ± 0.009	0.497 ± 0.038	0.436 ± 0.116	7.001 ± 0.270
	5	nd	1.527 ± 0.829	0.500 ± 0.222	0.456 ± 0.071	7.424 ± 0.472
	10	nd	1.799 ± 0.687	0.602 ± 0.228	0.528 ± 0.064	8.440 ±0.343
	20	nd	2.067 ± 0.595	0.633 ± 0.254	0.560 ± 0.065	9.359 ± 0.690
	40	nd	2.166 ± 0.703	0.664 ± 0.269	0.631 ± 0.083	10.594 ± 0.325
	80	0.059 ± 0.008	2.358 ± 0.535	0.820 ± 0.292	0.721 ± 0.027	12.075 ± 0.173
90	1	nd	1.583 ± 0.087	0.632 ± 0.007	0.477 ± 0.016	7.271 ± 0.040
	3	nd	1.853 ± 0.077	0.657 ± 0.017	0.549 ± 0.044	8.297 ± 0.451
	5	nd	2.047 ± 0.968	0.661 ± 0.057	0.567 ± 0.072	9.604 ± 0.799
	10	nd	2.186 ± 0.920	0.737 ± 0.062	0.594 ± 0.081	10.403 ± 0.984
	20	nd	2.702 ± 0.548	0.791 ± 0.053	0.619 ± 0.070	11.654 ± 0.338
	40	nd	2.889 ± 1.452	0.885 ± 0.105	0.673 ± 0.075	12.789 ± 1.637
	80	0.084 ± 0.036	3.963 ± 1.884	0.987 ± 0.271	0.727 ± 0.137	13.395 ± 2.127

* nd = not detected

C.3 Rate constant determination

Reaction rate constant and reaction order were measured by using the following equation. The graph of ln (C_∞/ (C_∞-C)) versus time was plotted with slope as reaction rate constant.

$$\ln\left(\frac{c_\infty}{c_\infty - c}\right) = k_{\text{obs}}t + a \qquad \text{(C.1)}$$

Where:

C : the concentration value at time t

C_{∞} : its concentration value when the equilibrium is reached

k_{obs} : an observed first order rate constant

t : extraction time

a : the intercept

C.3.1 Total polyphenols and tannin

Table C.5 Calculation of rate constant determination for total polyphenols and tannin contents

Condition		Total polyphenols				Tannin			
°C	Min	C (% w/w)	C_{∞}	C - C_{∞}	ln (C - C_{∞})	C (% w/w)	C_{∞}	C - C_{∞}	ln (C - C_{∞})
50	1	3.812	9.901	6.089	0.486	0.822	5.169	4.347	0.173
	3	5.265		4.636	0.759	3.358		1.811	1.049
	5	6.985		2.916	1.222	3.574		1.594	1.176
	10	8.802		1.099	2.199	3.775		1.393	1.311
60	1	4.277	10.281	6.004	0.538	1.667	5.824	4.157	0.337
	3	5.912		4.369	0.856	3.556		2.268	0.943
	5	7.851		2.430	1.443	3.644		2.180	0.983
	10	8.939		1.342	2.036	3.841		1.983	1.077
70	1	5.917	10.825	4.908	0.791	1.984	6.191	4.207	0.386
	3	8.727		2.098	1.641	3.724		2.466	0.920
	5	9.251		1.574	1.928	3.806		2.385	0.954
	10	10.133		0.692	2.750	3.880		2.311	0.986
80	1	6.575	11.089	4.515	0.899	2.003	7.283	5.280	0.322
	3	8.267		2.822	1.369	3.650		3.633	0.696
	5	9.320		1.769	1.835	3.876		3.407	0.760
	10	9.906		1.183	2.238	4.597		2.686	0.997
90	1	6.852	11.158	4.306	0.952	2.473	7.051	4.578	0.432
	3	8.199		2.959	1.327	4.019		3.032	0.844
	5	9.415		1.743	1.856	4.055		2.997	0.856
	10	10.767		0.391	3.352	4.968		2.084	1.219

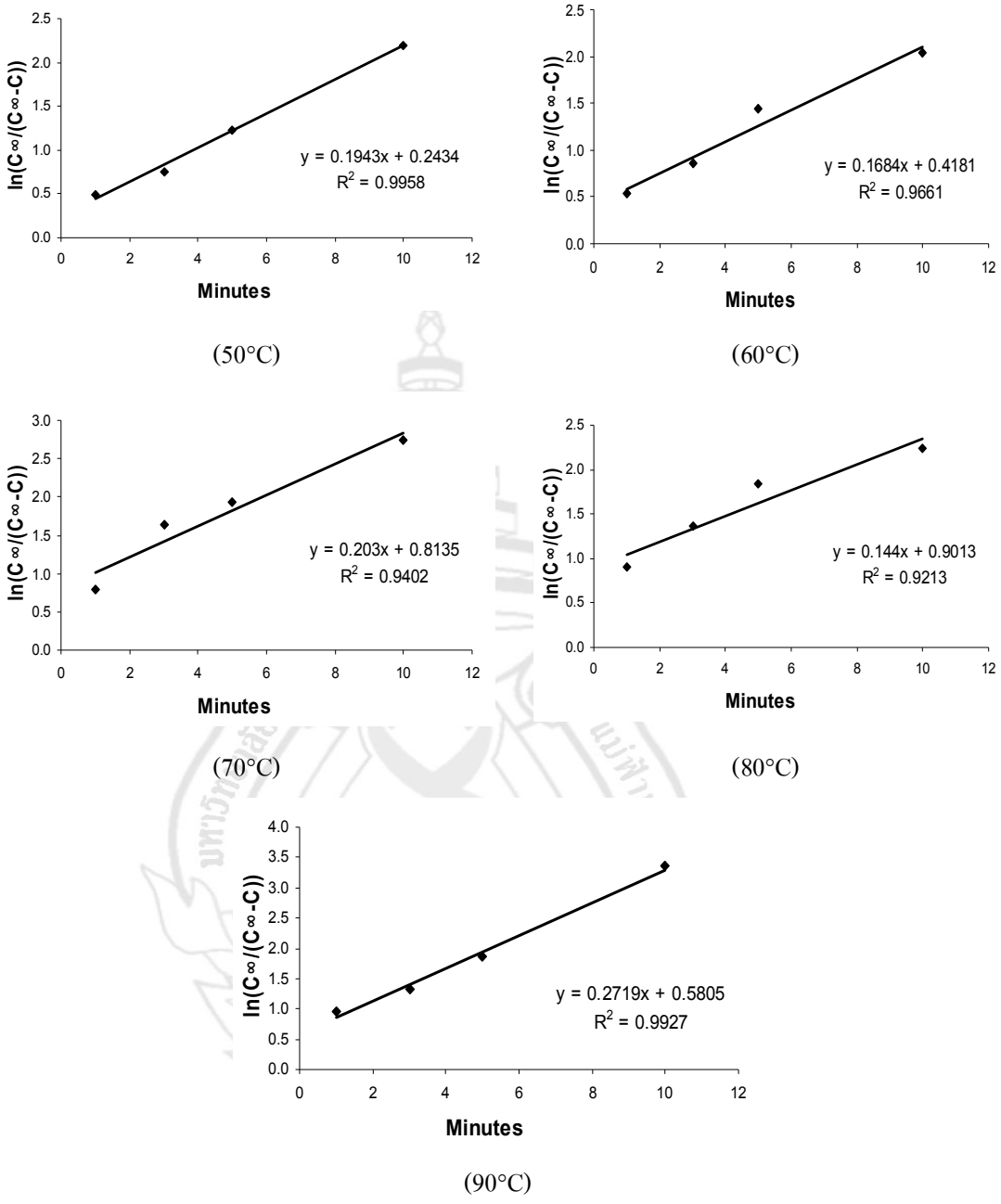
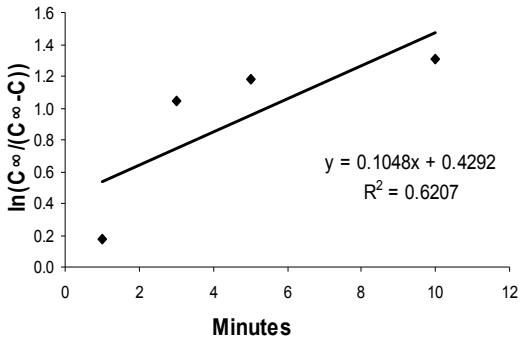
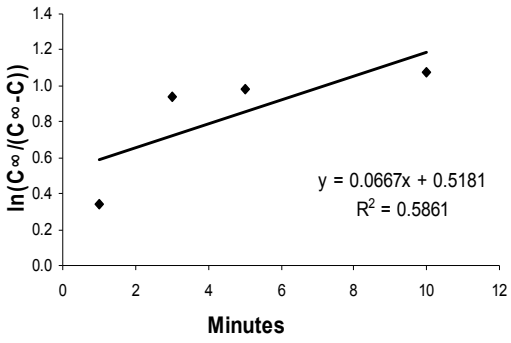


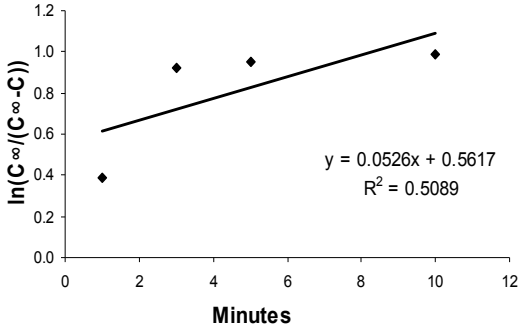
Figure C.1 First order plot for total polyphenols extraction from green tea at various times and temperatures



(50°C)



(60°C)



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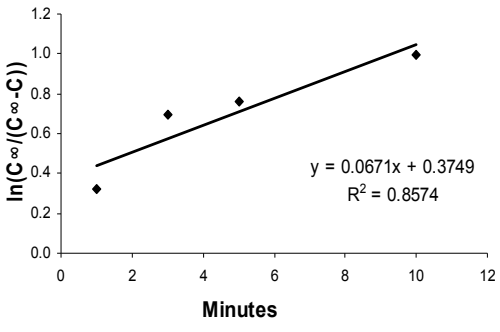
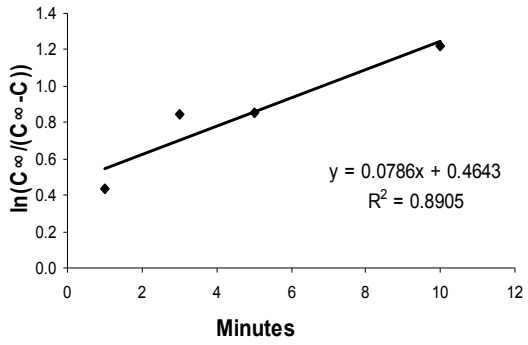


(80°C)



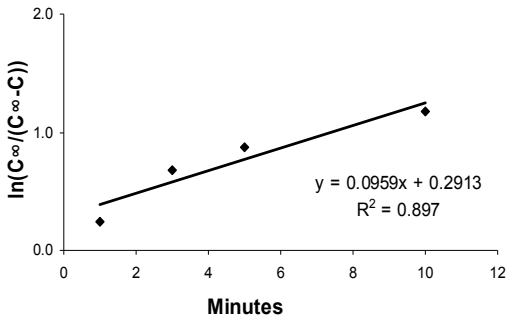
(90°C)

Figure C.2 First order plot for tannin extraction from green tea at various time and temperatures

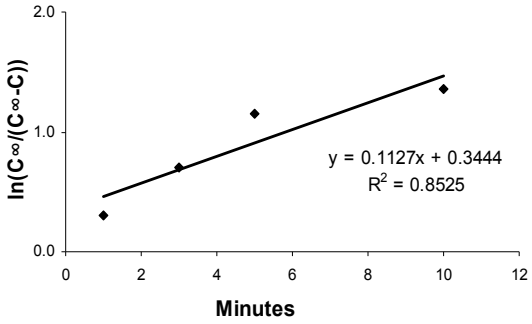
C.3.2 Total amino acids and caffeine

Table C.6 Calculation of rate constant determination for total amino acids and caffeine contents

Condition		Total amino acids				Caffeine			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
50	1	0.918	4.205	3.287	0.246	0.621	1.441	0.820	0.563
	3	2.083		2.121	0.684	1.005		0.436	1.195
	5	2.457		1.748	0.878	1.066		0.375	1.345
	10	2.913		1.292	1.180	1.142		0.299	1.571
60	1	1.117	4.281	3.163	0.302	0.826	1.643	0.817	0.699
	3	2.166		2.115	0.705	0.947		0.696	0.859
	5	2.932		1.349	1.155	1.118		0.525	1.140
	10	3.179		1.102	1.357	1.337		0.306	1.681
70	1	1.355	4.401	3.046	0.368	0.895	1.777	0.882	0.701
	3	2.207		2.194	0.696	1.187		0.590	1.103
	5	3.673		0.728	1.799	1.478		0.299	1.783
	10	3.888		0.513	2.149	1.509		0.268	1.892
80	1	1.662	4.534	2.872	0.457	1.301	1.886	0.585	1.171
	3	2.578		1.957	0.840	1.457		0.429	1.481
	5	3.895		0.640	1.958	1.545		0.342	1.709
	10	4.452		0.082	4.008	1.595		0.291	1.869
90	1	1.745	4.420	2.676	0.502	1.489	2.396	0.907	0.972
	3	2.913		1.507	1.076	1.749		0.647	1.309
	5	4.230		0.190	3.147	1.868		0.528	1.513
	10	4.414		0.006	6.548	2.077		0.320	2.014



(50°C)



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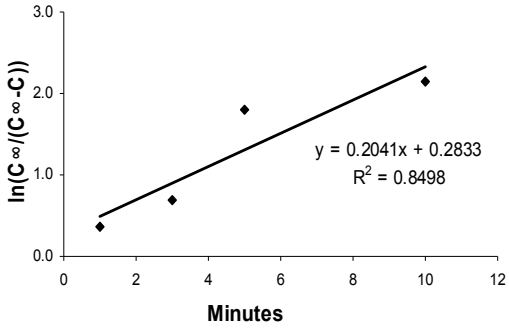

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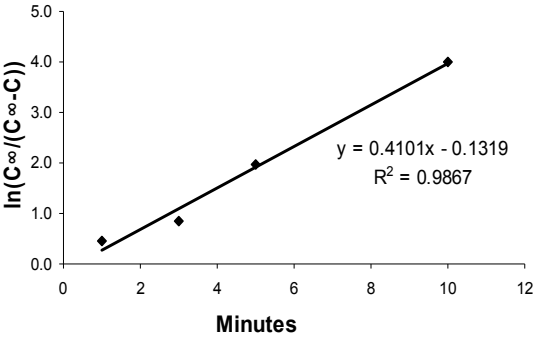

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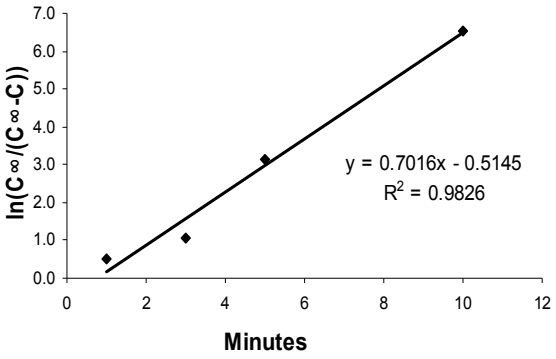
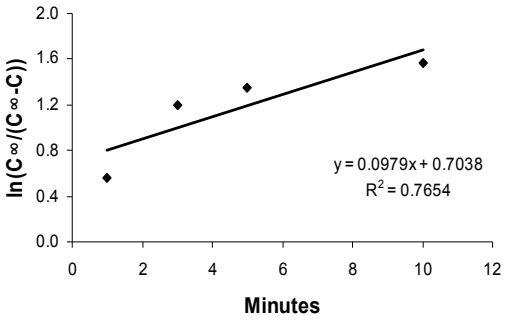


(90°C)

Figure C.3 First order plot for total amino acids extraction from green tea at various times and temperatures



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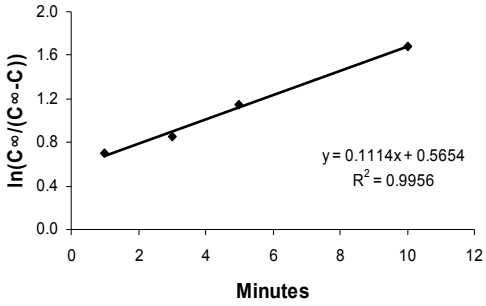

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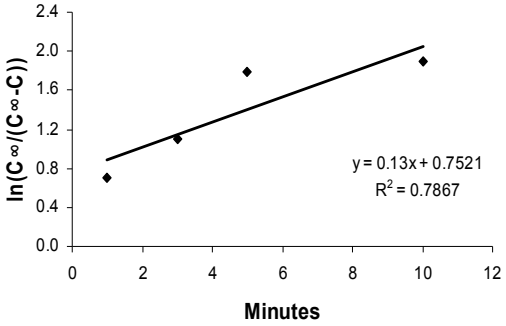

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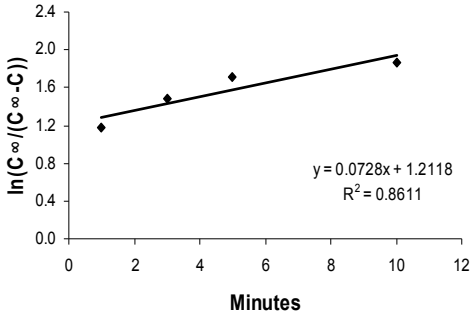

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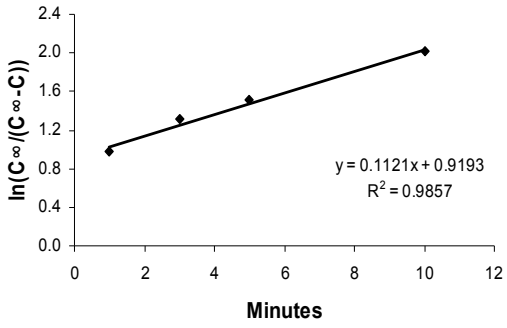


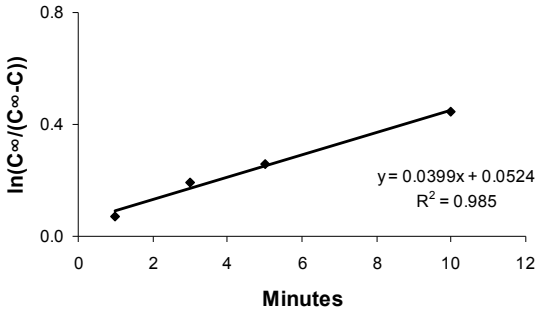
(90°C)

Figure C.4 First order plot for caffeine extraction from green tea at various times and temperatures

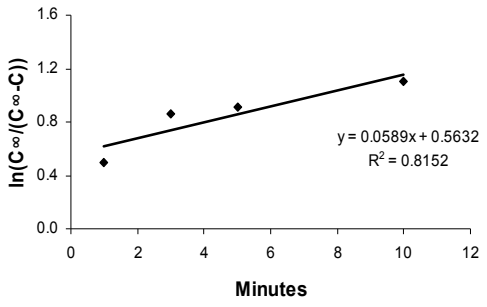
C.3.3 Individual catechins

Table C.7 Calculation of rate constant determination EGCG and EGC contents

Condition		EGCG				EGC			
°C	Min	C (% w/w)	C_∞	$C - C_\infty$	$\ln (C - C_\infty)$	C (% w/w)	C_∞	$C - C_\infty$	$\ln (C - C_\infty)$
50	1	0.031	0.456	0.425	0.071	0.713	1.266	0.553	0.829
	3	0.081		0.375	0.195	0.802		0.464	1.004
	5	0.104		0.352	0.259	0.835		0.432	1.076
	10	0.163		0.293	0.443	0.975		0.291	1.471
60	1	0.178	0.452	0.275	0.498	0.899	1.738	0.838	0.729
	3	0.261		0.191	0.859	1.078		0.659	0.969
	5	0.271		0.181	0.913	1.139		0.599	1.065
	10	0.302		0.150	1.101	1.290		0.447	1.357
70	1	0.173	0.605	0.431	0.338	0.992	2.015	1.023	0.678
	3	0.254		0.351	0.545	1.165		0.850	0.863
	5	0.304		0.301	0.698	1.372		0.642	1.143
	10	0.390		0.215	1.034	1.692		0.323	1.831
80	1	0.270	0.643	0.373	0.545	1.114	2.201	1.086	0.706
	3	0.368		0.274	0.851	1.184		1.017	0.772
	5	0.395		0.247	0.955	1.307		0.894	0.901
	10	0.436		0.206	1.135	1.531		0.670	1.190
90	1	0.323	0.664	0.341	0.667	1.015	2.842	1.826	0.442
	3	0.368		0.297	0.806	1.168		1.673	0.529
	5	0.414		0.250	0.976	1.895		0.947	1.099
	10	0.512		0.153	1.471	1.970		0.872	1.182



(50°C)



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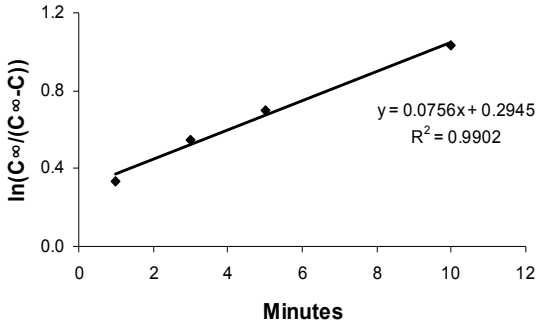

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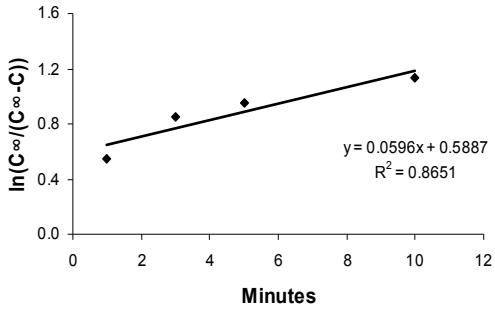

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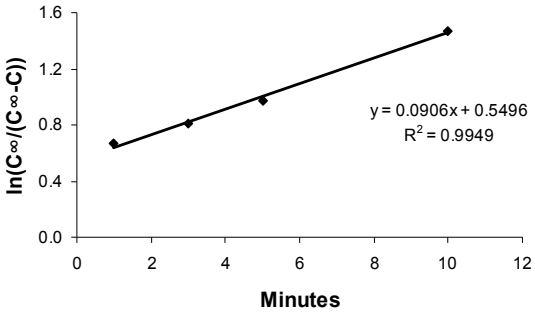
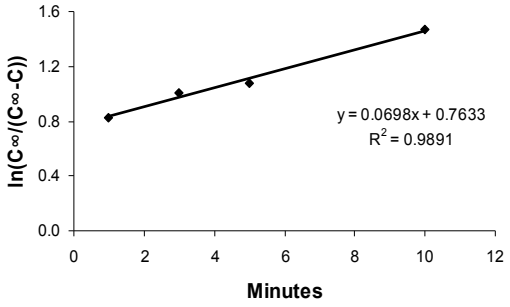


(90°C)

Figure C.5 First order plot for EGCG extraction from green tea at various time and temperatures



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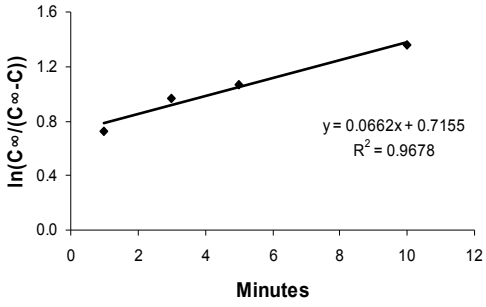

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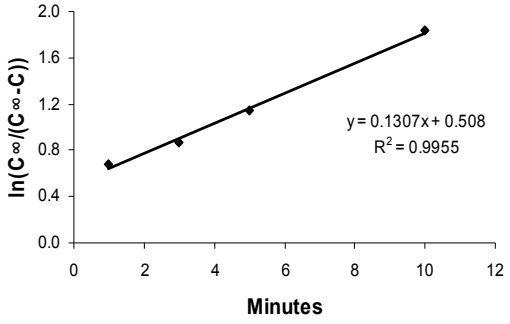

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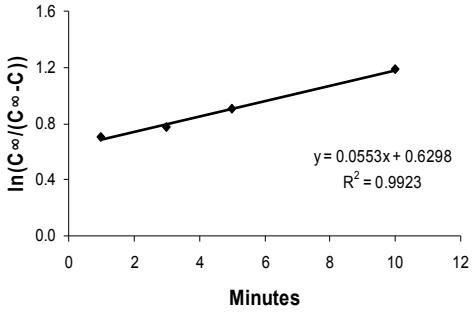

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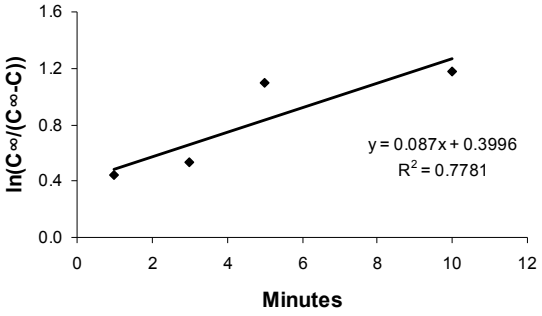


(90°C)

Figure C.6 First order plot for EGC extraction from green tea at various time and temperatures

Table C.8 Calculation of rate constant determination ECG and EC contents

Condition		ECG				EC			
°C	Min	C (% w/w)	C_∞	$C - C_\infty$	ln ($C - C_\infty$)	C (% w/w)	C_∞	$C - C_\infty$	ln ($C - C_\infty$)
50	1	0.151	1.008	0.857	0.163	0.348	0.773	0.425	0.598
	3	0.174		0.834	0.189	0.419		0.354	0.780
	5	0.252		0.756	0.288	0.442		0.331	0.848
	10	0.417		0.591	0.533	0.509		0.264	1.074
60	1	0.360	1.426	1.066	0.291	0.514	0.892	0.378	0.858
	3	0.410		1.016	0.339	0.576		0.316	1.037
	5	0.499		0.927	0.430	0.622		0.270	1.196
	10	0.546		0.880	0.483	0.627		0.265	1.214
70	1	0.801	1.891	1.090	0.551	0.547	1.037	0.490	0.750
	3	0.888		1.003	0.634	0.637		0.400	0.953
	5	0.989		0.903	0.740	0.647		0.389	0.979
	10	1.075		0.816	0.840	0.753		0.284	1.296
80	1	0.831	2.113	1.339	0.456	0.608	1.274	0.666	0.649
	3	0.929		1.184	0.579	0.653		0.621	0.719
	5	1.020		1.093	0.659	0.674		0.600	0.753
	10	1.090		1.023	0.725	0.858		0.416	1.119
90	1	0.896	2.185	1.289	0.528	0.855	1.281	0.425	1.102
	3	1.067		1.118	0.670	0.885		0.396	1.174
	5	1.252		0.933	0.851	0.900		0.381	1.212
	10	1.332		0.853	0.941	0.995		0.286	1.499

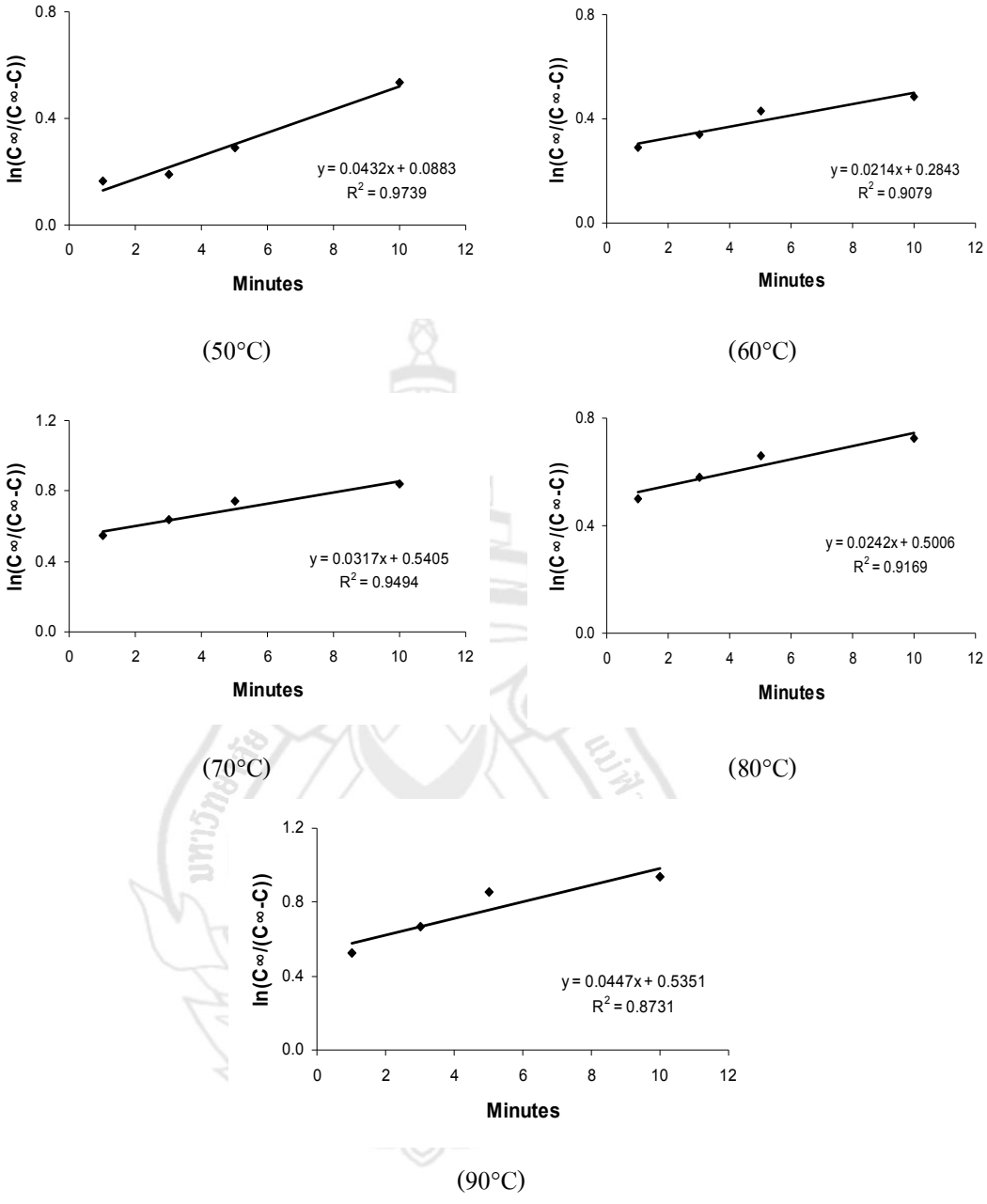


Figure C.7 First order plot for ECG extraction from green tea at various time and temperatures

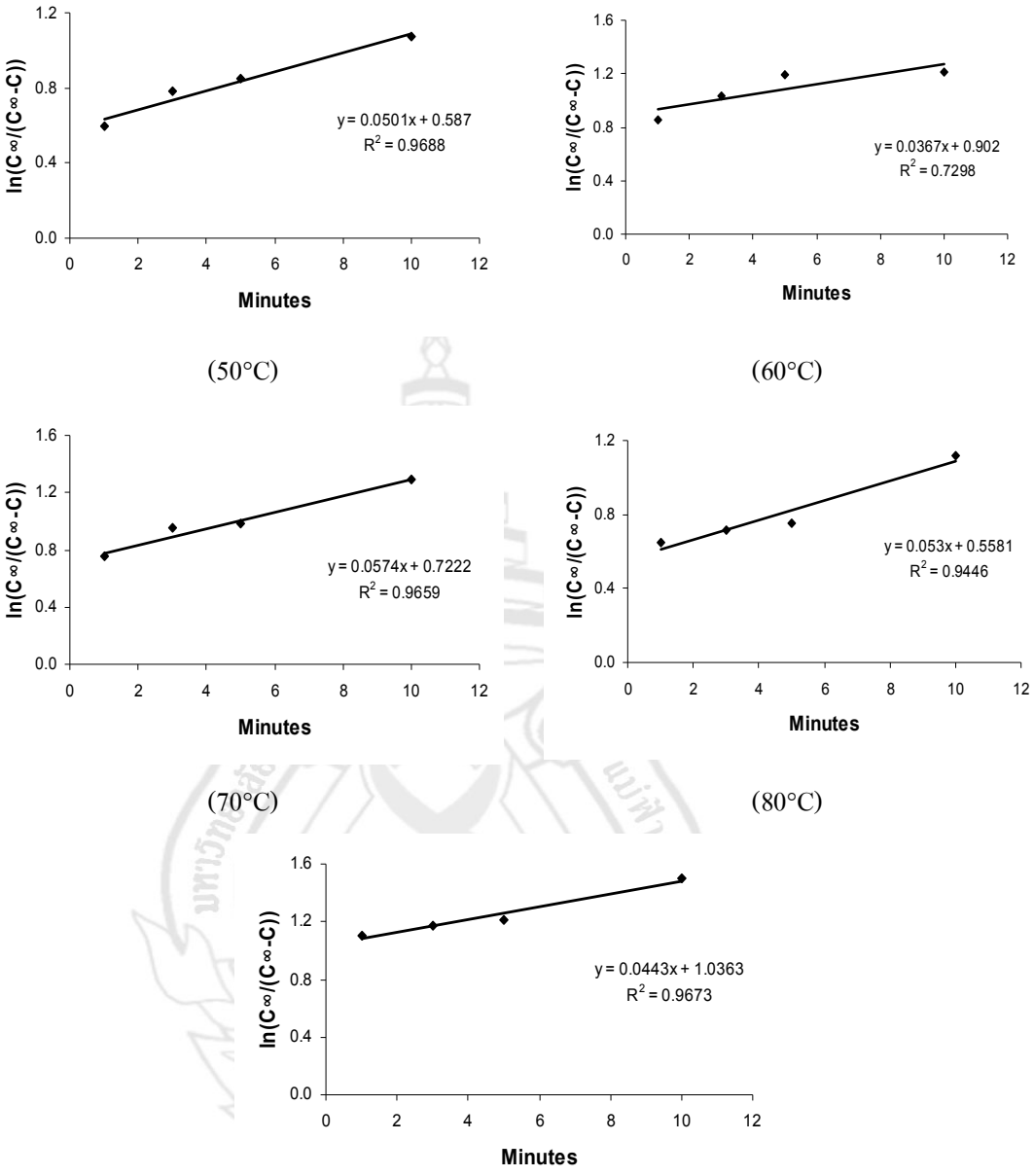
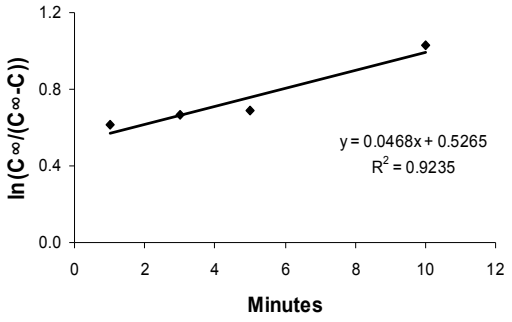


Figure C.8 First order plot for EC extraction from green tea at various time and temperatures

Table C.9 Calculation of rate constant determination GC and C contents

Condition		GC				C			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
50	1	0.618	1.344	0.726	0.616	0.242	0.544	0.302	0.588
	3	0.651		0.692	0.663	0.277		0.268	0.710
	5	0.669		0.675	0.688	0.248		0.296	0.608
	10	0.863		0.481	1.027	0.322		0.222	0.895
60	1	0.701	2.298	1.598	0.364	0.307	0.605	0.298	0.709
	3	0.943		1.355	0.528	0.331		0.274	0.792
	5	0.972		1.326	0.550	0.359		0.246	0.900
	10	1.156		1.142	0.699	0.412		0.193	1.144
70	1	0.775	2.363	1.588	0.398	0.413	0.700	0.287	0.891
	3	1.137		1.226	0.656	0.452		0.264	0.975
	5	1.559		0.804	1.078	0.453		0.247	1.043
	10	1.759		0.604	1.364	0.545		0.155	1.505
80	1	1.127	2.358	1.232	0.650	0.410	0.820	0.409	0.694
	3	1.477		0.882	0.984	0.497		0.322	0.934
	5	1.527		0.831	1.043	0.500		0.320	0.942
	10	1.799		0.559	1.439	0.602		0.217	1.328
90	1	1.583	3.963	2.380	0.510	0.632	0.987	0.355	1.022
	3	1.853		2.110	0.630	0.657		0.330	1.097
	5	2.047		1.916	0.727	0.661		0.326	1.108
	10	2.186		1.777	0.802	0.737		0.250	1.374



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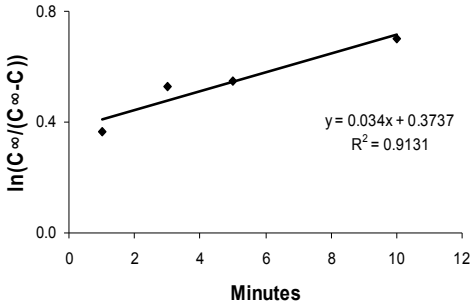

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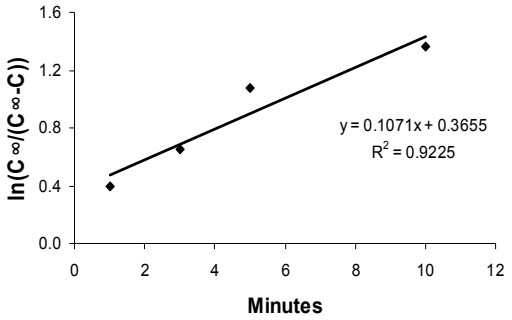

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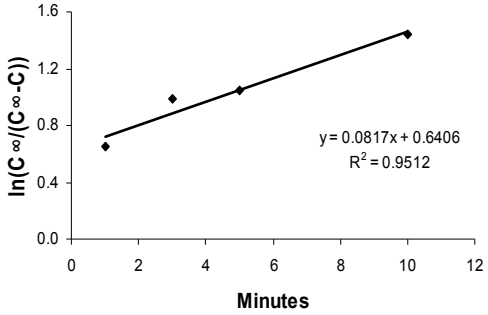

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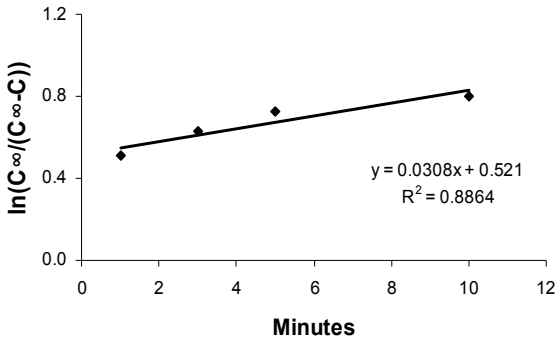


(90°C)

Figure C.9 First order plot for GC extraction from green tea at various time and temperatures

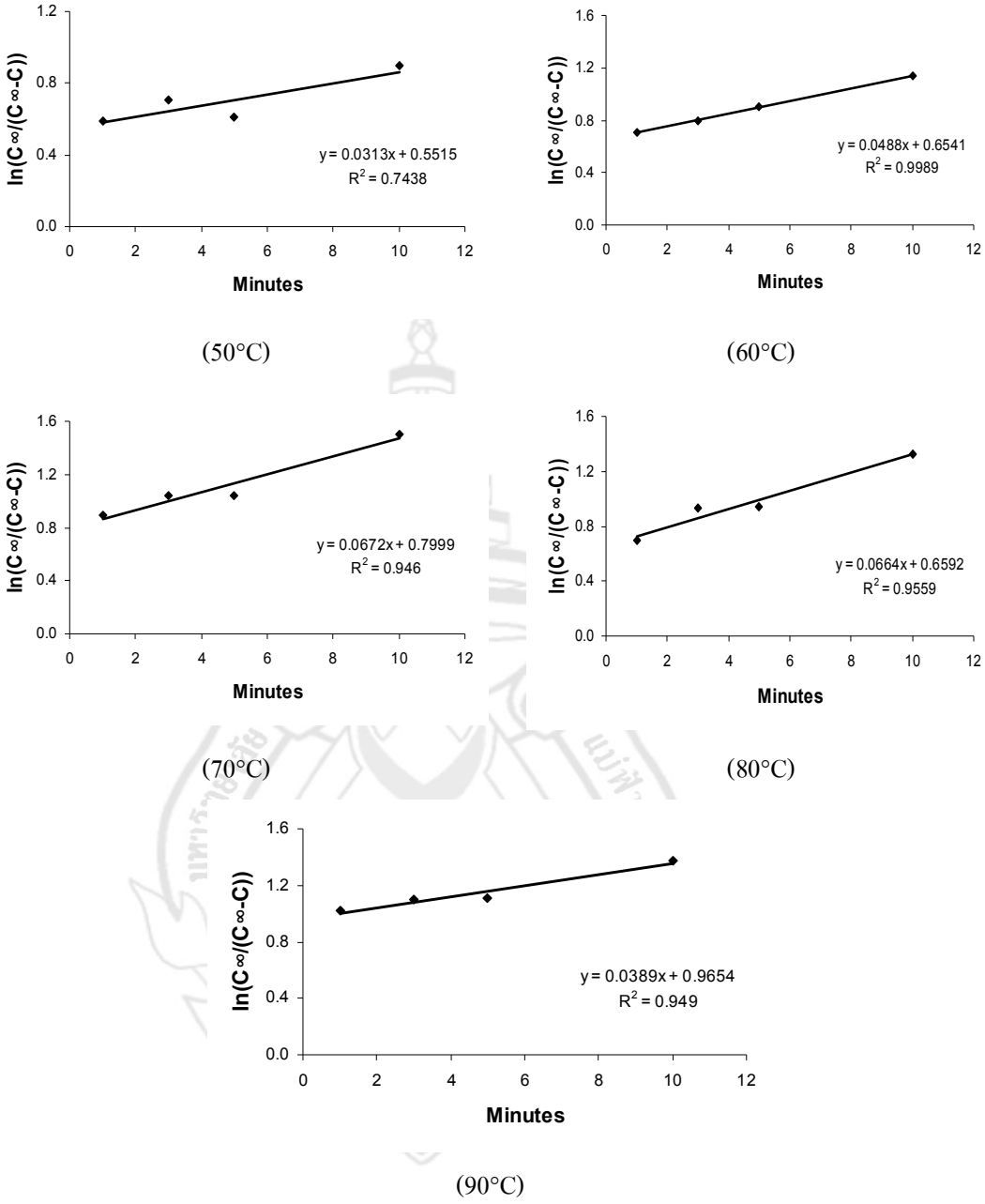
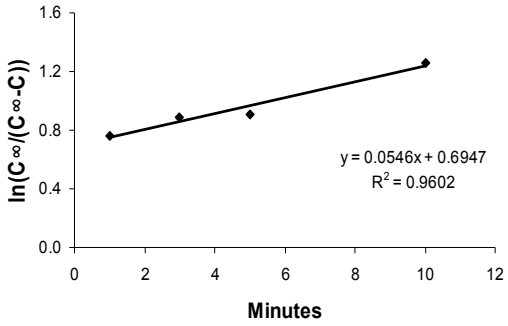


Figure C.10 First order plot for C extraction from green tea at various time and temperatures

Table C.10 Calculation of rate constant determination G and total catechins contents

Condition		G				Total catechins			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
50	1	0.279	0.523	0.244	0.761	3.003	7.355	4.352	0.525
	3	0.308		0.215	0.889	3.716		3.639	0.704
	5	0.311		0.212	0.904	3.927		3.429	0.763
	10	0.375		0.148	1.262	4.765		2.590	1.044
60	1	0.300	0.607	0.307	0.680	4.084	9.459	5.375	0.565
	3	0.353		0.254	0.872	4.898		4.560	0.730
	5	0.400		0.207	1.074	5.379		4.080	0.841
	10	0.441		0.166	1.298	6.112		3.347	1.039
70	1	0.297	0.708	0.411	0.544	4.894	11.142	6.248	0.578
	3	0.401		0.307	0.836	6.121		5.020	0.797
	5	0.430		0.278	0.934	7.233		3.909	1.047
	10	0.527		0.181	1.364	8.250		2.892	1.349
80	1	0.367	0.721	0.354	0.712	6.029	12.075	6.046	0.692
	3	0.436		0.285	0.927	7.001		5.074	0.867
	5	0.456		0.265	1.000	7.424		4.652	0.954
	10	0.528		0.193	1.318	8.440		3.636	1.200
90	1	0.477	0.727	0.250	1.067	7.271	13.395	6.124	0.783
	3	0.549		0.178	1.408	8.297		5.098	0.966
	5	0.567		0.160	1.513	9.604		3.791	1.262
	10	0.594		0.133	1.698	10.403		2.992	1.499



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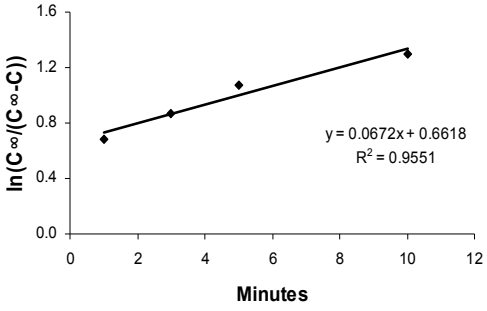

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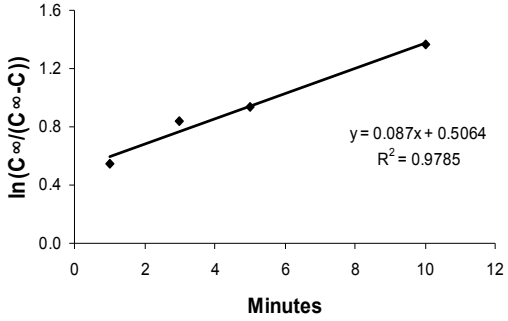

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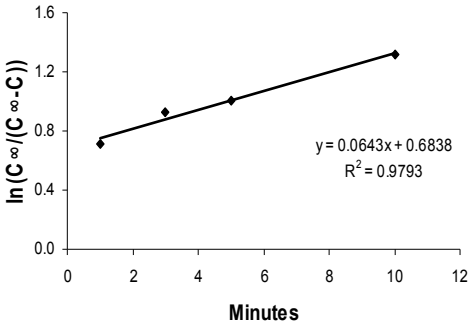

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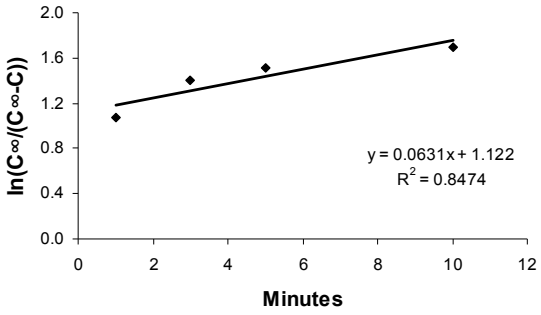

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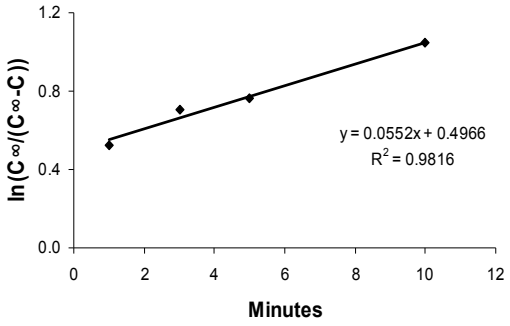
Figure C.11 First order plot for G extraction from green tea at various time and temperatures



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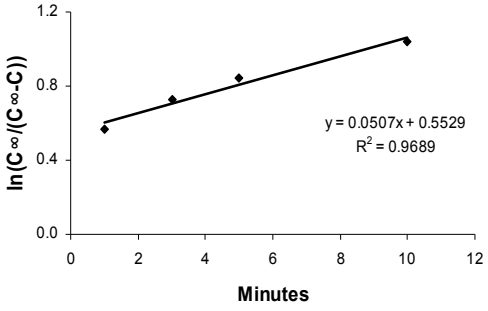

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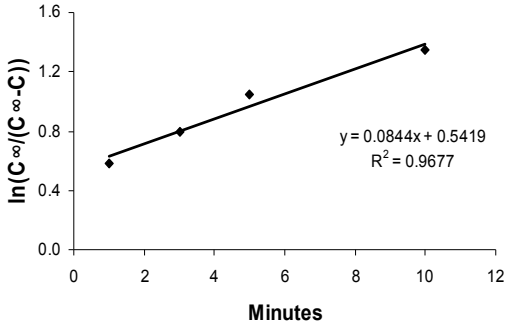

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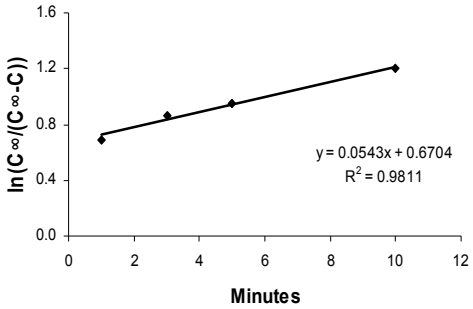

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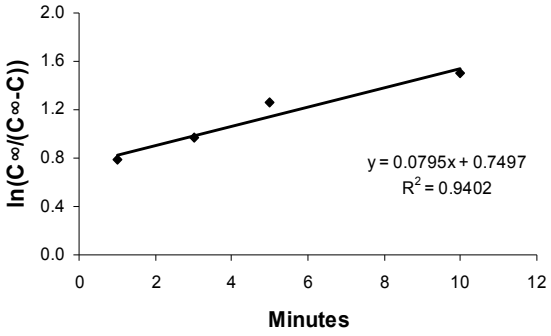

(90°C)

Figure C.12 First order plot for total catechins extraction from green tea at various times and temperatures

APPENDIX D

KINETIC STUDY IN PILOT PLANT SCALE

D.1 The design of tea extractor model unit

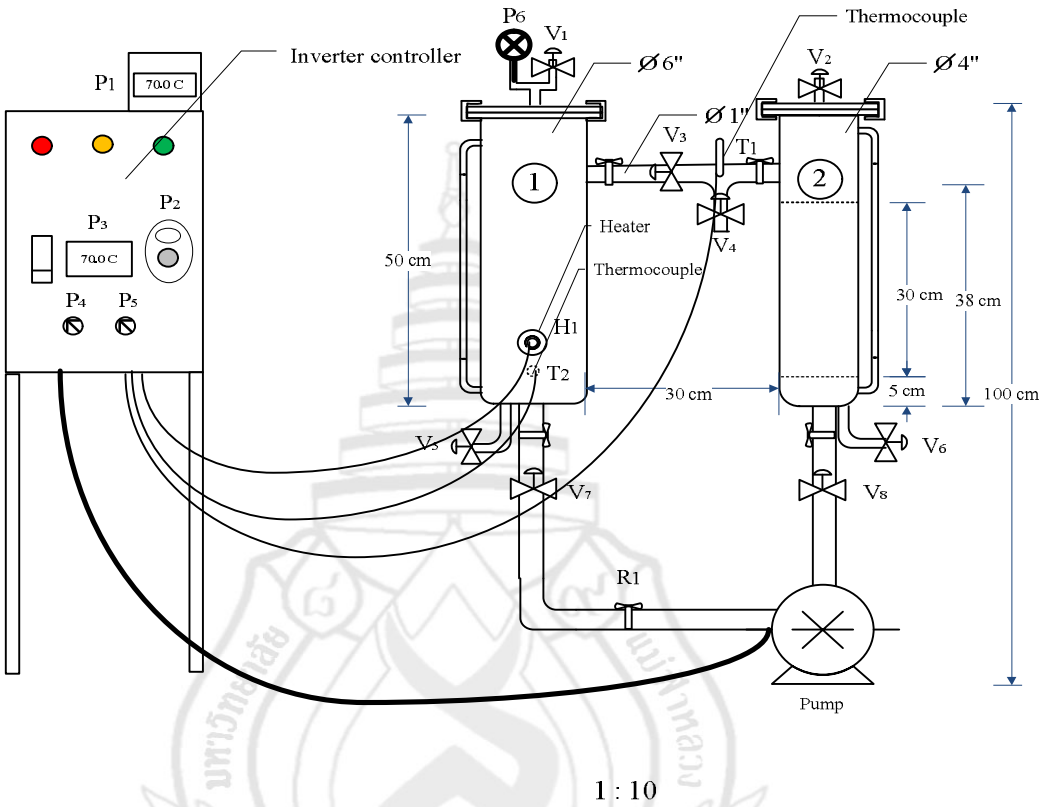

Figure D.1 Schematic diagram of tea extractor model unit (Wongsuwan, 2008)

D.1.1 Specification

1. Holding tank I : stainless steel, D = 15.25 cm, h = 50 cm, vol = 9.13 L
2. Holding tank II : stainless steel, D = 10.16 cm, h = 50 cm, vol = 4.05 L
3. Pipeline : D = 2.54 cm, vol = 1.01 L
4. Min. solvent at tank I : 1.65 L
5. Solvent volume (per batch) : 6.71 L
6. Max. tea leaves at tank II : 0.972 kg

During extraction process the tea leaves are extremely swelled and solvent penetrated into the tea leaves slowly. Due to that, it is necessary to used tea leaves less than 0.972 kg)

D.1.2 Design of Tea Extractor Model Unit

1. Holding tank

The extraction holding tank was designed from stainless steel. This material have a weak acidic resistance capability because acidic solvent was used in this study. Besides, based on GMP (Good Manufacturing Process), stainless steel is recommended to use in food industry. Tank I contain of immersion heater coil for applying heat to solvent under electrical power of 1,600 watts. The temperature during extraction was controlled by thermocouple type-K that installed inside tank I and at the pipe that connect tank I and tank II. Sight glass was installed in the outside part of each tank to monitor the level of solvent inside. Pressure gage (1-10 bar) was installed at the top of tank I to inform the pressure inside the tank. Besides, ball valve controllers were installed on the top of tank I and II to control the pressure.

2. Pipeline

The pipeline system was consisted of straight and elbow pipes. This system was used to connecting of each part of the unit and directing the fluid flow inside the pipes.

3. Pump

Centrifugal pump (Inoxpa, SIA model.8EF1003, Banyoles, Spain) was used in this unit. The gear motor was drive with 0.35kW, 220-240 V and 50 Hz (2870 rpm) of 3-phase circuits. The pump was designed from stainless steel, therefore it can be operated under hot and acidic system that used in this study.

4. Valve

Stainless steel butterfly valve and ball valve were installed to control the liquid flow rate in the system.

5. Thermocouple type K

Thermocouple type K was installed in the tank I and at the connection pipe between tank I and tank II. These thermocouple were connected to the display panel that shown the temperature.

6. Control panel

This unit consisted of two control panels. The first one was used for controlling and displaying the temperature inside the tank I and tank II, while the other one was used for monitoring the speed of the pump inverter.

D.2 Layout of experimental design

Table D.1 Layout of experimental design

Min	Temperature (^{0} C)					
	70		80		90	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	Y_{111}	Y_{112}	Y_{211}	Y_{212}	Y_{311}	Y_{312}
3	Y_{121}	Y_{122}	Y_{221}	Y_{222}	Y_{321}	Y_{322}
5	Y_{131}	Y_{132}	Y_{231}	Y_{232}	Y_{331}	Y_{332}
10	Y_{141}	Y_{142}	Y_{241}	Y_{242}	Y_{341}	Y_{342}
20	Y_{151}	Y_{152}	Y_{251}	Y_{252}	Y_{351}	Y_{352}
40	Y_{161}	Y_{162}	Y_{261}	Y_{262}	Y_{361}	Y_{362}
60	Y_{181}	Y_{182}	Y_{281}	Y_{282}	Y_{381}	Y_{382}
80	Y_{191}	Y_{192}	Y_{291}	Y_{292}	Y_{391}	Y_{392}
120	Y_{1121}	Y_{1122}	Y_{2121}	Y_{2122}	Y_{3121}	Y_{3122}

D.3 Raw data

Table D.2 Total polyphenols, tannin, theanine and caffeine content of green tea (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:28, pH 5)

Condition		Total polyphenols	Tannin	Theanine	Caffeine
^{0}C	Minute				
70	1	0.707 ± 0.146	0.340 ± 0.155	0.028 ± 0.004	0.082 ± 0.015
	3	2.972 ± 0.117	1.581 ± 0.099	1.170 ± 0.180	0.234 ± 0.008
	5	3.801 ± 0.064	1.602 ± 0.101	1.178 ± 0.074	0.265 ± 0.055
	10	3.975 ± 0.093	1.653 ± 0.162	1.319 ± 0.136	0.288 ± 0.105
	20	6.471 ± 0.125	3.614 ± 0.200	1.484 ± 0.122	0.328 ± 0.124
	40	7.213 ± 0.072	4.033 ± 0.220	1.685 ± 0.106	0.377 ± 0.131
	60	7.841 ± 0.323	4.657 ± 0.466	1.822 ± 0.096	0.408 ± 0.124
	80	9.106 ± 1.280	5.733 ± 0.983	1.920 ± 0.061	0.428 ± 0.128
	120	9.998 ± 0.602	6.180 ± 0.550	2.087 ± 0.077	0.540 ± 0.021
80	1	1.689 ± 0.077	0.963 ± 0.119	0.673 ± 0.038	0.146 ± 0.012
	3	2.956 ± 0.024	1.565 ± 0.066	0.920 ± 0.033	0.216 ± 0.006
	5	4.168 ± 0.053	2.041 ± 0.087	0.942 ± 0.155	0.224 ± 0.081
	10	4.504 ± 0.103	2.217 ± 0.095	1.098 ± 0.128	0.257 ± 0.104
	20	6.743 ± 0.371	4.170 ± 0.493	1.292 ± 0.055	0.306 ± 0.096
	40	8.017 ± 0.297	4.988 ± 0.362	1.575 ± 0.029	0.370 ± 0.102
	60	8.934 ± 0.114	5.789 ± 0.294	1.773 ± 0.080	0.433 ± 0.062
	80	9.848 ± 0.321	6.461 ± 0.288	1.875 ± 0.128	0.442 ± 0.095
	120	11.021 ± 0.050	7.007 ± 0.066	2.189 ± 0.238	0.564 ± 0.009

Table D.2 Total polyphenols, tannin, theanine and caffeine content of green tea (% w/w dried solid) extracted with water at various temperatures and times (tea-water ratio = 1:28, pH 5) (cont.)

Condition		Total polyphenols	Tannin	Theanine	Caffeine
^{0}C	Minute				
90	1	2.898 ± 0.080	1.670 ± 0.024	0.678 ± 0.097	0.252 ± 0.040
	3	3.610 ± 0.286	2.132 ± 0.232	1.150 ± 0.058	0.301 ± 0.013
	5	4.738 ± 0.557	2.965 ± 0.363	1.496 ± 0.287	0.361 ± 0.015
	10	5.757 ± 0.488	3.649 ± 0.451	1.575 ± 0.260	0.459 ± 0.090
	20	7.284 ± 0.268	4.968 ± 0.260	1.767 ± 0.235	0.493 ± 0.065
	40	9.396 ± 0.117	6.542 ± 0.069	1.969 ± 0.265	0.539 ± 0.198
	60	10.759 ± 0.511	7.239 ± 0.294	2.058 ± 0.254	0.617 ± 0.037
	80	11.002 ± 0.199	7.154 ± 0.200	2.135 ± 0.277	0.619 ± 0.173
	120	11.180 ± 0.000	7.364 ± 0.478	2.292 ± 0.349	0.695 ± 0.023

Table D.3 Individual catechins contents of green tea (% w/w dried solid) extracted with water at various temperatures and time (tea-water ratio = 1:28, pH 5)

Condition		EGCG	EGC	EC	GCG	GC	Total
^{0}C	Min						Catechins
	1	0.013 ± 0.004	0.025 ± 0.006	0.007 ± 0.001	nd	0.034 ± 0.022	0.079 ± 0.034
	3	0.332 ± 0.080	0.084 ± 0.021	0.082 ± 0.023	nd	0.148 ± 0.006	0.645 ± 0.042
	5	0.407 ± 0.091	0.357 ± 0.003	0.434 ± 0.535	nd	0.195 ± 0.004	1.393 ± 0.445
	10	0.477 ± 0.141	0.418 ± 0.042	0.491 ± 0.552	0.021 ± 0.002	0.246 ± 0.006	1.653 ± 0.374
70	20	0.563 ± 0.129	0.486 ± 0.046	0.582 ± 0.657	0.025 ± 0.002	0.285 ± 0.003	1.941 ± 0.483
	40	0.612 ± 0.171	0.525 ± 0.047	0.653 ± 0.728	0.040 ± 0.003	0.297 ± 0.030	2.126 ± 0.482
	60	0.639 ± 0.167	0.518 ± 0.057	0.676 ± 0.758	0.039 ± 0.004	0.311 ± 0.031	2.182 ± 0.498
	80	0.654 ± 0.174	0.517 ± 0.047	0.703 ± 0.803	0.042 ± 0.004	0.325 ± 0.030	2.242 ± 0.547
	120	0.645 ± 0.161	0.497 ± 0.006	0.735 ± 0.890	0.043 ± 0.006	0.341 ± 0.004	2.262 ± 0.715
	1	0.152 ± 0.009	0.054 ± 0.014	0.459 ± 0.070	nd	0.087 ± 0.004	0.752 ± 0.043
	3	0.269 ± 0.002	0.163 ± 0.011	0.520 ± 0.184	nd	0.179 ± 0.021	1.131 ± 0.217
	5	0.279 ± 0.074	0.273 ± 0.080	0.661 ± 0.149	nd	0.190 ± 0.010	1.402 ± 0.293
	10	0.358 ± 0.098	0.334 ± 0.080	0.772 ± 0.181	0.019 ± 0.001	0.263 ± 0.022	1.746 ± 0.339
80	20	0.419 ± 0.079	0.377 ± 0.060	0.892 ± 0.163	0.023 ± 0.003	0.320 ± 0.077	2.031 ± 0.228
	40	0.487 ± 0.078	0.433 ± 0.065	1.032 ± 0.149	0.029 ± 0.000	0.415 ± 0.117	2.396 ± 0.175
	60	0.500 ± 0.039	0.445 ± 0.037	1.106 ± 0.112	0.038 ± 0.003	0.472 ± 0.160	2.560 ± 0.031
	80	0.528 ± 0.002	0.458 ± 0.006	1.181 ± 0.071	0.038 ± 0.002	0.521 ± 0.207	2.727 ± 0.131
	120	0.570 ± 0.108	0.508 ± 0.087	1.311 ± 0.083	0.050 ± 0.006	0.637 ± 0.308	3.077 ± 0.592
	1	0.182 ± 0.079	0.082 ± 0.013	0.434 ± 0.007	nd	0.142 ± 0.039	0.840 ± 0.098
	3	0.146 ± 0.003	0.350 ± 0.094	0.645 ± 0.123	nd	0.170 ± 0.009	1.311 ± 0.035
	5	0.607 ± 0.164	0.575 ± 0.131	1.199 ± 0.282	0.018 ± 0.018	0.342 ± 0.055	2.741 ± 0.651
	10	0.642 ± 0.154	0.606 ± 0.118	1.275 ± 0.249	0.027 ± 0.017	0.384 ± 0.018	2.934 ± 0.521
90	20	0.669 ± 0.138	0.629 ± 0.122	1.370 ± 0.238	0.039 ± 0.011	0.486 ± 0.021	3.192 ± 0.529
	40	0.690 ± 0.168	0.624 ± 0.143	1.392 ± 0.299	0.046 ± 0.020	0.577 ± 0.017	3.330 ± 0.647
	60	0.705 ± 0.159	0.634 ± 0.135	1.416 ± 0.299	0.049 ± 0.011	0.630 ± 0.007	3.434 ± 0.597
	80	0.718 ± 0.161	0.741 ± 0.083	1.408 ± 0.354	0.048 ± 0.010	0.668 ± 0.008	3.583 ± 0.599
	120	0.725 ± 0.128	0.740 ± 0.115	1.395 ± 0.391	0.055 ± 0.017	0.745 ± 0.009	3.660 ± 0.661

D.4 Rate constant determination

D.4.1 Total polyphenols and tannin

Table D.4 Calculation of rate constant determination for total polyphenols and tannin contents

Condition		Total polyphenols				Tannin			
°C	Min	C (% w/w)	C_∞	$C - C_\infty$	$\ln (C - C_\infty)$	C (% w/w)	C_∞	$C - C_\infty$	$\ln (C - C_\infty)$
70	1	0.707	9.998	9.291	0.073	0.340	6.180	5.839	0.057
	3	2.972		7.026	0.353	1.581		4.599	0.295
	5	3.801		6.197	0.478	1.602		4.577	0.300
	10	3.975		6.023	0.507	1.653		4.527	0.311
80	1	1.689	11.021	9.332	0.166	0.963	7.007	6.044	0.148
	3	2.956		8.066	0.312	1.565		5.442	0.253
	5	4.168		6.853	0.475	2.041		4.966	0.344
	10	4.504		6.518	0.525	2.217		4.790	0.380
90	1	2.898	11.180	8.283	0.300	1.670	7.364	5.694	0.257
	3	3.610		7.571	0.390	2.132		5.232	0.342
	5	4.738		6.443	0.551	2.965		4.399	0.515
	10	5.757		5.423	0.723	3.649		3.715	0.684

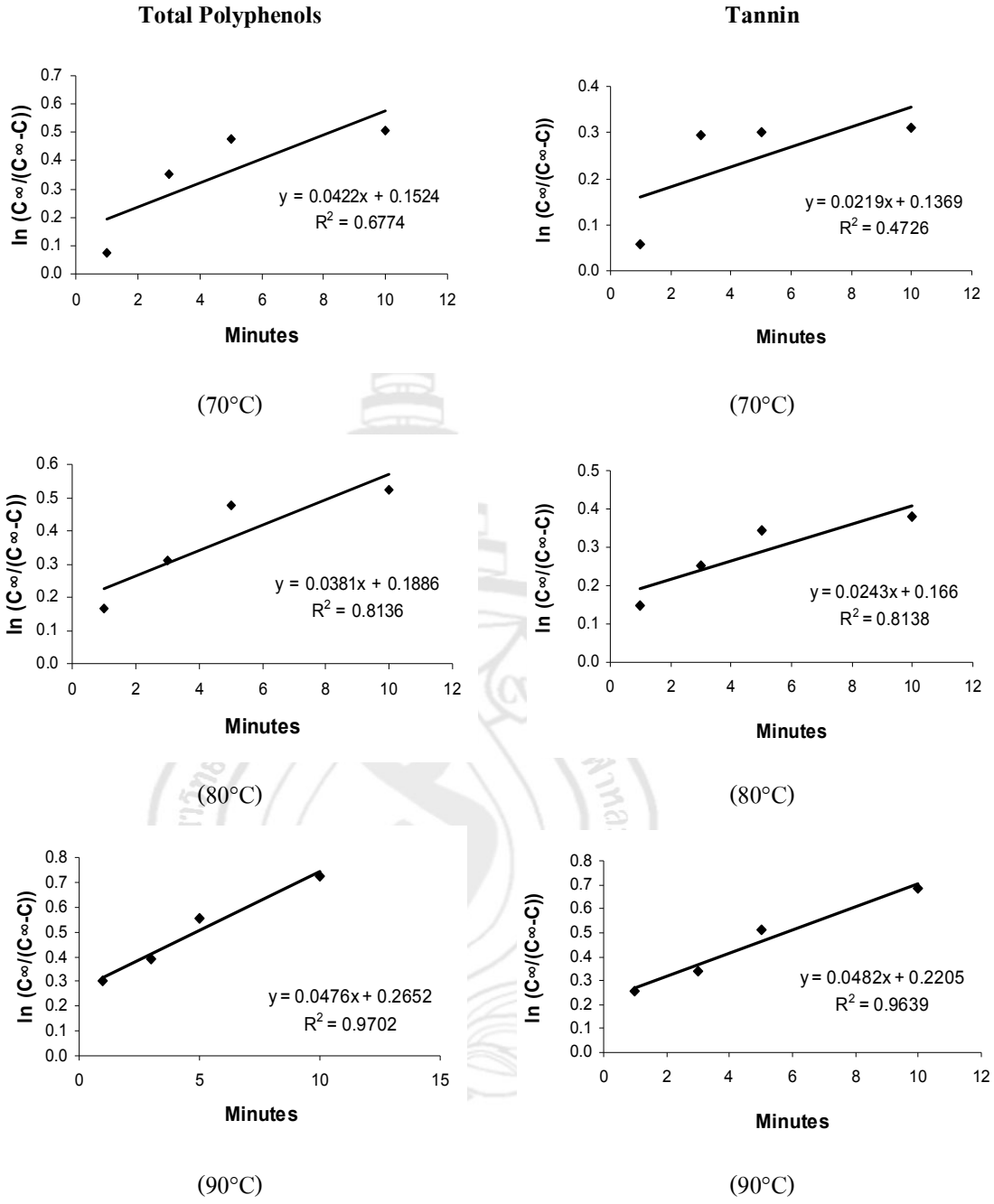

Figure D.2 First order plot for total polyphenols and tannin extraction from green tea at various time and temperatures

D.4.2 Theanine and caffeine

Table D.5 Calculation of rate constant determination for theanine and caffeine contents

Condition		Theanine				Caffeine			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
70	1	0.028	2.087	2.059	0.014	0.082	0.540	0.458	0.164
	3	1.170		0.917	0.823	0.234		0.306	0.567
	5	1.178		0.909	0.831	0.265		0.275	0.675
	10	1.319		0.768	1.000	0.288		0.251	0.764
80	1	0.673	2.189	1.516	0.368	0.146	0.564	0.418	0.300
	3	0.920		1.269	0.546	0.216		0.349	0.481
	5	0.942		1.247	0.563	0.224		0.340	0.506
	10	1.098		1.091	0.696	0.257		0.308	0.607
90	1	0.678	2.292	1.614	0.351	0.252	0.695	0.443	0.451
	3	1.150		1.142	0.697	0.301		0.394	0.567
	5	1.496		0.796	1.058	0.361		0.335	0.732
	10	1.575		0.717	1.162	0.459		0.236	1.079

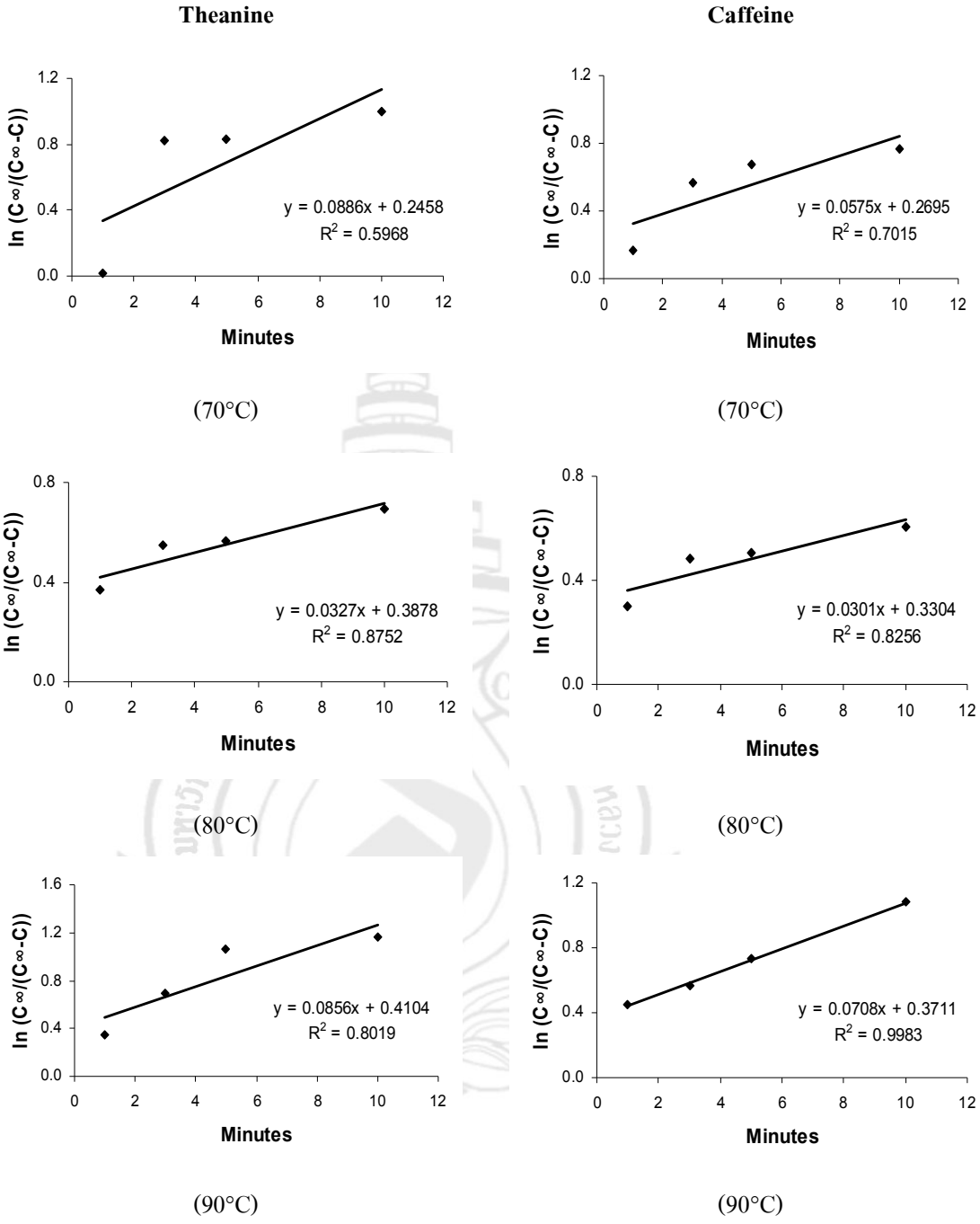

Figure D.3 First order plot for theanine and caffeine extraction from green tea at various time and temperatures

D.4.3 Individual catechins

Table D.6 Calculation of rate constant determination for EGCG and EGC contents

Condition		EGCG				EGC			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
70	1	0.013	0.654	0.641	0.020	0.025	0.525	0.500	0.048
	3	0.332		0.323	0.707	0.084		0.442	0.173
	5	0.407		0.248	0.971	0.357		0.168	1.141
	10	0.477		0.177	1.306	0.418		0.107	1.593
80	1	0.152	0.570	0.419	0.309	0.054	0.508	0.454	0.113
	3	0.269		0.301	0.638	0.163		0.345	0.388
	5	0.279		0.292	0.671	0.273		0.235	0.771
	10	0.358		0.213	0.986	0.334		0.174	1.070
90	1	0.182	0.725	0.536	0.293	0.082	0.741	0.658	0.118
	3	0.146		0.572	0.227	0.350		0.391	0.640
	5	0.607		0.111	1.864	0.575		0.165	1.499
	10	0.642		0.075	2.255	0.606		0.135	1.705

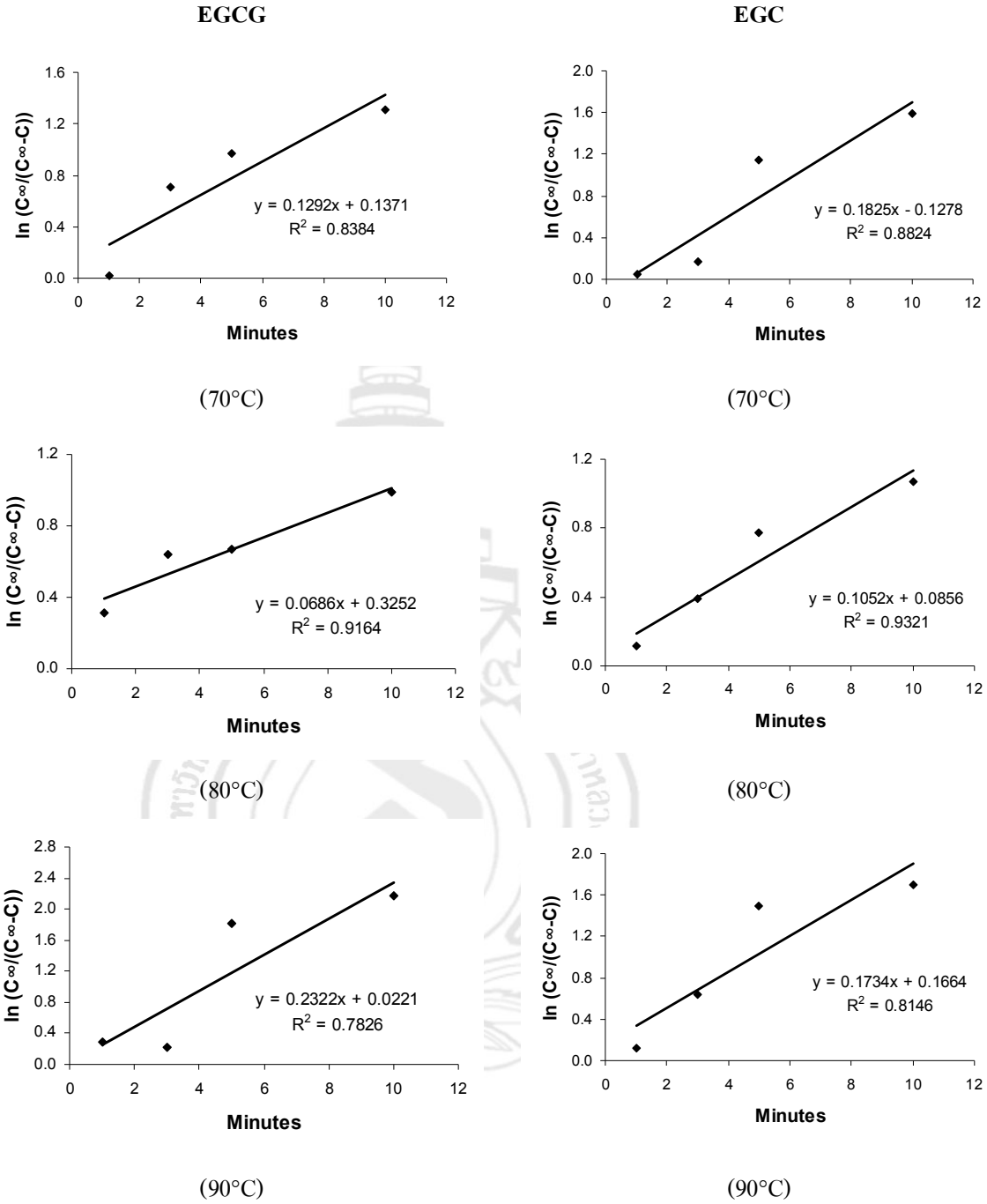

Figure D.4 First order plot for EGCG and EGC extraction from green tea at various time and temperatures

Table D.7 Calculation of rate constant determination for EC and GCG contents

Condition		EC				GCG			
°C	Min	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)	C (% w/w)	C_∞	C - C_∞	ln (C - C_∞)
70	1	0.007	0.735	0.728	0.010	1	0.000	0.043	0.043
	3	0.082		0.653	0.118	3	0.000		0.043
	5	0.434		0.301	0.893	5	0.000		0.043
	10	0.491		0.244	1.102	10	0.021		0.022
80	1	0.459	1.311	0.852	0.431	1	0.000	0.050	0.050
	3	0.520		0.791	0.505	3	0.000		0.050
	5	0.661		0.650	0.701	5	0.000		0.050
	10	0.772		0.539	0.889	10	0.019		0.031
90	1	0.434	1.416	0.982	0.366	1	0.000	0.055	0.055
	3	0.645		0.771	0.608	3	0.000		0.055
	5	1.199		0.217	1.876	5	0.018		0.037
	10	1.275		0.141	2.304	10	0.027		0.029

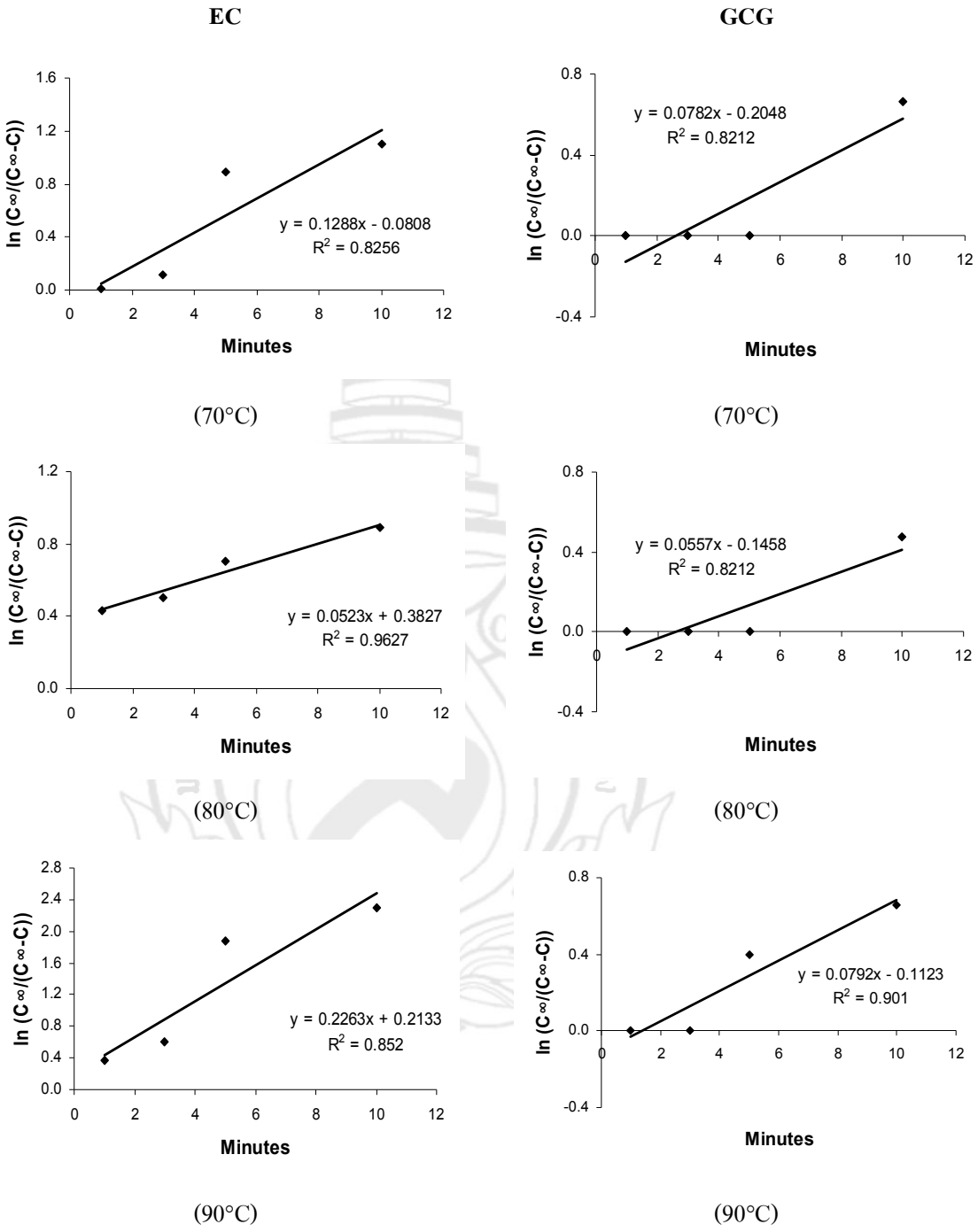

Figure D.5 First order plot for EC and GCG extraction from green tea at various time and temperatures

Table D.8 Calculation of rate constant determination for GC and total catechins contents

Condition		GC				Total catechins			
°C	Min	C (% w/w)	C_∞	$C - C_\infty$	ln ($C - C_\infty$)	C (% w/w)	C_∞	$C - C_\infty$	ln ($C - C_\infty$)
70	1	0.034	0.341	0.308	0.104	0.079	2.262	2.183	0.036
	3	0.148		0.193	0.568	0.645		1.617	0.336
	5	0.195		0.146	0.848	1.393		0.869	0.957
	10	0.246		0.095	1.277	1.653		0.609	1.313
80	1	0.087	0.637	0.549	0.148	0.752	3.077	2.325	0.280
	3	0.179		0.458	0.330	1.131		1.946	0.458
	5	0.190		0.447	0.354	1.402		1.675	0.608
	10	0.263		0.373	0.534	1.746		1.331	0.838
90	1	0.142	0.745	0.603	0.212	0.840	3.660	2.819	0.261
	3	0.170		0.574	0.260	1.311		2.348	0.444
	5	0.342		0.403	0.614	2.741		0.919	1.382
	10	0.384		0.360	0.726	2.934		0.725	1.618

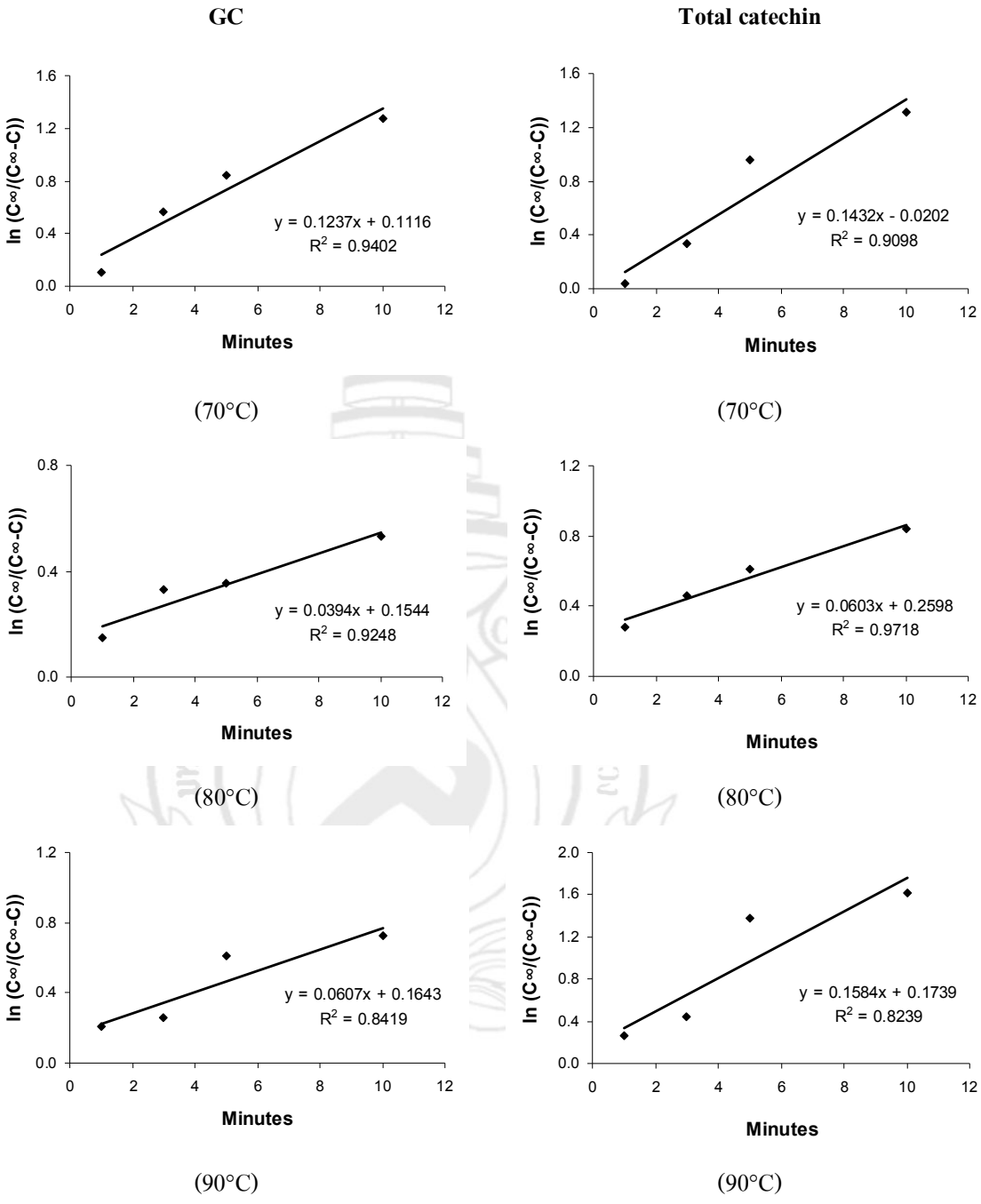

Figure D.6 First order plot for GC and total catechins extraction from green tea at various time and temperatures

APPENDIX E

FRACTIONATION OF GREEN TEA EXTRACT

E.1 Regeneration treatment (Bailey, 2001)

The regeneration treatment was performed to remove alkaloid compounds and other impurities from the resin. After fractionation was accomplished, 3%-5% sodium hydroxide solution was added to a level 10 cm above resin layer in the column and infiltrate for about 4 hours. This was repeated two times in order to make the regeneration treatment more complete. The resin then washed with water until neutral.

Table. E.1 Chemical compositions of fractions from residual green tea extract

Eluting agent	Fraction volume (ml)	ppm								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
Water	55-60	2447.9	396.729	nd	nd	nd	nd	nd	95.752	nd
	95-100	1996.112	396.420	nd	nd	nd	nd	nd	83.912	nd
	230-235	115.310	23.161	nd	nd	nd	nd	nd	nd	nd
50% EtOH	455-460	9.467	1.854	1.257	1.774	nd	1.991	nd	5.815	nd
	510-515	5996.559	602.245	520.104	458.800	57.772	835.569	2.808	1112.574	nd
	655-670	768.935	nd	248.060	nd	69.129	nd	218.657	nd	63.962
	920-925	70.121	nd	9.548	nd	nd	nd	34.496	nd	11.183
75% EtOH	1190-1195	107.529	nd	nd	nd	nd	nd	8.720	nd	1.847
95% EtOH	1610-1615	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1880-1885	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd = not detected

Table. E.2 Chemical compositions of combined fractions from residual green tea extract

Fraction (ml)	Volume (ml)	ppm								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
45-190	145	1096.367	328.369	nd	nd	nd	nd	nd	61.852	nd
190-455	265	72.672	4.582	nd	nd	nd	nd	nd	51.030	nd
455-1550	1095	nd	32.760	34.318	20.952	47.902	11.020	60.999	82.582	38.506

*nd = not detected

Table. E.3 Chemical compositions of fractions from green tea extract

Eluting Agent	Fraction Volume (ml)	ppm								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
Water	0-300	254.826	302.675	nd	nd	nd	nd	nd	93.493	nd
50% EtOH	400-450	17.920	83.592	nd	275.143	36.338	168.065	nd	225.035	nd
	750-800	574.066	nd	237.344	nd	101.432	nd	347.839	nd	108.442
75% EtOH	1150-1200	37.570	nd	8.562	nd	3.198	nd	24.115	nd	8.099
	1400-1450	54.491	nd	0.309	nd	nd	nd	10.088	nd	nd
	1750-1800	133.459	nd	nd	nd	nd	nd	nd	nd	nd

*nd = not detected

Table. E.4 Chemical compositions of combined fractions from green tea extract

Fraction (ml)	Volume (ml)	ppm								
		Theanine	**Caffeine**	**EGCG**	**EGC**	**ECG**	**EC**	**GCG**	**GC**	**CG**
300	300	254.826	302.675	nd	nd	nd	nd	nd	93.493	nd
300-600	300	1653.075	17.442	141.076	323.737	102.918	693.312	98.688	582.178	nd
600-1650	1050	143.956	37.096	12.068	nd	8.658	nd	21.310	38.305	17.259

* nd = not detected

Table. E.5 Chemical compositions of freeze dried of green tea extract combined fractions

Compound	Fraction	% w/w Dry basis of freeze dried fraction				
		(R0)	(R1)	(R2)	(R3)	(R4)
EGCG	I	nd	nd	nd	nd	nd
	II	7.234	6.552	6.469	6.182	6.319
	III	8.176	7.687	4.472	2.826	2.168
	Total	15.410	14.239	10.941	9.008	8.487
EGC	I	nd	nd	nd	nd	nd
	II	8.304	7.411	7.145	3.154	2.990
	III	nd	nd	nd	nd	nd
	Total	8.304	7.411	7.145	3.154	2.990
ECG	I	nd	nd	nd	nd	nd
	II	2.957	2.922	2.841	2.864	2.613
	III	6.123	6.464	4.538	2.969	2.258
	Total	9.080	9.386	7.379	5.815	4.871
EC	I	nd	nd	nd	nd	nd
	II	17.451	15.558	15.542	8.418	8.465
	III	nd	nd	nd	nd	nd
	Total	17.451	15.558	15.542	8.418	8.465
GCG	I	nd	nd	nd	nd	nd
	II	2.408	2.406	2.448	1.987	1.621
	III	13.877	9.871	7.790	6.871	6.425
	Total	16.285	12.277	10.238	8.858	8.046

*nd = not detected

Table. E.5 Chemical compositions of freeze dried of green tea extract combined fractions (cont.)

Compound	Fraction	% w/w Dry Basis of Freeze Dried Fraction				
		(R0)	(R1)	(R2)	(R3)	(R4)
GC	I	nd	nd	nd	nd	nd
	II	13.176	13.122	13.426	9.963	9.728
	III	nd	nd	nd	nd	nd
	Total	13.176	13.122	13.426	9.963	9.728
Theanine	I	12.305	9.895	9.946	8.810	8.079
	II	10.656	10.237	10.533	9.656	9.151
	III	nd	nd	nd	nd	nd
	Total	22.961	20.132	20.479	18.466	17.230
Caffeine	I	5.031	3.939	3.815	3.847	2.561
	II	7.562	7.513	6.536	5.556	6.696
	III	nd	nd	nd	nd	nd
	Total	12.593	11.452	10.351	9.403	9.257

*nd = not detected

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