Effect of Matcha green tea powder in shortbread biscuits on consumer acceptability and acute metabolic response

Benjapor Phongnarisorn

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Matcha green tea powder (MGTP) is made by finely grounding green tea leaves and is increasingly used to flavour food products. The aim of this thesis was to produce a shortbread biscuit product containing MGTP and to evaluate its consumer acceptability and the effect of its consumption on postprandial blood glucose, triglyceride, and satiety responses. In the first instance, the phytochemical composition of the MGTP was determined and compared to green tea extract powder (GTEP), another popular product, which is prepared by solvent extraction. HPLC analysis revealed that MGTP had higher total catechin content and lower caffeine content than GTEP. MGTP was incorporated into shortbread biscuits at 2, 4, 6 g per 100 g of flour. The effect of baking, storage, and the addition of sodium bicarbonate on the catechin stability was evaluated. Percentage of total catechin remaining in biscuits ranged from 81-89% when compared to catechin content in dough. Epimerization of epigallocatechin gallate (EGCG) to gallocatechin gallate (GCG) occurred during baking. Moreover, over one month storage, epigallocatechin (EGC) loss (30-50% loss) was significant and contributed to the significant loss of total catechins. Next, the sensory evaluation of shortbread biscuits formulated with three levels of MGTP (2, 4, 6 g per 100 g of flour) and three levels of sugar (25, 30, 35 g per 100 g of flour) was conducted through acceptability test with a 9-hedonic point scale. The biscuits that contained 2 g of MGTP and 25 g of sugar per 100 g of flour showed the highest acceptability, whereas the biscuit that contained 6 g of MGTP and 25 g of sugar per 100 g of flour received the lowest acceptability. The result indicated that consumers preferred the biscuits with low content of MGTP. Finally, a pilot study investigated the effect of MGTP, either incorporated into shortbread biscuits (6 g per 100 g of flour) or consumed as a drink (3 g dissolved in water) with a plain biscuit, on the postprandial glucose, triglyceride and satiety responses in healthy human subjects. The pilot study revealed that among Asian participants, the triglyceride response after consuming biscuits that incorporated with MGTP was significantly lowered, when compared with plain biscuits and plain biscuit with green tea drink meal. The result shows a possibility that a shortbread biscuits' matrix may enhance the triglyceride lowering effect of MGTP. Moreover, the sensory evaluation result indicated that familiarity of MGTP could increase the acceptability of shortbread biscuits incorporated with MGTP. Hence, this research shows that biscuits with MGTP have a potential to be put in the healthy food market.

Publication

Conference presentations

Phongnarisorn, B., Marshall, L. J., Holmes, M., and Orfila, C. Catechins in biscuits incorporated with Matcha green tea powder. The IFST's 50th Jubilee Conference 2014 poster showcase and competition, 14th -15th May 2014, London, UK. (Won the Bronze Prize for poster competition)

Phongnarisorn, B., Marshall, L. J., Holmes, M., and Orfila, C. Catechins in biscuits incorporated with Matcha green tea powder. Food Science & Nutrition 1st Annual PhD Conference, 24th September 2014, University House, University of Leeds, UK. (Won the second prize for poster competition)

Phongnarisorn, B., Marshall, L. J., Holmes, M., and Orfila, C. Catechins in biscuits incorporated with Matcha green tea powder. The fifth annual University of Leeds Postgraduate Research Conference, 8th December 2014, Parkinson building, University of Leeds, Leeds, UK.

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Table of contents

List of tables

List of figures

List of Abbreviations

CCK = Cholecystokinin

CG = (-)-Catechin gallate

CV = Coefficient of variation

E_a = Activation energy

EC = (-)-Epicatechin

ECG = (-)-Epicatechin gallate

EGC = (-)-Epigallocatechin

EGCG = (-)-Epigallocatechin gallate

ELSD = Evaporative light scattering detector

FRAP = Ferric reducing ability of plasma

GAE = Gallic acid equivalent

GC = (-)-Gallocatechin

GCG = (-)-Gallocatechin gallate

GI =Glycemic index

GLP-1= Glucagon-like peptide-1

GTEP = Green tea extract powder

HPLC = High performance liquid chromatography

iAUC = Incremental area under the curve

MGTP = Matcha green tea powder

PDA = Photo-diode array

PTFE = Polytetrafluoroethylene

PVPP = Polyvinylpolypyrrolidone

RSM = Response surface method

SEM = Standard error of mean

STD = Standard deviation

TE = Trolox equivalent

TPTZ = 2, 4, 6- Tri [2-pyridyl]-5-triazine

VAS = Visual analogue scales

1 Chapter one: General introduction and literature review

1.1 Tea and types of tea

Tea is one of the most commonly consumed beverages in the world. Tea is believed to originate in south China, nowadays, its cultivation has spread to many other countries. The main countries that produce tea are China, India, Japan, Sri Lanka, Indonesia, and Central African countries. According to the area of origin and processing methods, tea can be categorized into more than 300 different types of tea which are all derived from leaves and buds of tea (*Camellia sinensis*). Generally, tea can be classified into 4 major types of tea, white tea, green tea, oolong tea and black tea based on the variation of manufacturing process (Sang *et al.*, 2011; Pettigrew, 2004). The manufacturing process also makes the composition of nutrients in each type of tea differ from each other.

1.1.1 Tea processing

Black tea production accounts for 78% of worldwide tea production. Green tea, which is popular in Asia, especially in China and Japan, accounts for approximately 20% of worldwide production, while, oolong tea production accounts for 2%. (McKay and Blumberg, 2002). The brief process of different tea types is shown in Figure1.1. Black tea is made from tea leaves that have been through full oxidation to give unique flavour, aroma and color to the tea. While oolong is semi-oxidised tea and green tea is non-oxidised tea. During fermentation of black tea production, the oxidation and polymerization of catechins (antioxidant compounds found in tea leaves) occur and theaflavin and thearubigins are produces which contribute to the colour and aroma characteristics of black tea. Green tea production involves a steaming or pan frying process which destroys the polyphenol oxidase enzyme that oxidises the catechins to theaflavins and thearubigins. As green tea does not go through the oxidisation process, the green colour of the green tea leave and amount of catechins remains compared to black tea (McKay and Blumberg, 2002).

The production of white tea and green tea are similar, but white tea is made from newly grown buds and leaves, which are plucked in the early leaf growing stage before they are opened or matured (Lin *et al.*, 2008). Then tea leaves are withered in a controlled temperature room to lower moisture content (Rusak *et al.*, 2008; Pettigrew, 1997). White teas have been found to have higher amounts of caffeine than green tea, however are lower in catechins content than green tea because young buds and leaves have a lower polyphenol content than the matured leaves used in green tea production (Rusak

et al., 2008). Catechins are major polyphenols found in fresh tea leaves. These compounds have been shown to contribute to the health benefits of tea consumption, including the prevention of cancer, diabetes, and heart diseases (Thielecke and Boschmann, 2009; Cabrera *et al.*, 2006; Zaveri, 2006).

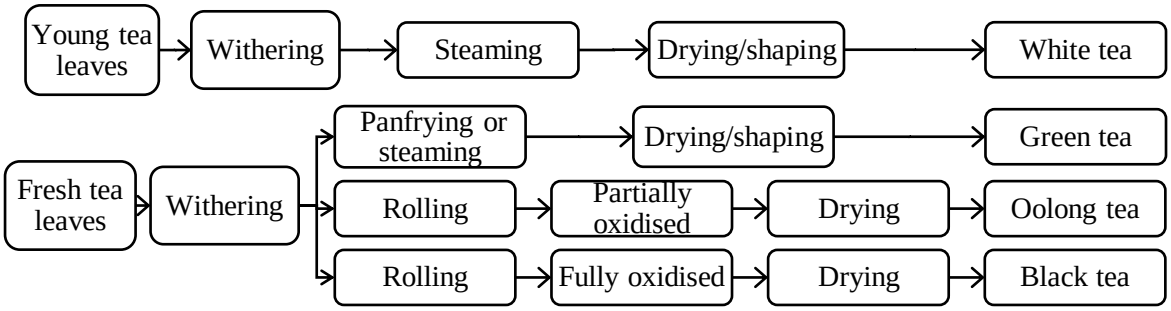


Figure 1.1: Tea processing

1.2 Green tea and its products

There are two plant varieties that are used in green tea production;

- *Camellia Sinensis* var. assamica, has the broad-leaf variety of the tea plant and is cultivated in tree or bush form. This variety is favourable and suitable for black tea production and is mainly grown in countries that have warm and tropical climates such as India, Sri Lanka, Africa and Argentina.
- *Camellia Sinensis* var. sinensis, has the small-leaf variety of the tea plant that is mainly grown in China and Japan which has the colder environment. This variety is often grown as shrubs or dwarf plant form and is commonly used in green tea and white tea production, because it has a sweeter taste than the broad-leaf variety.

In terms of genetics, these two varieties are differed by the matK rbcL nucleotide sequence polymorphism of the chloroplast locus (Stoeckle *et al.*, 2011; Katoh *et al.*, 2003). Nevertheless, both varieties can be used to produce all types of teas including white, green, yellow, red, black and oolong tea.

Optimum cultivating temperature of the tea plant is between 18-20 ^{o}C with 250 - 300 cm annual rainfall (Williges, 2005). Cultivation requires 5 hours of direct sunlight or 11

hours of indirect sunlight and takes place from sea level to 3,000 meters. Higher altitudes are involved with higher tea quality. Soils for cultivation should be well drained, sandy, thoroughly aired, low pH and nutritious with good layers of humus. Drought, water logging, excessive heat and frost have adverse effects on the growth of the tea plant and can lead to lower quality of tea products, regarding taste, aroma and bioactivity. During the first 2 to 4 years of cultivation, tea plants are not matured to harvest. They are often grown in controlled nursery condition. When tea plants are matured, they are transplanted to the field and are ready to harvest. The tender young leaves are harvested to produce most high-quality green tea. The high-quality green teas usually contain terminal buds, internodes and two to three leaves below the bud (Ahmed and Stepp, 2013; Sang *et al.*, 2011).

1.2.1 Origin of green tea production

China was the first to develop the cultivation, processing and preparation of green tea. The development of skilled practice and art of tea processing began to develop in the 8th century by Buddhist monks and spread to the public. Due to calming and stimulating effects of drinking tea, teas were drunk during meditation and were used as a tonic to maintain well-being. Nowadays, there are several types of green tea classified by area of cultivation and processing method. China is the largest producer and consumer of green tea and contributes to approximately 73% of world production (Ahmed and Stepp, 2013).

1.2.2 Chemistry and biochemical changes during green tea processing

Polyphenols and caffeine are secondary metabolites that serve as defence compounds which give plants resistance to pathogens and predators (Ahmed and Stepp, 2013; Ames *et al.*, 1990). Catechins are the major polyphenols in tea leaves that account for the health claims of green tea, involving its antioxidant and anti-inflammatory properties. Caffeine is a methylxanthine alkaloid, which gives the stimulant property to green tea. L-theanine is an amino acid found in green tea and is found to contribute to the relaxing property of tea. The content of catechins, caffeine and amino acids is varied in different green tea types according to environmental growth condition and processing.

Fresh tea leaves have a higher concentration of catechins than dried tea leaves. When the leaves are heated, rolled and dried, the content of catechins decreases because the heat stimulates hydrolysis, polymerization and transformation of catechins. For

instance, at least 15% of polyphenol content is lost from tea leaves due to the chemical changes during processing (Astill *et al.*, 2001). On the other hand, caffeine is relatively stable to heat and does not have substantial reduction during the process. Nevertheless, green tea processing is favourable for stabilising and increasing the shelf-life of catechins because the process deactivates the enzymes that would otherwise oxidise them (Astill *et al.*, 2001). High-temperature hydrolyses proteins to free amino acids which are essential aroma precursors and are transformed into volatile aromatic substances during green tea processing. For example, the L-theanine content was increased during rolling and drying. As a result, there is a higher amino acid content in processed tea leaves than fresh tea leaves (Ahmed and Stepp, 2013). Hence, the ratio of polyphenol to amino acid changes during processing.

The level of soluble carbohydrate is affected by the green tea process. During processing, under high temperature and humidity, the starch molecules in tea leaves are hydrolysed resulting in more soluble sugar. Chlorophyll is the main pigment in fresh and dried tea leaves. The content of chlorophyll in fresh shoots is decreased by half when fresh tea leaves are processed to dried shoots as a result of high temperature and hydrolysis (Xu and Chen, 2002). High altitude environments that have warm days and cool nights enhance the development of aromatic compounds. In addition, a higher application of nitrogen to soil has been shown to increase amino acids in the leaves (Ahmed and Stepp, 2013; Xu and Chen, 2002). Tea plants need to be grown under the shade, which encourages leaf tenderness and more intense green colour of tea leaves.

1.2.3 Matcha green tea powder (MGTP)

Matcha green tea powder (MGTP) is dried, ground green tea leaves which are mainly produced in Japan and China. Green tea powder is produced by pulverising the dried green tea leaves with a grinder, stone, ball or jet mill. To maintain good quality, low-temperature processing (20 °C) is applied during grinding. According to the study by Weiss and Anderton (2003), the amount of catechins extracted from MGTP is 137 times greater than the catechins content obtained from Chinese green tea and three times higher than the amount of other green teas from the largest of values found in the literature. MGTP is produced from shade-grown tea leaves for 20 days before harvesting; the tea bushes are covered to limit sunlight to reduce the rate of photosynthesis in the leaves. The shade-grown period is the important process that

decelerates the growth and changes the leaves to a darker green colour due the increased level of chlorophyll in the leaves. Therefore, the content of catechins in MGTP would be different from catechin levels from other green tea because sunlight influences the composition (Weiss and Anderton, 2003).

The consumption of MGTP is common in Asian countries, especially Japan. The market share of green tea consumption in western countries is more recent and continues to grow. On the other hand, MGTP is a new item on the market. In the UK, it is available via online shopping or in Asian shops. The normal green tea is produced by drying, rolling leaves and packing into bags. The tea bag is usually infused with boiling water and the infused water is consumed. However, Matcha tea is prepared by mixing finely green tea powder with warm water and the powder itself is consumed with the tea. As a result, Matcha tea would provide a greater amount of catechins in comparison with other green tea as the powdered leaves are ingested.

1.2.3.1 Health benefits of MGTP

In Japan, the increase of the consumption of powdered green tea was believed to be influenced by Kuwano *et al.* (1994) who proposed that a daily intake of 6 g of green tea contributed to healthy life. The production of MGTP for food processing in the marketplace is estimated to be approximately 2,500 tonnes per year in Japan (Sawamura *et al.*, 2009).

Green tea contains about 60-70% insoluble components including liposoluble vitamins, water-insoluble dietary fibres, chlorophylls, proteins, and 30-40% of water-soluble components, which are polyphenols, caffeine, amino acids, water-soluble vitamins, minerals and water-soluble dietary fibres. However, green tea is usually consumed as an extract with hot water, and all of the insoluble part is discarded as used tea leaves. The benefits of these insoluble components of green tea can be received by consuming green tea as a powder or mixing with food products as a flavouring agent (Maeda-Yamamoto *et al.*, 2013). Kuwano *et al.* (1994) recommended that consuming 6 g of green tea would provide 22% of vitamin A, 10% of vitamin B_2, 17% of niacin, 5% of magnesium/calcium, and 6% of potassium as a percentage of the daily requirement, and over 2 g of dietary fibres.

The average particle size of MGTP was reported to be about 20 µm (Sawamura *et al.*, 2009; Haraguchi *et al.*, 2003). The small size (less than 20 µm) of green tea powder is

more preferable due the effect of particle size on the mouthfeel and feeling on the throat when it is swallowed (Sawamura *et al.*, 2009; Pongsuwan *et al.*, 2008). A study on the effect of the particle size of green tea powder (called Benifuuki) on the absorption of ingested catechins found that smaller particles of green tea powder contributed to the highest absorption of ester-type catechins (EGCG and ECG) comparing with larger particles of green tea powder (Maeda-Yamamoto *et al.*, 2013). Hasegawa *et al.* (2003) studied the effect of powdered green tea on the lipid metabolism in male Zucker rats fed a 50% sucrose diet containing 15% butter. They found that the administration of 130 mg powdered green tea daily reduced the gain of body weight and various adipose tissue weight without affecting the amount of food intake. The study suggested that powdered green tea could lower the plasma total cholesterol by the inhibition of the synthesis of cholesterol in the liver (Hasegawa *et al.*, 2003).

1.2.3.2 Use of MGTP in food products

Typically, green tea powder is consumed by mixing in hot water. In Japan, green tea powder is mixed with foods such as mayonnaise, tartar sauce, potato salad, rice cake, tempura, cake, bread, hamburger steak and meatballs. Also, green tea is commonly used as a flavouring agent in bakery products. An example of its use in food is in a Korean traditional snack called Yukwa, which is sweet puffed waxy rice. Shen *et al.* (2014) conducted a study to investigate the effects of the addition of green tea powder on the quality and antioxidant properties of vacuum-puffed and deep-fried Yukwa. The study compared the loss of catechins and caffeine when Yukwa was either deep fried or vacuum puffed and found that the detectable amount of total catechins and caffeine were significantly higher in vacuum-puffed Yukwa than the deep-fried one. Also, the deep fried Yukwa with addition of 2 g per 100 g of waxy rice powder was more preferred than the Yukwa with no green tea powder and the most preferred in sensory evaluation test (Shen *et al.*, 2014). Therefore, MGTP may be an ingredient that provides new flavour to food products, improves shelf life and gives a healthier option to consumers. Nevertheless, there are a limited number of scientific studies on the stability of tea catechins during processing and storage, their interaction with other components in food matrix and the effect on the quality of the products.

1.2.4 Green tea extract powder (GTEP)

The green extract is made from concentrated green tea infusion by spraying drying to produce the powder to cover the application in nutraceutical supplement and food manufacturing.

1.2.4.1 Processing of tea extracts

There are 3 stages of tea extract processing as follows (Wang *et al.*, 2003a):

I. Strong infusion is produced by soaking green tea leaves in a solution of alcohol and water. The catechin content is approximately 2% w/v.
II. The strong infusion is further concentrated to a water content of 20-25% to produce a soft extract, which contains about 20% catechin.
III. Dry extract or powder is obtained by spray drying the strong infusions after it has been concentrated to 40-50% solid content. The extract is usually processed as a powder to become suitable for various uses (tablets, capsules, dry mixes)

The purified extract has higher contents of catechins due to further purification processes, for instance solvent extraction or column chromatography techniques. Membrane extraction is a new technique to separate catechins in order to obtain a higher content of catechins in the extract. A low-speed counter current chromatographic technique was also introduced to purify EGCG in order to produce tea extracts with higher content of EGCG.

1.2.4.2 Application of green tea extract

GTEP is commonly used in supplements and is advertised as effective for weight loss. Natural antioxidants, such as green tea extract powder, are also a great interest in food manufacturing. Synthetic antioxidants, for example, butylated hydroxyanisole and butylated hydroxytoluene are normally used to control oxidation and retain food quality. However, these synthetic antioxidants are found to be carcinogenic, and they are now restricted in food. Several studies show the application of green tea extract as a potential antioxidant in food products. For example, the addition of green tea extract into mooncakes, a traditional cake consumed during the Chinese Middle Autumn Festival, could improve the flavour and increase shelf life. Incorporation of tea extract in food products was also found to protect foods against microbial contamination (Wang *et al.*, 2003a).

1.3 Dietary polyphenols

Polyphenols are a group of organic compounds which are synthesized by plants as part of their defense mechanism against reactive oxygen species, which are induced by environmental and biological factors such as ultraviolet radiation or microbial infection (Tsao, 2010). Polyphenols have a strong antioxidant property, derived from the phenolic structure. Most polyphenols in nature are found as glycosides, which mean they are linked to a sugar moiety at different positions of the molecule. Polyphenols are categorised into subgroups, according to the chemical features of their aglycones, which are phenolic acids, flavonoids, tannins, stilbenes and lignans (Manach *et al.*, 2004). Phenolic acids are the mono-phenolic compounds that are classified into two main groups; benzoic acids and their derivatives, and cinnamic acids and their derivatives. The basic structure of each polyphenol sub-group is presented in Figure 1.2. Flavonoids are polyphenolic compounds that are found universally in plants. As shown in Figure 1.2, flavonoids have a C6-C3-C6 structure and can be classified into seven subclasses: flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanidins, and isoflavones (Manach *et al.*, 2004). Most flavonoids present in nature are linked with the sugar moiety via the β-glycosidic bond. However, flavanol is the only class of flavonoid that exists in a non-glycosylated form and is the main flavonoid found in green tea (Spencer, 2003).

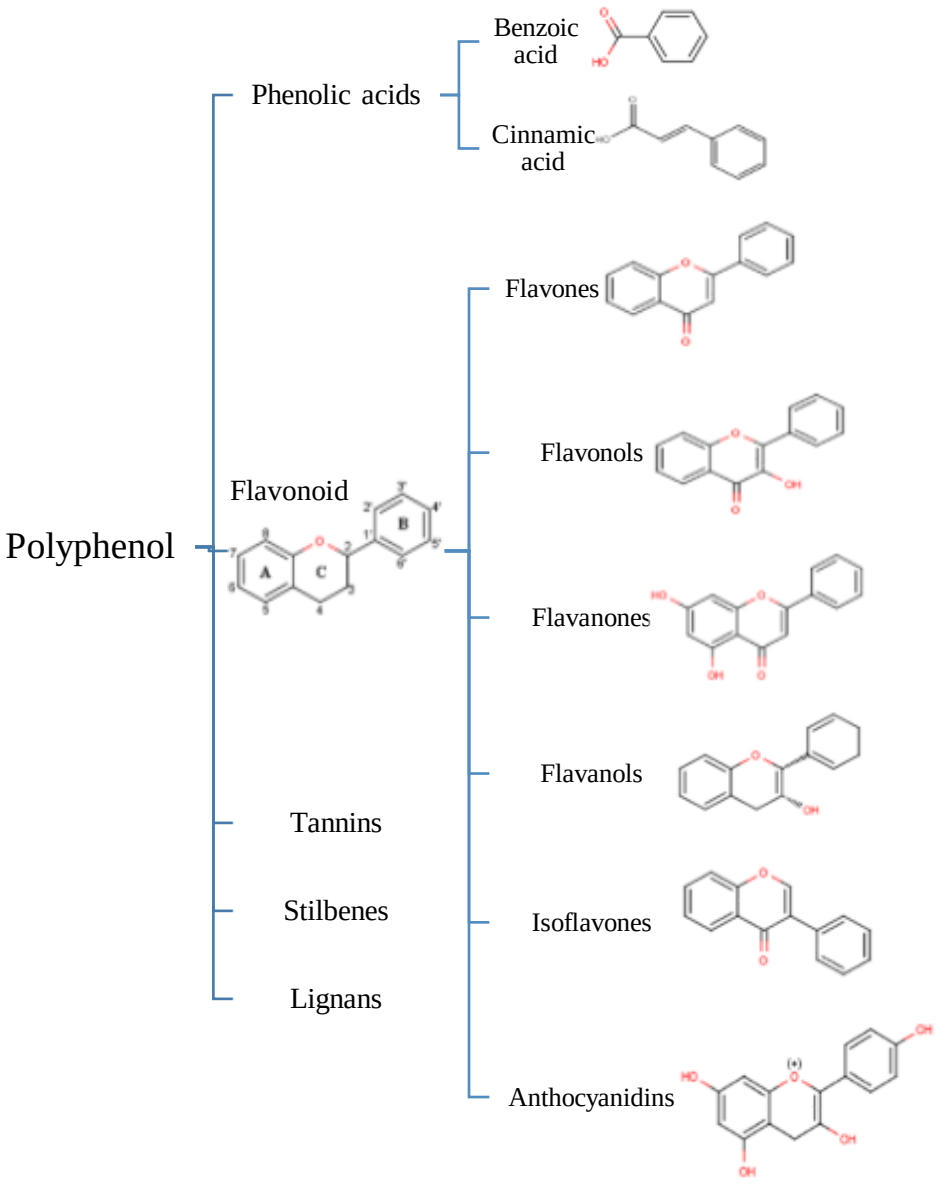
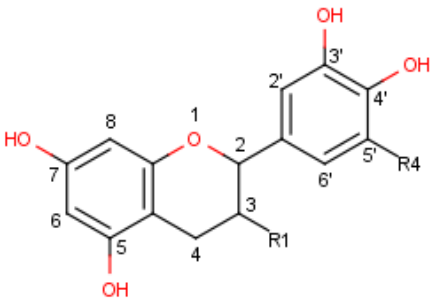


Figure 1.2: Classification of polyphenols adapted from Tsao (2010)

1.3.1 Flavanols (catechins) and their structures

Flavanols or catechins can be found in teas, wine and chocolate. The different types of catechin arise from changes in hydroxylation pattern of the B-ring at position 5' and the presence of gallic acid in position 3 (as shown in Figure 1.3). Gallic acid is a trihydroxybenzoic acid, a type of mono-phenolic acid which is often found in gallnuts, sumac, witch hazel, tea leaves and oak bark. Catechins (the main polyphenols found in green tea) are colourless and water soluble, which contribute to bitterness and

astringency of green tea. These are compounds that account for the health claims of green tea, involving its antioxidant and anti-inflammatory properties. The main catechin found in green tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) (Williamson *et al.*, 2011).



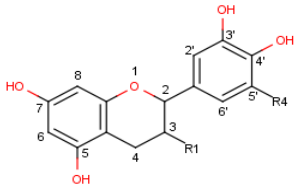
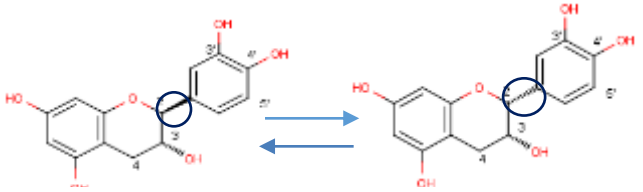
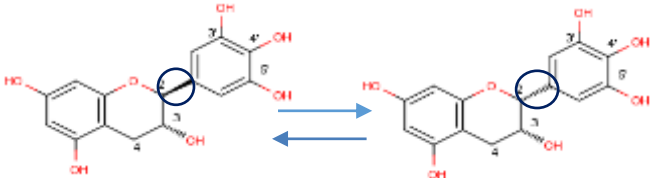
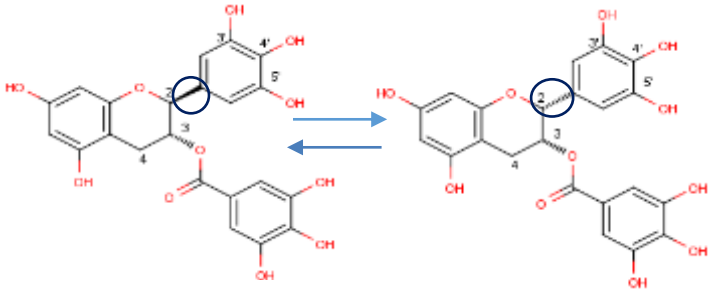
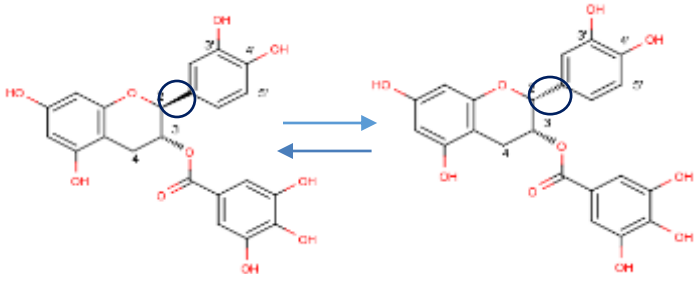
	R_1	R_4
Catechin	OH	H
Epicatechin (EC)	OH	H
Epigallocatechin (EGC)	OH	OH
Epicatechin gallate (ECG)	Gallate	H
Epigallocatechin gallate (EGCG)	Gallate	OH

Figure 1.3: Structure of Flavanol (Spencer et al, 2009), the structure was drawn by MarvinSketch progarmme (version 17.1)

EGC, EC, EGCG and ECG are catechins, which exist in the epimer form (as shown in Table 1.1). Their non-epimer forms are detected in a minor amount which are (+)-catechin, (+)-gallocatechin gallate (GCG), (+)-gallocatechin (GC) and (+)-catechin gallate (CG). The chemical difference in structure between epi-form and nonepi-form is the cis form (2R, 3R or 2, 3-cis, epi-form) and trans form (2S, 3R or 2, 3-trans, non epi-form) of catechins. The catechins in a 'cis' form can transform into their non-epimer forms. The transformation between pair catechins is called epimerization, and the reaction is reversible as shown in Table 1.1 (Wang *et al.*, 2008b).

Approximately 8-30% of total catechins are found in dried green tea leaves, and 29-80% are found in green tea extract (Amber *et al.*, 2010). Among all catechins found in tea, EGCG is the most abundant and only exists in tea. Hence, it has been used as a quality indicator of green tea products (Pelillo *et al.*, 2002).

Table 1.1: Structure of catechins; in non-epimer and epimer form

Basic catechins	
(+)-Catechin and **(-)- Epicatechin**	 (+)-Catechin (-)-Epicatechin
(+)-Gallocatechin and **(-)- Epigallocatechin**	 (+)-Gallocatechin (-)-Epigallocatechin
(+)-Gallocatechin gallate and **(-)- Epigallocatechin gallate**	 (+)-Gallocatechin gallate (-)-Epigallocatechin gallate
(+)-Catechin gallate and **(-)- Epicatechin gallate**	 (+)-Catechin gallate (-)-Epicatechin gallate

1.4 Health benefits of green tea catechins

Nowadays, tea beverages are widely consumed and have become the second most popular drink, only next to water in the world. Several scientific studies have reported that green tea polyphenols are associated with health benefits. They are reported to have antioxidant, antibacterial, anticarcinogenic, antiatherosclerotic and antidiabetic properties (Phung *et al.*, 2010; Thielecke and Boschmann, 2009; Cabrera *et al.*, 2006). Many studies found they could prevent oxidative DNA damage, lower the atherosclerotic index, and improve blood flow, liver function and oral function (Phung *et al.*, 2010; Thielecke and Boschmann, 2009). Epidemiological studies found that daily consumption of green tea promotes healthiness and long life (Thielecke and Boschmann, 2009; Cabrera *et al.*, 2006). Effect of green tea consumption is associated with the prevention of cancer (Kavanagh *et al.*, 2001) and cardiovascular disease (Sueoka *et al.*, 2001), and has also shown an effect on energy expenditure. For example, Komatsu (2003) found an increase in energy expenditure after a single administration of green tea (293 mg catechins; of which 156 mg was EGCG and 161 mg was caffeine) in humans. The cumulative increase in energy expenditure of 49.5±0.4 kJ per 2 hours was higher than the control that showed a cumulative increase of 11.2 ±1.1 kJ per 2 hours (Komatsu *et al.*, 2003). Therefore, green tea may be one of the solutions in preventing metabolic syndrome.

1.4.1 Bioavailability of polyphenols

Bioavailability is defined by the level of an ingested dose that is absorbed into the systemic circulation that reaches a specific site. The bioavailability of polyphenols is affected by biological and physiological factors in the gastrointestinal tract, such as pH, the digestive enzymes, and the microflora (D'Archivio *et al.*, 2010; Manach *et al.*, 2004). Extensive biotransformation and conjugation of polyphenols takes place after their absorption in the gastrointestinal tract and in the liver (Williamson and Clifford, 2010).

Most dietary polyphenols exist as glycoside conjugates except the flavanols. The absorption of some ingested polyphenols, especially flavanols, takes place in the small intestine. However, other polyphenols, which exist as glycoside forms need to be deglycosylated in order to be absorbed. There are two enzymes in the small intestine that can perform the polyphenol deglycosylation, which are lactase phloridizin

hydrolase (LPH) and cytosolic β-glucosidase (CBG). LPH is present in the small intestinal brush-border membrane and reacts on various substrates specifically for flavonoid-*O*-β-D-glucoside. The released aglycones are absorbed into the epithelial cells by passive diffusion (Day *et al.*, 2000). CBG is located within the epithelial cells, so the substrates are required to be transported into the cells prior to hydrolysis. The transporter that is involved is active sodium-dependent glucose transporter SGLT1 (Gee *et al.*, 2000). Therefore, the two possible pathways that allow the aglycones to enter the epithelial cells are known as "'LPH/diffusion' and 'transport/CBG' "(Crozier *et al.*, 2009).

Before entering the circulation, aglycones and microbial metabolites undergo conjugations, including methylation, sulfation or glucuronidation, which occur in small intestine and mainly in the liver, which is the main detoxifying organ. There are two stages of detoxification in the liver, called phase I and phase II (Grant, 1991). Phase I involves oxidation, reduction, or hydrolysis in order to functionalise a compound to be further transformed in phase II metabolism. Phase II involves adding polar hydrophilic molecules to the absorbed metabolites in the intestine or to molecules that have been transformed in the phase I metabolism in the liver. The purpose of the phase II metabolism is to change the metabolites (xenobiotics) to more water-soluble forms for excretion and elimination. Phase II activity can occur in the gut, whereas phase I activity mostly occurs in the liver. The reactions associated with phase II metabolism are glucuronidation, sulfation, and methylation. When polyphenols are absorbed in the small intestine, they mainly exist as conjugated forms with glucuronide, sulfate, or methyl group (Scalbert and Williamson, 2000).

The polyphenols that escape absorption in the small intestine reach the colon where they are deglycosylated and transformed by the microflora action. Colonic microflora cleaves the glycosides to aglycones that undergo ring fission resulting in production of phenolic acids and hydroxycinnamates. In the colon, the increase in the percentage of metabolites arises because phase 2 metabolites enter the colon either from enterocytes or with the bile (Crozier *et al.*, 2009). The excreted phase 2 metabolites and unabsorbed polyphenols, which are transformed by the gut micro flora, then the microbial catabolites are absorbed. Another study found that a greater proportion of dietary polyphenols (procyandin B2) is absorbed from the colon as microbial catabolites rather than from the small intestine (Renouf *et al.*, 2010). The colon contains the highest

concentration of bacteria in human body, which is about 10^{11}-10^{12} bacterial cells per gram of luminal content and these bacteria are mainly anaerobes which are able to hydrolyse and de-conjugate the moiety by their enzymes such as glycosidase (Selma *et al.*, 2009; Salminen *et al.*, 1998). Therefore, the colonic microflora plays an important role in absorption of polyphenols and may influence the biological activity of dietary polyphenols.

The bioavailability of polyphenols is not only influenced by the amount absorbed in the intestine and the conjugation with methyl, sulfate, or glucuronide group, but also by the microbial metabolism. The reactions determine the final form of polyphenols that contribute to the potential active compounds that benefits health.

1.4.1.1 Catechins and their absorption, metabolism and bioavailability

The main absorption site of catechins is in the intestines where some catechins are transformed to conjugated forms before reaching the blood stream. However, catechin and ECG are not present in the blood, whereas EGCG, EGC and EC are found in plasma as free aglycone or as conjugates with glucuronide, sulfate or methyl groups. The free form of EGCG accounts for 90% of the total EGCG absorbed, while the remaining catechins are largely sulfated conjugates rather than in the glucoronidated form (Chow *et al.*, 2003; Lee *et al.*, 2002; Lee *et al.*, 1995). In addition, both EGC and EC are mainly present as conjugates in blood. The peak plasma concentrations of EC and EGC conjugates occurs between 1.6-2.3 hours after green tea consumption as shown in Figure 1.5, the catechin conjugates appear in early T_{max} (time to reach the maximum concentration) suggesting that their absorption occurs in the small intestine (Stalmach *et al.*, 2010).

A review on the weighted average data from seven studies of green tea beverage consumption shows that EC is more highly absorbed than EGC. Moreover, EGCG is less absorbed than EC and EGC (Williamson *et al.*, 2011). Another study by Auger *et al.* (2008) also supported the finding that ECs have greater bioavailability in comparison with other flavonoids because it is more readily absorbed and excreted (Auger *et al.*, 2008).

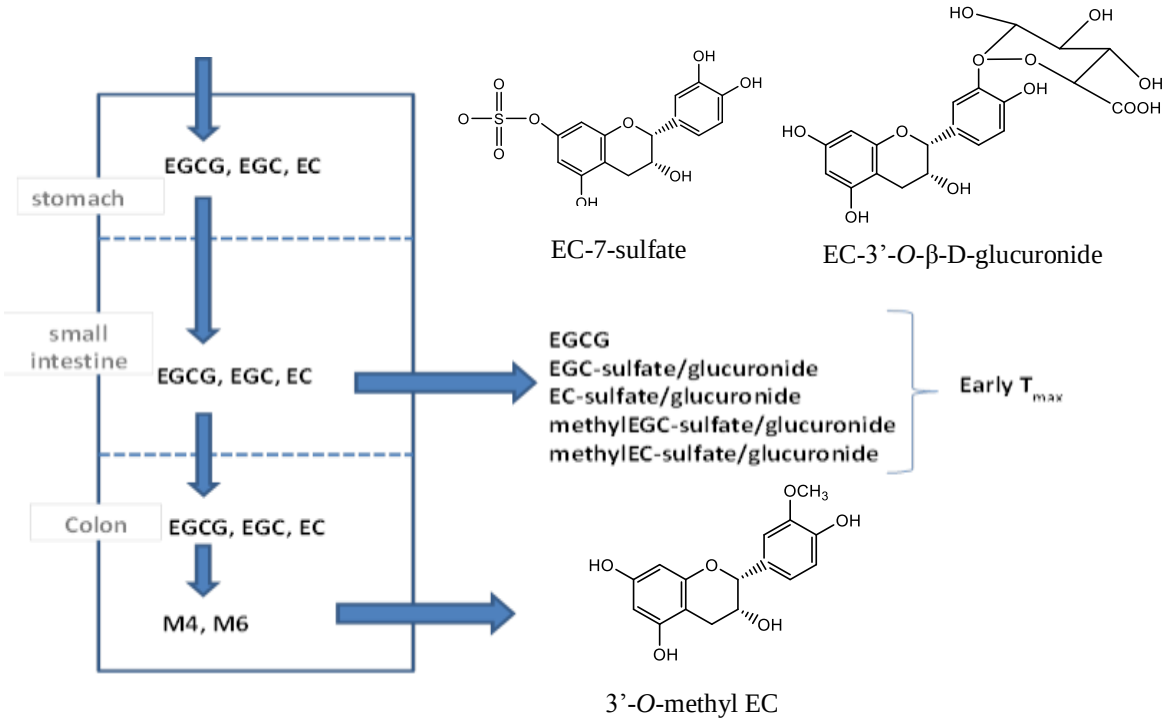


Figure 1.4: Absorption and metabolism of catechins (Williamson *et al.*, 2011)

The amount of catechin derivatives in urine can indicate the minimum amount of a compound that is absorbed (Williamson *et al.*, 2011). Following green tea consumption, low concentrations of free EGCG are found in the urine, whereas EGC and EC are presented as mono-glucuronide, monosulfate conjugates and methylated forms in the urine (Li *et al.*, 2000). The pharmacokinetic data of green tea polyphenol studies in healthy and ileostomist volunteers show similar data. Thus this supports the suggestion that the main absorption sites of catechins are in the small intestine. Approximately 4% bioavailability of consumed catechins from green tea, was revealed from small intestinal and hepatic metabolites that were quantified in 24 hours urine sample (Del Rio *et al.*, 2010). Another study showed further microbial metabolism of catechins in

the colon (Stalmach *et al.*, 2010). Majority of the parent catechins escape the absorption in the small intestine and enter the colon. Some metabolites return from the small intestine and enter into the lumen. The unabsorbed catechins and returned metabolites are hydrolysed and de-conjugated by microbial enzymes, then these aglycones are catabolized to phenylvalerolactones which can be found in the urine (Feng, 2006; Meng *et al.*, 2001; Meselhy *et al.*, 1997). Colonic catechin derivatives are (−)-5-(3,4,5-trihydroxyphenyl)-γ-valerolactone (M4) and (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (M6) which are found in the urine after green tea ingestion (Meng *et al.*, 2001; Li *et al.*, 2000). Microbial metabolism of EGC or EGCG provides M4 which is only found in the urine, while M6 comes from microbial metabolism of mostly EC and it is found in both the plasma and the urine (Lee *et al.*, 2002). The derived compounds from the colon may play a major role in the bioactivity of catechins, which confer an advantage to human health.

Base on Del Rio *et al.* (2010) study, micro-valerolactone metabolites were excreted, account for 36% of intake. The substantial amount of consumed catechins reaches the large intestine, where they are metabolized by microbial enzymes. Therefore, the unabsorbed catechin present in the small intestine may have an acute effect on digestion of carbohydrate and fat. When green tea is incorporated in food or consumed together with a meal, the acute effect of catechins on carbohydrate and lipid metabolism should be studied to monitor the response, as most ingested catechins are unabsorbed in the small intestine.

1.4.2 The effect of green tea and catechins on the risk of type 2 diabetes

Type 2 diabetes is caused by insulin deficiency or impaired effectiveness of insulin, which leads to hyperglycaemia and glucose intolerance. The prevalence of diabetes is increasing rapidly and type 2 diabetes has become a serious problem in most developed countries. It is predicted that by 2025, the prevalence of diabetes worldwide will increase to 333 million persons who account for 6.3% of the global population (Boyle *et al.*, 2001). There are positive relationships between green tea consumption and the decrease in risk of type 2 diabetes (Stote and Baer, 2008; Cabrera *et al.*, 2006; Greenberg *et al.*, 2006).

1.4.2.1 Epidemiology studies

Epidemiological studies have found that the consumption of green tea has a protective effect on the risk of developing type 2 diabetes, cardiovascular diseases and cancers (Stote and Baer, 2008). In the retrospective cohort study among Japanese adults, there was evidence that the consumption of green tea (more than 6 cups per day or 1440 ml per day) saw a 33% reduction in the risk of diabetes, where there was no relationship between the consumption of oolong tea or black teas with diabetes risk. More than 4 cups of green tea consumption (960 ml per day) saw a 30% reduced risk of developing type 2 diabetes in comparison with no tea consumption (Stote and Baer, 2008; Iso *et al.*, 2006).

1.4.2.2 Human and animal studies

Tsuneki *et al.* (2004) showed evidence of antidiabetic and antihyperglycemic effects of green tea consumption in diabetic mice and healthy humans. In the human study, they used the oral glucose tolerance test to investigate the acute change in blood glucose induced by 150 ml of green tea ingestion, which contained 108 mg of catechins. The average blood glucose levels of green tea administered samples at every time-point measured were lower than the mean blood glucose levels of control water samples. This result showed the acute antihyperglycemic effect of green tea after ingestion and indicated that green tea enhances glucose metabolism in healthy humans. The experiment in diabetic mice and normal mice showed that administration of green tea had a significant effect in lowering blood glucose in the diabetic mice compared to normal mice. The author suggested that several serum proteins might be affected by green tea ingestion, especially 4211 Da protein that was significantly reduced. This protein has a modulating effect and may be one of the underlying mechanisms involved in anti-diabetic and anti-hyperglycemic effects of green tea (Tsuneki *et al.*, 2004). In addition, Zhong *et al.* (2006) conducted a randomised, cross-over study on 20 healthy subjects, comparing the acute effects of tea extract (containing 300 mg EGCG) and placebo on carbohydrate absorption. The study found a 25% decrease in carbohydrate absorption after the single administration of green tea extract with a carbohydrate meal (Zhong *et al.*, 2006). Nevertheless, another study following four weeks consumption of green tea found no association between fasting glucose, insulin concentration and green tea consumption (Stote and Baer, 2008; Ryu *et al.*, 2006). The authors raised the point

of biomarker evaluation at fasting state after tea consumption and that the catechins are quickly metabolised. For instance, green tea catechins appear in plasma and peak within 1.5-2 hours and decline to baseline within a few hours. Measuring the effect of tea consumption after 12 hours may give inconsistent results (Stote and Baer, 2008). Hence, the acute effect of green tea should be investigated due to the bioavailability of green tea after consumption. The summary of human studies on the acute effect of green tea on carbohydrate absorption and metabolism is shown in Table 1.2.

Table 1.2: Summary of human studies on the acute effect of green tea on glucose response

Authors	Type of study and # of subjects	Methods	Reference and test meal	Outcomes
Tsuneki *et al.* (2004)	• A placebo-controlled crossover study • 22 healthy participants	• An oral glucose tolerance test before and after supplementation over 2 hours	**Reference meal:** 150 mL of hot water **Test meal:** 150 mL of green tea drink consisted of 1.5 of green tea powder with 108 mg of total catechins	✓ Study shows that glucose tolerance was improved with tea administration compared with hot water administration
Zhong *et al.* (2006)	• A randomised, placebo-controlled crossover study • 20 healthy participants	• Breath-hydrogen and $^{13}CO_2$ concentrations were assessed hourly for 8 hours	**Reference meal:** A meal contained 50 g carbohydrate as white rice, 10 g butter, and 0.2 g [^{13}C]triolein, and the beverages contained 10 g sucrose **Test meal:** The same meal with a beverage containing an extract of black (0.1 g), green (0.1 g), and mulberry (1.0 g) teas contained 100 mg of ECG, 300 mg of EGCG, and 100 mg theaflavin.	✓ With the carbohydrate-containing meal, the tea extract resulted in a highly significant increase in breath-hydrogen concentrations, which indicated significant carbohydrate malabsorption ✓ Tea extract induced malabsorption of 25% of the carbohydrate
Venables *et al.* (2008)	• A placebo-controlled crossover study • 11 healthy men	• An oral glucose tolerance test before and after supplementation	In 24 hour prior the experimental session, subjects consumed three capsules containing either corn flour as placebo or green tea extract with 890 mg of polyphenol including 366 mg of EGCG	✓ Green tea extract trial decreased the serum insulin concentration ✓ Acute green tea extract ingestion could improve insulin sensitivity and glucose tolerance in healthy young men

Continuation from Table 1.2

Authors	Type of study and # of subjects	Methods	Reference and test meal	Outcomes
Josic *et al.* (2010)	• A randomised, placebo-controlled crossover study • 14 healthy participants	• Glucose and insulin concentrations were measured at each time interval after a meal consumption	**Reference meal:** White bread and sliced turkey with 300 mL of water **Test meal:** White bread and sliced turkey with 300 mL of green tea (consisted of catechins, EC 25.5 mg, ECG 89.7 mg, EGC 3 mg and EGCG 32.4 mg)	✓ The study did not observe a reduction in glucose or insulin levels in participants who consumed 300 mL of green tea with a meal compared with control meal ✓ Increase of satiety and fullness were reported by participants after the meal consumption with green tea
Lochocka *et al.* (2015)	• A randomised, placebo-controlled crossover study • 28 healthy participants	• A $^{13}CO_2$ starch breath test was used • Starch digestion and absorption based on cumulative percentage ^{13}C dose recovery were measured by breath samples that were collected after a meal at time interval over 4 hours	**Reference meal:** ^{13}C-abundant cornflakes (50 g cornflakes with 100 mL low- fat milk) and an empty starch wafer (placebo control) **Test meal:** The same meal with a wafer that enclosed with 4 g of GTEP contained 257.6 mg of EGCG	✓ A single dose of green tea extract taken with a test meal decreased starch digestion and absorption ✓ The cumulative percentage dose recovery was significantly lower for the GTEP test than control test

1.4.2.3 The suggested mechanism of green tea and catechins on carbohydrate metabolism

The potential mechanisms of green tea, which may affect glucose metabolism and lowers the risk of diabetes are listed below.

1. Green tea can inhibit enzymes which digest carbohydrate, which lowers glucose production in the intestine, so the concentration of glucose and insulin in blood circulation is decreased (Coe *et al.*, 2013; Forester *et al.*, 2012; Yilmazer-Musa *et al.*, 2012; Liu *et al.*, 2011; Koh *et al.*, 2010; He *et al.*, 2007; Kobayashi *et al.*, 2000; Shimizu *et al.*, 2000). For example, Goh *et al.* (2015) found that bread fortified with 2% GTEP showed a significantly lower level of glucose release during the first 90 min of pancreatic enzyme digestion and a lower content of rapidly digested starch (RDS) content. The study suggested that GTEP fortified to bread may reduce the glycemic potential (glucose response) of bread (Goh *et al.*, 2015). Goh *et al.* (2015) proposed that the formation of catechins - α-amylase complex could be the potential mechanism of the enzyme inhibition, although the suppressing enzyme mechanism is not clearly elucidated yet. Some studies suggested the major interaction between catechins and the enzyme is hydrogen bonding or hydrophobic interaction (Goh *et al.*, 2015; Bandyopadhyay *et al.*, 2012; Siebert *et al.*, 1996), while others studies suggested the hydroxyl group on the 3-position or 5-position of A–C rings as well as the number of hydroxyl groups on the B-rings of catechins played a major role in the inhibition of enzyme activity (Miao *et al.*, 2014; Wang *et al.*, 2010). Forester *et al.* (2012) found that EGCG non-competitively inhibited pancreatic amylase activity by 34%. This evidence suggested that EGCG, which has more hydroxyl groups than other catechin, was the most active component in green tea that bind and precipitate protein, resulting in the enzyme inhibitory effect.
2. EGCG and ECG increase insulin sensitivity by promoting glucose uptake in adipocytes (Wu *et al.*, 2004a; Anderson and Polansky, 2002). For example, a study on the acute effects of green tea on blood glucose levels suggested that the promotion of insulin action in peripheral tissues, such as skeletal muscles and adipocytes via modification of serum protein from green tea administration (Tsuneki *et al.*, 2004).

1.4.3 The effect of green tea and catechins on risk of cardiovascular disease biomarkers

Serafini *et al*. (2000) found that after 30 minutes of a single dose of green tea (300 ml, containing 6 g green tea), the total antioxidant capacity values in plasma increased significantly compared with a control (300 ml water) ($P < 0.05$). Green tea showed the efficiency in protecting low-density lipoprotein from oxidation driven by peroxyl and ferril radicals, respectively (Serafini *et al.*, 2000). Due to green tea's health benefits in preventing the risk of cardiovascular diseases, human studies on the acute effect of green tea on triglycerides absorption and metabolisms were assessed to find the mechanisms of green tea on reducing the risk (Table 1.3).

Table 1.3: Human studies assessing the acute effect of green on triglyceride response

Authors	Type of study and # of subjects	Methods	Reference and test meal	Outcomes
Unno *et al.* (2005)	• A placebo-controlled crossover study • 9 male subjects with mild or borderline hypertriacylglycerolaemia	• Triglyceride concentrations were measured in each time interval after a meal consumption	**Reference meal**: A slice of bread with 20 g of butter with 10 mg of tea catechins (control) **Test meal:** A slice of bread with 20 g of butter with 224 mg of tea catechins (moderate dose) and 674 mg of tea catechins (high dose)	✓ The study showed that moderate and high doses of catechins reduced the iAUC of plasma triglyceride by 15.1% and 28.7% respectively compared with the control ✓ Tea catechins attenuated the postprandial increase in plasma triglyceride level after fat intake
Zhong *et al.* (2006)	• A randomised, placebo-controlled crossover study • 20 healthy participants	• Breath-hydrogen and $^{13}CO_2$ concentrations were assessed hourly for 8 hours	**Reference meal:** A meal contained 50 g carbohydrate as white rice, 10 g butter, and 0.2 g [^{13}C]triolein, and the beverages contained 10 g sucrose **Test meals:** The mentioned meals with a beverage containing an extract of black (0.1 g), green (0.1 g), and mulberry (1.0 g) teas	✓ The tea extract did not cause triglyceride malabsorption
Walkowiak *et al.* (2013)	• A randomised, placebo-controlled crossover study • 32 healthy participants	• A ^{13}C-labelled mixed triglyceride breath test was used. • Lipid digestion and absorption based on cumulative percentage ^{13}C dose recovery were measured by breathing samples that were collected after a meal at a time interval	**Reference meal**: A slice of bread with 12.5 g of butter **Test meal**: A piece of bread with 12.5 g of butter with green tea extract containing 257.6 mg of tea catechins	✓ A single dose of green tea extract consumed with a test meal decreased lipid digestion and absorption in humans

1.4.3.1 The suggested mechanism of green tea and catechins on fat/triglyceride metabolism

Due to poor absorption of parent catechins and their presence in the intestinal lumen as discussed in section 1.4.1.1, the lipid-lowering effects of green tea and catechins is likely to be mediated mainly via their influence on the intestinal process associated with digestion and absorption of dietary lipid (Koo and Noh, 2007; Ikeda *et al.*, 2005). Several *in vitro* studies indicate that green tea catechins, particularly EGCG, interfere with the emulsification, digestion, and micellar solubilization of lipids which are the essential steps involved in the intestinal absorption of dietary fat, cholesterol, and other lipids (Koo and Noh, 2007). The potential mechanisms of green tea, which may affect fat metabolism and lower triglycerides, are listed below (Koo and Noh, 2007).

1. Green tea and catechins inhibit intestinal lipid absorption, for example, Ikeda *et al.* (1992) found that mixtures of catechins extracted from Japanese green tea lowered the absorption of cholesterol and triglycerides in rats. The author suggested that the gallate esters of green tea catechins had more potential to inhibit cholesterol absorption because they observed that a mixture of EGCG and ECG was more effective than a mixture of EC and EGC in lowering the absorption of cholesterol. Later, Ikeda *et al.* (2003) observed that tea that contained high in GCG and CG were more effective in inhibiting cholesterol absorption than a catechin mixture high in EGCG and ECG.
2. Green tea and catechins inhibit luminal lipid hydrolysis (Thielecke and Boschmann, 2009). Catechins have been found to inhibit gastric and pancreatic lipase activities (Wang *et al.*, 2006b; Ikeda *et al.*, 2005) by preventing the emulsification of fat in the presence of bile acids (Juhel *et al.*, 2000) or by interacting with the emulsion formed, increasing the emulsion droplet size (Shishikura *et al.*, 2006).

1.4.4 The effect of green tea and catechins on the satiety

1.4.4.1 Satiety

Satiation involves several mechanisms including gut hormones signalling in the brain and gastric emptying process. Satiety is controlled by both acute (after eating a meal)

and long term signals. Gut hormones induce acute satiety signals. For example, ghrelin is one of the gut hormones, and its release is associated with hunger, and the release is suppressed after a meal intake. When the body obtains energy, a number of gut hormones are released from the gastrointestinal tract that enhancing satiety. Long term satiety signals give information about the amount of fat stored in the body. Hormones and their mechanism on satiety are listed in Table **1.4** (Benelam, 2009).

Table 1.4: Hormones and their actions in satiety (adapted from Benelam, 2009)

Hormones	Site of production	Effect on appetite	Mechanism	Addition effects
Ghrelin	Stomach	Increases hunger	Signals via ghrelin receptors in brain	✓ Has long-term effect on energy balance
Cholecystokinin (CCK)	Small intestine	Increases satiation in response to the presence of nutrients in the gut, particularly after fat rich meals	Signals via vagus nerves	✓ Delays gastric emptying ✓ Stimulates pancreatic lipase secretion ✓ Stimulates gall bladder contraction to release bile salt ✓ Acts as a neurotransmitter
Glucagon-like peptide-1 (GLP-1)	Small intestine and brain	Increases satiety	Signals via GLP-1 receptors in brain	✓ Stimulates insulin production ✓ Slows gastric emptying
Oxyntomodulin (OXM)	Small intestine and brain	Increases satiety	Signals via GLP-1 receptors in brain and reductions in Ghrelin	✓ Slows gastric emptying
Peptide YY (3-36) **(PYY 3-36)**	Small intestine and large intestine	Increases satiety	Signals via Y2 receptors in brain	✓ Slows gastric emptying and intestinal transport ✓ Reduces gastric secretions
Pancreatic polypeptide (PP)	Pancreas	Increase satiety	Signals via Y5 receptors in brain and via vagus nerve	✓ Reduces gastric secretions

1.4.4.2 The suggested mechanism of green tea and catechins on satiety

The satiation process involves hormones that are regulated in carbohydrate and lipid metabolism. For example, CCK is a hormone that increases satiation by signalling via vagus nerves, and it stimulates pancreatic lipase secretion and gall bladder contraction to release bile salt. GLP-1 that increases the satiety by signalling via the GLP-1 receptor in the brain, also stimulates insulin production. As green tea has the potential to reduce carbohydrate and fat digestion and absorption, it may have a side effect on satiety. For example, if catechins inhibit lipase, more lipase enzyme will be needed to digest the ingested fat. So the intestine may produce more CCK to signal the release the lipase and bile salt, and more CCK release means an increase in satiation (Benelam, 2009). A human study by Josic *et al.* (2010) found increased satiety and fullness were reported by the participants after the consumption of green tea with breakfast compared with a control meal. In addition, a study by Yang *et al.* (1992) found that after an administration of tea polyphenol in rat, the CCK level was significantly increased compared with control group (Yamamoto *et al.*, 1997). Another study found that GLP-1 concentration was significantly increased by daily intake of GTE which containing 856 mg of EGCG after 16 weeks (Liu *et al.*, 2014). Therefore, it is important to investigate satiety after consumption of green tea with a meal or green tea incorporated in food products to monitor the satiety increasing the effect of green tea.

1.5 L-theanine

1.5.1 Structure of L-theanine

L-theanine is gamma-glutamylethylamide, which is a derivative of glutamic acid. It is the predominant amino acid found in green tea and represents 50% of total free amino acids in tea leaves (Keenan *et al.*, 2011; Balentine *et al.*, 1997). L-theanine is a non-protein amino acid that only exists in free form. It was discovered in two natural sources, which are tea leaves (*Camellia sinensis*) and mushroom (*Xerocomus badius*) (Juneja *et al.*, 1999; Chu *et al.*, 1997).

The quality of green tea is often determined by the content of L-theanine because it gives the brothy sweet umami taste which is the unique characteristic taste of green tea. Umami is a Japanese-derived word which describes the fifth taste after sweetness,

saltiness, bitterness and sourness (Kawamura and Kare, 1987; Yamagushi, 1979). Due to its contribution to tea taste, the L-theanine content in tea leaves is correlated with tea quality and price (Vuong *et al.*, 2011). According to Keenan *et al.* (2011), the amount of L-theanine found in green tea available in the UK market ranged from 2.58–4.23 mg/g and has ranged from 1.31–4.16 mg/g in previous studies (Syu *et al.*, 2008; Alcázar *et al.*, 2007; Thippeswamy *et al.*, 2006).

L-theanine biosynthesis (Figure 1.5) takes place mostly in roots where glutamic acid and ethylamine are converted to L-theanine via the enzyme, theanine synthetase (Deng *et al.*, 2009). The L-theanine from roots is transferred via the phloem through its stems growing buds, which are further, stored in tea leaves. When tea leaves are exposed to sunlight and heat, L-theanine is hydrolysed back to its initial substrates, one of which is ethylamine. The free ethylamine in leaves can be utilised as a precursor for catechin synthesis. Therefore, the reduction of sunlight exposure with shade cloth has been shown to increase the concentration of L-theanine and lower the catechin content in tea leaves (Vuong *et al.*, 2011; Juneja *et al.*, 1999). Many previous studies found that the higher shade levels tended to increase the L-theanine and caffeine level in tea leaves (Song *et al.*, 2012; Hirai *et al.*, 2008; Ohta and Harada, 1996). Moreover, Song *et al.* (2012) found that as the tea leaf ages from bud to leaves, the content of L-theanine and caffeine in the leaf reduced, while the content of catechins is increased.

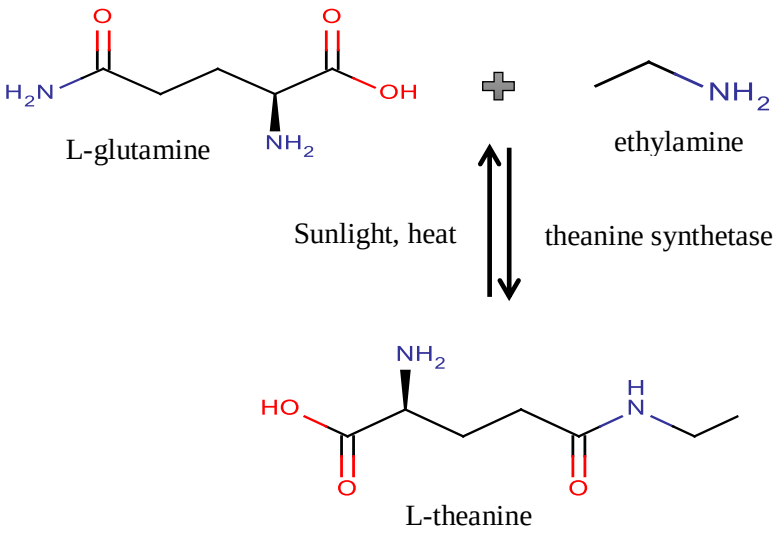


Figure 1.5: Synthesis of L-theanine (Vuong *et al.*, 2011)

1.5.2 Health benefits of L-theanine

Many *in vivo* and epidemiological studies have found that L-theanine consumption has several positive health effects (Miyagawa *et al.*, 2008; Cooper *et al.*, 2005; Kobayashi *et al.*, 1998). For example, consumption of L-theanine has been reported to enhance the generation of alpha brain waves which are related with a relaxed but alert mental state (Kobayashi *et al.*, 1998). Moreover, according to Kurihara *et al.* (2007), L-theanine could have potential as an immune system stimulant. Therefore, it is important to determine the L-theanine content in Matcha green tea to report its contribution to potential health benefits when utilised as a food ingredient.

1.6 Stability of catechins during processing and storage

The stability of catechins is mainly affected by pH and temperature. In aqueous solution, tea catechins are stable in the acidic systems (pH<4), while in near neutral or alkaline system (pH>6), the catechins degrade rapidly. Therefore, ascorbic acid has been found to exhibit a potentially protective effect on the stability of tea catechins. When the processing or storage temperature increases, tea catechins become less stable due to their thermal degradation, oxidation, epimerization, and polymerization (Wang *et al.*, 2003a; Chen *et al.*, 2000; Wang and Helliwell, 2000). In addition, oxygen present in the aqueous system affects the stability of catechins (Wang and Zhou, 2004). The oxidation would probably occur when the temperature was below 44 °C, which leads to degradation of catechins. However, Wang *et al.* (2000) found that catechin epimerization occurred in green tea infusion at 40 ^{o}C over prolonged storage. Hence, beside the temperature of the food system, the heating time also affected the epimerization of catechins (Ananingsih *et al.*, 2013; Wang *et al.*, 2000). Many researchers found that catechins degrade slowly at low temperature. Tea catechins were reduced by 29% after 3hours storage at 70 °C (Su *et al.*, 2003). On the other hand, when tea infusion was stored at 40 °C for 6 months, approximately the same reduction of total catechins (29.3%) was found (Wang and Helliwell, 2000). However, another study found more than 90% of tea catechins were lost after 6 months storage in ambient temperature (Chen *et al.*, 2000).

1.6.1 The stability of green tea catechins in food products

The antioxidant and radical scavenging properties of green tea catechins are structure-dependent and this is correlated with the stability of tea catechins (Ananingsih *et al.*,

2013). In terms of structure, the presence of the ortho -3', 4' –dihydroxyl moiety in the B ring of their molecules, can delocalize electron and stabilises the radical form (Ananingsih *et al.*, 2013). In addition, the three adjacent hydroxyl (-OH) groups at positions C-3', 4'and 5' in EGCG, GCG, and EGC have been reported to be more effective on antioxidant activity than the two adjacent hydroxyl (-OH) groups at C-3' and 4' in ECG, EC and CG. Moreover, the presence of gallate group in the B ring of catchins enhances the scavenging effect compared to the non-gallate catechins, for instance ECG>EC, EGCG>EGC (Amber *et al.*, 2010; Mandel and Youdim, 2004; Xu and Chen, 2002; Nanjo *et al.*, 1996). On the other hand, some studies found that some trans-catechins were more efficient in scavenging singlet oxygen than cis-forms (Ikeda *et al.*, 2003). According to the redox potentials of tea catechins, the order of antioxidant activity of catechins was reported as EGC > EGCG> GCG> EC> ECG (Balentine *et al.*, 1997). There have been several reports on order of scavenging ability of tea catechins. The order of their antioxidant activity was inconsistent in literature, which could be due to different methods, and food model system that used to evaluate antioxidant activity. For example, in an oil-in-water emulsion, EGCG and EC were lost completely in 15 and 30 days (Roedig-Penman and Gordon, 1997). In addition, a different complex matrix of products, play an important role in catechins' stability. Table 1.5 shows the order of catechins' stability in food processing and indicated that different food products contribute to different stability of each catechins.

Table 1.5: The order of catechins' stability in food processing/ food systems/ products. (adapted from Ananingsih *et al.* (2013))

Food System	Food Product	The order of catechins' stability (from the least stable to the most stable catechin)	Reference
Aqueous	Tea drinks	EGC=EGCG<ECG=EC	Chen *et al.* (2000)
Lipid and emulsion	Oil-in-water emulsions	EGC<EC<EGCG=ECG	Almajano *et al.* (2007)
	Oil-in-water emulsions	EGC<EGCG<ECG	Huang and Frankel (1997)
	Canola oil (rapeseed oil)	ECG<EC<EGCG<EGC	Chen and Chan (1996)
	Lard	EC<ECG<EGC<EGCG	Madhavi *et al.* (1996)
	Fish oil	EC<EGC<EGCG<ECG	Wanasundara and Shahidi (1996)
Solid and semi solid	Dried green tea leaves	EGC<GC<EC<ECG=EGCG	Friedman *et al.* (2009)
	Sponge cake	EC=ECG<EGCG=EGC	Lu *et al.* (2010)
	Biscuit	EGCG<ECG<GCG<CG	Sharma and Zhou (2011)
	Bread	EGC<EGCG,GCG<ECG<EC=GC	Wang and Zhou (2004)

1.6.2 Stability of catechins during baking

Understanding the stability of catechins and their interaction with other components in the food matrix is crucial in order to understand their effects in food products. This can be useful for the utilisation of green tea powder and extract as novel food ingredients in processed food.

1.6.2.1 Bread

Wang *et al*. (2004) studied the stability of catechins by adding green tea extract (150-500 mg per 100 g of flour) to bread dough and bread. They found that catechins were stable in the dough during freezing and frozen storage at -20 ℃ for up to 9 weeks. After the bread had been baked, the retention levels of EGCG and ECG were 83% and 91% respectively. During shelf-life (4 days storage at room temperature), tea catechins in bread were stable, and there were no significant losses in bread (Wang and Zhou, 2004). The study also revealed that EGCG and EGC were more likely to degrade than ECG and EC. In bread incorporated with green tea extract, the stability order of catechin ranked as GC > EC > ECG > GCG > EGCG > EGC (Wang and Zhou, 2004). The addition of ascorbic acid led to a lower pH of the dough, which contributes to the high recovery of catechin. A higher pH of system or more alkalinity causes more loss of polyphenols in the system. Several studies have shown a decrease in polyphenols when using alkalinizing agents such as sodium bicarbonate, or baking powder, which are often used as the leavening agent. Other studies have found that the phenolic content increased after baking when yeast was employed (Rosales-Soto *et al.*, 2012). Yeast is another agent that produces lactic or acetic acid, causing a lower pH of the dough when fermenting. For example, Wang *et al*. (2004) found that baking bread with addition of green tea extract using yeast provided good retention of green tea catechins (83-91%).

1.6.2.2 Biscuit

Sharma and Zhou (2011) found that the stability of catechins from green tea extract, incorporated in biscuits decreased as the baking progressed and increased as the concentration of green tea extract (150-300 mg per 100 g of flour) was increased in the biscuit formulation (Sharma and Zhou, 2011). Catechins become less stable when the temperature or pH of their system is increaesed. Moreover, the epimerization of EGCG to GCG was found to occur at 120 ℃ and pH 5-6 (Wang and Helliwell, 2000; Chen *et al.*, 2000). The process of making biscuits involves high temperature baking (160 ℃ for 10 min) and the addition of sodium bicarbonate, ammonium bicarbonate and baking powder, causing a higher pH in the dough. The high temperature contributed to sufficient energy for epimerization to occur in the biscuit system (Sharma and Zhou, 2011). The recovery percentages of ECG and EGCG in biscuits incorporated with green tea extract were 30% and 21%. While, the recovery percentages of ECG and EGCG in

bread were 83% and 91%. The difference in recovery percentage could be due to the core temperature in the bread and biscuits during baking and the pH of the food system. The core temperature during bread baking was between 80-101 ℃ for 8-9 min while during biscuits baking was between 120-130 ℃. Moreover, the addition of yeast to bread dough, led to a lower pH system of the dough.

1.7 The effect of green tea powder incorporated in food products on physical properties and acceptability

1.7.1 The effect of green tea on the hardness of food products

Hardness is an important quality index, and can be measured in both sensory acceptability test evaluation and instrumental analysis. Many studies found that hardness of food products was affected by the addition of green tea products. For example, Ahmad *et al.* (2015) found a significant decrease in hardness of cookies from and a significant decrease in texture acceptability as the level of green tea powder increased from 0 to 4% in cookie formulations. However, another study, on the effect of tea catechins on the sensory quality of bread, found that the hardness of bread increased with an increase in GTEP (Wang *et al.*, 2006). Therefore, increasing GTEP was found to have a negative effect on the sensory quality of both cookies and bread. The level of GTEP incorporated played an important part on texture of bread. For instance, 150 mg per 100 g flour formulation led to a significant negative effect on bread volume and texture, whereas at 100 mg of GTEP per 100 g of flour did not affect the changes (Wang *et al.*, 2006a). The sensory evaluation with both trained and untrained panellist showed that with the increased amount of GTEP in the formulation, the brightness and sweetness of the bread decreased, while hardness, stickiness and astringency increased (Wang *et al.*, 2007). The authors suggested that the increase of hardness in the bread incorporated with green tea extract might be explained by the effects of green tea polyphenols on the enzyme and yeast activities (Wang *et al.*, 2007). Catechins may depress amylase activity leading to a reduction in maltose produced in the dough, which consequently reduces the yeast activity during fermentation. The reduced yeast activity leads to less gas produced, hence, a smaller volume with relatively harder and denser texture of the bread. A study on quality and antioxidant property of green tea sponge cake showed that the cake became harder with increasing levels of green tea powder (Lu *et al.*, 2010). The results are in agreement with the findings of a previous study (Lee and

Lin, 2008), which reported an increase in chiffon cake hardness with the increase of GABA (aminobutyric acid) tea. On the other hand, the hardness of biscuits is affected by the content of shortening and sugar (Hoseney and Rogers, 1994). Shortbread dough contained the high content of shortening (butter) and sugar with a low amount of water. These ingredients of shortbread prevent the gluten development, which contributed to crumbly properties of biscuits. Regarding to thes previous studies, MGTP may have a role in the texture, this need to be investigated further. Hence, the hardness of biscuits incorporated with different levels of MGTP was measured to investigate the effect of MGTP on the texture of shortbread biscuits

1.7.2 The effect of green tea incorporated in food products on acceptability

MGTP contributes green colour and bitterness to incorporated food products. Thus, the incorporation of green tea powder to food products will affect the acceptability of products. For example, Lu *et al.* (2010) used green tea powder to substitute 0%, 10%, 20%, and 30% of wheat flour to make sponge cakes. They found that the cake with 30% of flour replaced with green tea powder were rated lower in all sensory liking results. Another study on noodles with addition of green tea powder found that the highest level of green tea (3 g of green tea powder per 100 g of flour) incorporated in noodle formulation, received the lowest mean score of overall acceptability (Li *et al.*, 2012). The noodles with 3 g of green tea powder per 100 g of flour incorporated was described as slightly astrictive by the panellists (Li *et al.*, 2012). A study on the acceptability of cookies that had green tea powder added to wheat flour at different levels (1, 2 and 4%) found that the addition of 4% green tea powder to wheat flour scored significantly higher (P <0.05) values for colour, aroma, and taste acceptability compared to the control. However, the increased addition of green tea powder significantly decreased appearance and texture acceptability (Ahmad *et al.*, 2015). Ahmad *et al.* (2015) suggested that green tea powder can be incorporated only at lower levels as higher levels may have adverse effect on hardness and appearance. The familiarity of green tea can also affect acceptability. A study investigated the perception of Korean and French consumers of green tea and found that familiarity affected their perception. The sensory properties seemed to be the main factors for the Korean consumers. On the other hand, the French consumers who were less familiar with green teas perceived the differences of green teas based on their acceptability (Kim *et al.*, 2013).

1.8 Justification and aims of research

The utilisation of green tea powder in food such as bakery products, may provide a healthier appeal and functional food to consumers (Amber *et al.*, 2010). Hence, the marketing opportunity for these products can be developed by the presence of green tea polyphenols. As shortbread biscuits are widely known and consumed in Europe, a shortbread recipe with the addition of MGTP is developed in order to investigate the effect of the ingredients and processing on the stability of catechins in MGTP. To develop a novel biscuit recipe with MGTP, the acceptability of biscuits with increasing level of MGTP and sugar incorporated have to be evaluated to observe the effect of these ingredients and their relationship on the acceptability of biscuits, and optimize the level that consumers are willing accept. As mentioned in section 1.4 on the health effect of green tea and catechins, the acute metabolic response after consumption of biscuits with MGTP need to be investigated whether the accepted levels would provide the beneficial effect. The aims of this research were therefore:

I. To compare the total polyphenol content and catechins present in two green tea products: MGTP and GTEP
II. To investigate the stability of catechins, caffeine and L-theanine in MGTP incorporated in biscuits after baking and over storage time.
III. To investigate the change of physical properties in biscuits containing MGTP
IV. To observe the trend of consumer acceptability on different ratios of MGTP and sugar incorporated in shortbread biscuit recipes
V. To conduct a pilot trial investigating the effect of MGTP incorporated in biscuits on glucose, triglyceride response and satiety levels in healthy human subjects in comparison to plain biscuits consumed either with green tea drink or water

1.8.1 Objectives

- To measure the total polyphenol content and antioxidant capacity of MGTP and GTEP using Folin-ciocalteu assay and FRAP assay
- To determine the amount and composition of catechins and caffeine in MGTP and GTEP using HPLC analysis
- To optimize the L-theanine extraction and quantification method used for green tea powder using HPLC analysis

- To determine the catechin profile, caffeine and L-theanine content in dough, baked biscuits, and biscuits that have been stored for 1 month by HPLC analysis
- To evaluate consumer acceptability of shortbread biscuit incorporated with MGTP using 9-point hedonic scale and analyse the data by response surface methodology
- To measure the hardness of biscuits using a texture analyser
- To measure the glucose, triglyceride response and satiety level in human subjects over 3 hours using a finger prick glucometer, CardioChek® meter and by using a self-reporting visual analog scale, after consuming a provided portion of three food samples:
    a. plain biscuits consumed with water (control),
    b. green tea enriched biscuits consumed with water,
    c. plain biscuits consumed with green tea drink

2 Chapter two: Materials and methods

All the materials and analytical methods applied throughout the research are described in this chapter.

2.1 Raw materials

2.1.1 Green tea products

- Matcha factory® Matcha green tea powder- Chah Ltd (Solihull, UK)
- Pure source Nutrition® Green tea extract powder -Pure source Nutrition (Hertfordshire, UK)

2.1.2 Biscuits

- Tesco® Plain flour – Tesco supermarket (Leeds, UK)
- Tate Lyle® Caster sugar – Tesco supermarkets Ltd (Leeds, UK)
- Anchor® Unsalted butter – Tesco supermarkets Ltd (Leeds, UK)
- Marvel® Dried skimmed milk powder – Tesco supermarkets Ltd (Leeds, UK)
- Dr.Oetker® Bicarbonate of Soda - Tesco supermarkets Ltd (Leeds, UK)
- Tesco® Table salt – Tesco supermarket (Leeds, UK)

2.2 General instruments and equipment

The following instruments and equipment were used throughout the research:

- Analytical balance (KERN KB) – KERN & SOHN GmbH Co. Ltd (Balingen, Germany)
- Analytical balance (Mettler Toledo Xs 104) – Mettler–Toledo Ltd (Beaumont Leys Leicester, UK)
- Conventional oven of 90 cm single cavity gas range cooker (BC190.2TCSS)- Baumatic Ltd (Merthyr Tydfil, UK)
- Evaporator (Genevac EZ-2) – Genevac Ltd (Ipswich, UK)
- Freeze dryer (Christ Alpha 1-4 LD) - SciQuip Ltd (Shropshire, UK)
- Heated circulating water bath (Grant TxF 200) - Grant Instruments (Cambridge, UK)
- Magnetic stirrer – Stuart Scientific Co. Ltd (Surrey, UK)
- Mixing machine (Kenwood Chef Classic KM336) – Kenwood Ltd (Havant, UK)
- Morrisons® Oven tray (35 cm) - Morrisons supermarket (Leeds, UK)
- Morrisons® biscuit cutter (4.8 cm) - Morrisons supermarket (Leeds, UK)

- pH Meter (Hanna Basic HI 2210) – Hanna Instruments Ltd (Bedfordshire, UK)
- Pestle and mortar (Haldenwanger™ 190 mL) - Fisher Scientific Ltd (Leicestershire, UK)
- Spectrophotometer (Cecil CE 3021) – Cecil Instruments Ltd (Cambridge, UK)
- Ultrasonic water bath (Grant OLS 200) – Grant Instruments (Cambridge, UK)
- Vortex mixer (FB 15013) – Fisher Scientific Ltd (Loughborough, UK)
- Vacuum sealing machine –The Food Machinery Company Ltd (Kent, UK)
- Tempscan® Scanning Thermometer – Comark Ltd (Hertfordshire, UK)

2.3 Determination of total polyphenols and antioxidant capacity of MGTP and GTEP

2.3.1 Principles of Folin-Ciocalteu assay and Ferric reducing ability of plasma (FRAP) assay

Total polyphenol content can be determined by Folin-Ciocalteau assay. Folin-Ciocalteau reagent is a mixture of phosphomolybdate and phosphotungstate which is used to react with any reducing substances (Singleton *et al.,* 1999). The colour intensity in the test reaction mixture is measured. Total polyphenol values are determined at a wavelength of 765 nm. Gallic acid is often used as a standard to quantify the total polyphenol content. Hence, the results are presented as gallic acid equivalents (GAE).

The antioxidant capacity of samples can be measured by the FRAP assay. The assay measures the antioxidant capacity of samples by their ferric reducing ability. Ferric ion is reduced to ferrous ion at low pH causing a ferrous-tripyridyltriazine complex to form an intense blue colour. FRAP values are measured by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Trolox is commonly used as a standard.

2.3.2 Materials for Folin-Ciocalteu and FRAP assay

2.3.2.1 Folin-Ciocalteu assay materials

- Folin & Ciocalteu's phenol reagent - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Formic acid (99 %) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Gallic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Methanol HPLC grade - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Sodium carbonate - Fisher Scientific Co. Ltd (New Jersey, USA)

2.3.2.2 Ferric Reducing Ability of Plasma (FRAP) assay materials

- Glacial acetic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Hydrochloric acid 37 % - Fisher Scientific Co. Ltd (Leicestershire, UK)
- Iron (III) chloride hexahydrate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Sodium acetate trihydrate - - Fisher Scientific Co. Ltd (Leicestershire, UK)
- 2, 4, 6- Tri [2-pyridyl]-5-triazine (TPTZ) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Trolox - Calibiochem Co. Ltd (Darmstadt, Germany)

2.3.3 Preparation of samples for both Folin-Ciocalteu and FRAP assay

2.3.3.1 Extracting solution (70% methanol with 0.3%formic acid)

Methanol (700 mL) and formic acid (3 mL) were added to a 1000 mL volumetric flask. The solution was made up to 1000 mL with water.

2.3.3.2 Extraction procedure

Approximately 100 mg of sample (MGTP and GTEP) was weighed into a 15 mL falcon tube and 5 mL of the extract solution at 70 ℃ was added. The tubes were vortexed for 15 s then incubated in a heated circulating water bath at 70 ℃ for 5 min. After that, the tubes were removed from the water bath and placed in racks in room temperature water to cool down. Then the extract was centrifuged at 3000 rpm for 10 min. The supernatant was collected in another falcon tube, and then the extraction repeated. The supernatant was collected, pooled together and the volume was adjusted to 10 mL with the extracting solution. The extracts were diluted 1:10 in water prior to both the Folin-Ciocalteu and FRAP assays. To determine the effect of time on total polyphenol

extraction of MGTP, the extraction was carried out at 5 different extraction times, of 5, 10, 15, 20 and 30 min.

2.3.4 Determination of total polyphenols using Folin-Ciocalteu assay

2.3.4.1 Preparation of reagents for Folin-Ciocalteu assay

1. Folin-Ciocalteu reagent

   Folin-Ciocalteu reagent (1 mL) was diluted to 10 mL with water in the appropriate volumetric flask. The reagent was freshly prepared for each experiment.

2. Sodium carbonate solution (7.5% w/v)

   Sodium carbonate (75 g) was dissolved with water (1 L) in a volumetric flask. For every sample, 4 mL of the end solution is required.

3. Gallic acid standard

   Gallic acid (0.1 g) was dissolved in 100 mL of water. The standard solution was diluted to produce a gallic acid standard curve with concentration from 25 µg/mL to 150 µg/mL. Water was used for concentration 0 µg/mL.

2.3.4.2 Procedure and quantification of total polyphenols

Total polyphenol content was determined according to the method by ISO 14502-1: 2005. The extracted sample or standard solution (1 mL) was added to a falcon tube, containing 5 mL of a 1:10 dilution of Folin-Ciocalteu reagent in water. The mixture was vortexed for 5 s, and then 4 mL of sodium carbonate solution was added within 5 min. The mixture was vortexed and incubated in a 26 °C water bath for 2 hours. After the incubation, the absorbance at 765 nm was measured. Water was used as a blank. The total polyphenol content was obtained from a standard curve of gallic acid ranging between 0 to 150 µg/mL ($R^2 > 0.99$), the standard was prepared fresh on the experiment day. The total polyphenol content was expressed as gallic acid equivalents (GAE) in µg/mg of material. The result was reported as a mean value of 3 extractions. The total polyphenol content was calculated using the following equation:

$$\text{Total polyphenol} = \frac{A \times 10}{m}, \quad \text{Equation 1}$$

where, A is the compound concentration in sample solution calculated from the standard curve (µg/mL); 10 is the dilution factor, and m is the mass (mg) of tea.

2.3.5 Determination of antioxidant capacity by FRAP assay

2.3.5.1 Preparation of reagents for FRAP assay

1. Acetate buffer, (300 mM, pH 3.6)
   Sodium acetate trihydrate (3.1 g) was dissolved with 900 mL of water in a volumetric flask, followed by addition of 16 mL of glacial acetic acid. The total volume was made up to 1 L with water.
2. TPTZ (2, 4, 6 – Tripyridyl – s – triazine) solution (10 mM):
   TPTZ (312.3 mg) was dissolved in 100 mL of HCl (40 mM).
3. Iron (III) chloride hexahydrate (20 mM)

   Iron (III) chloride hexahydrate (540 mg) was weighted and dissolved in 100 mL of distilled water.

4. FRAP reagent
   FRAP reagent was prepared by the mixture of acetate buffer, TPTZ and $FeCl_3$ with the ratio of 10:1:1
5. Trolox standard
   Trolox (25 mg) was dissolved in 10 mL of ethanol and made up to 100 mL by distilled water. The standard solution was diluted to the concentrations of Trolox standard curve ranged from 10 to 160 µg/mL. FRAP reagent was used as a blank.

2.3.5.2 Procedure and quantification of antioxidant capacity

The extract of sample or standard solution (200 µL) was added to a falcon tube, containing 6 mL of a FRAP reagent (ratio of 1:30). The tube was vortexed and allowed to stand in a dark place for 10 min. The absorbance was read at 593 nm. FRAP reagent was used as a blank. The Trolox standard ranged from 10 to 160 µg/mL ($R^2 > 0.99$). The antioxidant activity was expressed as Trolox equivalents (TE) in µg/mg of material.

The result was reported as a mean value of 3 extractions. The FRAP was calculated using the following equation:

$$\text{Antioxidant capacity} = \frac{A \times 10}{m}, \quad \text{Equation 2}$$

where, A is the compound concentration in sample solution calculated from standard curve (µg/mL); 10 is the dilution factor, and m is the mass (mg) of tea.

2.4 Determination of individual catechins, caffeine and L-theanine using high performance liquid chromatography (HPLC)

2.4.1 Principle of HPLC

This method combines the process of separation and quantification of phenolic compounds in tea. HPLC consists of several parts including binary pump, auto sampler, column and detector. Liquid chromatography systems are equipped with detectors, such as photo diode array (PDA), ultraviolent-vis (UV-Vis), electrochemical (ED), mass spectrometry (MS), and chemilumifluorescence (FD) detectors and these detectors have been used for the analysis of polyphenols in tea (Ananingsih *et al.*, 2013). PDA and UV-Vis detector are most widely used with detection wavelengths of 200-400 nm to detect phenolic compounds. A reversed phase column (C18) is most often used to separate polyphenols. For the eluting mobile phase, methanol or acetonitrile in aqueous solution is often used. The addition of a small amount of acetic acid, phosphate buffer or formic acid in the mobile phase has been found to improve polyphenol separation significantly. Goto *et al.* (1996) found that the temperature of the column significantly influences the separation of catechins. When the column temperature increased, the retention time of each analysed compound decreased. Filtration of extract prior to injection for HPLC analysis is recommended to prevent a contamination in a HPLC column. Sometimes, solid phase extraction is used as pre-treatment for clean-up of tea samples before HPLC analysis. Wang and Zhou (2004) developed a method for the analysis of tea catechins and caffeine in bread matrix using a water-methanol-formic acid solvent system. A C18 reversed-phase column was employed to separate the catechins, which were detected at 275 nm by a PDA detector, and a similar method was applied for the detection of tea catechins in the biscuit matrix (Wang and Zhou, 2004).

Therefore, the method of Wang and Zhou (2004) was adapted to determine the catechin content in each sample.

Many analytical methods have been developed to identify and determine the content of L-theanine, such as HPLC coupled with either an evaporative light scattering detector (ELSD), PDA or fluorescence detector (Csupor *et al.*, 2014; Song *et al.*, 2012; Thippeswamy *et al.*, 2006). ELSDs can detect most of all components that are less volatile than the mobile phase. It is used to detect food components such as sugars, fats and surfactants. The main limitations of ELSD are the non-linear response of the standard curve. The general limit of detection of ELSD is 50 – 100 nanogram, whereas, the UV-Vis and fluorescence detectors excel the limits of detection in fentogram range. Csuper *et al.* (2014), Keenan *et al.* (2010) and Song *et al.* (2012) determined L-theanine by using a binary gradient HPLC method with photodiode array detector (PDA); the wavelength used to detect L-theanine is 200-210 nm which can detect polyphenolic compounds as well (Csupor *et al.*, 2014; Song *et al.*, 2012; Keenan *et al.*, 2011). Rand (2009) developed a method to determine L-theanine using HPLC equipped with a C18 column and ELSD. The HPLC method was isocratic elution with 100% water as the mobile phase and the tea extract samples were pre-treated with polyvinylpolypyrrolidone (PVPP) to eliminate the polyphenols. An isocratic run was used in this method because the samples were treated with PVPP prior to HPLC injection. When samples are not pre-treated with PVPP, gradient elution on HPLC should be used in order to remove polyphenolic compounds that accumulate in the column.

2.4.2 Materials and equipment for HPLC analysis

2.4.2.1 Materials

- Absolute ethanol - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Acetonitrile HPLC gradient - VWR International S.A.S (France)
- Caffeine – Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Catechin- Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Catechin gallate – Insight Biotechnology Ltd (Middlesex, UK)
- Epicatechin - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Epicatechin gallate – Cambridge Bioscience Ltd (Cambridge, UK)
- Epigallocatechin – Cambridge Bioscience Ltd (Cambridge, UK)

- Epigallocatechin gallate – Cambridge Bioscience Ltd (Cambridge, UK)
- Folin & Ciocalteu's phenol reagent - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Formic acid (99 %) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Gallic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Glacial acetic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Gallocatechin gallate – Insight Biotechnology Ltd (Middlesex, UK)
- Gallocatechin – Cambridge Bioscience Ltd (Cambridge, UK)
- Hexane - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- L-theanine (99%)– Cambridge Bioscience Ltd (Cambridge, UK)
- Leucine (99 %) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Methanol HPLC grade - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Polyvinylpolypyrrolidone (PVPP) - Sigma-Aldrich Co. Ltd (St. Louis, USA)

2.4.2.2 Equipment

2.4.2.2.1 HPLC

- **Conventional HPLC and UV-Vis detector**

The HPLC consists of a binary pump, a solvent delivery module (LC – 20AD) coupled with an online unit degasser (DGU – 20A5), a thermostated autosampler/injector unit (SIL – 20AC) and a UV-Vis detector (SPD – 20A) – Shimadzu Corporation (Kyoto, Japan).

- **Ultrafast – High Performance Liquid Chromatography with Evaporative Light Scattering Detector (ELSD)**

The HPLC ($UFLC_{XR}$) consists of a binary pump, a solvent delivery module (LC – 20AD) coupled with an online unit degasser (DGU – 20A3), a thermostated autosampler/injector unit (SIL – 20AC) and a photodiode array (PDA) with multiple wavelength detector (SPD – M20A) and a Shimadzu ELSD-LT – Shimadzu Corporation (Kyoto, Japan).

2.4.2.2.2 Column

- Chromatography column, reverse phase C_{18}; 5µm, 250 x 4.6 mm (Gemini 5µ C18 110A, S/NO 540974-22) – Phenomenex® (Cheshire, UK)

2.4.3 Catechins and caffeine analysis and quantification using HPLC

2.4.3.1 Preparation of samples for HPLC method

Matcha green tea powder (MGTP; 10 mg), and Green tea extract powder (GTEP; 10 mg) were weighed into falcon tubes, and 5 mL of 70% methanol with 0.3% formic acid was added to each tube. The sample tubes were placed in a water bath at 70 °C for 5 min to equilibrate to temperature and another 5 min to extract. The tubes were removed and placed in a rack to cool down at room temperature. After cooling down, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant was collected. The extraction was repeated again, the supernatant was pooled together, and its volume was made to 10 mL with the extracting solvent. The extract sample was filtered through a 0.2 µm PTFE syringe filter and put in an amber vial. The sample was futher analysed by HPLC. The comparison between extraction time was carried out with 5 extractions at durations of 5, 10, 15, 20 and 30 min with MGTP with 3 extractions at each time.

After a pack of MGTP had been opened, the green tea powder was immediately extracted. The powder was then stored in its bag that was sealed and stored at room temperature. The MGTP was then extracted again after 3 months and 5 months to monitor the stability over storage at room temperature. The catechins and caffeine of three packages of MGTP were analysed to observe the difference of catechins content in each package.

2.4.3.2 Standards preparation for HPLC analysis

Each standard compound was accurately weighed (1 mg) and was dissolved in 1 mL ethanol to prepare 1 mg/mL standard stock solutions, then vortexed and aliquoted to 100 µL in an eppendorf micro-centrifuge tube. All stocks were dried down on the vacuum centrifugal evaporator at a setting of low b.p. (boiling point) for 15 min and kept at -20 ^{o}C until further use. Standard curves were prepared with standards' stock by dissolving in extracting solvent (70% methanol with 0.3% formic acid) at concentrations between 2.5-100 µg/mL. The standard mixture contained caffeine, catechin, EC, EGCG, ECG, EGC, GCG and gallic acid. Standard solutions were

injected into HPLC and the corresponding peak area of each compound was used to plot the standard calibration curve.

2.4.3.3 HPLC-PDA analysis of samples and standards

A method for HPLC analysis was adapted from Wang and Zhou, (2004) and developed to suit the HPLC system available in the laboratory. The catechins and caffeine were analysed by a HPLC system, consisting of PDA detector (Shimadzu, SPD-20A). A 10 µL sample was injected onto a Phenomenex C18 reverse-phase column (5 µm, 250 × 4.6 mm) running a binary gradient of water (Millipore, UK) with 0.1% formic acid (eluent A) and acetonitrile with 0.1 % formic acid (eluent B), at a flow rate of 0.5 mL/min. The gradient initiated at 10 % (eluent B), remained at 10% for 5 min, and increased to 15% over 9 min, then increased to 60% over 23 min, then to 95% over 3 min and held at 95% for 2 min to wash the column before it was returned to 10% over 2 min and re-equilibrated over 6 min. The total method length was 45 min. The column temperature was set at 25 ˚C. Catechins and caffeine were detected at 275 nm. Identification of each compound in samples was performed by comparing the retention time and spectrum with the standard mixture of 8 compounds. Each extracted sample was performed on HPLC with duplicate run.

2.4.3.4 Quantification of catechins and caffeine

The concentration of each catechin and caffeine of MGTP and GTEP were calculated using the equation obtained from the calibration curve of each compound. The content of (µg/mg of powder) of each catechin and caffeine was calculated from the following equation:

$$\text{The content of each catechin and caffeine } = \frac{A \times V}{m}, \qquad \text{Equation 3}$$

where, A is the compound concentration in sample solution (µg /mL) of extract; V is the volume of the sample solution, and m is the mass (mg) of powder.

2.4.3.5 Method validation for HPLC analysis of green tea samples

The method was validated in terms of selectivity, linearity, recovery, detection, quantification limits and precision. Calibration curves were constructed on 4-6 data points for standards ranging from 2.5-100 µg/mL and the calibration curves were forced through zero. The limit of detections (LODs) and limit of quantifications (LOQs) were

determined based on the standard deviation of the y-intercept from the regression line (standard error of estimate) and slope using the calibration curve data, using the following equation:

$$LOD = 3S_{y/x}/b, \quad \text{Equation 4}$$

$$LOQ = 10S_{y/x}/b, \quad \text{Equation 5}$$

where, $S_{y/x}$ is the standard error of estimate, b is the slope of the calibration curve.

The LODs and LOQs results were expressed as detectable and quantifiable concentrations in µg/mL.

Recovery studies were conducted by spiking MGTP extract samples with 10 µg/mL of each standard. The percentage of recovery was calculated by the following equation:

$$\%\ \text{Recovery} = \frac{A - B}{C} \times 100, \quad \text{Equation 6}$$

where, A is amount of the analysed compound in the extract solution with spiked, B is amount of the analysed compound in the extract solution without spiked, C is amount of analysed compound added.

For the intra-day assay, 5 replicate analyses of a mixture of standards (10 µg/mL) were carried out and on different days for inter-day precision studies. Precision at each concentration was presented as the % coefficient of variation (CV) of the average measured peak area.

2.4.4 L-theanine analysis and quantification using HPLC

2.4.4.1 Preparation of samples for L-theanine quantification

MGTP was weighed (100 mg) in 15 mL centrifuge tubes, then 10 mL of water was added, and tubes were vortexed for 1 min then heated in a water bath at 80 °C to equilibrate (5 min) and continued for 5 min. After cooling down at room temperature, the sample tubes were centrifuged at 3000 rpm for 10 min, and the supernatant was collected. The pellet was extracted by repeating the same procedure. The supernatant was collected together, and the total volume was made up to 20 mL. The extract was analysed with HLPC with both isocratic and binary gradient elution methods. The

extraction time was conducted at 5, 10, 15 and 20 min to observe the effect of extraction time on L-theanine released. Three extractions were conducted for each time.

2.4.4.2 L-theanine standard

The stock standard was prepared by weighing 1 mg of L-theanine standard and dissolving it in 1 mL of water. The stock was vortexed several times to ensure that all standard was dissolved. The stock standard was diluted with water to make a series of dilutions covering the range 5-100 µg/mL for L-theanine quantification in tea samples and covering the range of 0.5-40 µg/mL for L-theanine quantification in biscuit samples.

2.4.4.3 HPLC method optimisation for L-theanine quantification

According to the literature review, ELSD and PDA detectors have been used to detect L-theanine in tea samples. Therefore, in our study, we compare both of detectors with isocratic and binary HPLC condition in L-theanine determination.

2.4.4.3.1 HPLC method with isocratic elution and ELSD

2.4.4.3.1.1 PVPP pre-treatment of extract samples

PVPP was weighed (250 mg) in 50 mL centrifuge tubes, and then 30 mL of water was added. PVPP was allowed to swell overnight in the tubes and then the water was decanted. 10 mL of tea extract was added to the swollen PVPP. The samples were vortexed and incubated at room temperature for 10 min. The samples were centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered using a 0.2µm PTFE syringe filter into an amber vial for HPLC analysis.

2.4.4.3.1.2 Optimisation of HLPC assay for L-theanine determination

The isocratic run was performed on a Shimadzu UFLC equipped with PDA detector (SPD-M20A) and a Shimadzu ELSD-LT. The sample was injected and run for 10 min. To detect L-theanine with ELSD during method development, the gain was set to 10 and the gas pressure at 350 kPA. The drift tube and nebuliser temperatures were set at 70 °C. The mobile phase consisted of 100% water. The flow rate was 0.8 mL/min. The column temperature was set at 35 °C and the autosampler temperature was set at 10 °C. For each analysis, 30µL of sample or standard was injected.

2.4.4.3.2 HPLC method with binary elution

The binary gradient elution was performed on a conventional HPLC, Shimadzu (SPD-20A series). The mobile phases consisted of water/formic acid (99.9:0.1 v/v), and acetonitrile/formic acid (99.9:0.1 v/v) for reverse phase chromatography. Both mobile phase A and B were prepared fresh for every set of runs and degassed before use. The C18 column was eluted with 100% A for 10 min then eluted to 60% B on a linear gradient over 5 min. The 60% B elution was held for 3 min, returned to 0% over 2 min and allowed to re-equilibrate for 10 min. The total run was 30 min at a constant flow rate of 0.8 mL/min. The analyte was monitored by UV detection at 200 nm. The column temperature was set at 35 ℃ and the autosampler temperature was set at 10 ℃. For each analysis, 30µL of sample or standard was injected.

2.4.4.4 L-Theanine quantification

The content (µg/ mg of green tea) of L-theanine was calculated from the equation:

$$\mathrm{L-theanine\ in\ mg\ \ per\ 100mg\ of\ greentea} = \frac{A \times V}{m}, \quad \text{Equation 7}$$

where, A is the compound concentration in sample solution from standard curve (µg /mL) of extract, V is the volume of the sample solution (20mL) and m is the mass (mg) of powder.

2.4.4.5 Method validation

For linearity, solutions of standards were prepared at seven concentrations within the range of 5 – 100 µg/mL for L-theanine quantification in tea and within the range of 0.5-40 µg/mL for L-theanine quantification in biscuits. The results were plotted to obtain the calibration curves and coefficient of determination. All the calibration curves were forced through zero. The LOD and LOQ were determined based on the standard deviation of the y-intercept of the regression line (standard error of estimate) and slope using the calibration curve data. The formulation used to calculate LOD and LOQ were mentioned earlier in section 2.4.3.5 (Equation 4 and 5).

Recovery studies were conducted by spiking extract samples with 10 µg/mL of L-theanine standard. For the intra-day assay, 4 replicate analyses of a mixture of standards (10 µg/mL) were carried out and on different days for inter-day precision studies. The recovery study was performed in both tea extract and biscuit extract samples. Recovery percentages were calculated by comparing the measured L-theanine to the added L-theanine (10µg/mL). Precision at each concentration was presented as the %CV of

measured peak area from mean peak area. LODs and LOQs were determined based on the concentration of standards and results were expressed as detectable and quantifiable concentrations in µg/mL.

2.4.4.6 The effect of extraction temperature on L-theanine quantification

The comparison of extraction temperature between room temperature (18 °C) and 80 °C temperature was determined. The room temperature water extraction was similar to the method in section 2.4.4.1, but the samples were not incubated in the water bath, but were at room temperature for 5 min.

2.5 Biscuit preparation and HPLC analysis for catechins, caffeine and L-theanine in biscuit samples

2.5.1 Materials

The ingredients for biscuits with/without sodium bicarbonate and for shortbread biscuit are listed in Table 2.1.

2.5.1.1 Biscuit formulation with/without sodium bicarbonate

The recipe was adapted from Laguna *et al.,* (2013). The formulas were prepared with and without the addition of alkaline agent (sodium bicarbonate) and named with sodium bicarbonate and without sodium bicarbonate

2.5.1.2 Shortbread formulation

Three formulations of shortbread biscuits were prepared using the same quantity of all the ingredients except the MGTP. The proportions of MGTP and flour were 2, 4, and 6 g of green tea powder per 100 g of flour; the three samples were named 2g MGTP, 4g MGTP, and 6g MGTP respectively. The plain shortbread biscuits were prepared using the same quantity of all the ingredients without adding green tea powder.

Table 2.1: Ingredients for biscuit formulations

	Biscuit formulation		Shortbread biscuit formulation
Ingredients (g)	**with sodium bicarbonate**	**without sodium bicarbonate**	
Flour	100	100	100
Butter	60	60	83.3
Sugar	30	30	25, 30, 35
Matcha Green tea powder (MGTP)	2	2	2, 4, 6
Water	9.3	9.3	-
Sodium bicarbonate	0.5	-	-
Milk powder	1.8	1.8	-
Salt	1	1	-

2.5.2 Preparation of biscuits incorporated with MGTP

2.5.2.1 Biscuit formulation with/without sodium bicarbonate

The butter was creamed in a mixing machine for 4 min at minimum speed to obtain a homogenous cream. After this, the sugar was added and mixed in for 2 min at speed 4. The milk powder, previously dissolved in all the water, was added and mixed in for 2 min at the minimum speed. Finally, the flour, MGTP, and sodium bicarbonate if used were mixed in together at minimum speed for 2 min. The dough was rested for 1 hour in a fridge. The dough was then sheeted to 0.4 mm thickness and the biscuits were cut into a circle shape (4.8 cm diameter). Biscuit pieces were placed on an aluminium tray and were rested for 10 min, at 4 °C, then baked in a conventional oven for 5 min at 180 °C.

Then the trays were turned bringing the side that had been at the back to the front of the oven to ensure homogenous cooking and baked for a further 5 min at the same temperature. The oven and the oven trays were always the same; the trays were placed at the same level in the oven and the number of biscuits baked was always the same. After cooling, the biscuits were packed and stored in vacuum-sealed bags until HPLC analysis.

2.5.2.2 Shortbread biscuits

The dough was prepared by a creaming technique, butter and sugar were beaten together with a mixing machine for 5 min, and then flour and green tea were added, followed by mixing for 5 min. The dough was rested for 1 hour in a fridge. The dough was rolled to 0.4 mm thickness, the biscuits were cut into a circle shape (4.8 cm diameter) and were rested for 10 min at 4 °C, before being placed in the conventional oven at 180 °C for 7 min. Then the oven trays were rotated and the biscuits were baked for another 8 min. After baking, the biscuits were left to cool down at room temperature. The biscuits were packed and stored in vacuum-sealed bags until HPLC analysis.

2.5.3 Freeze drying of samples

Dough and biscuits were ground by pestle and mortar and freeze-dried by a freeze dryer with the vacuum system set at 0.040 mbar and the condenser at -50 °C for 24 hours. All samples for HPLC analysis were weighed before and after freeze drying.

2.5.4 HPLC analysis of catechins in dough and biscuits samples

2.5.4.1 Defatting of samples and extraction of catechins

A freeze-dried sample was weighed (1 g) in a 50 mL centrifuge tube and was defatted by adding 30 mL of hexane and incubated in a water bath at 70 °C for 20 min. After cooling down at room temperature, the sample was centrifuged at 3000 rpm for 10 min. The hexane fraction was decanted. The sample was left in the fume cupboard with the light off for 2-3 hours to evaporate the remaining hexane from the sample. Defatted sample was extracted by adding 25 mL of 70% methanol with 0.3% formic acid. The same tube was incubated in a water bath at 70 °C for 5 min to equilibrate to the temperature and another 45 min to extract. The sample was removed and cooled down at room temperature for 10 min. After cooling down, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant was collected. The extraction was repeated, then the

supernatant was pooled together, and its volume was made to 50 mL with the extracting solvent. The sample was filtered with a 0.2 µm PTFE syringe filter and put in an amber vial HPLC analysis.

2.5.4.2 HPLC-PDA quantification of catechins and caffeine in samples

The standard preparation and HPLC analysis of catechins and caffeine were the same method as outlined in sections 2.4.3.2 - 2.4.3.4. The amount of catechins and caffeine in the dough and biscuit samples were calculated from the weight of samples after freeze-drying and reported as mg/biscuit.

2.5.4.2.1 Biscuits with and without sodium bicarbonate

The catechins and caffeine in dough and biscuits (n=4) were compared to investigate the effect of sodium bicarbonate on the recovery percentage of catechins and caffeine after baking. After 3 months of storage, the catechins and caffeine in biscuits were measured again to determine the stability of catechins and caffeine during storage.

2.5.4.2.2 Shortbread biscuits

The catechins and caffeine in dough and biscuits (n=12) were compared to determine the recovery percentage of catechins and caffeine after baking in biscuits with 3 levels of MGTP incorporated. After 1 month of storage, the catechins and caffeine in biscuits were measured again in order to determine the stability of catechins and caffeine during storage.

2.5.4.3 Recovery rate

Recovery studies were conducted by spiking blank biscuit extract samples with 10 µg/mL of each standard (EGCG, EGC. EC, ECG, GCG, and caffeine) with 5 replicate analyses.

2.5.5 L-theanine determination in dough and biscuits

2.5.5.1 L-theanine extraction from dough and biscuits incorporated with MGTP

Afreeze-dried sample was weighed (1 g) in a falcon tube. The sample was defatted by adding hexane to samples and incubated at 70 °C for 20 min. After that, the sample was cooled down in room temperature for 10 min, then it was centrifuged at 3000 rpm for 15 min. The hexane fraction was decanted. The sample was dried in the fume cupboard for

3 hours. The dried defatted sample was extracted with 10 mL of water and incubated at room temperature for 10 min. The sample was centrifuged at 3000 rpm for 15 min. The extract was collected. The extraction was repeated again, and the extract was collected together and the made up to a total volume of 20 mL with water.

2.5.5.2 HPLC methods for L-theanine quantification in dough and biscuits

The standard preparation and HPLC method for L-theanine in biscuits was the same as for L-theanine determination in green tea (section 2.4.4.2 and 2.4.4.3.2). Each dough and biscuit that analysed with HPLC was weighed before and after freeze drying. The amount of L-theanine in sample were calculated with the weight of samples after freeze-drying and reported as mg/biscuit.

2.6 Physical characteristics and sensory evaluation for acceptability of shortbread biscuits incorporated with MGTP

For the sensory evaluation, shortbread biscuits preparation was according to the procedure mentioned in section 2.5.2.2. The biscuits were formulated with MGTP at the level of 2, 4, 6 g per 100 g of flour and with sugar at the level of 25, 30, or 35 g per 100 g of flour.

2.6.1 Materials and equipments

2.6.1.1 Consumables for sensory evaluation

- Vacuum sealing plastic bags –The Food Machinery Company Ltd (Kent, UK)
- Morrisons® Plastic glass - Morrisons supermarket (Leeds, UK)
- Morrisons® Paper plates - Morrisons supermarket (Leeds, UK)

2.6.1.2 Apparatus for physical evaluation

- Brazier blade probe (HDP/BS) - Stable Micro Systems Ltd (Surrey, UK)
- Computerised system and sensory software (Compusense 5.6) - Compusense Inc – (Ontario, Canada)
- Mettler Toledo Infrared Moisture Analyser, (MJ33) - Mettler-Toledo Ltd (Leicester, UK)
- Texture Analyser (TA.XT*plus*) - Stable Micro Systems Ltd (Surrey, UK)

2.6.2 Physical characteristics of the shortbread biscuits

2.6.2.1 Hardness of shortbread biscuits

Hardness was measured using a texture analyser equipped with the blade set consisting of a Warner Bratzler blade, a slotted blade insert and a blade holder. Biscuits were cut using the brazier blade probe (HDP/BS). The experimental condition was 1.5mm/s of pre-test speed, 2.0 mm/s of test speed, 10.0 mm/s of post-test speed, and a 10 mm of probe travel distance. The force at break (kg) was measured. Hardness of 6 biscuits was determined for each formulation.

2.6.2.2 Diameter, thickness and spread ratio of shortbread biscuits

Biscuit width was measured by placing 5 biscuits edge-to-edge (both vertically and horizontally). The biscuits thickness was measured by stacking 5 biscuits. The measured value is divided by 5 to obtain the average value. Measurements were expressed in cm as the mean of average measured values of three different trials. The spread ratio was calculated by dividing biscuit diameter with thickness.

2.6.2.3 Moisture measurement of biscuits and weight loss during baking

The moisture content of samples was determined in four replicates for each formulation. The moisture analyser was set on the biscuit mode at drying temperature of 100 °C. An aluminium sample pan was placed in the moisture analyser. Each sample of ground biscuits and dough was weighed (1 g) in the aluminium pan. The heating module of the moisture analyser was closed to dry the samples and measure the moisture content. When drying was complete, the moisture content was recorded. The weight of biscuits was measured before and after baking to calculate weight loss during baking.

2.6.3 Ethics for sensory evaluation

Ethical approval for this study was granted by the Mathematics and Physical Sciences, and Engineering Ethical Committee at University of Leeds (MEEC 13-026). The research was conducted at the school of Food Science and Nutrition at the University of Leeds (Appendix B).

2.6.4 Sensory evaluation

2.6.4.1 Sample size

Fifty participants were recruited to attend the study. This sample size is sufficient to perform non-parametric ranking tests for preference. The recommended number of untrained assessors for product acceptability test is 25 – 100 participants (Lawless and Heymann, 2010; Lawless and Heymann, 1999).

The power calculation is listed below:

$$n = \frac{(Z)^2(\sigma)^2}{(e)^2}, \quad \text{Equation 8}$$

where n is the required sample size, Z is the value for the standard normal curve for 95% confidence (1.96), σ is the standard deviation of samples (assumed that is 2, from the hedonic scale), e is the margin of error for the sample mean (assumed that is 0.5). From the calculation, 31 participants were required. However, 54 participants were recruited to improve the test.

2.6.4.2 Recruitment

The study poster was posted on advertising boards in the Food Science building. Any interested person could contact the researcher by e-mail as informed in the poster. Interested potential participants were invited to come to the Food Technology Laboratory, where the researcher explained the study and gave the participants the information sheet and consent form (Appendix B.2) for them to read and sign.

2.6.4.3 Experimental design of sensory evaluation

Sensory evaluation of biscuits was conducted by the acceptability test with 9-point hedonic scale from 'dislike extremely' to 'like extremely'. The testing acceptability attributes were overall acceptability, appearance, aroma, colour, texture, bitterness and sweetness. All sensory evaluation's sessions were carried out in separate booths equipped with a computerised system and sensory software (Compusense Inc. Copyright © 2013), where sensory data was recorded directly by the participants.

The study was conducted to evaluate the consumer acceptability of 9 formulations of 3 levels of MGTP and 3 levels of sugar incorporated in shortbread biscuits. Samples were assigned with 3-digit codes, and the software randomised their serving orders. Plain

water was provided to rinse the mouth between samples. There were two sessions of sensory evaluation performed by the panellists. Each panelist was asked to sit in a sensory booth and a biscuit was given for a set of questionnaires. Before tasting, the panelist was asked to observe appearance and colour of a given biscuit to rate the appearance and colour acceptability. After that, the panelist tasted a given biscuit and answered the acceptability questions. At the first session, the evaluation was conducted with 54 untrained panellists with 6 biscuit samples. The second session was conducted to confirm the significant differences of the acceptability among the biscuit formulations and to construct response surface plot. Forty-six participants repeated the test in the second session with 7 biscuits samples. The same content of MGTP and sugar incorporated in the biscuits were tested in both sessions. The 4 biscuits with repeated formulation that were tested in both sessions were biscuits with 2 g of MGTP, 25 g and 30 g of sugar, with 4 g of MGTP, 30g and 35 g of sugar per 100 g of flour. The design of the sensory evaluation, abbreviated names of each formulation, coded variables, and actual variables were listed in Table 2.2.

R program (R version 3.2.5, with R Commander package) was used to plot the response surface analysis, and analyse the data for the regression coefficients used to form the mathematical models that explained the relationship between the independent variable: MGTP (X_1) and sugar (X_2), response variables: testing acceptability attributes (Y): overall, appearance, aroma, colour, texture, bitterness and sweetness of the biscuit samples. The mathematical models of the relationship between the independent variables: MGTP (X_1) and sugar (X_2), response variables on the acceptability response (Y) is listed below:

$$Y = \beta Session + \beta X_1 + \beta X_2 + \beta X_1 X_2 + \beta X_1^2 + \beta X_2^2, \quad \text{Equation 9}$$

where y is the response (acceptability); β is the parameter estimate coefficient for each linear and cross product term for the prediction model; X_1 , X_2 , X_1X_2 , are the linear terms for MGTP and sugar and their cross product terms; X_1^2,X_2^2, are the quadratic terms for MGTP and sugar.

Table 2.2: Design for the sensory evaluation study of MGTP and sugar incorporated in biscuits

Session	Experiment number and abbreviated name	Coded variable		Actual variable	
		X_1	X_2	MGTP (M) (g per 100 g of flour)	Sugar (S) (g per 100 g of flour)
1st session (54 participants)	1: 2M25S1	-1	-1	2	25
	2: 2M30S1	-1	0	2	30
	3: 2M35S1	-1	1	2	35
	4: 4M25S1	0	-1	4	25
	5: 4M30S1	0	0	4	30
	6: 4M35S1	0	1	4	35
2nd session (46 participants repeated)	7: 2M25S2	-1	-1	2	25
	8: 2M30S2	-1	0	2	30
	9: 4M30S2	0	0	4	30
	10: 4M35S2	0	1	4	35
	11: 6M25S2	1	-1	6	25
	12: 6M30S2	1	0	6	30
	13: 6M35S2	1	1	6	35

The abbreviation name for each formulation was named from the amount of MGTP, sugar incorporated and the session that biscuits were tested. The name begins with 2, 4, 6M which is the amount of MGTP per 100 g of flour in the biscuit formulations, following by 25, 30, 35S which is the amount of sugar per 100 g of flour in the biscuit formulation and the last number (1 and 2) is the session number.

2.7 Human intervention pilot study

2.7.1 Materials and equipment

2.7.2 Consumables for human study

- Antiseptic wipes (Alcotip 70% Isopropyl Alcohol Pre-Injection Swabs) – Amazon.co.uk
- Disposable lancets (Accu chek Safe T pro plus disposable lancets) - Roche diagnostic Ltd (West Sussex, UK)
- Blood glucose test strip (Accu-Chek compact 17-drum test strips) - Roche diagnostic Ltd (West Sussex, UK)
- Sterile disposable pipette (15µL Safetec Pipettes), - BHR Pharmaceutical Ltd (Nuneuton, UK)
- Triglyceride test strips (CardioChek® PTS Panel triglyceride test strips) - BHR Pharmaceutical Ltd (Nuneuton, UK)

2.7.3 Apparatus for human study

- Glucometer (Accu-Chek® Aviva blood glucose meter) - Roche diagnostic Ltd (West Sussex, UK)
- Cardiochek Professional analyser (CardioChek® PA Blood Analyser), -BHR Pharmaceutical Ltd (Nuneuton, UK)

2.7.4 Ethics

Ethical approval for this study was granted by the Mathematics and Physical Sciences, and Engineering Ethical Committee at University of Leeds (MEEC 14-040). The research was conducted at the school of Food Science and Nutrition at the University of Leeds (Appendix C).

2.7.5 Sample size and subjects

According to Brouns *et al.* (2005), 10 subjects are sufficient to calculate the GI of foods. Hence, 10 healthy adults were recruited to participate in the study.

2.7.6 Recruitment

Advertisements were put on notice boards at the School of Food Science and Nutrition and other places at the University of Leeds. Interested volunteers contacted the

researcher, and a face-to-face meeting was arranged. At the meeting, interested volunteers were given an information sheet and were asked to complete a health screening questionnaire (Appendix C.3). Questionnaires used in this study were adapted from the National Health and Nutrition Examination Survey (NHANES) and modified to reflect the cultural and socio-economic specificities of the recruitment.

Any volunteers who met the inclusion criteria (18-60 years; not allergic to any food, not pregnant or lactating, not diagnosed with chronic diseases such as diabetes, cancer, cardiovascular or digestive diseases, and not taking any medication that affect the glucose response) were asked to consider participation. Participants had the opportunity to ask questions and one week to decide whether to take part. Written informed consent (Appendix C.1) was obtained from those who decided to take part. Participants could withdraw from the study at any time during the experimental session.

2.7.7 Study protocols

2.7.7.1 Reference and test food

Three types of food meals (one reference and two test meals) were tested for this study. They were 100 g of plain shortbread biscuits with 300 mL of water, 100 g of green tea biscuits with 300 of water and 100 g of plain shortbread biscuits with 300 mL of Matcha green tea drink. The test meals contained 54-55g of average carbohydrate.

The green tea biscuit was prepared as described in section 2.5.2.2 with 6 g of MGTP incorporated in the biscuit and plain biscuits were prepared without addition of MGTP. Once the biscuits were baked and cooled down, 100 g of biscuits were weighed and packed in vacuum-seal plastic bags and used within 1 month for the human study. Matcha green tea drink was made before serving by weighing MGTP (3 g) and mixed with 200 mL of water. After consuming the Matcha green tea, another glass of 100 mL of water was provided for participant to drink.

2.7.7.2 Study design

Ten healthy adults were recruited to the study after they met the criteria from the health-screening questionnaire. The design of the study was a randomised control study without blinding. Each participant was asked to attend 3 times.

2.7.7.3 Subjects preparation

The participants were asked to arrive at the School of Food Science and Nutrition after overnight fasting (10-14 hours). Before baseline blood measurement, the participants were asked to answer the pre-study questionnaires and 24-hour food recall questions. The pre-study questionnaires were designed to gather demographic information, ethnicity, diet and fitness activity of each participant (Appendix C.4).

2.7.7.4 Glucose and triglyceride measurement

Before testing, the participant was made comfortable. Then, the baseline blood glucose and triglyceride was measured in a finger-prick blood sample. Antiseptic wipes were used to sanitise the finger before and after collecting blood. The finger was rubbed gently using a gloved hand before pricking to stimulate the blood flow and then a finger-prick blood sample was pricked using a Safe T pro Accu chek disposable lancet to obtain a blood droplet. The blood droplet was placed on the glucose test strip and inserted into a glucometer that returned the blood glucose concentration in mmol/L. After obtaining a blood droplet for the glucose measurement, the finger was held on the side and pressed to enhance the blood flow to obtain another blood droplet for the triglyceride measurement. Then a sterile disposable pipette was used to collect 15µl of blood (the black line marked on the pipette). The extracted blood was transferred to the triglyceride test strip, which was inserted, into a triglyceride meter to measure the blood triglyceride concentration in mmol/L.

After baseline fasting blood glucose and blood triglycerides were taken, then the subject was given a test food to be consumed within a period of 10 min and only water was permitted during the tests. Eight blood glucose measurements were taken at 15, 30, 45, 60, 90, 120, 150, 180 min after the start of the food consumption. Three blood triglyceride measurements were taken at 60, 120, 180 min after the start of the food consumption.

2.7.7.5 Satiety level measurement

2.7.7.5.1 Principles

Satiety can be measured via energy intake record and indirectly by using self-reported measures. Visual analogue scales (VAS) are the most commonly used method of self-reporting appetite. VAS is a simple tool that asks subjects to rate their level of hunger,

fullness, or desire to eat. The scale consists of a line, usually 100 or 150 mm, anchored from ‘extremely hungry’ to ‘extremely full’. The distance of the mark is measured to indicate the satiety levels VAS is easy to use and measure. It was found to be reproducible and valid on a short term basis, in that the satiety/hunger ratings correlate with energy intake (Benelam, 2009; Flint *et al.*, 2000). With VAS, there may be a tendency for subjects to avoid extremes. The inter-individual difference affects the satiety measurement. Hence, a within-subject (cross-over) design is preferable, and a test meal or a control meal is given in each study session, followed by the VAS self-reporting at each selected interval. VAS is completed at intervals throughout a study session to monitor change in appetite.

2.7.7.5.2 Protocols

Participants answered the satiety questionnaires before meal (0 min) and 15, 30, 45, 60, 90, 120, 150 and 180 min after the start of the meal consumption. A VAS was used to assess the participants’ satiety scoring scale from ‘extreme hunger’ to ‘extremely full’.

After participants had finished with blood measurement and satiety questionnaires, they were provided with a light breakfast. Participants were asked to repeat the procedure above another two times to gain information of each food sample (eg., plain biscuit, green tea biscuit, plain biscuit and green tea drink). The order, in which the food sample provided, was randomised according to the Latin-square calculation for each participant (the table of randomisation is provided in Appendix C.2).

2.7.7.6 Incremental area under the curve calculation

The incremental area under the curve (iAUC) of the glucose response was calculated according to FAO/WHO1998, using the trapezoidal rule where all the areas of GRs collected during the three hours period are added together, by ignoring area beneath the baseline. As shown in Figure 2.1, the area segment 7 and 8 are below the baseline, which means these areas will be ignored.

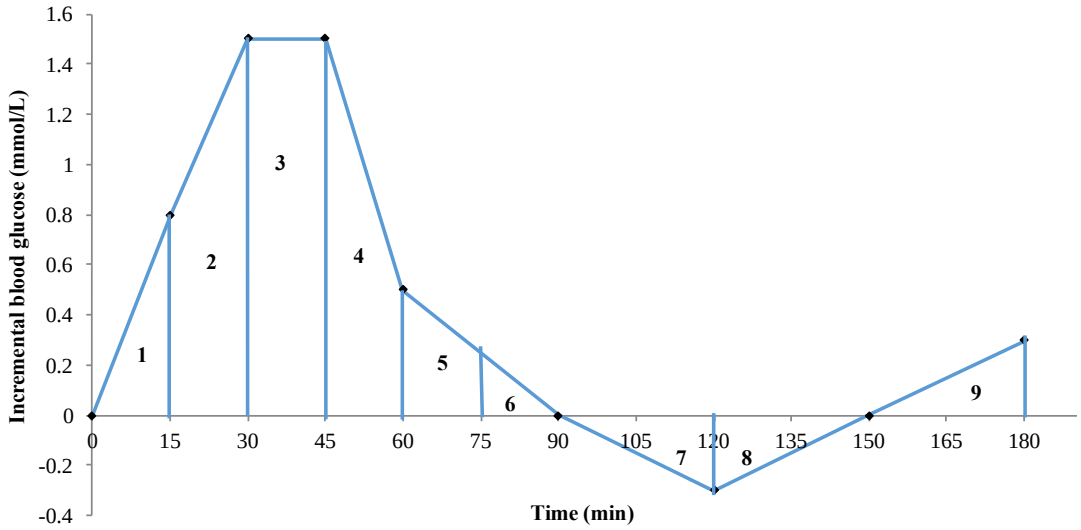


Figure 2.1: Calculation of iAUC

Accordingly, the iAUC can be calculated using the following equations:

For each area under the curve,

$$A_1 = (G_0 + G_1)/2 \times (T_1 - T_0)$$

$$A_2 = (G_1 + G_2)/2 \times (T_2 - T_1)$$

$$A_3 = (G_2 + G_3)/2 \times (T_3 - T_2), \text{ continue to } A_4 \ldots A_9$$

$$iAUC = A_1 + A_2 + A_3 + A_4 + A_5 + A_6 + A_9, \qquad \text{Equation 10}$$

where, G is the glucose concentration in mmol/L, T is the time in minutes, and A is the area under curve of the glucose response of certain food. Assuming that at time T_0, $T_1 \ldots T_9$ (equaling 0, 15 ...180 min, respectively), the blood glucose concentration are, G_0, $G_1 \ldots G_9$, respectively, where A_x is the iAUC for the X_{th} time interval, and the X_{th} time interval is the interval between times T_{x-1} and T_x (Brouns *et al.*, 2005). The same calculation is applied for iAUC of triglyceride response and satiety level. The mean, standard deviation, standard error, and coefficient of variation of iAUC were calculated.

2.8 Statistical analysis

IBM SPSS Statistics software version 22 was used for statistical testing.

2.8.1 Statistical analysis for comparison of total polyphenol and antioxidant activity of green tea product

Results were analysed statistically to determine mean values and standard deviations (STDs). The one tail t-test was conducted to determine the difference between total polyphenol and antioxidant activity of green tea products (MGTP and GTEP).

2.8.2 Statistical analysis for the effect of extraction time on total polyphenol extracted

Analysis of variance (ANOVA) and Tukey's-b *post hoc* test were performed to determine the effect of extraction time (5, 10, 15, 20, 30 min) on polyphenol extracted which were determined by Folin-Ciocalteu assay.

2.8.3 Statistical analysis for HPLC of catechins

Results were analysed statistically to determine mean values, STDs, and standard error of means (SEMs) of quantified masses of compounds obtained from HPLC-PDA in duplicate runs. The total catechins content was presented by the sum of the amounts of individual catechins (EGCG, ECG, EGC, GCG, catechin and EC). ANOVA and Tukey's-b *post hoc* test were performed to determine the difference between the amount of catechins in each pack of MGTP and the stability of catechins over storage time at room temperature. Differences with $P < 0.05$ were considered significant. Mann-Whitney U Test was performed to determine the difference between before and after baking, and after baking and after 1 month storage.

2.8.4 Statistical analysis for HLPC of L-theanine

Results were analysed statistically using SPSS Statistics to determine mean values, STDs, and SEMs of quantified masses of compounds obtained from HPLC analysis. Mann-Whitney U Test was performed to determine the difference between the amount of L-theanine determined with the different detector (ELSD vs. PDA), extraction time, extraction temperature, in dough and biscuit, and during 1 month storage.

2.8.5 Statistical analysis for physical characteristic and acceptability of biscuits

Results were analysed statistically to determine mean values and STD. One-way ANOVA and Turkey's-b *post hoc* test was performed to determine the difference in scores between biscuits' formulation. Two-way ANOVA and Turkey's-b *post hoc* test was conducted to determine the difference of the acceptability between MGTP levels and sugar levels and their interaction effects.

2.8.6 Statistical analysis for the human intervention pilot study

Results were analysed statistically using to determine mean values, STDs, and SEMs of glucose response, triglyceride response and satiety levels. ANOVA and Turkey's-b *post hoc* was conducted to investigate the difference between test meals and demographic variation factors between the subjects that may affect the glucose response, triglyceride response and satiety levels.

3 Chapter three: Determination of total polyphenols, individual catechins, caffeine and L-theanine in green tea products

3.1 Introduction

Catechins are the most important components in green tea that contribute to health effect. The determination of catechins content in MGTP and GTEP is required to select the suitable green tea product to incorporate to biscuits in order to study the stability of catechins, the acceptability and health effects of biscuits. The Folin-Ciocalteu assay that used to measure the total polyphenol of samples, while FRAP assay is used to measure the antioxidant capacity of samples. Hence, FRAP was used to ensure the results of total polyphenol content of samples from Folin-Ciocalteu assay, as the result of both assay should be correlated.

Catechins and caffeine are the secondary metabolites of tea for a defence mechanism. These compounds reduce oxidative stress in plants and provide the plants with resistance to pathogens and predators in order to protect it from environmental condition. Hence, the content of caffeine and catechins is varied depending on the environmental conditions (Ahmed and Stepp, 2013; Ames *et al.*, 1990). For example, Unachukwu *et al.* (2010) revealed that the total catechins content found in various types of green tea were 10-fold different from 21.38 to 228.20 mg/g of dried tea leaves which were extracted by water infusion. The same green tea types that came from a different producer were shown to have different amounts of catechins and antioxidant activity (Unachukwu *et al.*, 2010). Therefore, the content of catechins and caffeine in different packages of MGTP should be determined to observe the variation in catechins and caffeine content.

Additionally, there have been few reports on the quantification of L-theanine content in MGTP. L-theanine has been identified by different detectors (ELSD and PDA), different HPLC elution methods, different extraction times and temperatures. There is no standard method used for L-theanine quantification in the literature. A suitable method for L-theanine was therefore developed for this research.

3.1.1 Aim

I. To compare the total polyphenols, antioxidant capacity and composition of catechins and caffeine in green tea products (MGTP and GTEP)

II. To optimise the HPLC condition in order to find the suitable method in L-theanine determination in MGTP

3.1.2 Objectives

- To measure the total polyphenol and antioxidant capacity of MGTP and GTEP using Folin-Ciocalteu assay and FRAP assay
- To meausure the amount and composition of catechins and caffeine in green tea products using HPLC
- To optimise the maximum extraction time of polyphenols from MGTP
- To optimise the method used to determine L-theanine by investigating the differences in detectors used; PVPP treated and untreated samples, HPLC elution, extraction time and temperature

3.2 Results

3.2.1 Comparison of total polyphenols and antioxidant activity of green tea products using Folin-Ciocalteu assay and FRAP assay

As shown in Figure 3.1, the GTEP extract had higher total phenolic content expressed as gallic acid equivalent (GAE) µg/mg of samples (206±12 µg/mg) and higher antioxidant activity expressed as Trolox equivalent (TE) µg/mg of samples (464 ±32µg/mg) than extracts of MGTP (total phenolic content: 132±2 µg/mg and antioxidant activity: 315±17 µg/mg). The calibration curves of Folin-Ciocalteu assay and FRAP assay were plotted to calculate the total polyphenols and antioxidant activity of MGTP and GTEP (Appendix A.1, Figure 1 and 2).

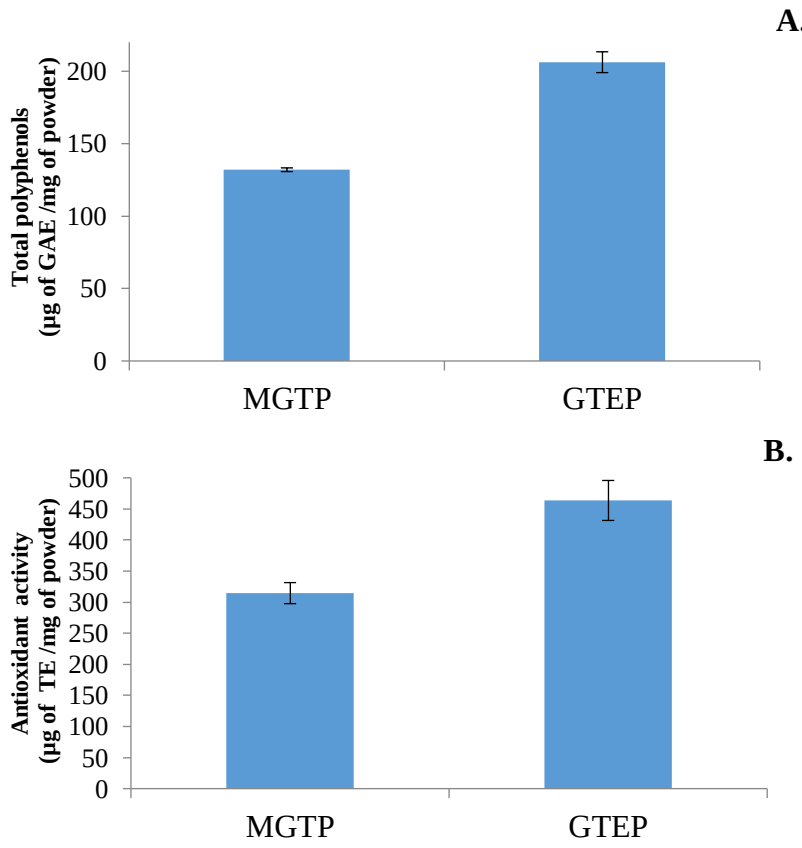


Figure 3.1: A. Comparison of total polyphenol between MGTP and GTEP extracts, using Folin-Ciocalteu assay. Error bars represent the STD (n=3 extractions) B. Comparison of antioxidant activity between MGTP, and GTEP extracts using FRAP assay. Error bars represent the STD (n=3 extractions).

3.2.2 Effect of extraction time on total polyphenols extracted

The data from Folin-Ciocalteu assay (Figure 3.2) indicated that the increased extraction time did not significantly lower the total polyphenol extracted from MGTP according to ANOVA (P >0.05).

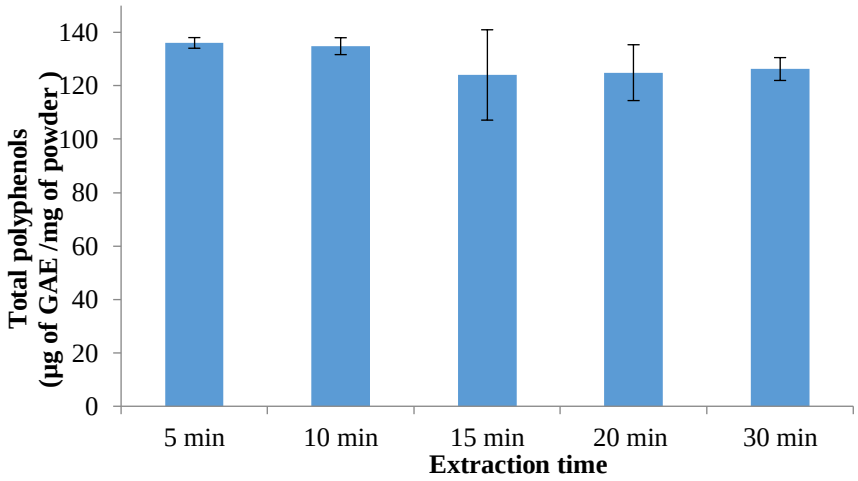


Figure 3.2: Comparison of extraction time on the total polyphenol of MGTP, using Folin-Ciocalteu assay and the error bars represent the STD (n=3 extractions).

3.2.3 HPLC analysis of catechins and caffeine in MGTP and GTEP

The catechins and caffeine were detected by HPLC with UV-Vis detector at a wavelength of 275 nm. The chromatogram of standards and samples is presented in Figure 3.3.

The retention times of 6 catechins, gallic acid and caffeine are summarised in Table 3.1. All catechins and caffeine were well resolved and eluted within 30 min of running time.

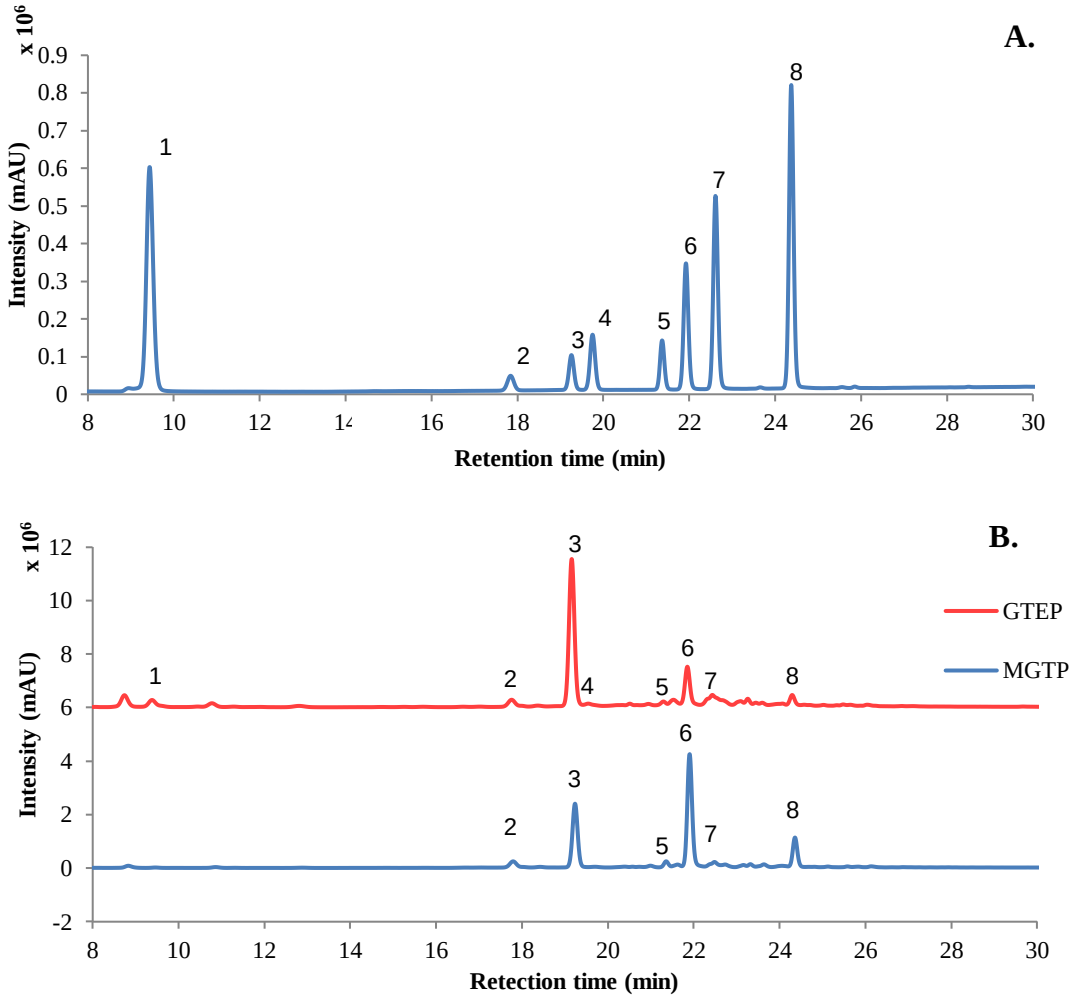


Figure 3.3: A. Chromatograms of standards mixture at a concentration of 10ug/mL per standard. B. Chromatograms of MGTP and GTEP samples. mAU is the intensity units of UV-Vis detector. Detection was performed with UV at a wavelength at 275 nm. Peak identification and approximate retention times in parentheses are as follows: 1. Gallic acid (9.3 min); 2. EGC (17.4 min); 3.Caffeine (19.0 min); 4.Catechin (19.4 min); 5. Epicatechin (21.4 min); 6.EGCG (21.9 min); 7.GCG (22.4 min); and 8.ECG (24.4 min), separated by Phenomenex C18 reverse phase column 5 µm, 250 × 4.6 mm.

3.2.3.1 Validation of HPLC method for catechins and caffeine

Precision at each standard concentration was presented as % coefficient of variation (CV) of a measured peak area from mean peak area. LODs and LOQ were determined based on the concentration of standards and results were expressed as detectable and quantifiable concentrations in µg/mL, as shown in Table 3.1.

The calibration curves of each compound were plotted and are presented in Figure 3 in Appendix A.2. All the calibration curves were forced through zero. Good linearity was observed for all catechins, gallic acid and caffeine (R^2 >0.99). The LODs and LOQs of 8 standards were found to be in the range 0.03 µg/mL to 7.11 µg/mL and 0.09 µg/mL to 23.71 µg/mL, as summarised in Table 3.1. Intra-day analysis of a standard mixture of compounds ranged between 0.48-0.70% for repeatability precision experiments, while inter-day analysis of the same standards over 2 different days yielded %CV ranging from 0.60 to 2.56%. The percent recovery of standards spiking in MGTP extract samples was expressed in Table 3.2, ranging from 95.3% to 109.8%.

Table 3.1: Data for retention time, linearity, LOD and LOQ of catechins, gallic acid and caffeine

Analytes	Retention time	Precision of retention time (%CV)	Range (µg/mL)	Slope	R^2	LOD (ug/mL)	LOQ (ug/mL)	Precision Intra-day (%CV) n=5	Precision Inter-day (%CV) n=10
Gallic acid	9.3±0.01	0.11	1-20	9519.2	0.9998	0.03	0.09	n.d.	n.d.
EGC	17.4±0.03	0.17	2.5-80	408.06	0.9992	7.11	23.71	0.48	1.48
Catechin	19.4±0.02	0.10	1-20	1403.6	0.999	0.51	1.71	0.65	2.20
EC	21.4±0.03	0.14	2.5-50	1382.2	0.9994	1.11	3.70	0.64	2.00
ECGC	21.9±0.01	0.07	2.5-100	2492.2	0.9997	1.83	6.12	0.49	2.56
GCG	22.4±0.03	0.12	1-40	3978	0.9997	0.70	2.32	0.63	0.60
ECG	24.4±0.04	0.17	2.5-15	5513.4	0.9998	0.40	1.33	0.70	0.94
Caffeine	18.8±0.02	0.33	1-100	5975.8	0.9999	0.85	2.72	n.d.	n.d.

Table 3.2: Percent recovery of each catechin (10 µg/mL) in MGTP extract samples

Analytes	%Recovery of spiking
EGC	102.1±7.4
Catechin	103.3±2.5
EC	99.5±6.5
EGCG	102.4±1.5
GCG	95.3±0.6
ECG	109.8±1.9

3.2.4 The effect of time on catechin extractions using HPLC analysis

Table 3.3 and 3.4 show the comparison of catechins content and caffeine content at different extraction times. A slight degradation occurred with prolonged extraction (10, 15, 20, 30 min). However, the difference of each extraction time was not significant using ANOVA analysis (P>0.05). The standard deviation and %CV of prolonged extraction time tend to be smaller than the shorter time extraction as shown in Table 3.3 and 3.4.

Table 3.3: Comparison of concentrations measured by HPLC of individual catechins and caffeine extracted for different times (n=3).

Analytes	Time of extraction (min)	Average (µg/mg)	Precision (%CV)
EGC	5	36.22±5.17	14.28
	10	33.24±3.54	10.66
	15	33.36±3.50	10.50
	20	33.02±2.35	7.13
	30	34.26±1.53	4.45
EC	5	5.16±0.65	12.62
	10	4.83±0.37	7.75
	15	4.82±0.65	13.48
	20	4.71±0.14	2.96
	30	4.82±0.10	2.16
EGCG	5	67.21±8.77	13.05
	10	62.10±5.92	9.53
	15	61.76±7.10	11.49
	20	61.57±4.18	6.78
	30	63.05±2.83	4.48
GCG	5	1.24±0.16	12.59
	10	1.22±0.11	9.04
	15	1.21±0.16	13.30
	20	1.16±0.12	10.03
	30	1.26±0.06	4.96
ECG	5	7.27±1.00	13.78
	10	6.73±0.59	8.72
	15	6.72±0.79	11.79
	20	6.65±0.45	6.84
	30	6.81±0.31	4.56

Continuation of Table 3.3

Analytes	Time of extraction (min)	Average (µg/mg)	Precision (%CV)
Caffeine	5	20.49±2.60	12.67
	10	19.03±1.84	9.69
	15	18.98±2.20	11.60
	20	18.84±1.26	6.70
	30	19.44±0.83	4.27

3.2.5 Quantification of catechins and caffeine in MGTP and GTEP

The content of catechins and caffeine of MGTP was analysed from 3 packages and the results shown in Table 3.4. ANOVA and Tukey's-b *post hoc* test were applied to determine the statistical difference of each catechin and caffeine in different packages of MGTP. The content of EGCG in each package was significantly different. The average total catechins content in MGTP was 107 µg per mg of powder. Table 3.5 shows the content of catechins and caffeine in GTEP. The total catechins content found in GTEP was 87 µg per mg of powder.

Table 3.4: The content of catechins and caffeine in 3 packages of MGTP with intra-day and Inter-day precision analysed by HPLC

Analytes/Package		Average (µg/mg) (n=9)	Precision (% CV)	
			Intra-day, (n=3)	Inter-day, (n=9)
EGC	1	40.39±6.95	17.07	17.21
	2	34.52±9.15	19.46	26.50
	3	23.63±1.94[a]	6.17	8.19
Catechin	1	0.84±0.13	15.88	16.01
	2	0.64±0.25	27.60	39.45
	3	0.19±0.06[a]	20.86	32.74
EC	1	5.83±0.93	15.70	16.02
	2	5.88±1.51	19.29	25.72
	3	5.53±0.28	4.93	5.14
EGCG	1	76.86±13.56	17.29	17.64
	2	55.55±15.08[a]	18.86	27.14
	3	44.22±2.51[b]	5.47	5.67
GCG	1	2.50±1.17	20.77	46.97
	2	1.55±0.55[a]	16.95	35.77
	3	1.90±0.20[a]	7.83	10.40
ECG	1	8.28±1.42	17.98	17.20
	2	6.29±1.61[a]	18.82	25.57
	3	6.25±0.60[a]	4.95	9.56
Caffeine	1	19.48±3.17	16.60	16.27
	2	15.85±4.25	18.80	26.78
	3	5.30±0.38[a]	4.73	7.14

The letter (a and b) above the average content of each catechin and caffeine indicates a significant difference content of each package (P<0.05).

Table 3.5: The content of catechins and caffeine in GTEP

Analytes	Amount (µg/mg) (n=9)	Precision (%CV)	
		Intra-day (n=3)	Inter-day (n=9)
Gallic acid	3.14± 0.26	8.29	8.30
EGC	41.48±3.40	6.60	8.20
Catechin	1.90±0.59	22.90	30.80
EC	4.41±0.88	15.19	19.99
EGCG	30.70±2.28	7.73	7.44
GCG	5.07±1.01	14.38	19.99
ECG	3.73±0.26	7.02	6.90
Caffeine	51.61±4.04	7.72	7.82

Figure 3.4 shows the regression line and positive correlation between caffeine content (x-axis) and total catechins content (y-axis) (A), and between caffeine content and EGCG content (y-axis) (B) of each sample measured. The results indicate a linear relationship between caffeine content and total catechins content, and between caffeine content and EGCG content in MGTP samples. The coefficients of correlation were 0.83 between caffeine content and total catechins and 0.80 between caffeine content and EGCG content. The coefficient of correlation was close to 1, which indicates the strong relationship between these variables. Because the EGCG content accounts for about 50% of total catechins, therefore, the regression line of Figure 3.4A and B look similar.

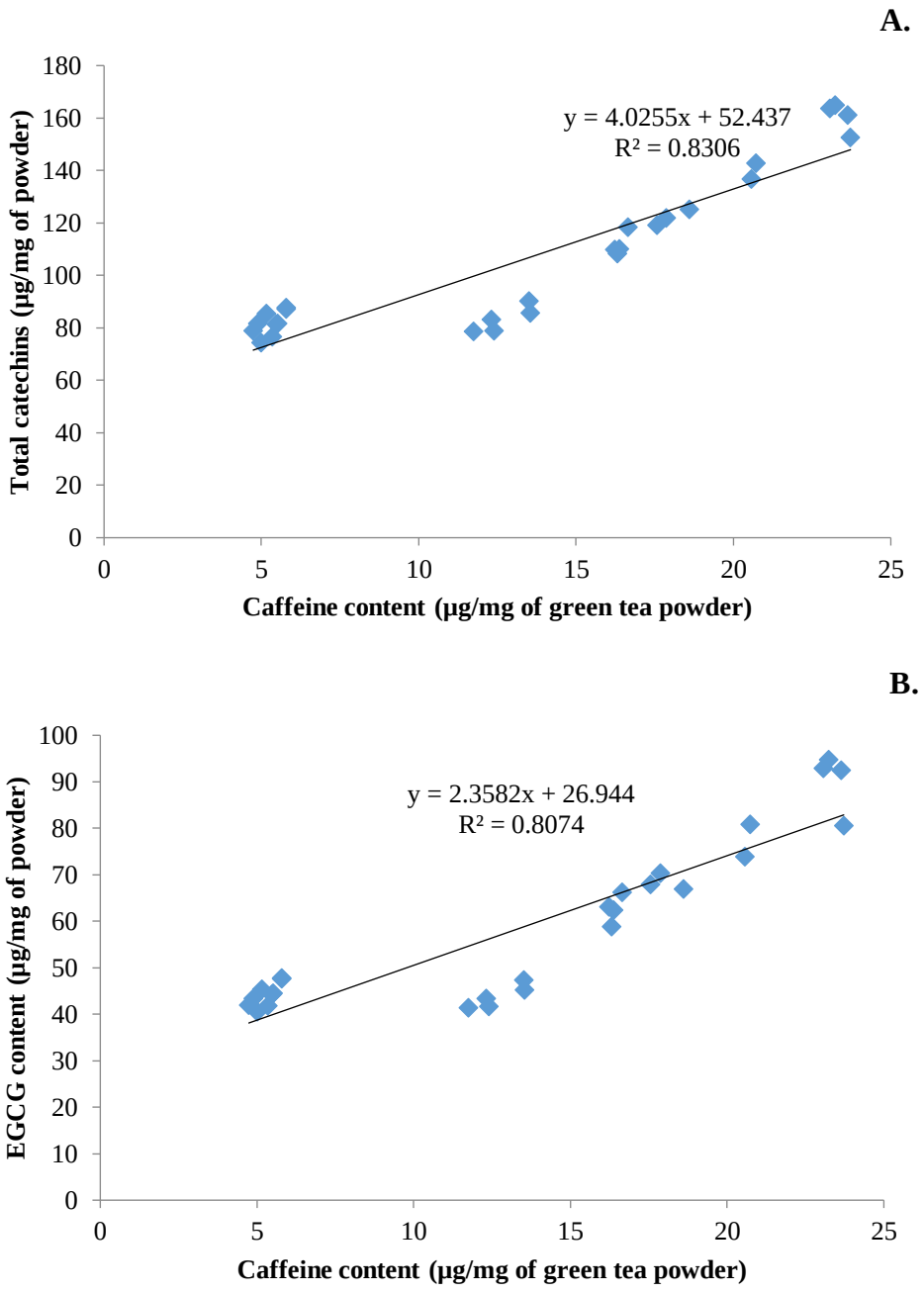


Figure 3.4: A. Correlation between caffeine content and total catechins content. B. Correlation between caffeine content and EGCG content from 3 packages of MGTP.

3.2.6 Stability of catechins during storage

Figure 3.5 shows the change in total catechins content (A) and individual catechins and caffeine (B) in MGTP over 3 months and 5 months after the package was opened. According to ANOVA, there was no significant difference in the total catechins and each major catechin between opening the package, 3 months and 5 months storage in the package.

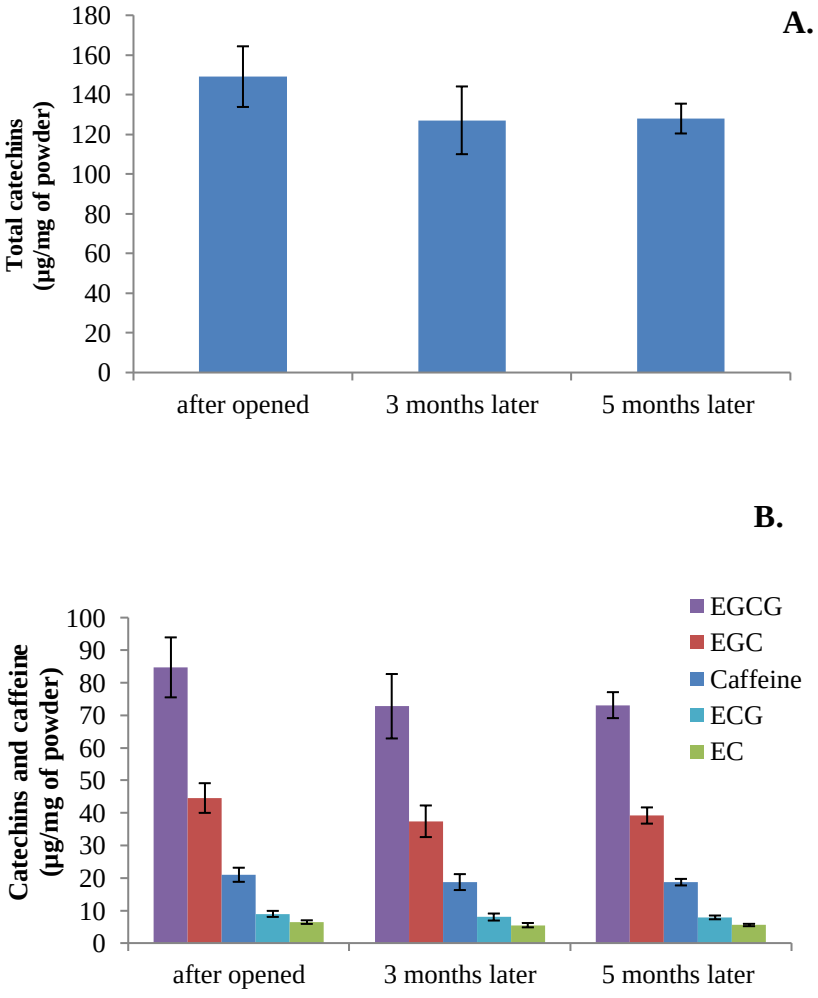


Figure 3.5: A. The stability of total catechins in MGTP. B. The stability of individual catechins and caffeine in MGTP measured by HPLC during storage at room temperature over 3 and 5 months. The error bars represent the SEM (n=3)

3.2.7 Determination of L-theanine in MGTP

Two different detectors, which are photodiode array (PDA), and evaporative light scattering detector (ELSD) used to measure L-theanine standard. The chromatogram of L-theanine standard detected by both ELSD and PDA detector is shown in Figure. The chromatogram of L-theanine detected by ELSD showed that a lower temperature of drift tube provided a lower peak height of L-theanine.

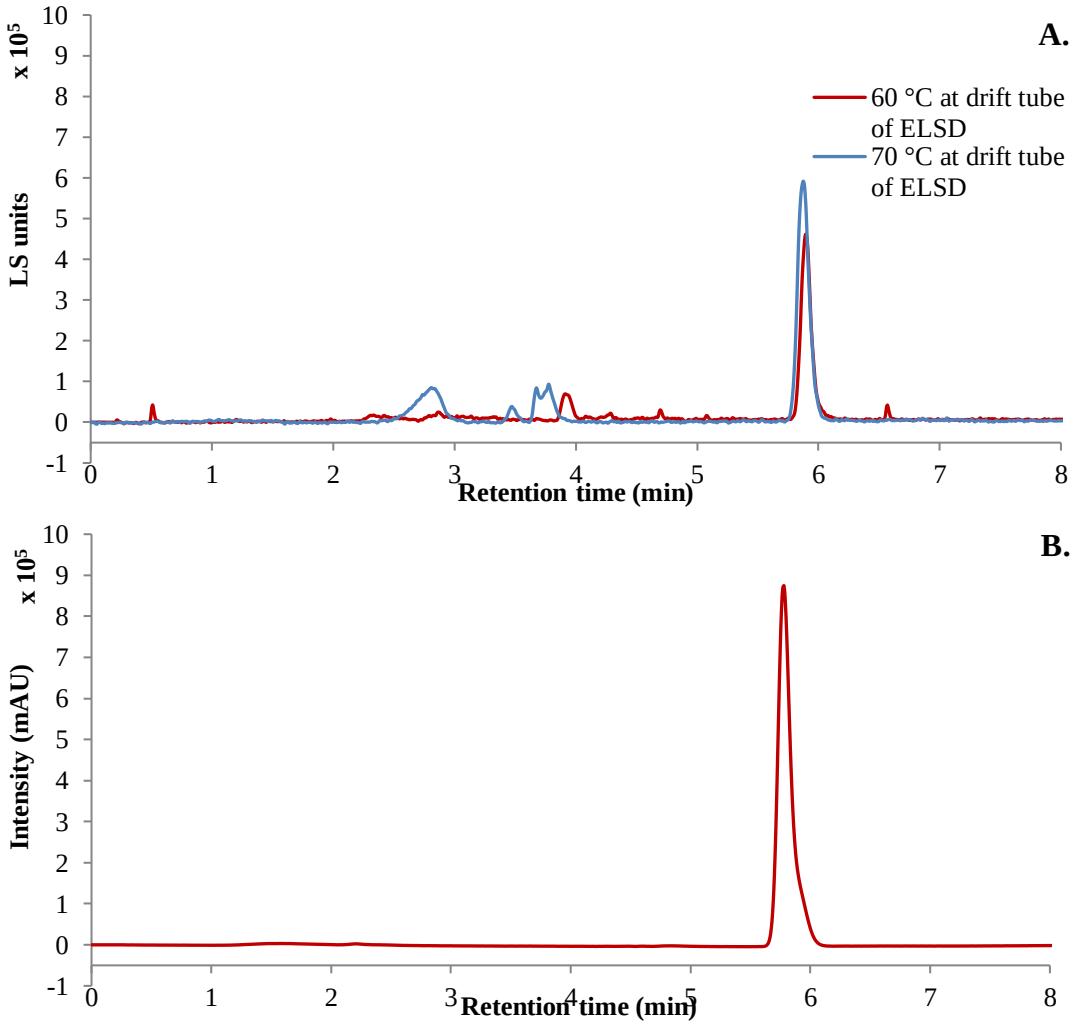


Figure 3.6: A. HPLC chromatogram of the L-theanine standard solution (10µg/mL) using isocratic water elution with C18 column, flow rate: 0.8 mL/min, injection volume: 30 µL, detected with ELSD, detector drift tube temperature 60 and 70°C, gas pressure 350 kPA, signal gain: 10. *LS unit: light scattering unit. B. HPLC chromatogram of the L-theanine standard solution (10µg/mL) using isocratic water elution with a C18 column, flow rate: 0.8 mL/min, injection volume: 30 µL, detected with PDA detector, at 200 nm, mAU is the intensity units of PDA detector.

3.2.7.1 Comparison of between ELSD and PDA detector used for L-theanine determination in MGTP

The chromatogram of L-theanine in MGTP samples detected by ELSD and PDA detector is shown in Figure 3.7. The L-theanine was quantified and the result showed that the ELSD and PDA detector did not have a significant difference in determining the L-theanine content (Appendix A.3.1, Figure 6). The calibration curve obtained from the ELSD detector was a polynomial curve (Appendix A.3, Figure 4) which was not suitable for quantification. The logarithmic values of x and y values were required to obtain the linear calibration curve. Due to this, the PDA detector was chosen to detect the L-theanine because the detector produced linear calibration curve, which can be used directly to calculate the L-theanine content (Appendix A.3, Figure 5). The linearity study of calibration curves used to determine L-theanine content with different HPLC elution and detector is shown in Appendix A.3, Table1.

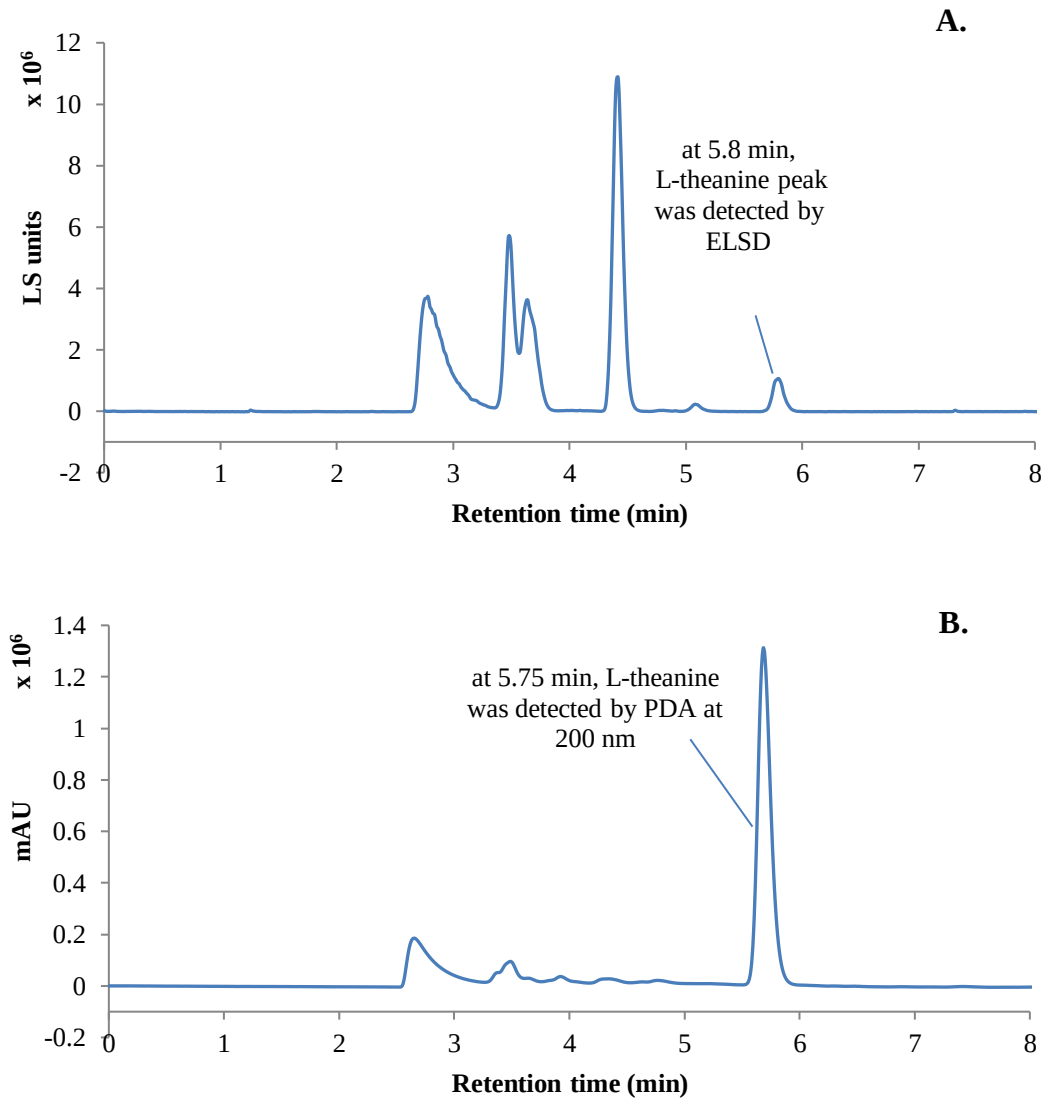


Figure 3.7: A. HPLC chromatogram of L-theanine in the MGTP extract (100 mg of powder in 20 mL of water), detected with ELSD detector, detector drift tube temperature 70°C, gas pressure 350 kPA, signal gain: 10, *LS unit: light scattering unit. B: HPLC chromatogram of L-theanine in the MGTP extract (100 mg of powder in 20 mL of water) detected with PDA detector, at 200 nm, mAU is the intensity units of PDA detector.

3.2.7.2 Comparison of PVPP pre-treatment with isocratic and binary elution of HPLC

The binary HPLC method with samples that were not treated with PVPP prior to injection gave significantly higher L-theanine (3.84± 0.05µg/mg of powder, average of 3 extraction samples) content than the isocratic HPLC method with PVPP pre-treatment of samples (3.19±0.05 µg/mg of powder) at 5 min extraction time point (Appendix A.3, Figure 7). There was no significant difference in L-theanine content with different extraction time (5, 10, 15, 20 min). Based on this result, the HPLC binary elution without PVPP treatment on samples was chosen to determine L-theanine content.

3.2.7.3 Precision of the method (Binary gradient run with PDA detector)

The precision of the HPLC binary gradient method for L-theanine was assessed by intra-day and inter-day injections of the same sample (n=4). Four samples were analysed per day and on the three consecutive days. Intra-day and inter-day variations were 0.98% and 1.39%, respectively, showing the precise performance of the method. The recovery of L-theanine spiking in the extract was 100.61% with 1.03 % intra-day variation of 3 extracted samples, which indicates a good accuracy of this method and was in accordance with recovery values of the previously published methods (Csupor *et al*, 2014).

3.2.7.4 Effect of extraction temperature on L-theanine determination

The comparison of extraction temperature on L-theanine determination between room temperature (18 °C) and 80 °C temperature was determined using the HPLC binary elution on the sample without use of PVPP. The room temperature and 80 °C temperature extraction did not have a significant effect on L-theanine determination. With room temperature extraction (18 °C), the average L-theanine content found in MGTP was 3.85±0.11 µg/mg of powder and 3.77± 0.10 µg/mg with 80 °C temperature extraction**.**

3.3 Discussion

3.3.1 Total polyphenols and catechins in MGTP and GTEP

According to Folin-Ciocalteu assay and FRAP assay (Figure 3.1), GTEP had a higher total phenolic content expressed as gallic acid equivalent (GAE) µg/mg of samples and a higher antioxidant activity expressed as Trolox equivalent (TE) µg/mg of samples than MGTP. However, the results obtained from HPLC-PDA method showed that MGTP had a higher EGCG, EC, and ECG content than GTEP. From HPLC analysis, GTEP had a higher caffeine and GCG content than MGTP. Moreover, gallic acid was found in GTEP, but was not found in MGTP. The average total catechins (sum up the catechin content analysed by HPLC) found in MGTP was 107 µg/mg of powder and in GTEP was 87 µg/mg of powder. According to Folin-Ciocalteu assay, GTEP contained 206 µg/mg of total phenolic content expressed as GAE. Therefore, GTEP contained other phenolic compounds besides catechins that contribute to higher total phenolic content and higher antioxidant activity in Folin-Ciocalteu assay and FRAP assay.

Even though MGTP and GTEP are both a powder form of green tea product, the processing of these products is the key to the difference in polyphenol content. MGTP is made by using stone grinding of dried tea leaves at 20 ℃ and friction temperature during stone grinding is controlled to be 40-50 ℃. Therefore, the polyphenol content of MGTP is retained due to the low-temperature processing. Whereas, the GTEP is produced by extracting polyphenols in the solvent (mainly alcohol and water) and produces the powder by evaporating the solvent, often using a high-temperature processing method such as spray drying. The spray drying processing is required to evaporate the extracting solvent in order to produce green tea extract powder. Hence, high heat of spray drying (100-150 ℃) may decompose the catechins or epimerise the compounds, resulting in higher gallic acid and GCG (the epimerized form of EGCG) content in GTEP. Moreover, Folin-Ciocalteu assay and FRAP assay are spectrophometric methods which are non-specific. For instance, Everette *et al.* (2010) indicated that the Folin- Ciocalteu reagent was found to be reactive towards many compounds such as thiols, many vitamins, the nucleotide base guanine, dihydroxyacetone and some inorganic ions. Therefore, the reagent may interact with

some compounds in GTEP, which lead to over-estimation of total polyphenol content and antioxidant capacity of GTEP.

The average total phenolic content of MGTP was 132 μg/ mg of MGTP determined by Folin-Ciocalteu assay. While, total catechins content analysed by HPLC-PDA method was 107 μg/ mg of MGTP (calculated by summing up all the catechins analysed). The result indicated that most of the phenolic content found in MGTP was mostly catechins, which were made up to approximately 10% of dry weight of the material. There are a few published studies on the content of catechins in MGTP. For example, Weiss and Anderton (2003) used micellar electrokinetic chromatography to separate and quantify the catechins in MGTP. The value was presented in mg catechin per g of powder. The average value of ECGC found in MGTP was 5.4% of dry weight. Another study by Nishitani and Sagesaka (2004) used HPLC method to analyse the catechin in MGTP and found the average EGCG in MGTP accounted for 5.24% dry weight. Both studies show a similar content with to this study result on MGTP. Moreover, Komes *et al*. (2010) extracted 2 g of Matcha tea with 200 mL of distilled water at 80 °C for 3 min. The result from Komes *et al*. (2010) found 3.5% of EGCG and 9.9 % of total catechins in Matcha tea. However, the result of EGCG in MGTP in this study (ranged from 4.3 – 7.8% of EGCG in dry weight) was higher than EGCG found in Matcha tea (3.5% of EGCG) used in Komes *et al*. (2010).

Some difference in values of other catechins (EGC, ECG, ECG, EC) were found in the literature, and compared to the result obtained here, which could be due to different extracting condition, such as extracting solvent, pH of extracting solvent, extraction time and temperature and different source of the tea. Cheong *et al*. (2005) reported a higher extraction yield of tea in the powdered form (at 100 ^{o}C), compared with loose leaf tea, because the smaller size of green tea powder provided a larger surface area, thus providing a faster extraction efficiency of various tea constituents.

Each of catechin has its favourable extraction conditions (Labbe *et al.*, 2006). Labbe *et al.* (2006) suggested categorising catechins into two groups according to their behaviour during brewing. The first group of was time dependent catechins (such as EGC and EC), and another group was time/temperature dependent catechins (EGCG, GCG, and ECG). They proposed a two-step extraction procedure in order to maximise catechins extraction efficiency. They suggested that a decrease of EGCG occurs at 90 °C.

Therefore, their experiment was carried out with two extraction procedures; the first extraction was brewing tea at 30 °C for 30 min. This procedure aimed to obtain the maximum amount of EGC, and most of the EGCG remained in the leaves. Then, the second extraction was to extract the maximum amount of EGCG, which was carried out at 75 ℃ for 40 min. They found that the EGCG content in the second extraction was higher than the first extraction by 41-55.5%. The study suggested the catechins have a favourable temperature and time that maximise the content of catechins extracted in water during brewing. Hence, the comparison of the results with literature may be difficult due to the lack of uniformity in the conditions used to prepare tea infusion (ratio leaves/water, extraction time, water temperature) and the value of the units of the results.

3.3.2 The effect of extraction time on release of polyphenols and individual catechins

Zimmermann and Gleichenhagen (2011) revealed that the prolonged steeping time of green tea infusion resulted in a higher content of catechins released from tea leaves to water. They found that tea leaf infusion at 100 ℃ for 7 min gave a three-fold increase in catechins in comparison with infusion at 70 ℃ for 3 min. In addition, reduction of pH in infused water increased the EGCG released. They found that addition of lemon juice (pH=3.0) increased EGCG extracted by 20%. Hence, in this study, different time extraction with an acidic extracting agent (70% methanol with 0.3% Formic acid) at 70 ℃ was tested to monitor the effect of prolonged extraction time to see whether this factor could increase or decrease the amount of catechin extracted.

The result (Figure 3.2) from the comparison of extraction time on the total polyphenols (expressed as GAE) using the Folin-Ciocalteu assay showed that 5 min extraction yielded the highest content of total polyphenols with the smallest variation in value than the other extraction times. The result was in agreement with a previous study which found that a higher amount of phenolic compounds and flavonoids in the first 5 min of extraction of green tea (Rusak *et al.*, 2008). On the other hand, El-Shahawi *et al.* (2012) studied the influence of infusion time (5, 15, 30 min) on the content of catechins and caffeine released. The study found that the rate of release of green tea catechins and caffeine into boiling water increased with prolonged infusion time. The amount of

EGCG significantly release increased on longer infusion time in boiling water with 90 °C incubation. The ECGC content after 5 min infusion time was 7 mg/g of tea, while after 30 min infusion time, 25 mg/g of tea was released (El-Shahawi *et al.*, 2012). The observed data suggested that catechins and caffeine had a greater extraction from green tea leaves with the prolonged infusing time up to 30min.

Table **3.3** showed the comparison of major catechins content measured by HPLC and extracted with different extracting times. There was no significant difference in the content of major catechins (EGCG, EGC, ECG and EC) in prolonged extraction times. The result of the content of total polyphenols from Folin-Ciocalteu assay and HPLC analysis were in an agreement that 5 min extracting time yielded the highest average catechins extracted from MGTP. This finding was in accordance with Komes *et al.* (2010) who suggested that extraction efficiency of studied catechins from green tea depends on the extraction conditions and type of green tea. Their study found that maximum extraction efficiency of an aqueous extraction at 80 °C, was 5 min for Matcha powder, 15 min for bagged green tea and 30 min for loose leaf green tea. Moreover, the finding in this chapter revealed that prolonged extraction time resulted in slightly lower total catechins content in MGTP aqueous extract. Perva-Uzunalic *et al.* (2006) found that catechins in green tea powder were likely to degrade during longer extraction time, along with using a higher water temperature for extraction. The maximum extraction efficiency of catechins with water was obtained at 80 °C after 20 min (97%) and at 95 °C after 10 min of extraction (90%), higher extraction temperature and prolonged extraction time resulted in degradation of catechins observed (Perva-Uzunalic *et al.*, 2006). Another study by Cheong *et al.* (2005) also showed that prolonged extraction time may degrade the catechin content. The study compared the catechins extraction rate between green tea powder and green tea leaves using water at 100 °C and found that the extraction rate of catechins of green tea powder was higher than of green tea leaves at the same extraction time. However, the decomposition of catechins from green tea powder was faster than that of green tea leaves as the content of catechins from the powder was lower than the content from green tea leaves after 30 min of extraction (Cheong *et al.*, 2005). Their findings were in agreement with the results in this study that prolonged extraction time at high temperature can lead to catechin degradation.

3.3.3 Relationship between caffeine and catechins content in MGTP

The result found that the content of catechins in different packages of MGTP was significant different (Table **3.4**). The content of catechins in MGTP in tea leave is varied due the cultivation area and sunlight exposure. The older tea leaves tend to have higher catechins content than younger leaves, because more sunlight exposure lead to development of catechins (Vuong *et al.*, 2011).

Our result showed that the content of caffeine found in MGTP samples was positively correlated with total catechins and EGCG content (Figure 3.4). The catechins content also is affected by tea production process and storage. During general tea processing, at least 15% of polyphenols are reduced from the original content in freshly picked tea leaves as the polyphenols undergo oxidation, hydrolysis, polymerization, epimerization (Astill *et al.*, 2001). On the other hand, caffeine is less sensitive to heat and the content is not substantially reduced during tea processing. Green tea processing involves deactivating the polyphenol oxidase enzyme that is responsible for the oxidation of catechins. Hence, the catechins in green tea are stable during storage and therefore correlate well to the caffeine content.

3.3.4 Stability of catechins during storage

As shown in Figure 3.5, the average total catechins and EGCG in MGTP 3 months and 5 months after opening the package were slightly lower than the initial amount measured, but the difference was not significant according to ANOVA. The cause of catechins reduction during storage could be due to the introduction of moisture and oxygen after the package opened, following by the chemical reaction such as oxidation. Therefore, the condition of storage did not have an effect on the catechins meaning that MGTP from the same package after opening could be used for experimentation. Studies have found that high temperature during storage decreased the tea catechins (Wang and Zhou, 2004; Wang and Helliwell, 2000). However, Ortiz *et al.* (2008) demonstrated that catechins content in dried green tea powder were maintained under the storage condition of ≤43% Relative humidity, at 22 °C for 3 months. This finding is in agreement with the result in this chapter that the catechins were stable over 3 months storage in its own package at room temperature. In addition, the degradation of catechins in solid from (e.g., green tea powder) was 1000 times lower than the

degradation of catechins in tea extract solution (Lomovsky, 2012). The catechins degradation in solid form depends on ambient humidity, storage temperature, and type of food co-ingredients. The reaction rate constant (k) of catechins increased with increased water activity (water mobility). For instance, the storage at a water activity of 0.75 had the highest impact on catechin stability than other lower points of water activity (Corey *et al.*, 2011).

3.3.5 Determination of L-theanine in MGTP

Kvasnička and Krátká (2006) suggested that the boiling temperature provides better extraction of L-theanine content than 20 ℃ (room temperature water), whereas Sari and Velioglu (2011) showed that the L-theanine extracted with 80 ℃ temperature had significantly higher yield than other temperatures used to extract L-theanine. Therefore, the 80 ℃ was used to extract the L-theanine from MGTP samples in this study. The comparison of the room temperature (18 ℃) and 80 ℃ temperature extraction of L-theanine was also studied. From the literature review, it was assumed that the 80 ℃ temperature extraction would yield higher L-theanine than room temperature. However, in our experiment, the room temperature (18 ℃) and 80 ℃ temperature extraction did not have a significant difference on the L-theanine determination. The literature reviews were not in agreement with our finding, which could be due to the particle size of the green tea samples. As stated by Wan *et al.* (2009), L-theanine is soluble in water, where 1 g of L-theanine can dissolve in 2.6 mL of water at 0 ℃ or 1 g of L-theanine can dissolve in 1.8 mL of water at 100 ℃. However, due to the small particle size of MGTP, the release of L-theanine to extract water is faster, therefore the prolonged high-temperature extraction may lead to decomposition of L-theanine extracted. However, the insignificant effect of extraction time on the determination of L-theanine showed that the prolonged temperature of 80 ℃ up to 20 min did not degrade the L-theanine content.

Previous studies suggested that the daily dose of L-theanine to exert beneficial effects was at least 50 mg (Vuong *et al.*, 2011; Keenan *et al.*, 2011). Our experiment found 3.8 µg/mg of MGTP. Therefore, in order to gain the benefits of L-theanine, approximately 12.82 g of the MGTP needs to be consumed per day. This is a large amount of green tea powder to be consumed, as one cup of tea is around 2 g of tea, hence around 6-7

cups of tea made by green tea powder need to be consumed in order to gain benefits of L-theanine.

3.4 Conclusion

The comparison of total phenolic content, antioxidant capacity and individual catechins in MGTP and GTEP were determined by Folin-Ciocalteu assay, FRAP assay and HPLC-PDA method. The result showed that GTEP had significantly higher total polyphenol content and antioxidant capacity than MGTP, determined by Folin-Ciocalteu assay and FRAP assay. However, with HPLC analysis where the individual catechins and caffeine content were determined, MGTP had higher catechins content than GTEP, and had lower caffeine content than GTEP. Because the Folin-Ciocalteu assay and FRAP assay are the non-specific method and the reagent may react with other compounds that lead to an over estimation of total polyphenol. Therefore, the result from the HPLC method was more reliable as the method measures the content of each individual catechin.

All of the major catechins found in MGTP showed no significant change in average content extracted with prolonged extraction time. However, prolonged extraction time has a potential to degrade catechins extracted from MGTP. Hence, the suitable time for extraction catechins in MGTP is 5 min. The catechin content in MGTP varied from 75 - 165 µg/mg of MGTP in 3 different packages. The level of catechins found in MGTP followed a similar order with infused green tea; EGCG > EGC > ECG > EC > GCG > catechin. The analytical method for L-theanine was developed in this chapter to provide a simple and reliable method without using PVPP pre-treatment with good accuracy and precision (more than 97% recovery and less than 5% coefficient variance), and was able to analyse L-theanine in biscuits that contained MGTP. MGTP was chosen to incorporate in biscuits in the next chapter to investigate the stability of catechins and caffeine during baking.

4 Chapter four: Stability of catechins during the biscuits making process

4.1 Introduction

Temperature and pH of a food system during processing play an important role in the stability of catechins. Sharma and Zhou (2011) found that the catechins content of green tea extract, incorporated into biscuits, decreased as the temperature of the biscuits increased while baking. On the other hand, a higher pH of the food system causes more loss of polyphenol in the systems. Several findings have shown a decrease in polyphenol induced by the use of alkalinising agents such as sodium bicarbonate, and baking powder, which are often used as the leavening agents in bakery products. For instance, baking soda (and baking at 177 ℃ for 12 min) contributed a total loss of anthocyanin content in muffins enriched with purple wheat bran (Rosales-Soto *et al.*, 2012; Li *et al.*, 2007). Also, the baking powder contributed to 75-80% loss of cyanidin-3-galactoside in muffins enriched with apple skin powder (Rodriguez-Mateos *et al.*, 2013; Rupasinghe *et al.*, 2008). Similar to other polyphenols, catechins are less stable when the temperature and pH of their system are higher. The process of making biscuits involves high temperature baking (160 °C for 10 min) and the addition of alkalinity – inducing agent which were sodium bicarbonate, ammonium bicarbonate and baking powder, leading to a higher pH in the dough (Sharma and Zhou, 2011). The retention rate results (2-21% of EGCG) obtained for biscuits incorporated with green tea extract was significantly lower than bread (80-83% of EGCG) (Sharma and Zhou, 2011; Wang and Zhou, 2004). This can be explained by the pH of the dough and higher core temperature of biscuit while baking (120-130 ℃) which was higher than the core temperature of bread baking (80-101 ℃). Also, the different complex matrix of ingredients, consisting wheat protein, starch, fat and sugar of bread and biscuits contributed to the varied stability of catechins. The antioxidant activity and stability of tea catechins vary according to the lipid/emulsion system, as well as temperature and pH of the food. Therefore, it is crucial to investigate the stability of catechins from MGTP incorporated in biscuits. The stability study of catechins can be useful for the utilisation of MGTP as a novel food ingredient in processed food.

L-theanine is an essential component of green tea that contributes to the umami taste, which can influence the taste of biscuits incorporated with MGTP. Therefore, the stability of L-theanine in MGTP incorporated into biscuits after baking was investigated

in this chapter in order to observe the effect of baking temperature on the degradation of L-theanine. The method developed in chapter 3 was used to measure the amount of L-theanine in biscuits incorporated with MGTP. The result of L-theanine stability can also be utilised for the application of MGTP in bakery products.

4.1.1 Aims of this chapter

I. To investigate the stability of catechins and caffeine in MGTP incorporated in the following biscuits
    a. Biscuits with/without sodium bicarbonate
    b. Shortbread biscuits

II. To investigate the stability of catechins and caffeine in MGTP incorporated in the following biscuits over 1-3 months storage
    a. Biscuits with/without sodium bicarbonate
    b. Shortbread biscuits

III. To investigate the L-theanine stability in MGTP incorporated in the shortbread biscuits

4.1.2 Objectives

To determine the individual catechins, caffeine and L-theanine content in dough and biscuit incorporated with MGTP using HPLC

4.2 Results

4.2.1 HPLC analysis of catechins in biscuits with MGTP

In chapter 3, it was determined that the MGTP employed in this study had approximately a total catechins content of 10% which consisted of EGCG, EGC, EGC, EC, GCG, and 5% of caffeine. Of all catechins analysed, EGCG and EGC were the two major compounds in the MGTP. The results were similar to nutrition information provided by the producer (Matcha Factory), as well as to a previous studied by Weiss and Anderton (2003). The injection of standard to construct the calibration curve was performed to determine the major catechins and caffeine content in dough and biscuits. Figure 4.1 shows the chromatogram of catechins and caffeine found in shortbread biscuits and dough. The recovery rate of catechins in shortbread spiking with 10 µg/mL of each catechin was measured to ensure that the peak found in chromatograph of biscuit is the analysed compound. The spiking recovery is reported in Table 4.1.

Table 4.1:The recovery rate of catechins in shortbread spiking with 10 µg/mL of each catechin

Analytes	% Recovery of spiking
EGC	107.2±3.5
EC	101.2±0.9
EGCG	104.9±4.0
GCG	103.5±0.3
ECG	100.3±2.0

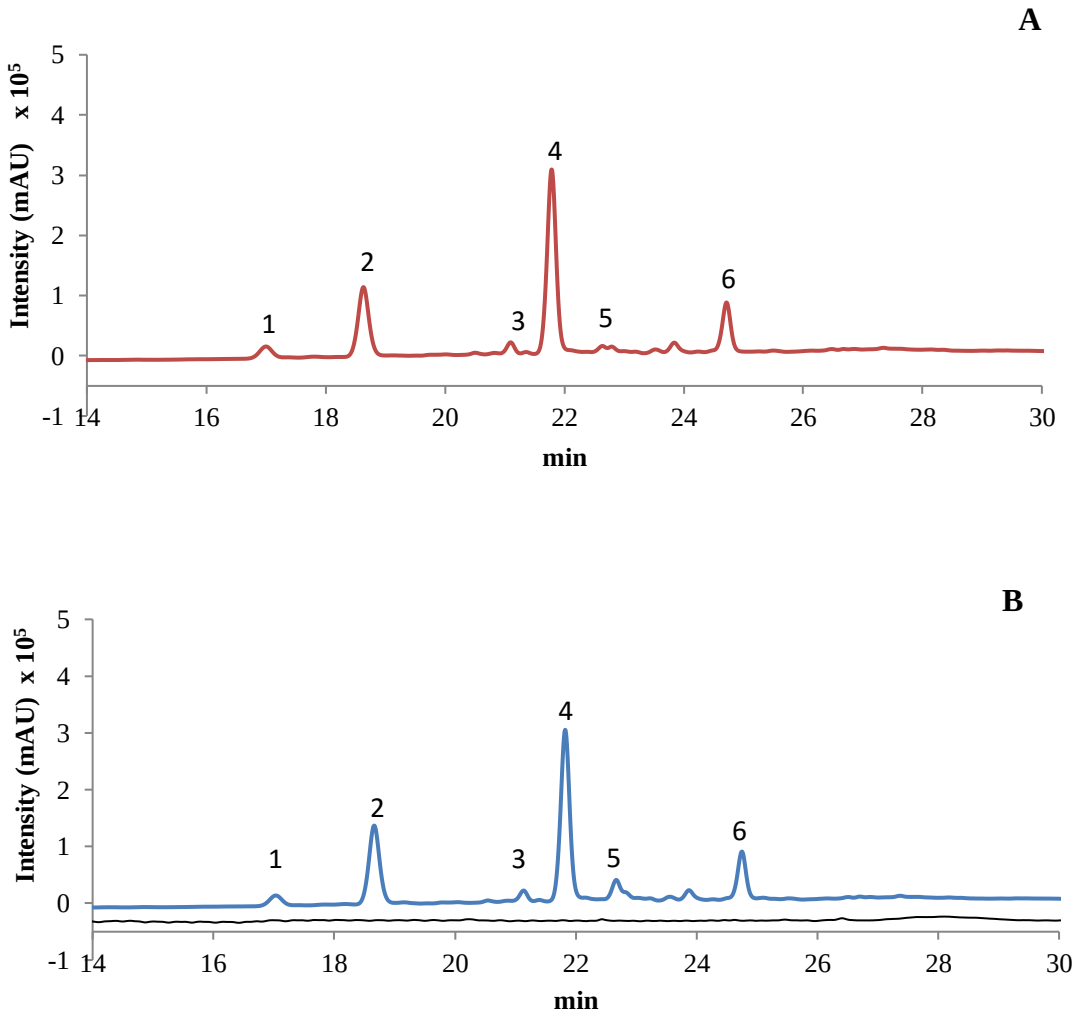


Figure 4.1: Chromatograph profiles of tea catechins, A. in dough; B. in plain and green tea biscuits: 1.EGC; 2. Caffeine; 3.EC; 4. EGCG; 5.GCG and 6.ECG.

4.2.2 Catechin profile in dough and biscuits with and without an alkaline-induced agent

The effect of an alkalizing agent addition to biscuits on the catechins profile was studied. The biscuit formulation was adapted from Laguna *et al.*, (2013) with the addition of 2 g of MGTP per 100 g of flour. The alkalizing agent used in this formulation was sodium bicarbonate, which was added at 0.5 g per 100 g of flour. To investigate the effect of the addition of an alkalizing agent, another batch was conducted without addition of sodium bicarbonate to compare the effect. The catechin profiles in biscuit dough and baked biscuits of both formulations with and without sodium bicarbonate were determined.

As shown in Figure 4.2 and

Table **4.2**, there was no significant difference in the content of catechins between biscuits with and without sodium carbonate added in the dough. A significant decrease in catechin content after baking of both biscuits was observed in EGC, caffeine, EGCG, ECG and total catechins. A significant increase in the content of GCG was found in both biscuits.

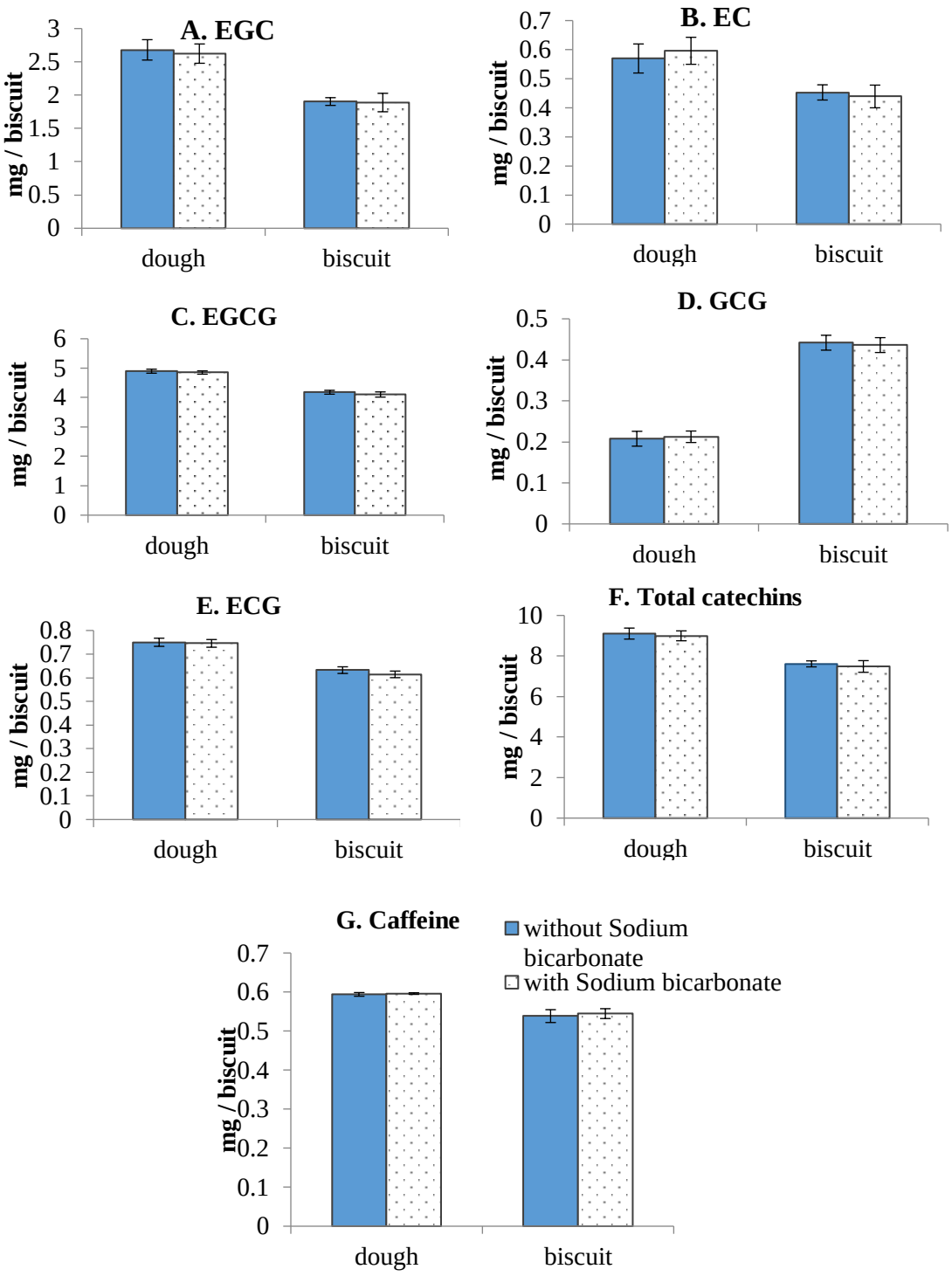


Figure 4.2: Comparison of catechins in the dough and after baking of biscuits with and without sodium bicarbonate in dough, incorporated at 2 g per 100 g of flour: A. EGC; B. EC; C. EGCG; D. GCG; E. ECG; F. total catechins; and G. caffeine. Results are expressed as mg per biscuit. The error bars represent the SEM (n=4)

Table 4.2: Average remaining percentage of tea catechins in biscuits after baking with and without addition of sodium bicarbonate with addition of 2 g MGTP per 100 g of flour

	Without sodium bicarbonate (%)	With sodium bicarbonate (%)
EGC	71.5±5.7	71.8±2.7
EC	80.2±6.0	78.2±2.8
EGCG	85.3±0.8	84.6±1.8
GCG	213.3±12.2	206.7±17.1
ECG	84.3±1.8	82.0±1.8
Total catechins	83.7±3.0	83.1±2.0
Caffeine	90.6±4.6	91.4±3.6

4.2.3 Stability of tea catechins in biscuit during shelf life storage

The biscuits with and without an addition of sodium bicarbonate were kept in vacuum sealed aluminum bags for 3 months to investigate the catechins profile after 3 months storage. As shown in Figure 4.3, a significant loss of total catechins after 3 months storage was found in both biscuits. The percentage of total catechin loss of biscuit with sodium bicarbonate was 15.16% whereas the total catechin loss in biscuit without the addition of sodium bicarbonate was 5.72%. Comparing the loss between both formulations, the significant loss of catechins in biscuits with the addition of sodium bicarbonate was higher in EGC, EC, and EGCG than the biscuit without the addition of sodium bicarbonate. There was a significant difference in total catechins between biscuits without (7.0±0.1 mg per biscuit) and with sodium bicarbonate (6.7±0.1 mg per biscuit).

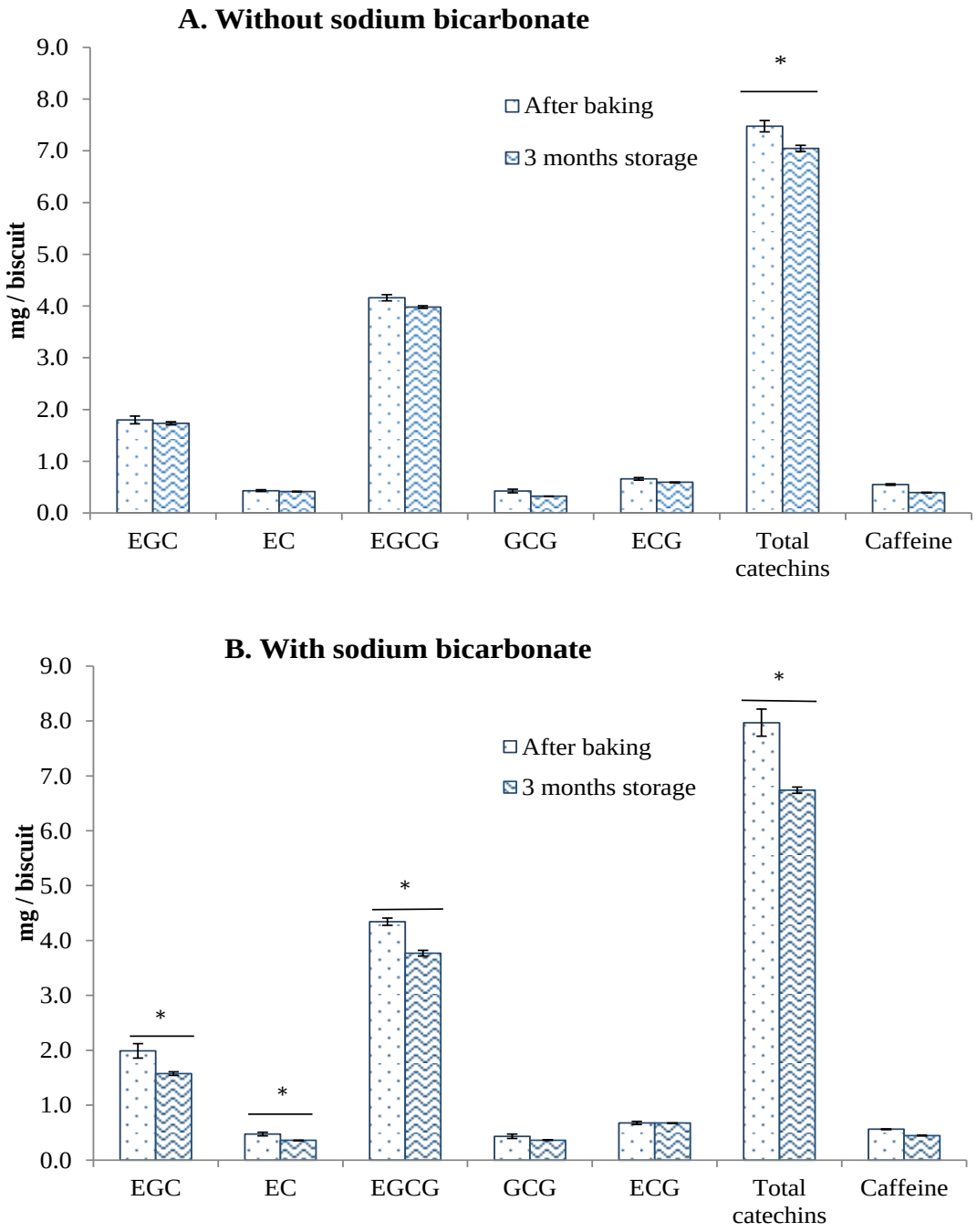


Figure 4.3: Stability of catechins and caffeine in biscuits (2 g MGTP per 100 g flour formulation) during shelf storage in room temperature, A. biscuits without sodium bicarbonate and B. biscuits with sodium bicarbonate. Results are expressed as mg per a biscuit, * indicates a significant difference ($P < 0.05$) between after baking and 3 months storage and the error bars represent the SEM (n=4)

4.2.4 Stability of shortbread biscuits after baking

Figure 4.4 shows the loss of catechins and caffeine in shortbread biscuits incorporated with 3 levels of MGTP (2, 4, 6 g per 100 g of flour). These are the same biscuits as used for sensory evaluation in chapter 5. The sample size was 12 pieces of biscuits and dough in order to monitor the distribution of catechins in biscuits and remaining rate of these compounds. Table 4.3 shows the retention rate of catechins and caffeine found in biscuits after baking, compared with the initial amount found in biscuit dough. A significant loss of total catechins was found after baking. EGC was found to be the least stable catechin, which lost 19-31% of its initial content after baking. While, ECG was found to be relative stable with 5-11% loss after biscuit baking. The remaining percentage of GCG was found to be over 100% with all 3 levels of MGTP incorporated. The total catechin remaining percentage ranged from 81-89%.

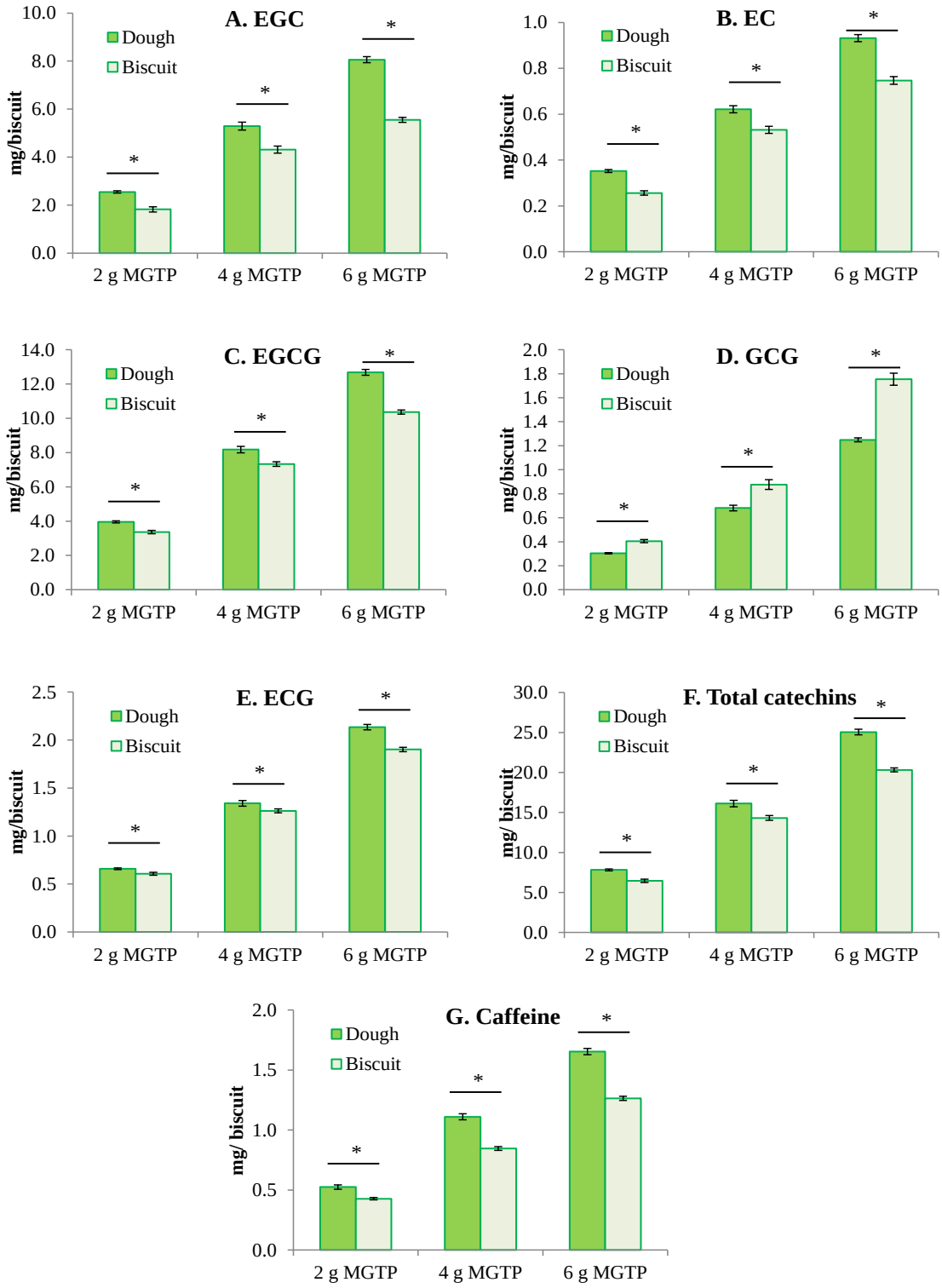


Figure 4.4: Amount of catechins in dough and shortbread after baking incorporated with 3 levels of MGTP (2, 4, 6 g per 100 g of flour): A. EGC ; B. EC; C. EGCG; D. GCG; E. ECG; F. total catechins; and G. Caffeine. Results are expressed as mg per biscuit. * indicates a significant difference ($P < 0.05$) before and after baking. The error bars represent the SEM (n=12)

Table 4.3: Average remaining percentage of catechins and caffeine in shortbread after baking

	2 g MGTP (%)	4 g MGTP (%)	6 g MGTP (%)
EGC	70.9±9.5	81.5±2.5	68.9±1.6
EC	72.6±4.5	85.7±3.5	80.2±1.7
EGCG	84.9±4.0	89.7±2.1	81.8±1.4
GCG	133.0±7.3	128.6±11.0	140.3±8.6
ECG	92.2±3.9	94.3±2.4	89.1±1.2
Total catechins	82.4±5.7	88.9±2.5	81.1±1.4
Caffeine	81.9±5.0	76.3±3.4	76.4±1.1

4.2.5 Stability of catechins in shortbread during shelf life storage

The catechins content in shortbread biscuit during shelf life storage for 1 month at room temperature in plastic bags was determined. There was slight loss during storage of the catechins observed, as shown in Figure 4.5. In 2 g of MGTP shortbread recipe, a significant decrease in EGC was observed, compared to the amount in freshly baked shortbread. The loss of EGC contributed to the significant difference of the total catechins after 1 month storage of the biscuits (Figure 4.5A).

Similarly, significant loss of EGC was found in biscuits with 4 g of MGTP added. However, other catechins' content in biscuits with 4 g of MGTP biscuits was not significantly changed after 1 month storage. As shown in Figure 4.5B, the total catechin content after 1 month storage was significant different mainly due to the loss of EGC.

As shown in Figure 4.5C, a significant decrease was observed in EGC and EGCG in 6 g of MGTP shortbread recipe. The average total catechin remaining was 80.8%. In biscuits with 4 g and 6 g of MGTP, a slight increase of GCG was observed in the biscuits with an average of 17 % and 13 % increase of GCG content respectively. As shown in Figure 4.5, there was no significant difference in caffeine content in

shortbread biscuits after 1 month storage. Remaining percentage of tea catechins and caffeine in shortbread after 1 month room temperature storage is shown in Table **4.4**.

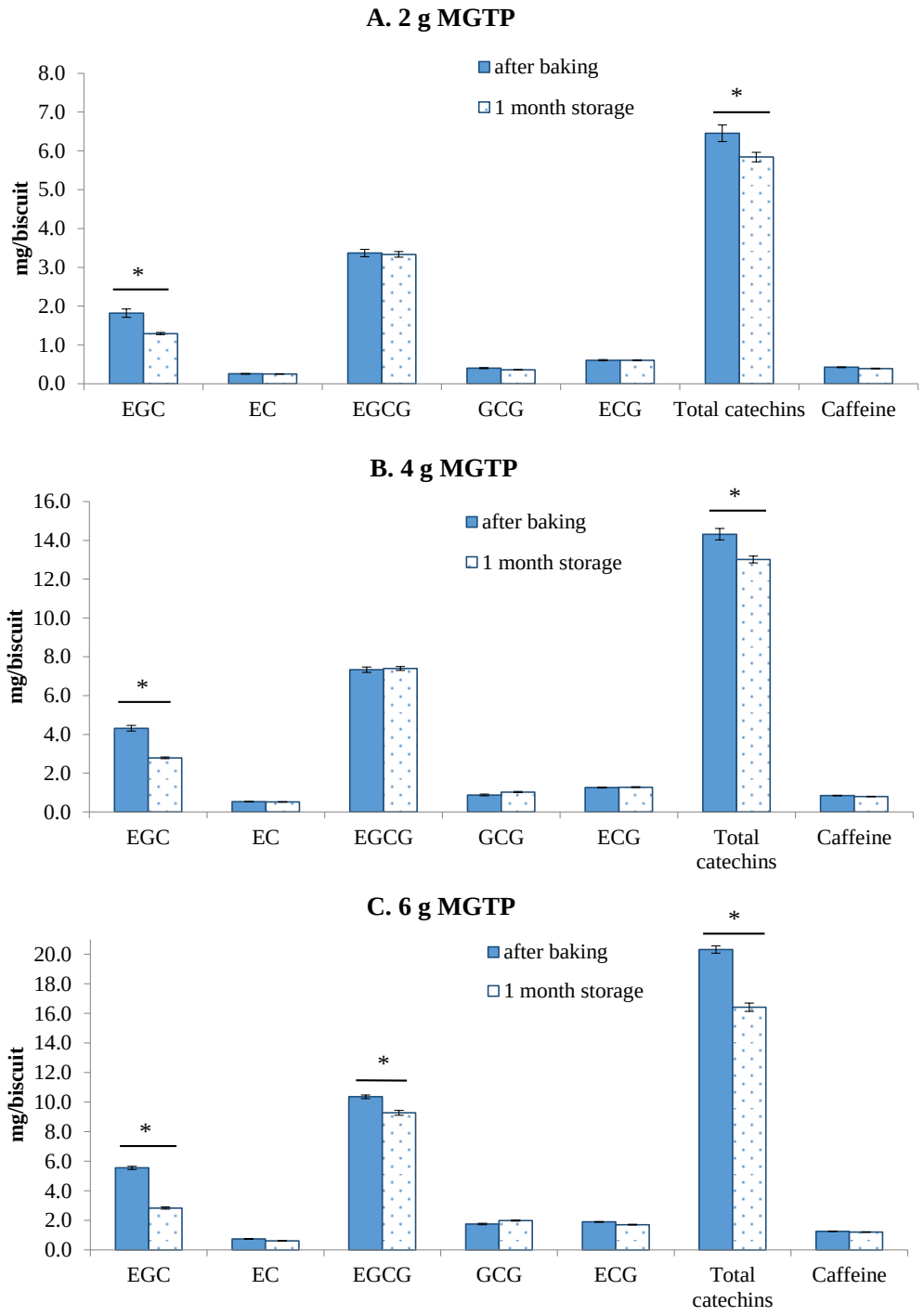


Figure 4.5: Stability of catechins in shortbread during shelf storage at room temperature over, A. biscuits with 2g MGTP per 100 g flour formulation, B. biscuits with 4g MGTP per 100 g flour formulation, and C. biscuits with 6g MGTP per 100 g flour formulation. Results are expressed as mg per a biscuit. * indicates a significant difference ($P < 0.05$) between after baking and 1 month storage and the error bars represent the SEM (n=12)

Table 4.4: Average remaining percentage of tea catechins and caffeine in shortbread after 1 month room temperature storage

	2g MGTP (%)	4g MGTP (%)	6g MGTP (%)
EGC	72.9±10.7	65.2±5.0	51.0±2.3
EC	97.7±3.8	97.9±3.8	83.2±3.5
EGCG	99.2±3.5	101.0±2.7	89.4±2.3
GCG	89.1±5.1	118.4±10.0	113.6±5.4
ECG	99.7±2.7	101.2±1.5	89.9±1.4
Total catechins	90.9±4.6	91.1±3.3	80.8±1.8
Caffeine	92.2±2.1	94.7±3.5	95.7±1.1

4.2.6 Determination of L-theanine content in shortbread biscuits incorporated with green tea

Figure 4.6 shows that L-theanine in dough and biscuit extracts was clearly separated. The HPLC method gave an excellent linearity (correlation R^2 = 1) of the calibration curve of L-theanine standard which ranged from 0.5-40 µg/mL. The LOD and LOQ of the L-theanine calibration curve were 0.38 µg/mL and 1.28 µg/mL.

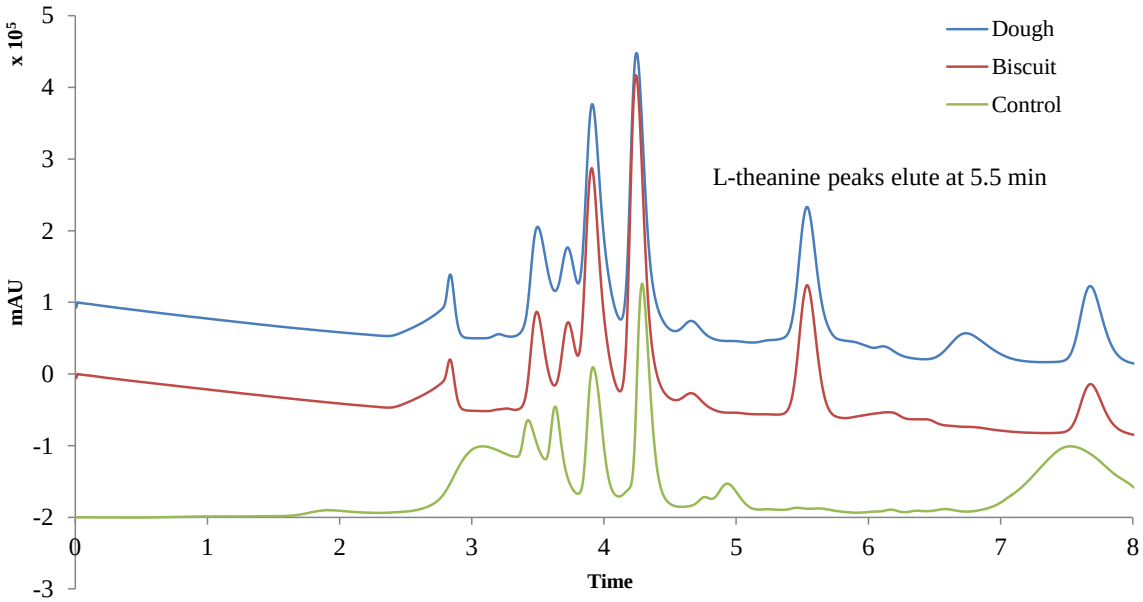


Figure 4.6: HPLC chromatogram of dough extract, biscuit extract and control (in 20 mL of water) using binary gradient elution with C18 column, flow rate: 0.8 mL/min, injection volume: 30 µL, detected, at 200 nm

L-theanine extraction in biscuit using water with heat (80 °C), caused the starch to swell and absorb water, becoming a gel. The gel was dispersed after centrifugation, but the extract had to be filtered twice prior to HPLC analysis. In addition, the previous result (section 3.2.7.4) showed that room temperature extraction did not have a significant effect on the L-theanine determination in MGTP. Hence, the room temperature extraction was also used to determine L-theanine in dough and biscuit that was incorporated with MGTP in order to compare the L-theanine content with 80 °C temperature extraction. The result showed that there was no difference ($P > 0.05$) in L-theanine content extracted with room temperature (118.5±1.1 µg/g of freeze-dried sample) and 80 °C extraction temperature (120.8±3.0 µg/g of freeze-dried sample). Therefore, the extraction at the room temperature was used to extract the L-theanine

from dough and biscuit in further experiment in order to avoid the gelatinization of starch during extraction. The precision of extraction methods was determined by intra-day and inter-day (3 days) of three extracted samples. Intra-day and inter-day variations were 2.08% and 7.04%, respectively. The remaining percentage of L-theanine spiking in the extract was 95.22% with 0.66% intra-day variation of three extracted samples, indicating that the method was both accurate and precise.

4.2.7 Stability of L-theanine during baking process

The change of L-theanine after baking is shown in Figure 4.7. The remaining percentage was calculated by comparing the L-theanine in dough and biscuit after baking. The remaining percentage of L –theanine in biscuits incorporated with 2 and 4 g of MGTP per 100 g of flour were 82.1±4.4%, and 97.1±1.5% respectively. The remaining percentage of L-theanine of biscuit with addition of 2 and 4 g of MGTP per 100 g of flour after 1 month storage were 93.7±3.8% and 96.6±2.5%.

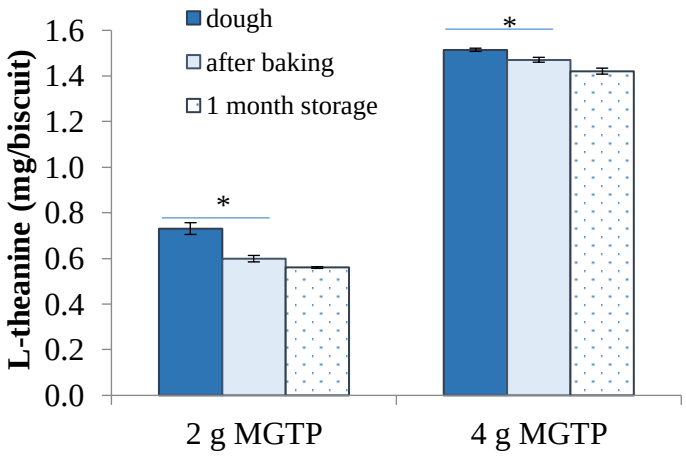


Figure 4.7: Change in L-theanine after baking of shortbread and 1 month storage. Biscuits were incorporated with 2 levels of MGTP at 2 and 4 g per 100 g of flour: Results are expressed as mg per shortbread. * indicates significant differences ($P < 0.05$) before and after baking and the error bars represent SEM (n=4).

4.2.8 Temperature during baking

Temperature of the oven and shortbread biscuits during baking was monitored as shown in Figure 4.8.

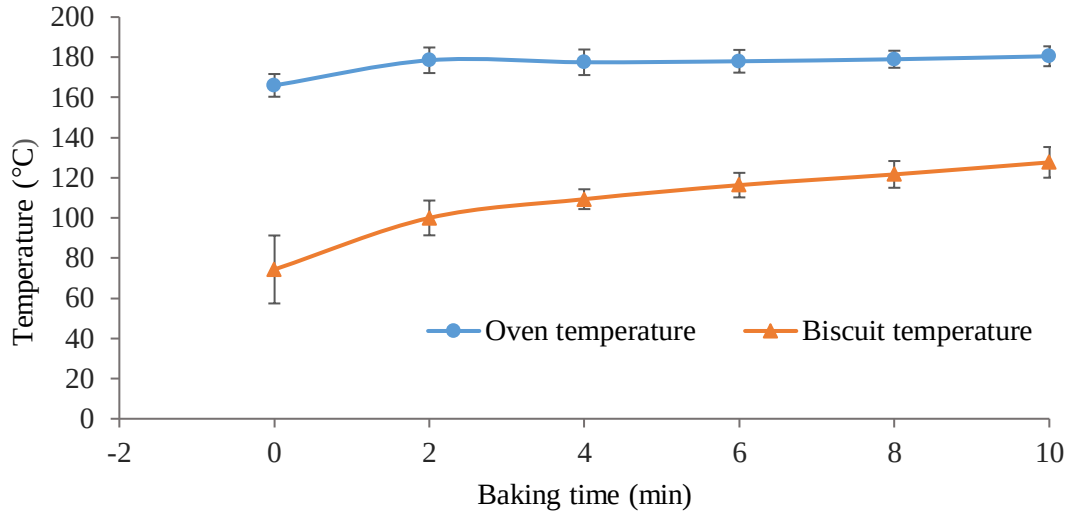


Figure 4.8: Temperature of oven and shortbread biscuits during baking, measured by Tempscan® Scanning Thermometer. The error bars represent the STDs (n=3).

4.3 Discussion

4.3.1 Comparison of catechin stability in biscuits

The remaining percentage of catechins in biscuits with/without sodium bicarbonate (except GCG) after baking ranged from 71% to 85% and the stability after baking ranged as EGCG>ECG>EC>EGC (Table **4.2**). EGC exhibited the lowest remaining percentage. Catechins and caffeine in 12 samples of shortbread biscuits dough and baked biscuits also were determined, with 3 levels of MGTP (2, 4, 6 g per 100 g of flour) incorporated. EGC was the least stable catechin during the baking process of biscuits. This result correlated with the remaining percentage result in biscuits with/without sodium bicarbonate. The remaining percentage of EGCG and ECG in shortbread biscuits ranged approximately 80-94% and the stability ranged as ECG>EGCG>EC>EGC (Table 4.3). This result was in agreement with the study on the stability of catechins using green tea extract in bread making process (Wang and Zhou, 2004). In bread that was incorporated with GTEP, a good retention of catechins was

also observed, the average retention was 83% for EGCG and 91% for ECG, evaluated in freshly baked bread (Wang and Zhou, 2004). On the other hand, our result showed much higher remaining percentage of catechins than the study on the stability of catechins during biscuit making using GTEP which had a retention rate between 2-21% for EGCG and 7-40% for ECG (Sharma and Zhou, 2011). The biscuits incorporated with GTEP showed a higher remaining percentage of ECG than EGCG which was in agreement with this study's result of shortbread biscuits (Sharma and Zhou, 2011). EGC was the least stable catechin in both bread and biscuit incorporated with GTEP (Sharma and Zhou, 2011; Wang and Zhou, 2004), as well as was the least stable catechin in biscuit making in this study. However, the stability order of catechins in biscuits with sodium bicarbonate and shortbread was slightly different. The ECG was more stable than EGCG in shortbread biscuits, whereas in biscuit with/without sodium bicarbonate, the EGCG was more stable than ECG. The difference in stability of catechins could be due to the different food matrix. Food products such as bread and biscuits contain a starch-gluten matrix as the major component, binding with lipid, water and sugar that form a multiphase matrix that can affect catechins due to interactions with these components (Ananingsih *et al.*, 2013). The comparison of catechins stability in different food systems is shown in Table **1.5**.

According to Sharma and Zhou (2011), the degradation and epimerization of tea catechins followed pseudo first-order kinetics. The activation energy remained unchanged in different food systems, such as an aqueous solution or bread and biscuit system. However, a higher rate was found for the degradation and epimerization of EGCG and GCG than ECG and CG. This evidence could explain how ECG was more stable after baking process. Furthermore, the rate could be due to the difference structure of each catechin and its interaction with the food matrix. Ananingsih *et al.* (2013) suggested a hypothesis that the configuration –ortho and number of hydroxyl groups can significantly affect the activity of antioxidants and stability of them. The author also suggested that GCG was more stable than EGCG due to its smaller steric hindrance structure. EGC could be less stable than other catechins because it has stronger ability than EC to scavenge free radicals due to its hydroxyl group at the 5' position of the B ring as shown in Figure 1.3.

4.3.2 Epimerization of catechins

The increase of GCG was observed after the baking process as the retention percentage exceeded 100% in all biscuits (Table **4.2** and Table 4.3). These results indicate that epimerization of EGCG to GCG occurs during baking. A previous study used GTEP incorporate into biscuits to determine the impact of the baking process on green tea catechins (Sharma and Zhou, 2011). The study suggested that epimerization occurred during baking as the amount of GCG exceeded 100% retention rate (Sharma and Zhou, 2011). The core temperature of biscuits in this study reached 120-130 ℃ during baking (Figure 4.8) which would provide sufficient energy for epimerization of EGCG to GCG. The rate of the epimerization from EGCG to GCG is varied in different food systems during food processing and the higher rate leads to an increase in GCG in the food product (Wang *et al.*, 2008a; Wang *et al.*, 2008b). For instance, Wang *et al.* (2008a) observed a higher rate of the epimerization from EGCG to GCG in bread than that the epimerization from EGCG to GCG in aqueous system resulting in an increase in GCG found in bread.

4.3.3 Effect of the alkalinity-inducing agent incorporated into biscuits on the stability of catechins

The study results from this study showed that after baking the sodium bicarbonate incorporated in biscuit dough did not have a significant effect on lowering the catechins level when compared with the dough without sodium bicarbonate (Figure 4.2 and Table **4.2**). This result could be explained by the small amount of alkaline agent (0.5 g of sodium bicarbonate per 100 g of flour) not significantly increasing the pH of the dough which was the one the major factors of loss in catechin content (Sharma and Zhou, 2011). The pH of biscuit dough without sodium bicarbonate addition was 6.3 whereas the pH of biscuit dough with sodium bicarbonate addition was 6.8. However, the previous study by Sharma and Zhou (2011) had a combination of sodium bicarbonate, ammonium bicarbonate and baking powder at 0.5, 1, and 0.3 g per 100 g of flour added to the dough mixture. This combination could lead to a higher pH of biscuit dough in that study and therefore a higher loss of catechins after baking.

In this study, it was found that over 3 month storage, the biscuits with the addition of sodium bicarbonate had lost significantly higher total catechins including EGC, EC, and EGCG, than the biscuits without sodium bicarbonate. The result showed that the sodium

bicarbonate may have a significant effect on the degradation of catechins during storage, as the pH of biscuit with sodium bicarbonate was slightly higher than the biscuit without sodium bicarbonate. To our knowledge, this is the first study that found a decrease in catechins in biscuits with addition of MGTP over storage.

4.3.4 Stability of catechins in shortbread biscuits during storage

Changes in catechin content were evaluated after 1 month storage at room temperature in shortbread biscuits. After 1 month storage of biscuits, EGC content had decreased the most among all catechins. The average remaining percentages were 73%, 65% and 51% for biscuits with 2, 4 and 6 g of MGTP incorporated respectively. EGC loss was significant and contributed to the significant loss of total catechins of all shortbread biscuits during storage.

The results showed an increase in GCG after 1 month storage in biscuits incorporated with 4 g and 6 g of MGTP per 100 g of flour. The EGCG after 1 month storage of biscuits with 4 g of MGTP remained almost the same content, whereas EGCG of biscuits with 6 g of MGTP decreased about 10%. Friedman *et al.* (2009) suggested that during storage oxidation was the major cause of EGCG degradation because there was no increase in GCG, which is the product of EGCG epimerization. However, the result showed the increase of GCG content after 1 month storage. The degradation of catechins is caused by these reactions: oxidation, epimerization and polymerization that can form other compounds. The formation of homo- and hetero- catechin autoxidation dimers was the result of the loss of catechins (Friedman *et al.*, 2009; Tanaka *et al.*, 2002). Friedman *et al.* (2009) found the same continuously decreasing trend of catechins over 4 months storage of bagged tea, but after 6 month storage, an increase in catechins was observed. Therefore, an increase in catechins could be the result of the oxidation, epimerization, and polymerization. Lomovsky (2011) studied the effect of air access to catechin degradation and revealed that the degradation of catechin in aqueous solution was reduced in the absence of air. The total concentration of catechin in aqueous solution decreased 7-9 folds from the initial concentration due to the degradation of EGC and EGCG. With the absence of air, EGC degraded rapidly and faster than other catechins, whereas, under the air access condition, EGCG was the most reactive component to degrade (Lomovsky, 2012). Hence, during storage in this study, the degradation of EGC occurred more than other catechins because the biscuits were stored in vacuum-sealed bag and hence in the absence of air.

4.3.5 L-theanine extraction from biscuit and dough

Keenan *et al.* (2011) studied the effect of milk addition on the content of L-theanine in tea infusion and found that the high volume of milk (50 mL of semi-skimmed milk), added to tea infusion, significantly reduced the amount of L-theanine extracted and determined by HPLC coupled with DAD detector ($P< 0.0001$). The study suggested that the amount of L-theanine decreased because it bonded with other components in milk as seen by the peak of L-theanine was co-eluting closely with other peaks. The peaks of L-theanine extracted from the standard tea preparation and tea with 12 mL of semi-skimmed milk did not co-elute with other peaks (Keenan *et al.*, 2011). As the in dough and biscuit have the milk protein, and fat obtained from butter, the L-theanine may bind with other components, however, the peaks of L-theanine in dough and biscuit extracts were clearly separated from other peaks (Figure 4.6). This allowed us to investigate the stability of L-theanine after baking in biscuits. There was a significant difference in L-theanine content in dough and biscuit (Figure 4.7). The loss of L-theanine (3-18%) could be due to the baking temperature. The core temperature during baking was 120-130 ℃, which can degrade L-theanine content. Sari and Velioglu, (2011) observed that boiling point water (approximately 96 ℃) used in L-theanine extraction result in significant losses of L-theanine content during 25 min extraction. The amounts of L-theanine found in biscuits incorporated with 2 and 4 g of MGTP were approximately 0.6 and 1.3 mg per a biscuit respectively which is about 7.5% and 16.5% of the L-theanine found in a standard cup of green tea (200ml with 2 g of tea leaves) (Keenan *et al.*, 2011). Even though the L-theanine contents found in biscuits incorporated with MGTP were small, the consumption of biscuits could increase the amount of L-theanine consumed.

4.4 Conclusion

The addition of sodium bicarbonate (0.5 g per 100 g of flour) did not change the total catechins content in both dough and biscuits with and without sodium bicarbonate. However, over 3 months storage, the total catechins of biscuits with addition of sodium bicarbonate was significantly lowered than biscuits without addition of sodium bicarbonate. Catechins in MGTP incorporated in biscuit dough were relatively stable after the shortbread biscuits baking process (180 ℃, 10 min) with 81-89% of total catechins retained in biscuits after baking. The increase in GCG content in baked biscuit

and reduction in EGCG showed that epimerization occurred during the baking process. After 1 month storage, 80-91% of total catechins content after baking remained in the biscuits. EGC was the least stable catechin during baking and storage due to its increase in loss in the absence of air in the vacuum-sealed packaging. The results contribute to information about the impact of baking on catechin, caffeine and L-theanine content of MGTP, which may be relevant to epidemiological and human intervention studies that investigate the health benefits of catechin rich products. The shortbread biscuit was selected for further study in sensory evaluation because biscuits with addition of sodium bicarbonate may lead to higher decrease the catechins content during storage. A shortbread biscuit (~10 g) with 2-6 g of MGTP contains 6-20 mg of total catechins, which is 4.3-14.3% of cup of green tea (140 mg of catechins per a cup of green tea). The addition of MGTP to bakery products, such as biscuits, may turn the biscuits into a functional food. To create a functional food, the next steps in this research would be to analyse the texture and consumer acceptance of the product. Following this, it is important to study the health benefits of such a product, such as measuring the antidiabetic effect after consumption.

5 Chapter five: Acceptability of shortbread biscuits incorporated with Matcha green tea powder

5.1 Introduction

Over the last few decades, the health benefits of green tea have been investigated by many scientific studies (Ahmed and Stepp, 2013; Cabrera *et al.*, 2006), providing a positive conclusion which has promoted the consumption of green tea globally. Green tea products are often sold as a health food in European countries and the USA. In the UK, the market share of green tea is still low (2.7%), although these markets are opening up. Between 2010 and 2014, the market share of green tea in the UK increased from 1.8% to 2.7% (approximately 2700 tons to 3700 tons) (*CBI Market Intelligence*, 2016).

MGTP was introduced recently into the European market and is a relatively new product for most European consumers. The expansion of Matcha green tea consumption is increasing, especially as a flavouring agent incorporated into bakery products. This usage has stimulated researchers to investigate and clarify the perception and preference of consumers. Research has suggested that even though green tea is unfamiliar to consumers, their desire of drinking green tea has increased due to its health benefits (Kim *et al.*, 2013; Mennen *et al.*, 2007). Moreover, in the past few decades, the demand for wheat-based products (i.e., biscuits) with value-added components has grown rapidly (Carrillo *et al.*, 2012; Šebečić *et al.*, 2007). Incorporating MGTP into bakery products may mask the unacceptable flavour and reduce bitterness or astringency of green tea as perceived by consumers (Jaeger *et al.*, 2009). The consideration that MGTP may be introduced in Western countries by reducing negative sensory attributes via incorporating it into bakery products is therefore explored in this chapter.

Fine green tea powder is relatively easy to incorporate into foods. Its dispersibility is good in food products and solubility of the nutritional components are increased after grinding, contributing to better absorption by the body (Zhao *et al.*, 2009). Hence, it is a suitable candidate for developing novel food products. At present, little scientific information is available on the function of MGTP incorporated into food products. A few studies have investigated the incorporation of GTEP into the bakery products, such as bread and biscuits (Sharma and Zhou, 2011; Wang *et al*., 2007). There are limited studies on the acceptability

of green tea powder incorporated into the wheat-based product, such as noodles, cakes and biscuits (Ahmad *et al.*, 2015; Li *et al.*, 2012; Lu *et al.*, 2010).

The contribution of MGTP towards the sensory characteristics associated with biscuit quality as well as consumer perception level is unknown at present. Therefore, there is a need to carry out a sensory study on the acceptability of biscuits with the addition of MGTP. From the previous chapter, the remaining percentage of catechins in MGTP was relatively high. Therefore, higher incorporation would increase the catechins intake. However, the acceptability of these products require evaluation to find the highest level that consumers are willing to accept and the associated increase of sugar content needed to ensure acceptability.

5.1.1 Aim

I. To evaluate the consumer acceptability of varying ratios of MGTP and sugar incorporated into a shortbread biscuit recipe

5.1.2 Objective

- To observe the trend in consumer acceptability on different ratios of MGTP and sugar incorporated in shortbread biscuit recipes using statistical test and response surface methodology
- To measure physical properties of biscuit e.g. the hardness of biscuits using a texture analyser

5.1.3 Statistical analysis and response surface method

Response surface methodology (RSM) is a collection of mathematical and statistical techniques to form a response surface plot and build empirical models between output response and variables (Khuri and Mukhopadhyay, 2010). The method is used in our study to construct mathematical models that explained the relationship between the independent variable: MGTP (X_1) and sugar (X_2) response variables on the response (acceptability). RSM and statistical analysis were outlined earlier in sections 2.6.4.3 and 2.8.5. The abbreviated names of each formulation, coded variables, and actual variables are listed in Table 5.1.

Table 5.1: Design for the study on acceptability of MGTP and sugar incorporated in shortbread biscuits

Session	Experiment number and abbreviated name	Coded variable		Actual variable	
		X_1	X_2	MGTP (M) (g per 100 g of flour)	Sugar (S) (g per 100 g of flour)
1st session (54 participants)	1: 2M25S1	-1	-1	2	25
	2: 2M30S1	-1	0	2	30
	3: 2M35S1	-1	1	2	35
	4: 4M25S1	0	-1	4	25
	5: 4M30S1	0	0	4	30
	6: 4M35S1	0	1	4	35
2nd session (46 participants repeated)	7: 2M25S2	-1	-1	2	25
	8: 2M30S2	-1	0	2	30
	9: 4M30S2	0	0	4	30
	10: 4M35S2	0	1	4	35
	11: 6M25S2	1	-1	6	25
	12: 6M30S2	1	0	6	30
	13: 6M35S2	1	1	6	35

5.2 Results

5.2.1 Physical quality of biscuits

The physical attributes of biscuits are presented in Table 5.2.

Table 5.2: Physical quality of biscuits used in the acceptability test, providing hardness, diameter, thickness, moisture content, spread ratio, and weight loss after baking

Experiment number: Abbreviated name	Hardness (N) n=6	Diameter (cm) n=5	Thickness (cm) n=3	Moisture content (%) n=4	Spread ratio n=3	Weight of biscuits (g) n=6
1: 2M25S1	9.47±2.15^{a}	5.30±0.08^{bc}	0.60±0.01^{ab}	1.68±0.02^{bc}	8.88±0.25^{bcd}	9.11±0.19^{de}
2: 2M30S1	7.69±2.49^{a}	5.40±0.07^{bc}	0.59±0.01^{ab}	1.27±0.17^{ab}	9.31±0.17^{de}	8.40±0.36^{a}
3: 2M35S1	10.69±3.26^{ab}	5.62±0.11^{d}	0.58±0.02^{ab}	2.26±0.49^{e}	9.94±0.10^{f}	8.74±0.22^{abcd}
4: 4M25S1	11.14±3.39^{ab}	5.38±0.08^{bc}	0.59±0.01^{ab}	1.84±0.14^{cd}	9.05±0.24^{cde}	8.65±0.09^{abc}
5: 4M30S1	14.37±4.89^{bc}	5.58±0.11^{d}	0.59 ±0.01^{ab}	2.53±0.14^{e}	9.49±0.17^{def}	8.57±0.07^{ab}
6: 4M35S1	14.63±3.87^{bc}	5.60±0.11^{d}	0.60±0.02^{abc}	1.38±0.15^{ab}	9.40±0.37^{def}	8.78±0.20^{abcd}
7: 2M25S2	11.08±1.40^{ab}	5.26±0.09^{b}	0.62±0.02^{bc}	1.87±0.07^{cd}	8.55±0.11^{abc}	8.56±0.23^{ab}
8: 2M30S2	14.33±2.26^{bc}	5.46±0.05^{cd}	0.57±0.02^{a}	1.23±0.04^{a}	9.54±0.34^{ef}	9.08±0.30^{de}
9: 4M30S2	16.82±2.32^{cd}	5.36±0.11^{bc}	0.64±0.02^{c}	2.17±0.11^{de}	8.33±0.05^{ab}	9.02±0.14^{cde}
10: 4M35S2	15.11±1.42^{bc}	5.34±0.05^{bc}	0.58±0.02^{ab}	1.36±0.05^{ab}	9.20±0.33^{de}	9.24±0.26^{ef}
11: 6M25S2	20.49±1.21^{d}	5.06±0.05^{a}	0.61±0.01^{abc}	1.54±0.07^{abc}	8.17±0.09^{a}	8.92±0.16^{bcd}
12: 6M30S2	17.97±0.96^{cd}	5.22±0.08^{b}	0.61±0.01^{abc}	1.67±0.07^{bc}	8.57±0.23^{abc}	9.48±0.17^{f}
13: 6M35S2	16.55±0.68^{cd}	5.38±0.08^{bc}	0.61±0.01^{abc}	1.38±0.11^{a}	9.00±0.17^{cde}	8.91±0.26^{bcd}

The mean values (±STDs) that are followed by the same letter within a column are not significantly different at $P < 0.05$ with one-way ANOVA following with Turkey's-b *post hoc* test.

The hardness of biscuits was measured with a texture analyser using a blade probe to cut the biscuits. The method imitated the evaluation of hardness by consumer holding the biscuit in hands and breaking by bending (Tyagi *et al.*, 2007). As shown in Figure 5.1, the hardness of biscuits was measured by the peak force that required breaking biscuits (Bourne, 2002).

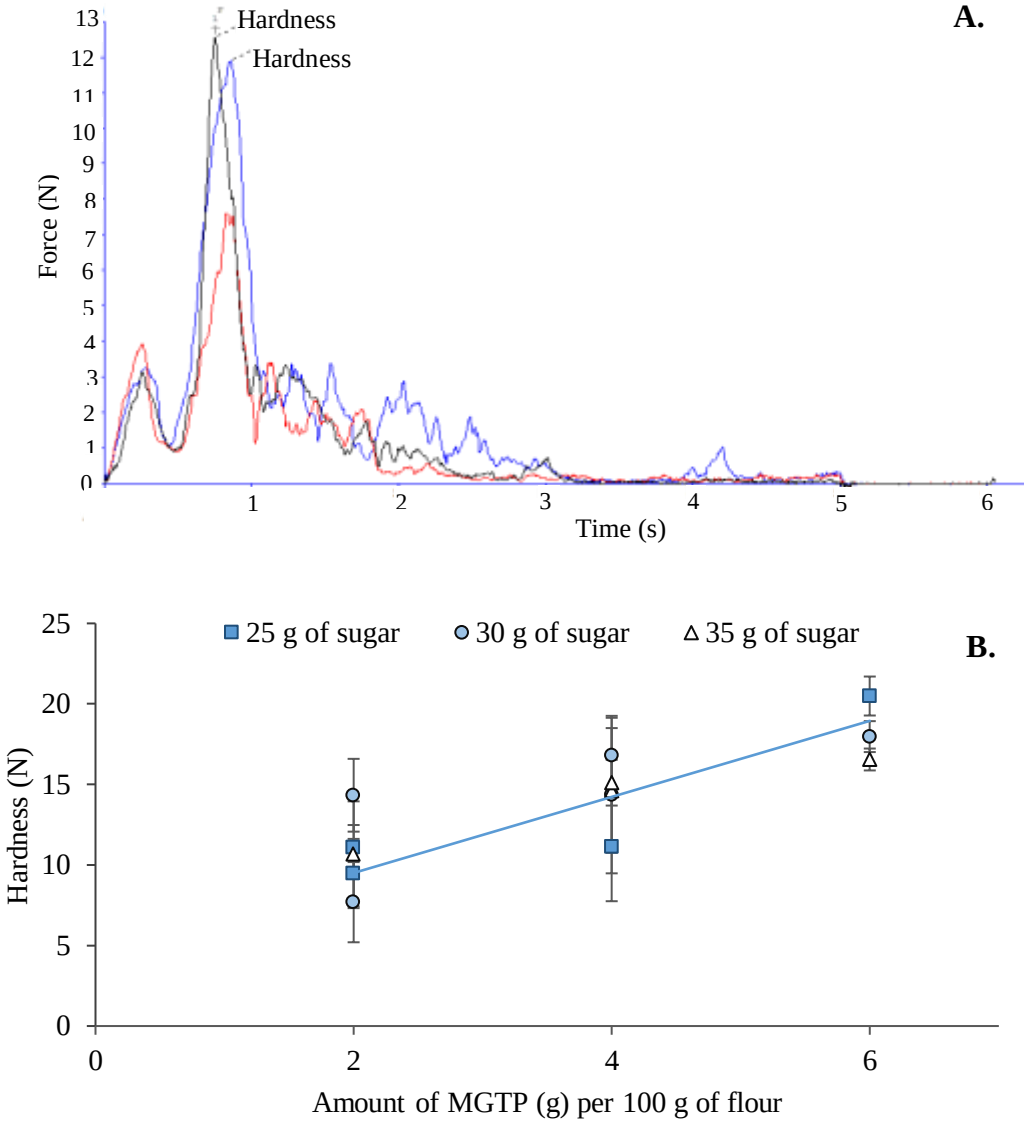


Figure 5.1: A. Example of texture analysis plot of the hardness of biscuits with 2 g of MGTP per 100 g of flour incorporated, B. Hardness plot of biscuits with 3 levels of MGTP and sugar incorporated. The error bars represent the STDs (n=6).

With a higher level of MGTP incorporated in biscuits, the hardness was found to be increased significantly in some cases, especially between biscuits with 2 g and 6 g of MGTP as shown in Table 5.2 and Figure 5.1B. The highest level of hardness found in 6M25S2 biscuits was 20.49±1.21 N, the lowest level of hardness found in 2M30S1 biscuits was 7.69±2.49 N. With increased level of sugar content, the diameters of biscuits were increased. Hence, the spread ratio of biscuits was increased with the increased level of sugar content. The difference in hardness of 6M25S2 and 2M30S1 could be due to the spread ratio and weight of biscuits. The average spread ratio of 2M30S1 was significantly higher than 6M25S2, may be due to higher sugar content and the average weight of 2M30S1, which was significantly lower than 6M25S2. Hence, these factors (sugar content and weight of biscuits) could contribute to a significant difference in hardness of both biscuits. The weights of biscuits also play an important role in increasing the hardness. For example, the hardness of 2M30S2 was significantly higher than 2M30S1 could be due to weight of 2M30S2 was significantly higher. In addition, the weight of biscuit is likely to affect the hardness more than moisture and spread ratio. For example, the 2M25S2 significantly higher moisture and lower spread ratio than 2M30S2, but the hardness of both were not significantly different. This could be largely due to the weight of 2M30S2, which was significantly higher than 2M25S2. In order to compare the hardness more accurately, the weight of all biscuit should be uniform. However, with the same level of sugar incorporated (25 g per 100 g of flour), the hardness of 2M25S1 (also 2M25S2) and 6M25S2 was significantly different, while the moisture and weight of both biscuits were not significant different. Nevertheless, the spread ratio of 6M25S2 was significantly lower than 2M25S1. Hence, the increasing level of MGTP may reduce the spread ability of biscuit, which further affect the hardness of biscuits. In addition, the difference in moisture content of biscuits did not significantly change the hardness of biscuits as shown in Table 5.2.

5.2.2 Acceptability of biscuits

5.2.2.1 Participants' information

Participants were in the age ranged from 20 to 50 years old. The same participants attended two sessions for the acceptability tests. In the first session, 54 participants were recruited, whereas, in the second session, 46 participants attended. Gender and ethnicity are shown in Table 5.3. Among all the participants who attended both sessions, there were 9 participants who eat biscuits daily and 3 participants who never or eat biscuits less than once a month.

Table 5.3: Participants' information for acceptability test of biscuits

	Session 1	**Session 2**
No. Of participant	54	46
Gender	(F: M) 36:18	(F: M) 32:14
Age (years)	26±7	27±7
Ethnicity	Asian: 28	Asian: 23
	European: 21	European: 18
	Mixed: 3	Mixed: 3
	African: 2	African: 2
Biscuits consumption frequency	Less/never: 3	Less/never: 3
	Daily: 9	Daily: 9
	Weekly: 29	Weekly: 22
	Monthly: 13	Monthly: 12

The acceptability of biscuits, which were measured using hedonic-scale as mention in 2.6.4.3, are reported in Table 5.4. One-way ANOVA and Turkey's-b *post hoc* test was performed to determine the difference in acceptability scores between 13 biscuits Two-way ANOVA and Turkey's-b *post hoc* test was conducted to determine the difference of the acceptability between MGTP and sugar levels, and their interaction effects.

Table 5.4: Acceptability of 13 biscuits using 9-point hedonic scale, providing overall, appearance, aroma, colour, texture, bitterness, and sweetness acceptability

Experiment number: Abbreviated name	**Acceptability**						
	Overall	Appearance	Aroma	Colour	Texture	Bitterness	Sweetness
1: 2M25S1	5.87±1.80^{abc}	5.81±1.57^{abc}	6.06±1.57^{a}	5.59±1.60^{ab}	6.15±1.80^{a}	5.78±1.71^{a}	5.80±1.77^{a}
2: 2M30S1	5.96±1.75^{abc}	5.59±2.06^{ab}	6.04±1.77^{a}	5.65±2.06^{ab}	6.35±1.54^{a}	5.44±1.84^{a}	6.19±1.79^{a}
3: 2M35S1	6.13±2.14^{abc}	5.91±1.67^{abc}	5.87±1.86^{a}	5.72±1.86^{ab}	6.33±1.89^{a}	5.46±2.00^{a}	5.96±2.00^{a}
4: 4M25S1	5.20±2.17^{bc}	5.11±1.95^{abc}	5.65±1.76^{a}	4.89±2.10^{b}	5.89±1.84^{a}	4.93±2.05^{a}	5.46±1.79^{a}
5: 4M30S1	5.87±1.98^{abc}	5.31±1.75^{abc}	6.19±1.40^{a}	5.24±1.95^{ab}	6.22±1.46^{a}	5.65±1.80^{a}	5.98±1.84^{a}
6: 4M35S1	5.59±1.96^{abc}	5.11±1.78^{abc}	6.06±1.49^{a}	4.85±1.89^{b}	6.33±1.58^{a}	5.43±2.06^{a}	5.74±1.80^{a}
7: 2M25S2	6.63±1.61^{a}	6.28±1.63^{a}	6.61±1.51^{a}	6.17±1.74^{a}	6.72±1.33^{a}	6.15±1.74^{a}	6.46±1.50^{a}
8: 2M30S2	6.37±1.50^{ab}	6.22±1.65^{ab}	6.50±1.55^{a}	6.26±1.77^{a}	6.67±1.56^{a}	6.15±1.70^{a}	6.28±1.54^{a}
9: 4M30S2	6.15±1.51^{abc}	5.87±1.59^{abc}	6.37±1.22^{a}	5.76±1.68^{ab}	6.43±1.41^{a}	5.89±1.68^{a}	6.39±1.48^{a}
10: 4M35S2	6.22±1.50^{abc}	5.54±1.71^{abc}	6.11±1.61^{a}	5.35±1.75^{ab}	6.67±1.45^{a}	6.11±1.60^{a}	6.00±1.70^{a}
11: 6M25S2	5.09±1.85^{c}	4.87±2.13^{c}	5.91±1.38^{a}	4.83±2.09^{b}	6.04±1.52^{a}	4.93±2.24^{a}	5.50±1.81^{a}
12: 6M30S2	5.33±2.08^{bc}	5.02±2.09^{bc}	5.96±1.40^{a}	5.02±2.17^{ab}	6.15±1.70^{a}	4.98±2.31^{a}	5.98±1.93^{a}
13: 6M35S2	5.85±1.94^{abc}	5.59±2.15^{abc}	6.00±1.66^{a}	5.52±2.19^{ab}	6.46±1.49^{a}	5.17±2.06^{a}	6.09±1.68^{a}

The mean values (±STDs) that are followed by the same letter within a column are not significantly different at $P < 0.05$ with one-way ANOVA following with Turkey's-b *post hoc* test.

5.2.2.2 Overall acceptability

As shown in Table 5.4, the overall acceptability was decreased with higher level of MGTP incorporated in the biscuits. Higher sugar level increased the overall acceptability of biscuits with 6 g MGTP content incorporated. According to the one-way ANOVA following with a Turkey's-b *post hoc* test, the 2M25S2 biscuits, which received the highest acceptability, had significantly higher overall acceptability than 6M25S2 biscuits, which gained the lowest overall acceptability. According to the two-way ANOVA following with a Turkey's-b *post hoc* test, the overall acceptability of biscuits of biscuits with 2 g of MGTP was significantly different from biscuits with 6 g of MGTP. The increased sugar content did not significantly increase the overall acceptability of biscuits. There was no interaction effect of MGTP and sugar on the overall acceptability.

5.2.2.3 Appearance acceptability

The highest overall appearance acceptability was found in 2M25S2 biscuits. The lowest overall appearance acceptability was found in 6M25S2 biscuits. The difference of these two biscuits was significant. As shown in Table 5.4, the overall appearance acceptability of biscuits with 4 g and 6 g of MGTP was significantly lower than biscuits with 2g of MGTP content. In addition, the overall appearance acceptability of biscuits with different sugar content was not significantly different.

5.2.2.4 Aroma acceptability

According to one-way ANOVA, there was no significant difference in aroma acceptability between each biscuit formulation. Moreover, with two-way ANOVA, no significant difference in aroma acceptability between biscuits with different tea levels and sugars levels incorporated was found.

5.2.2.5 Colour acceptability

As shown in Table 5.4, 2M25S2 and 2M30S2 biscuits had significantly higher colour acceptability response than 4M25S1, 4M35S1 and 6M25S2 biscuits using one-way ANOVA. The biscuits contained 2 g of MGTP per 100 g of flour had significantly higher colour acceptability response than biscuits that contained 4 g and 6 g of MGTP per 100 g of flour and sugar content did not have a significant effect on colour acceptability.

5.2.2.6 Texture acceptability

There was no difference in texture acceptability between biscuit formulations. MGTP and sugar content did not significantly affect the texture acceptability of biscuits. Although the higher content of MGTP mixed in the biscuits formulation decreased the average texture acceptability, the change was not significant.

5.2.2.7 Bitterness acceptability

There was no significant difference in bitterness acceptability between biscuit formulations. However, there was a significant difference in bitterness acceptability between biscuits with low content of MGTP (2 g) and biscuits with a high content of MGTP (6 g). The sugar content did not significantly affect the bitterness acceptability.

5.2.2.8 Sweetness acceptability

There was no difference in sweetness acceptability between biscuit formulations using one-way ANOVA and no significant difference in levels of tea and sugar incorporated using two-way ANOVA.

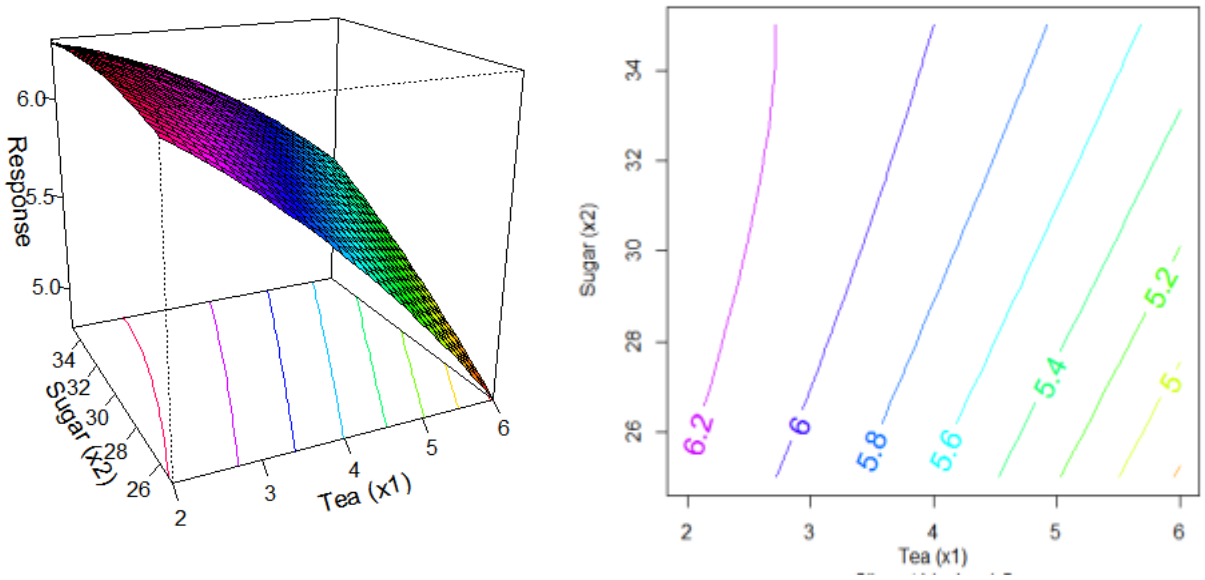
5.2.3 Response surface of acceptability data

The data of acceptability was used to plot the response surface and analyse the data for the regression coefficients used to form the mathematical models (Equation 9) that explained the relationship between the independent variable: MGTP (X_1) and sugar (X_2) response variables. The estimated coefficients for each acceptability are listed in Table 5.5.

Table 5.5: Estimated coefficients for regression equation for acceptability response of biscuits with 3 levels of MGTP and sugar content where *, **, and * indicates significant difference at *P*<0.05, *P*<0.01 and *P*<0.001 respectively using response surface methodology**

Variables	**Acceptability**						
	Overall	Appearance	Aroma	Colour	Texture	Bitterness	Sweetness
Session	0.52**	0.46**	0.38*	0.50**	0.39*	0.59**	0.35*
$\mathbf{X_1}$	-0.54***	-0.54***	-0.21*	-0.52***	-0.24*	-0.56***	-0.24*
$\mathbf{X_2}$	0.20*	0.10	0.00	0.10	0.16	0.09	0.13
$\mathbf{X_1X_2}$	0.16	0.15	0.13	0.14	0.08	0.14	0.15
$\mathbf{X_1^2}$	-0.11	0.05	-0.13	0.15	-0.06	-0.35*	0.01
$\mathbf{X_2^2}$	-0.05	-0.01	-0.15	-0.15	-0.003	-0.04	-0.26

A.



B.

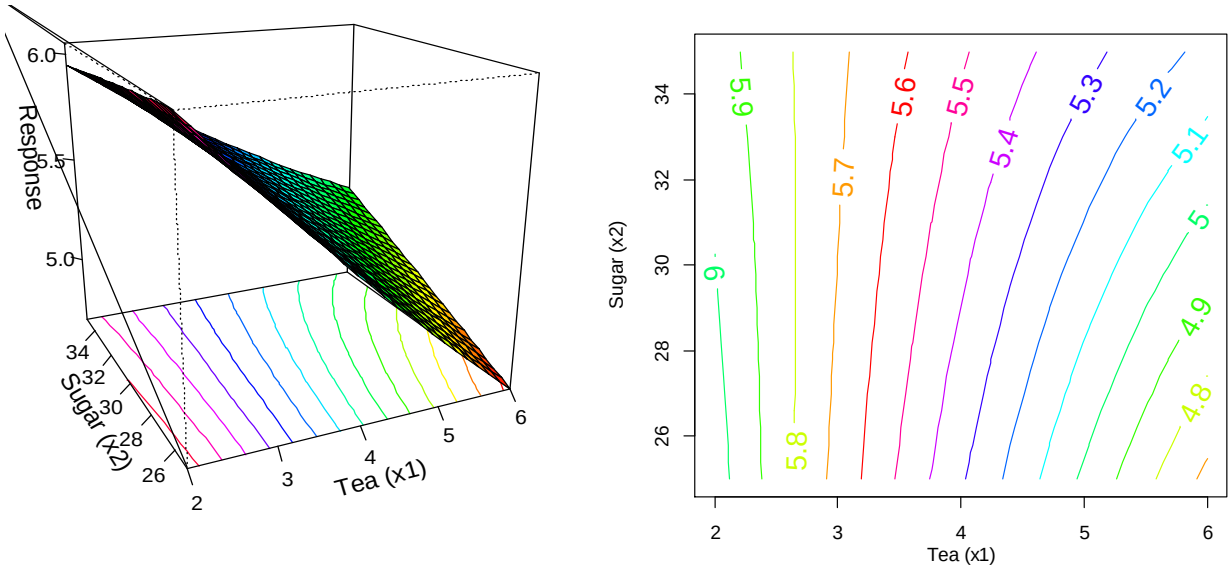
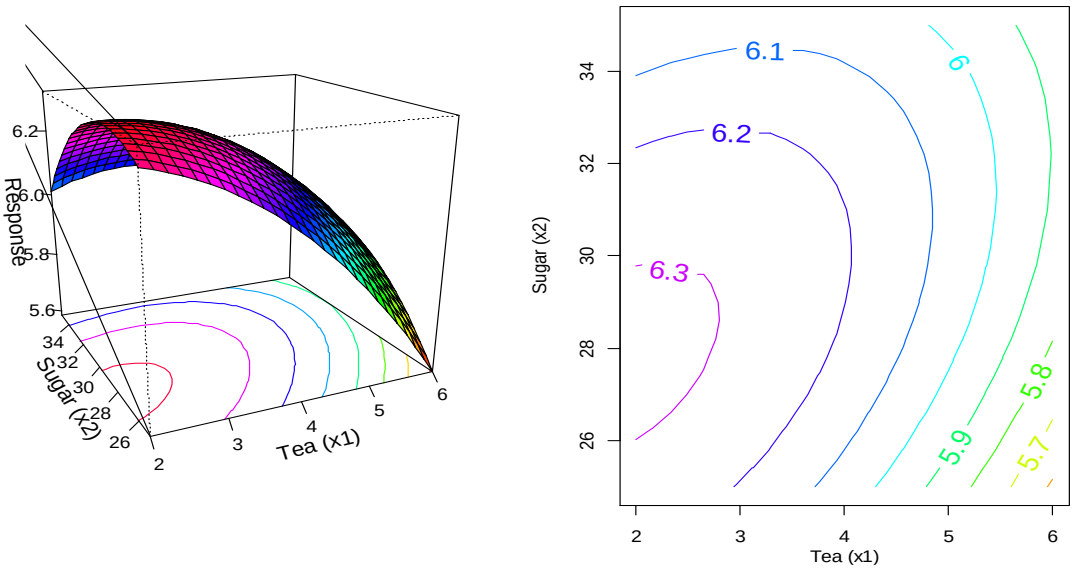


Figure 5.2: Response surfaces and contour plots of the effect of MGTP and sugar content incorporated in shortbread biscuit where A. is overall acceptability and B. is appearance acceptability.

A.



B.

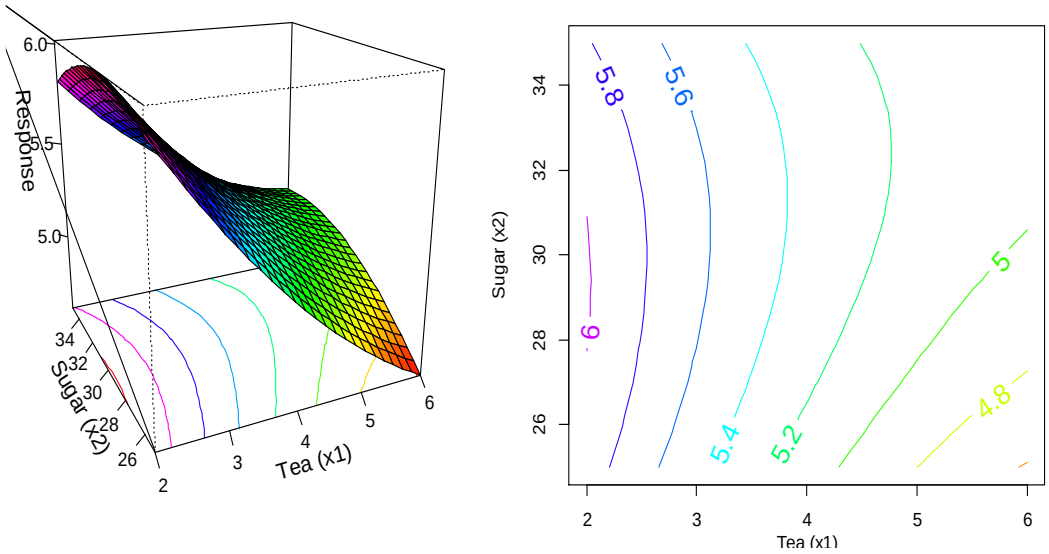
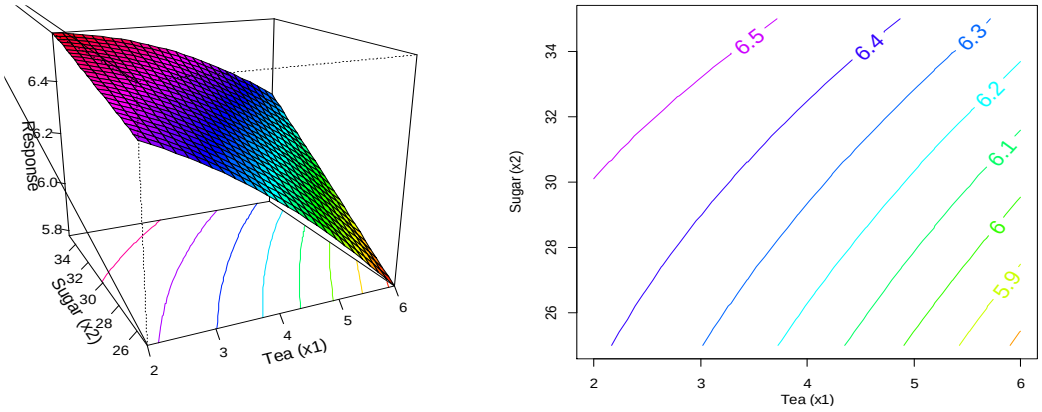
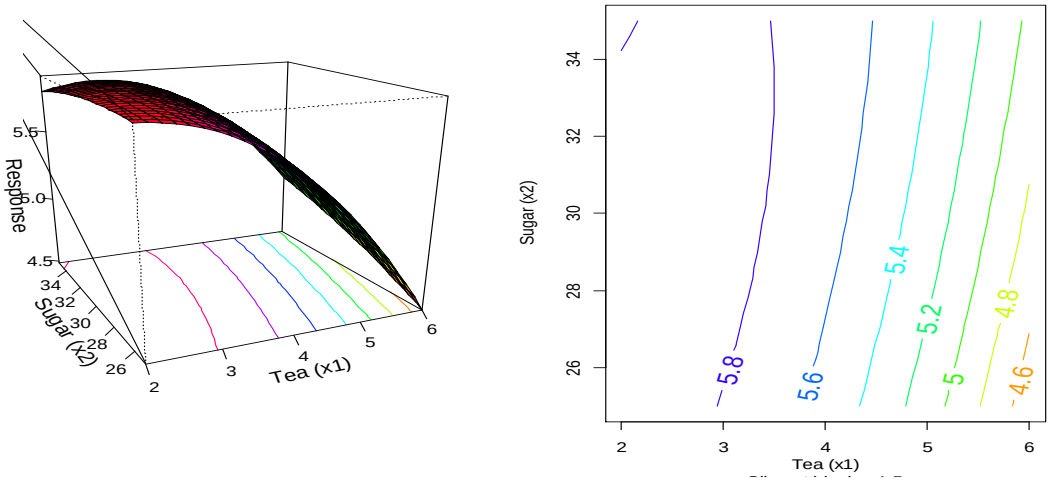


Figure 5.3: Response surfaces and contour plots of the effect of MGTP and sugar content incorporated in shortbread biscuit where A. is aroma and B. colour acceptability.

A.



B.



C.

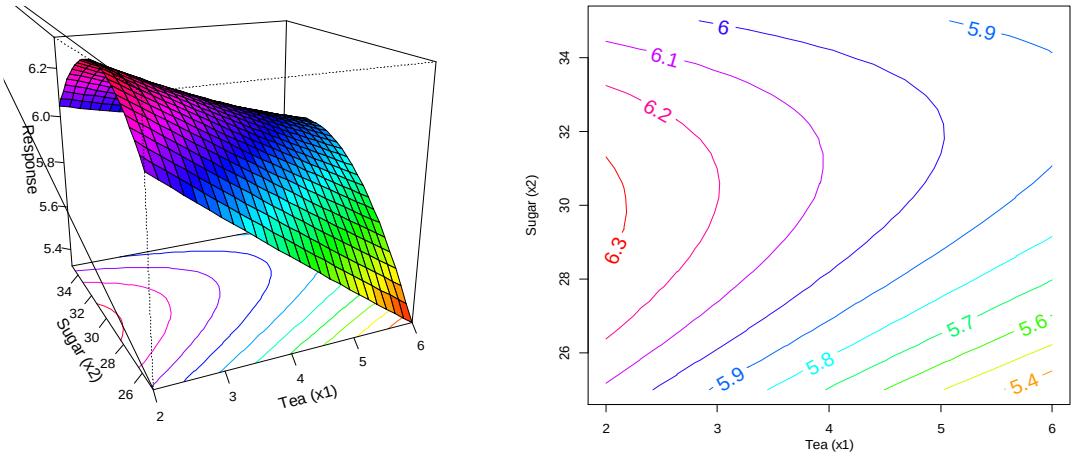


Figure 5.4: Response surfaces and contour plots of the effect of MGTP and sugar content incorporated in shortbread biscuit where A. is texture, B. is bitterness, and C. is sweetness acceptability.

As shown in Table 5.5, the estimated coefficients of MGTP (X_1) for all acceptability tested were negative. Hence, all of the acceptability measures were significantly decreased by a higher MGTP content incorporated. The estimated coefficients of sugar (X_2) for all acceptability tested were positive. However, the linear term of sugar (X_2) significantly affected only the overall acceptability. The sugar content significantly increased the overall acceptability. Other acceptability attributes were not affected by sugar content. The interaction between 2 variables (X_1X_2) and their quadratic terms of these variables did not significantly affect most of the acceptability of biscuits. The quadratic effect of MGTP content (X_1^2) incorporated significantly affected only the bitterness acceptability (P <0.05).

The overall acceptability was significantly affected by the linear (P<0.001) effect of MGTP content incorporated and affected by the linear (P <0.05) effect of sugar content incorporated in biscuits. Moreover, the session had a significant positive effect (P<0.01) on the overall acceptability. As shown in Figure 5.2A overall acceptability was decreased significantly with the increase of MGTP.

5.2.3.1 Appearance acceptability

The appearance acceptability was affected significantly by the linear term (X_1) MGTP content (P<0.001). The surface response and contour plot showed the overall appearance acceptability trend that low content of MGTP (2 g per 100 g of flour) received higher acceptability response.

5.2.3.2 Aroma acceptability

From the response surface analysis, the aroma acceptability was significantly affected by the linear term (X_1) of Matcha content incorporated (P<0.05). As shown in Figure 5.3A, the surface response and contour plot showed that the highest response of aroma acceptability was achieved around a sugar level of 30 g per 100 g of flour. The surface response method suggested the stationary point at 1.84 g of MGTP and 27.73 g of sugar per 100 g of flour to obtain the highest response.

5.2.3.3 Colour acceptability

From the response surface analysis, the colour acceptability was significantly affected by the linear term (X_1) of Matcha content incorporated (P<0.001). As shown in Figure 5.3B, the surface response and contour plot showed that the highest response of colour acceptability was clustered around the biscuits with a sugar level of 30 g per 100 g of flour.

5.2.3.4 Texture acceptability

From the response surface analysis, the texture acceptability was significantly affected by the linear term (X_1) of matcha content incorporated (P<0.05). The response surface and contour plot suggested that high sugar and low content of MGTP increased the texture acceptability (Figure 5.4A). The stationary point from the response surface suggested that sugar content at 30 g per 100 g of flour is the optimum level to obtain the highest texture acceptability.

5.2.3.5 Bitterness acceptability

From the response surface analysis, the bitterness acceptability was significantly affected by the linear term (X_1) and the quadratic term (X_1^2) of Matcha content incorporated (P<0.001 and P<0.05). As shown in the Figure 5.4B, the bitterness acceptability received a high response with biscuits that had a low level of MGTP.

5.2.3.6 Sweetness acceptability

From the response surface analysis, the sweetness acceptability was significantly affected by the linear term (X_1) of Matcha content incorporated (P<0.05). As shown in the Figure 5.4C, the sweetness acceptability obtained the highest score with the biscuits that had sugar level at 30 g per 100 g of flour and at 2 g of MGTP per 100 g of flour.

5.2.4 Comparison of Asian and other ethnic groups on overall acceptability

When the overall acceptability of biscuits was compared between Asian and other ethnic participants, it was found that Asian participants had given a higher score of overall acceptability of all biscuits tested than other ethnic participants (Table 5.6). However, the difference was not significant ($P<0.05$), due to the large variation.

Table 5.6: Comparison of overall acceptability of biscuits between Asian and other ethnic groups

Experiment number and abbreviated name	Overall Acceptability	
	Asian	Other ethnic groups
1: 2M25S1	5.96±1.80	5.77±1.84
2: 2M30S1	6.14±1.35	5.77±2.10
3: 2M35S1	6.32±2.04	5.92±2.26
4: 4M25S1	5.61±1.87	4.77±2.41
5: 4M30S1	6.36±1.47	5.35±2.33
6: 4M35S1	6.07±1.63	5.08±2.17
7: 2M25S2	6.74±1.60	6.52±1.65
8: 2M30S2	6.43±1.27	6.30±1.72
9: 4M30S2	6.43±1.20	5.87±1.74
10: 4M35S2	6.52±1.41	5.91±1.56
11: 6M25S2	5.39±1.90	4.78±1.78
12: 6M30S2	5.87±1.87	4.78±2.17
13: 6M35S2	6.30±1.64	5.39±2.15

5.3 Discussion

This study did not include a control biscuit to compare the result because the aim was to assess the effect of increasing MGTP and sugar in biscuit on the acceptability and their interaction effect on the acceptability. Hardness, as an important quality index measure, was determined in shortbread biscuits incorporated with MGTP in both sensory acceptability test evaluation and instrumental analysis. The results are shown in Table 5.2 and 5.4. The hardness increased with the increase in MGTP content in the instrumental analysis. This result is agreement with previous studies on cake, noodlea and a traditional korean rice snack (Yukwa) that were incorporated with green tea powder where they found an increase of hardness with increasing levels of green tea powder (Shen *et al.,* 2014; Li *et al.,* 2012; Lu *et al.,* 2010). On the other hand, the result from this study contrasts with a study by Ahmad *et al.,* (2015) that found a decrease in hardness of cookies with an increasing level of green tea powder added to formulations. This could be due to the fibre content of green teas used in the studies. Fibre can influence the hardness of biscuits is the content of fibre. Previous studies have observed increased breaking strength when flour is replaced by fibre (Laguna *et al.*, 2014; Tosh and Yada, 2010; Gujral *et al.*, 2003). Brennan and Samyue (2004) studied different soluble and insoluble fibres for flour replacement in biscuits, observing a slight increase in the breaking strength values for those containing potato peel (insoluble) and no increase for inulin and beta-glucans (soluble). In addition, Laguna *et al.* (2013) suggested that longer wheat fibre gave harder texture to biscuits compared to the medium length wheat fibre. Therefore, the types of fibres found in food components could have a different effect on the overall hardnesswhen incorporated into biscuits. MGTP used in our study contained 2.6% of fibre which could affect the hardness of biscuits. Both origin of tea and processing may have an effect on the types of fibres and content of fibres found in tea leaves. Ahmad *et al*. (2015) used green tea leaves purchased in India, whereas this study used MGTP produced in China. Therefore, the fibre content in green tea needs to be investigated further to understand the impact on texture.

The high sugar incorporation in the dough has contributed to a glassy texture and high spread ratio (Manley, 1998). The increase in diameter, length and width during baking is related to the quantity of sugar and its particle size. High sugar content increases spread

because the sugar liquefies at baking temperatures. Granulated sugar is most likely to retain a crisp texture because sugar is hygroscopic (It readily takes up water) and white sugar makes crisper cookies than brown sugar or honey. In addition, the small particle size of sugar contributes to more spread than a coarse particle. In our study, the caster sugar was use in the biscuit making. The caster sugar is refined. Hence, it increased the spread of biscuits. As the results in Table 5.2 shown, the higher sugar incorporation led to higher spread ratio. The biscuit in this study also contained a high amount of fat which may play an important part in the interaction of protein and starch which affects the hardness. The fat in shortbread inhibits the formation of long protein strand called gluten, which is found in wheat flour and gives elasticity to dough leading to a chewy texture in final products. As a result, the inhibition leads to crumbling texture of shortbread (Manley, 2001). The high content of fat in biscuits leads to a decrease in hardness of biscuits. A decrease level in MGTP in the biscuit formulation contributed to higher fat content in biscuits. Hence, this may decrease the formation of gluten and lead to lower hardness in biscuits, as shown in the result that the hardness of biscuits with 2 g of MGTP was lower than the hardness of biscuits with 6 g of MGTP. The result in this study showed that low moisture content (1.2-2.5%) in the biscuits did not affect the hardness. This result is in an agreement with a study by Mandala *et al.* (2006) indicated that biscuits with low moisture content ranging from 0.4-3% did not contribute to a significant difference in hardness.

Even though MGTP incorporation significantly increased the hardness of biscuits, the texture acceptability of each biscuit was not significantly different from each other. This could be due to the principal difference between a sensation and a mechanical test (Wang *et al.*, 2007), also, the varied perception of participants, which is difficult to keep uniform among participants. The hardness of bread was found to increase with the increase of GTEP incorporated in bread by both a texture analyser and sensory analysis with trained and untrained panellists (Wang *et al.*, 2007). The hardness obtained from the panellists was more varied than the texture analyser due to the varied perception of each individual. In addition, the participants rated the acceptability according to their own preference, hence the variation in result could occur.

The ANOVA and RSM analysis of acceptability data showed a similar trend that low MGTP and sugar content received the highest acceptability. The increase in sugar in biscuit

formulation did not significantly affect the acceptability according to two-way ANOVA. However, with RSM analysis, the overall acceptability was significantly affected by sugar content ($P<0.05$). Moreover, appearance, colour and bitterness acceptability was significantly different between 2 g and 6 g MGTP incorporated in biscuits according to two-way ANOVA. These attributes are the main factors that affected the overall acceptability of the biscuits.

It has been revealed that tea catechins are responsible for tastes of astringency and bitterness. EGCG and ECG are more astringent than EC, EGC, and catechin (Narukawa *et al.*, 2010; Scharbert and Hofmann, 2005). EGCG was the most abundant catechin (approximately 50% of total catechins) in the MGTP used, and according to the earlier study in chapter 4. There was approximate 80% of EGCG remaining in the biscuit after baking. This means that there were about 3.4, 7.3, 10.4 mg of EGCG in each biscuit with MGTP at the level of 2, 4, 6 g per 100 g of flour, respectively. Another component that contributes the bitterness to green tea is caffeine, which was found in biscuits at 0.4, 0.9 and 1.3 mg in each biscuit with MGTP. Therefore, the EGCG as well as other catechins and caffeine in MGTP enable the participants to detect astringency and bitterness, which affects the acceptability. Wang *et al.* (2007) suggested that variation in the astringency perception and rating could be due to the different salivary flow produced in individual participants. Participants who produce a greater amount of saliva are expected to perceive a lower level of astringency intensity (Drobna *et al.*, 2004). This could be the reason for the significant variation of acceptability.

~~From two-way ANOVA and RSM result,~~ the bitterness acceptability was decreased as the level of MGTP incorporated in biscuits increased. This is possibly due to the increase of catechins and caffeine that contribute to bitterness in biscuits. In the second session of sensory evaluation, the increase of sugar increased sweetness acceptability of biscuits with 6 g of MGTP per 100 g of flour. However, the increase of sugar did not increase the sweetness acceptability in the biscuits with 2 and 4 g of MGTP per 100 g of flour. Bitterness can significantly suppress the sense of sweetness. In other words, the sense of sweetness is reduced when astringency and bitterness are perceived (Wang *et al.*, 2007). It was found that bitterness can be perceived at much lower concentration than sweetness at ratio of 1:31 magnitude (Green *et al.*, 2010). The author suggested that that the tasting

system of human has evolved to detect bitterness, which regards as a potential toxin at a small quantity against the sweetness, which represents the need to find and consume the high-energy food. Therefore, the sweetness of sugar was less detectable in biscuits with a high level of MGTP incorporated. This may explain the increase of the acceptability of biscuits with 6 g of MGTP per 100 g of flour when the sugar content was increased in the biscuits.

The RSM result suggested the highest aroma acceptability received from 2 g of MGTP. A higher level of MGTP incorporated in biscuits may contribute to higher undesirable aroma due to the heat treatment. Heat treatment can cause the undesirable smell of green tea. The trained panelist detected more undesirable (described as wet wood smell) aroma of heat processed green tea at 121℃ for 1 min comparing to unprocessed green tea (Wang *et al.*, 2003b). Therefore, the aroma of shortbread biscuits MGTP may be affected by baking temperature at 180℃ that cause the undesirable aroma. However, Ahmed found the increasing in green tea powder in cookies (1-4%) increase the aroma acceptability of cookies, this could be due to the difference in aroma components found in tea variety that grown in India and China.

The higher amount of MGTP added to biscuits contributed to a more intense green colour from chlorophyll in green tea leaves (Wang *et al.*, 2004). Therefore, the colour and appearance acceptability was significantly decreased by incorporation of MGTP. The baking temperature could change the colour of chlorophyll in MGTP. Kim *et al.* (2007) suggested that green tea solution became less green and deeper yellow after heating 120℃ for 4 min. Therefore, masking the colour of biscuits by adding fresh green food colour may increase the acceptability of biscuits.

RSM plots of aroma, colour and sweetness acceptability suggested that 2 g of MGTP per 100 g of flour and the sugar level at 30 g per 100 g of flour to give highest response. Therefore, this sugar level (equally 15.5 g of sugar per 100 g of shortbread) can be used as a guide to develop a green tea shortbread formulation with sugar substitutes, such as stevia in order to receive good acceptability. The commercial shortbread (Walker) in the market contained almost the same amount of sugar (15.8 g of sugar per 100 g of shortbread) with shortbread with MGTP.

The RSM result suggested that the biscuits that were repeated the test in the second sessions received a higher average score in every acceptability tested. Moreover, the Asian participants were likely to give a higher score in acceptability than other ethnic participants (Table 5.6). These results correlated with a study on the comparison of green tea products between Korean and French consumers (Kim *et al.*, 2013). The study found that familiarity of products influenced consumers' perception and acceptability of green teas. Therefore, the Asian participants who are more familiar with green tea products rated the biscuits higher than other ethnic participants did. This could be due to the familiarity of the product leading to a higher score. Therefore, increase in familiarity of MGTP may increase the acceptability of biscuit. The session explaining about the beneficial information of MGTP prior to the test may help participants to get familiar with MGTP and rate higher acceptability.

5.4 Conclusion

Hardness is likely be affected by the increase of MGTP, as the hardness of biscuits with 6 g of MGTP was significantly higher than the biscuits with 2 g of MGTP. The effect of MGTP on shortbread biscuit acceptability was assessed using a 9-hedonic point scale and the data was analysed with response surface methodology. The results of RSM found that the MGTP significantly affects the acceptability of biscuits including appearance, aroma, colour, texture, bitterness and sweetness. The RSM analysis showed that sugar significantly increased the overall acceptability of biscuit. Therefore, sweetness may mask the undesirable taste of green tea in biscuits that contained a high amount of green tea.

The results from this chapter together with the chapters 3 and 4 provide useful information on the selection of the appropriate amount of MGTP in biscuits flavouring and enrichment. With high levels of MGTP added to biscuits, the acceptability of the biscuits was not significantly compromised and if promoted as a functional health food may have some success. Meanwhile, the MGTP incorporated into biscuits may produce a functional food product with additional health benefits. This study provides a good guide for the addition of MGTP in biscuits for the bakery manufacturers who are to pursue the production of biscuits incorporated with MGTP.

6 Chapter six*:* A pilot study investigating the effect of MGTP on postprandial glucose, triglyceride and satiety responses in healthy subjects (randomised triple – crossover design trial)

6.1 Introduction

Nutritional research has shown that the major antioxidant compounds (catechins) found in green tea have potential roles in preventing cancer, diabetes and cardiovascular diseases (Yang *et al.*, 2014; Thielecke and Boschmann, 2009). For instance, Zhong *et al*. (2006) found a 25% decrease in carbohydrate absorption after a single consumption of green tea extract containing 300 mg of EGCG, 100 mg of ECG and 100 mg of theaflavin with a carbohydrate meal (Zhong *et al.*, 2006). Hence, the study suggested that green tea may reduce the amount of glucose absorbed into the bloodstream, which may lower the risk of developing diabetes (Zhong *et al.*, 2006). Moreover, a review by Koo and Noh (2007) suggested that green tea reduces the absorption of dietary fat by interfering with the processes of lipid digestion in the intestine. For example, a study in an animal model on the effect of antioxidant compounds found in green tea on the absorption of dietary lipids suggested that the compounds can slow down lipid absorption by inhibiting the activity of pancreatic lipase (Ikeda *et al.*, 2005; Unno *et al.*, 2005). Unno *et al*. (2005) has shown that 224 mg of green tea catechins can lower the concentration of lipids in the blood by 15% after meal consumption and therefore may lower the risk of developing the cardiovascular diseases. Moreover, another study found the increased satiety after the consumption of green tea with a meal (Josic *et al.*, 2010). The increased satiety may be another solution in energy intake control and weight management. Due to evidence that green tea has the potential to lower the risk of developing diseases such as diabetes and cardiovascular diseases, MGTP is used in this study to investigate its effect on glucose, triglyceride, and satiety responses upon consumption of shortbread biscuits containing MGTP.

Shortbread biscuits have a high fat content from butter (36%), high starch content from flour (37.7%) and sucrose content from added sugar (16.3%). MGTP at a level of 6 g per 100 g of flour or approximately 2.7% was added to the formulation as explained in chapter 2 (section 2.5.2.2). The response to these biscuits on consumption will be compared to that of plain shortbread biscuits consumed with a green tea drink or water. In addition, the effect of MGTP on satiety level after consumption of shortbread biscuits will be assessed.

A cross-over design was adopted so that each individual's response to the different test foods could be assessed independently of the intra-individual variation.

Despite the growing interest in the effect of green tea on postprandial plasma glucose, triglyceride responses, and their metabolism, to our knowledge, this is the first study that investigates the effect of MGTP incorporated in a bakery product on the postprandial glucose, triglyceride, and satiety responses.

6.1.1 Aim

I. To conduct a pilot study to study the effect of MGTP in shortbread biscuits on glucose response, triglyceride response and satiety levels in healthy human subjects in comparison to consuming a shortbread plain biscuits consumed with either green tea drink or water

6.1.2 Objective

- To measure the glucose response and triglyceride response in human subjects after consuming a provided portion of three food samples: a. plain shortbread biscuits consumed with water (control), b. green tea enriched shortbread biscuits consumed with water, c. plain shortbread biscuits consumed with green tea drink, over 3 hours using a finger prick glucometer, or cardiochek® meter respectively
- To measure the satiety level in human subjects after consuming a provided portion of the mentioned food samples over 3 hours by self-reporting visual analog scale provide for participant to mark their hunger/satiety on the scale
- To explore the effect of demographic characteristics like, gender, BMI, and ethnicity on glucose response using ANOVA followed by Tukey's-b *post hoc* test where required

6.2 Results

6.2.1 Demographic characteristics

Table **6.1** shows the recorded characteristics of subjects; 10 subjects were recruited from different backgrounds (n = 4 from Asian countries, n = 3 from South American countries, n = 2 from Europe, n = 1 from Africa) with ages ranging from 27 – 44 years. The mean BMI of subjects was 26.67±4.48 kg/m^2. Among the 10 subjects, 1 subject was obese, 5 subjects were overweight. Sixty percent of participants were female subjects. Sixty percent was alcohol consumers. Seventy percent reported that they performed physical activity at least 30 min weekly and the physical activities reported were walking, bicycling and swimming.

Table 6.1: Characteristics of the subjects (n=10), providing with age, ethnicity, BMI status, gender, smoking status, alcohol consumption and physical activity

#	Age (year)	Ethnicity	BMI (kg/m^2)	Gender	Smoking	Alcohol consumption	Physical activity
1	44	European	25.4	Female	No	Yes	Yes
2	30	European	24.5	Male	No	Yes	Yes
3	36	African countries	37.1	Female	No	Yes	Yes
4	30	Mixed-South American countries	24.6	Female	No	Yes	Yes
5	27	Mixed-South American countries	21.9	Male	No	Yes	Yes
6	30	Mixed-South American countries	28.3	Female	No	Yes	Yes
7	30	Asian countries	25.7	Female	No	No	No
8	32	Asian countries	29.4	Female	No	No	No
9	31	Asian countries	28.3	Male	No	No	No
10	28	Asian countries	21.6	Male	No	No	Yes

[1] Body Mass Index (BMI) ranges:
Underweight 18.5 or below
Normal 18.5-24.9
Overweight 25-29.9
Obese 30 and over

6.2.2 Food samples

The nutritional information of food samples (Table 6.2) were calculated by using data from nutritional labels of ingredients and compensating with moisture loss after baking. Figure 6.1 shows the portions of biscuits in a plastic bag given to participants. Table 6.3 presents the amount of catechins and caffeine in MGTP used to produce green tea biscuits and drink for this study and the content of catechins and caffeine found in biscuits. The content of catechins and caffeine was determined by using HPLC method described in chapter 2.

One portion of green tea biscuit samples (100 g) contained approximately 232.5 mg of total catechins, whereas one portion of green tea drink made with MGTP contained 256.8 mg of total catechins.

Figure 6.1: Packages of green tea biscuits and plain biscuits (100 g per pack) served to the subjects.

Table 6.2: Estimated nutritional data of food samples

	Control	Green tea biscuits	Plain biscuits with green tea drink
Food samples	100 g of plain biscuits with 300 mL of water	100 g of green tea biscuits with 300 mL of water	100 g of plain biscuits with green tea drink (used 3 g of MGTP dissolved in 200 ml of warm water) and 100 ml of water
Carbohydrate (g)	54.0	54.6	55.0
Sugar (g)	16.0	15.5	16.0
Fat (g)	36.0	35.15	36.15
Protein (g)	6.0	7.0	6.9
Estimated energy (kcal)	550	554	559
Total catechins (mg)	0	232.5	256.8

Table 6.3: Average catechins and caffeine content in MGTP and one biscuit (10 g)

Catechins and caffeine	mg/g	mg/biscuit
EGC	18.4±2.2	4.6±0.6
EC	6.9±1.3	1.2±0.1
EGCG	45.9±5.5	12.4±1.1
GCG	6.8±0.9	2.7±0.3
ECG	7.6±0.9	2.1±0.2
Total catechins	85.6±10.6	23.3±2.0
Caffeine	6.5±0.8	1.9±0.3

6.2.3 Fasting blood glucose, triglycerides and satiety level of the participants

Table 6.4 shows the average fasting blood glucose, triglycerides, and satiety level over three measurements for each participant. To reduce the intra-individual variation, participants were required to fast 12-14 hours prior to the study visits. The fasting blood glucose and triglyceride levels were within the normal ranges (3.9-5.5 mmol/L for glucose and <1.70 mmol/L for triglycerides) (Roche, 2011; BHR). The average fasting blood glucose and triglycerides were 4.9±0.4 and 1.09±0.25 mmol/L while the average fasting satiety level was -4.1±2.6 cm. The highest fasting blood glucose was associated with participant #8 (female and Asian subject), 5.6±0.3 mmol/L, while the lowest was associated with participant #4 (female and mixed ethnicity), 4.3±0.1 mmol/L. For the fasting blood triglycerides, the highest was associated with participant #6 (female and mixed ethnicity), 1.50±0.27 mmol/L, whereas the lowest was associated with participant #2 (male and European subject), 0.71±0.04 mmol/L. For the fasting satiety level, the highest was associated with participant #1 (female and European subject), 0.7±2.8 cm, whereas the lowest was associated with participant #7 (female and Asian subject), -7.5±4.3 cm.

Table 6.4:Baseline blood glucose, triglyceride and satiety level of the participants, data express as the mean ± STD (n=10)

#	Fasting blood glucose[1] (mmol/L)	Fasting blood triglyceride[2] (mmol/L)	Fasting satiety level[3] (cm)
1	5.0±0.1	0.9±0.2	0.7±2.8
2	4.7±0.2	0.7±0.0	-3.7±1.3
3	4.8±0.2	1.3±0.5	-5.8±1.4
4	4.3±0.1	1.0±0.4	-2.1±3.2
5	5.1±0.1	0.9±0.1	-3.3±1.4
6	5.4±0.2	1.5±0.3	-3.3±1.4
7	4.8±0.1	1.4±0.2	-7.5±4.3
8	5.6±0.3	1.1±0.2	-2.9±0.7
9	5.0±0.1	1.0±0.1	-7.5±2.5
10	4.5±0.2	1.2±0.5	-5.8±1.4
Total average	4.9±0.4	1.1±0.3	-4.1±2.6

[1] Mean of fasting blood glucose (n=3)

[2] Mean of fasting blood triglycerides (n=3)

[3] Mean of fasting satiety level (n=3) , The scale of satiety level was rating from extremely hungry to extremely full (measuring from -10 cm to 10 cm scale line)

The coefficient of variation (CV) of fasting blood glucose, blood triglycerides and satiety level of all of participants were 8%, 23%, and 62% respectively. The CV of satiety level was large, reflecting the high variation in satiety level between study visit and between individuals. High variation of fasting satiety level could be because the participants were allowed to rate the scale by themselves. Each person rates their own satiety based on the keyword (‘extremely hungry’ to ‘extremely full’) on the scale line.

One way ANOVA using Tukey’s-b analysis was conducted to investigate the demographic variation between the subjects that may affect the glucose response, triglyceride response and satiety level. There was no significant difference in fasting blood glucose, fasting blood triglycerides, and fasting satiety level among ethnic groups (Table 6.5).

Table 6.5: Comparison of BMI and fasting blood glucose, triglyceride, and satiety level of the participants in different ethnic groups. Data expressed as the mean ±STD.

Ethnicity	All (n=10)	Asian (n=4)	European (n=2)	Mixed – South American (n=3)	*P*[3]
Age	31±5	30±2	37±10	27±3	0.13
Gender[1]	6F:4M	2F:2M	1F:1M	2F:1M	-
BMI[2]	26.7±4.5	26.3±3.5	25.0±0.6	24.9±3.2	0.81
Fasting blood glucose (mmol/L)	4.9±0.4	5.0±0.5	4.9±0.2	4.9±0.5	0.97
Fasting blood triglycerides (mmol/L)	1.1±0.3	1.2±0.2	0.8±0.1	1.1±0.4	0.26
Fasting satiety level (cm)	-4.1±2.6	-5.9±2.2	-1.5±3.1	-2.9±2.0	0.09

[1] F:M = #Female: #Male

[2] Body Mass Index

[3] Significant value (significant $P < 0.05$) between ethnic groups

6.2.4 Glucose response

Figure 6.2 represents the mean values of plasma glucose curves of the 10 participants over 180 min period after consumption of the 3 test meals. The average incremental area under the curve (iAUC) of glucose response over 180 min was calculated according to equation 10 mentioned in section 2.7.7.6. Consumption of plain biscuits gave the highest average iAUC (106.1±63.0 mmol.min/L) and the consumption of plain biscuits with green tea had the lowest average iAUC (81.9±36.0 mmol.min/L). The glucose response of all of the food samples peaked at 30 min after the intake. Plain biscuits with green tea gave the highest maximum incremental blood glucose peak at 1.21±0.22 mmol/L, whereas green tea biscuits gave the lowest maximum peak at 1.06±0.10 mmol/L. Glucose response of all food samples reached back to the baseline within a 180 min period. No significant differences were observed in glucose levels or the iAUCs between food samples (Table 6.6). The glucose response of each subject after consumption of food samples over 180 minutes is presented in Figure 6.3.

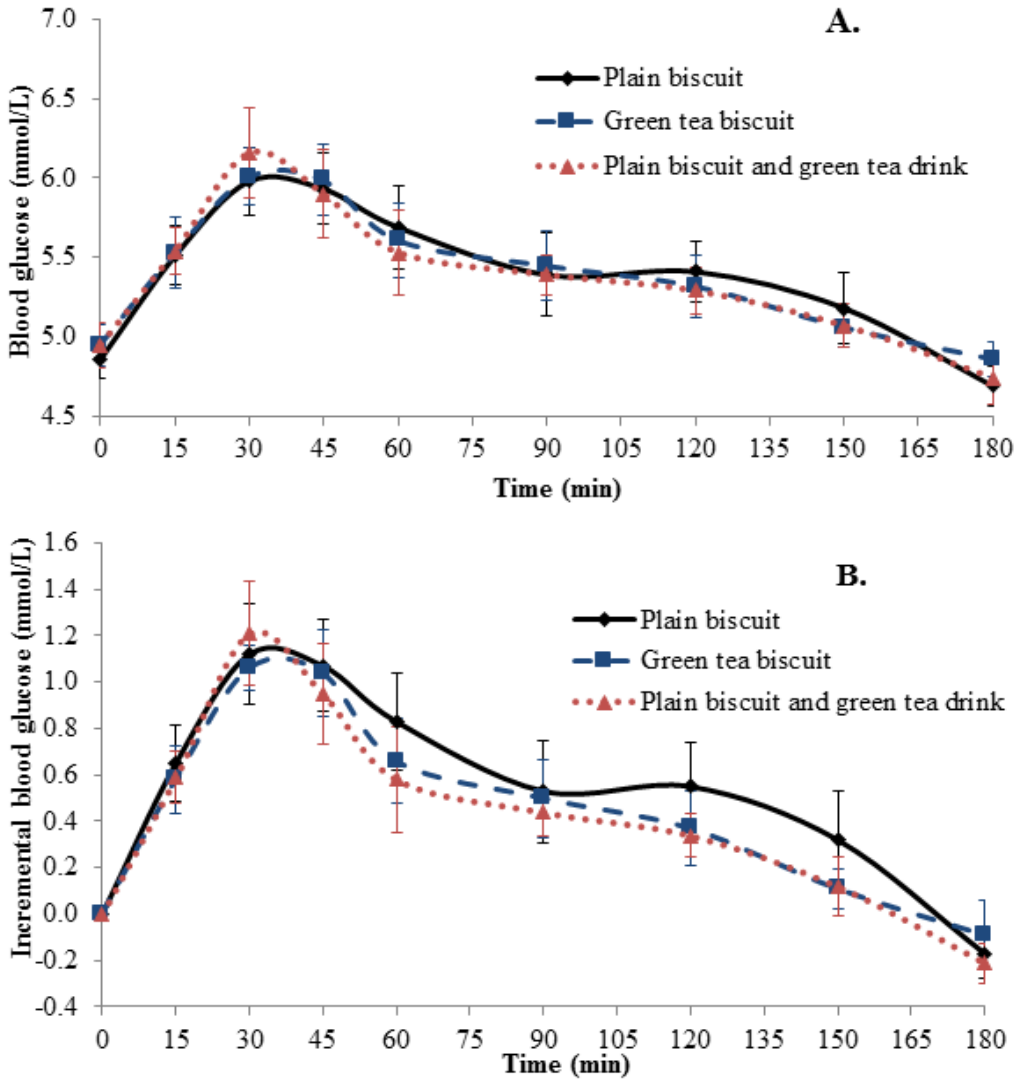


Figure 6.2: A. The mean blood glucose response curves and B. The mean incremental blood glucose response curves after ingestion of food samples over 180 minutes. (-◆-) represents the blood glucose curve of plain biscuit, (-■-) represents the blood glucose curve of green tea biscuit, and (..▲..) represents the blood glucose curve of plain biscuit consumed with green tea drink. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the SEM (n=10 participants).

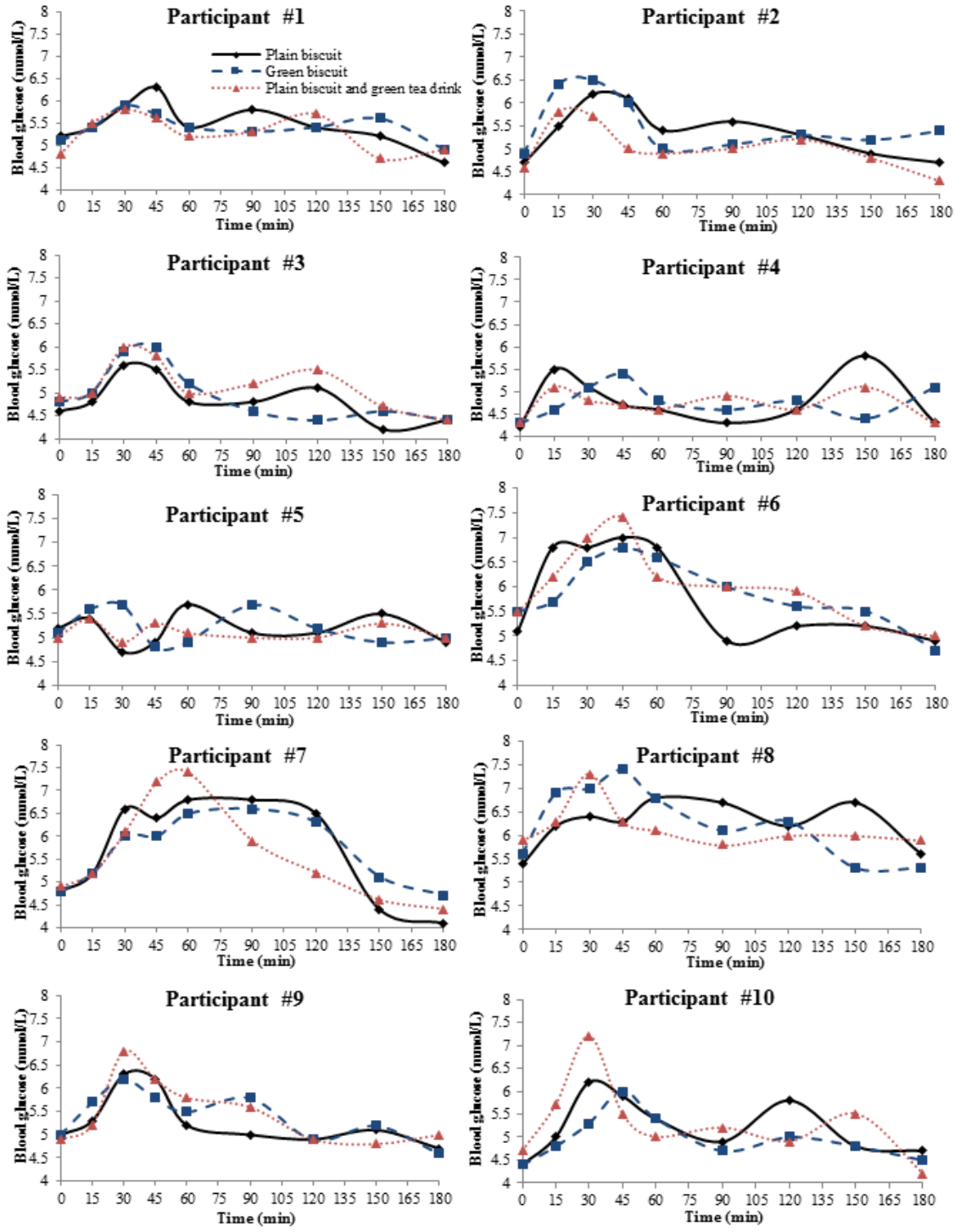


Figure 6.3: The individual profiles of glucose response after consumption of food samples over 180 minutes. (-◆-) represents the blood glucose curve of plain biscuits, (-■-) represents the blood glucose curve of green tea biscuits, and (..▲..) represents the blood glucose of plain biscuits consumed with green tea drink.

6.2.4.1 Inter-individual variation in glucose response and factors affecting glucose response

One-way ANOVA using Tukey's-b analysis was performed to investigate the variation that may affect the glucose response. There was no significant difference in fasting blood glucose and iAUC after consumption of plain biscuits (reference food) between the three ethnic groups (Table **6.5**).

6.2.4.1.1 Demographic factors and glucose response

Participant #1 was the oldest among the other participants (44 years) and the iAUC (58.5 mmol.min/L) was almost 4 times higher than participant #5 who was the youngest (27 years) with an iAUC of (12 mmol.min/L), after ingestion of plain biscuits. In addition, the number of males recruited in this study was 4 and the average iAUC was 83.8±65.9 mmol.min/L, while 6 females were recruited and the average iAUC was 120.9±62.3 mmol.min/L, which was 44% higher than the males. However, as shown in Table **6.6**, there was no significant difference of iAUC between gender and food samples.

Table 6.6: Incremental area under curve (mmol.min/L) observed in the subjects after consumption of the food samples. Data expressed as mean of iAUC±STD.

Food samples	All	Female (n=6)	Male (n=4)	P^1
Plain biscuit	106.1±63.0	120.9±62.3	83.8±65.9	0.39
Green tea biscuit	89.2±44.7	96.5±52.3	78.2±34.3	0.56
Plain biscuit with green tea drink	81.9±36.0	86.0±37.1	75.8±38.8	0.68
P^2	0.54	0.50	0.97	

[1] Significant value (significant $P < 0.05$) between female and male subjects

[2] Significant value (significant $P < 0.05$) between food samples

6.2.4.1.2 Lifestyle factors and glucose response

Subject #3 had the highest BMI (37.0 kg/m^2) and the iAUC of plain biscuits (51 mmol.min/L) was 67 % lower than subject #10 who had the lowest BMI (21.63 kg/m^2) and an iAUC of 154.4 mmol.min/L. Furthermore, subjects # 7 and # 5 had the highest and the lowest iAUC of plain biscuits (207 and 12 mmol.min/L respectively) were both physically active. After consumption of plain biscuits, the average iAUC of normal BMI participants (n=4) was 100.7±61.7 mmol.min/L, whereas the average iAUC of overweight BMI participants (n=5) was 121.4±70.7 mmol.min/L. The average iAUC of overweight BMI participants was higher than the average iAUC of normal BMI in all the tested food samples; however, there was no significant relationship between the iAUC of normal and overweight BMI subjects (Table **6.7**).

Table 6.7 : Incremental area under curve (mmol.min/L) observed in normal BMI and overweight BMI subjects after consumption of the food samples. Data expressed as mean of iAUC±STD.

Food samples	Normal BMI (n=4)	Overweight BMI (n=5)	P^1
Plain biscuit	100.7±61.7	121.4±70.7	0.66
Green tea biscuit	80.1±34.3	105.6±51.7	0.43
Plain biscuit with green tea drink	73.1±37.5	93.8±38.2	0.44
P^2	0.69	0.74	

[1] Significant value (significant $P < 0.05$) between BMI groups

[2] Significant value (significant $P < 0.05$) between food samples

6.2.4.1.3 Effects of ethnicity on glucose response

Table **6.8** represents the iAUC (mmol.min/L) observed in the subjects from different ethnic groups after consumption of the food samples. The glucose response to plain biscuits for the Asian subjects was the highest iAUC (146.3±69.9 mmol.min/L) while the iAUC of Europe and Mixed ethnic (South American countries) subjects were 90.4±45.1 and 81.25±60.0 mmol.min/L respectively. However, there was no significant

difference in iAUC of the 100 g of plain biscuits, green tea biscuits and plain biscuits with green tea drink between three ethnic groups (*P*=0.40, 0.20, 0.64).

Table 6.8: Incremental area under curve (mmol.min/L) observed in the subjects from different background after consumption of the food samples. Data expressed as mean of iAUC ±STD.

Food samples /Ethnicity	Asian (n=4)	European (n=2)	Mixed – South American (n=3)	*P*[1]
Plain biscuit	146.3±69.9	90.4±45.1	81.25±60.0	0.40
Green tea biscuit	123.8±46.8	79.5±28.6	64.8±29.5	0.20
Plain biscuit with green tea drink	97.7±44.4	83.6±4.8	67.8±42.0	0.64
***P*[2]**	0.49	0.94	0.90	

[1] Significant value (significant *P* < 0.05) between ethnic group

[2] Significant value (significant *P* < 0.05) between food samples

6.2.5 Triglyceride response

Normally, triglyceride response is studied for 6 hours because it takes 6-8 hours for triglycerides to peak and return to baseline after food consumption. However, due to the limited time and difficulty in participant recruitment, this study aimed to monitor the triglyceride response for 3 hours as a pilot study to observe the trend in triglyceride response due to the effect of green tea. There was no significant difference in the baseline level of plasma triglycerides among the three ethnic groups (Table **6.5**). There was also no difference in fasting triglyceride levels among the participants between test meals and control meal.

The incremental postprandial response of plasma triglycerides are illustrated in Figure 6.4. When the participants consumed the test meal, plasma triglycerides rose from the baseline (0 min) and reached the highest level at 120 min after the ingestion of test

meal. The concentration of triglycerides almost reached back to baseline after 180 min of the consumption. As shown in Figure 6.4, the concentration of triglycerides after the consumption of green tea biscuits remained close to the baseline value after 60 min, then rose to the highest level at 120 min after the intake and returned to baseline at 180 min.

Over 180 minutes, the control meal (plain biscuits) induced an average 0.29 mmol/L increase in plasma triglycerides over the baseline level at 120 min. While green tea biscuits induced an average 0.25 mmol/L increase in plasma triglycerides and the plain biscuits consumed with green tea drink induced an average 0.41 mmol/L increase in plasma triglycerides. The average reduction in iAUC after green tea biscuits consumption (35.6 ± 44.6 mmol.min/L) was 20.2%, compared with the plain biscuits consumption (44.6 ± 32.1 mmol.min/L). While iAUC after plain biscuits consumed with green tea drink (39.4 ± 35.7mmol.min/L) was 11.7% lower than iAUC of plain biscuits consumption. The one-way ANOVA with comparison using Tukey's-b test was applied. The analysis found that there was no significant difference in iAUC between all three food samples ($P = 0.87$).

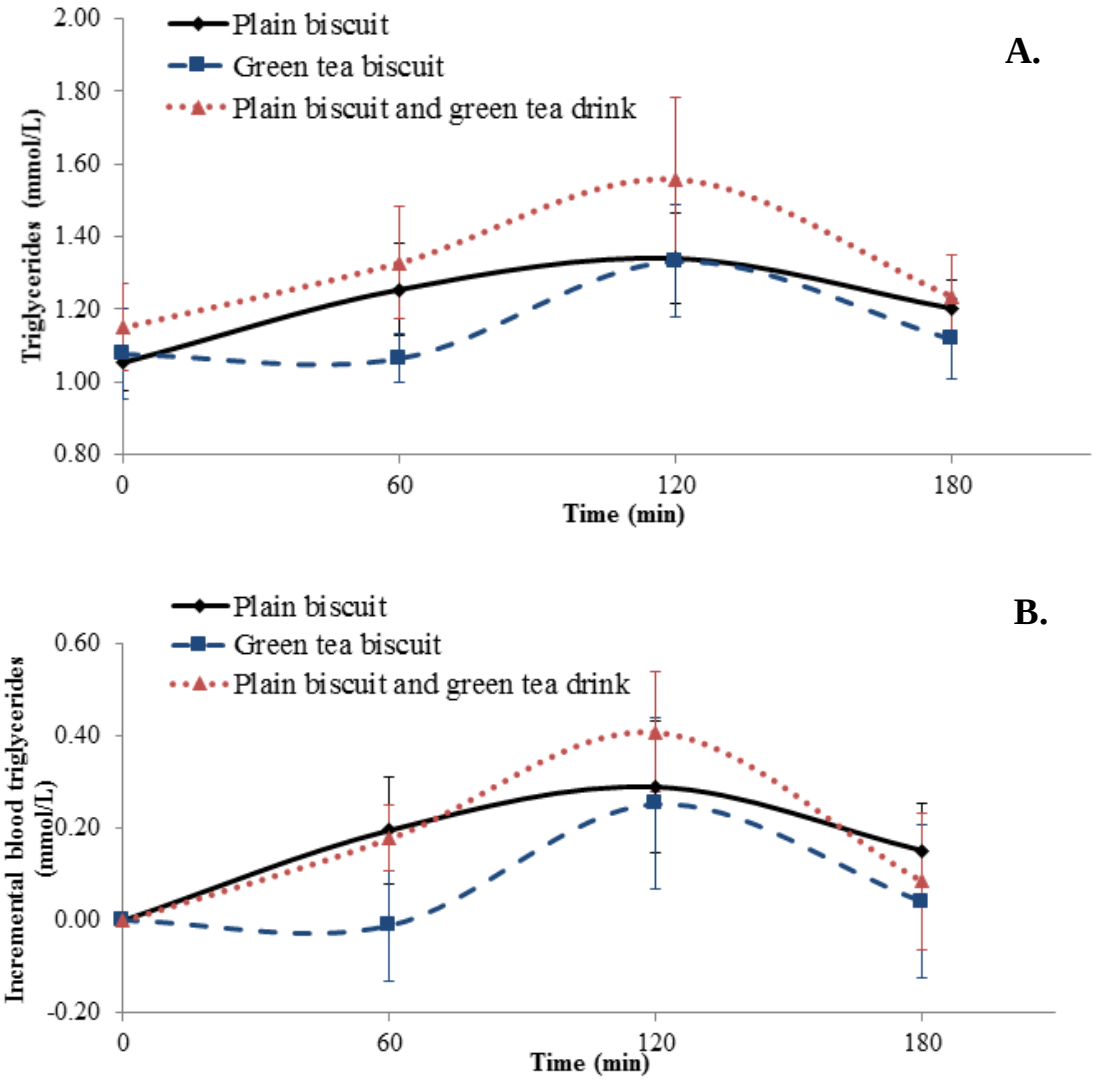


Figure 6.4: A. The mean blood triglyceride response curves and B. the mean incremental blood triglyceride response curves after ingestion of food samples over 180 minutes. (-◆-) represents the blood triglyceride curve of plain biscuits, (-■-) represents the blood triglyceride curve of green tea biscuits, and (..▲..) represents the blood triglyceride curve of plain biscuits consumed with green tea drink. Data expressed as the amount of triglyceride in mmol/L and the error bars represent the SEM (n=10 participants).

6.2.5.1 Triglyceride response in Asian subjects

Figure 6.5 shows the incremental postprandial levels of plasma triglycerides after ingestion of three food samples among Asian subjects. The maximum level of plasma triglycerides after consumption of plain biscuits (control) in Asian subjects was reached at 60 min and dropped slightly, then remained the same until 180min, whereas the maximum level of plasma triglycerides after consumption of plain biscuits with green tea drink was reached at 120 min and returned to baseline at 180 min. Unlike the control, the triglyceride response after ingestion of green tea biscuits fell slightly lower than the baseline value at 60 min after intake, returned to baseline at 120 min and slightly dropped again at 180 min after ingestion. Among Asian subjects, an average 0.26 mmol/L decrease in plasma triglycerides over the baseline level at 60 min was found in the green tea biscuit meal, whereas, the plain biscuits induced an average 0.44 mmol/L increase in plasma triglycerides over the baseline level at 60 min. At 60 min after ingestion of food sample among Asian subject, the incremental triglycerides of green tea biscuits was significantly lower than control (plain biscuits) and plain biscuits with green tea drink ($P = 0.005$).

The iAUC of green tea biscuits (8.0 ± 12.5 mmol.min/L) was significantly lower ($P = 0.02$) than other food samples; (iAUC of plain biscuits, 51.7 ±22.3 mmol.min/L and iAUC of plain biscuits and green tea drink, 32.7 ± 17.6 mmol.min/L). Compared with the iAUC of the ingestion of plain biscuits, the average reductions in iAUC after a consumption of green tea biscuits was 84.5%, and after consumption of plain biscuits with green tea drink was 36.8%. However, there was no significant effect on iAUC observed between control and plain biscuits with green tea drink.

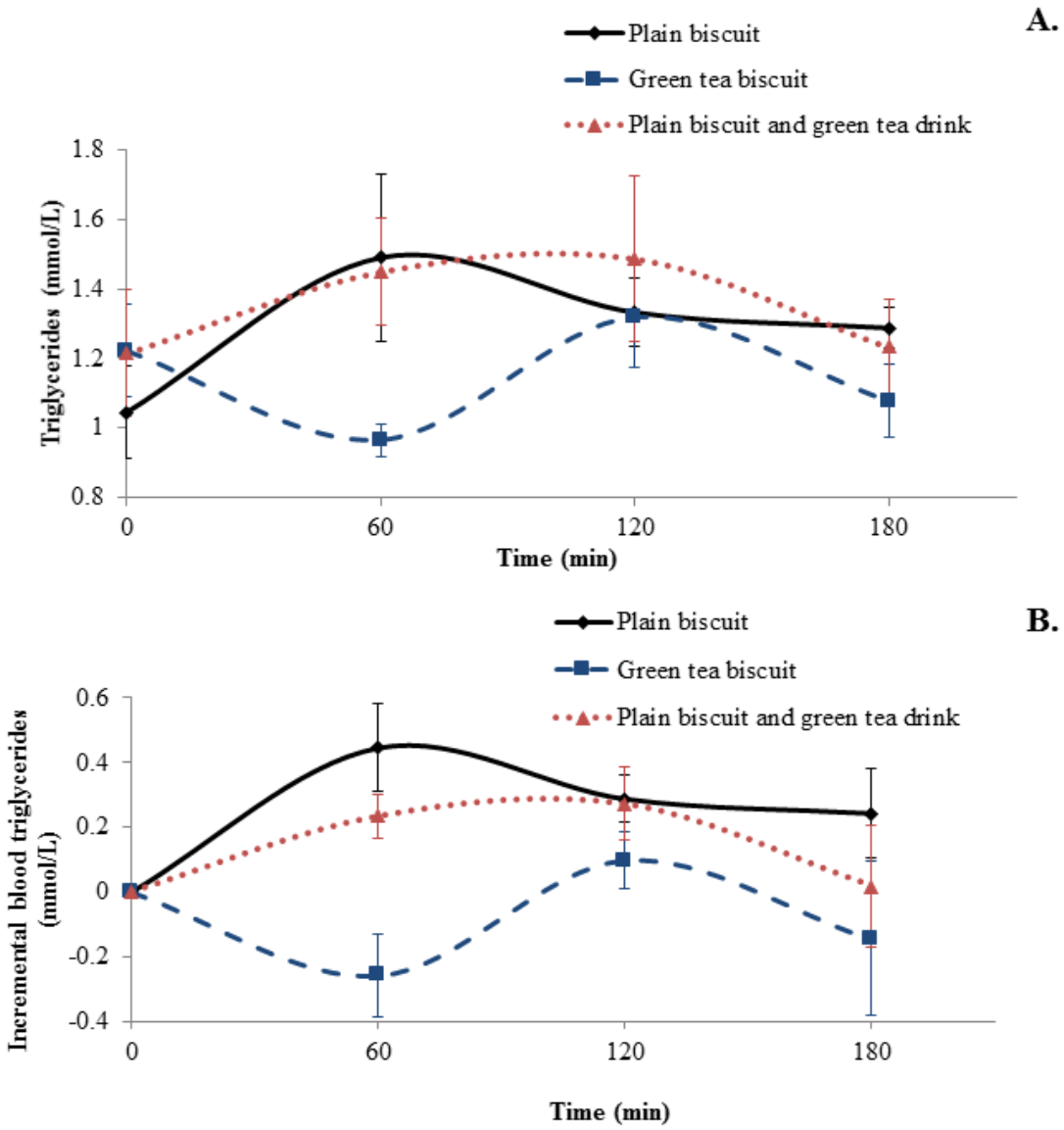


Figure 6.5: A. The mean blood triglyceride response curves and B. the mean incremental blood triglyceride response curves after ingestion of food samples over 180 minutes among Asian subjects. (-◆-) represents the blood triglyceride curve of plain biscuits, (-■-) represents the blood triglyceride curve of green tea biscuits, and (..▲..) represents the blood triglyceride curve of plain biscuits consumed with green tea drink. Data expressed as the amount of triglyceride in mmol/L and the error bars represent the SEM (n=4 Asian participants).

6.2.6 Satiety response

The satiety levels of all test food meals peaked at 15 min after consuming a meal and gradually decreased over the 180 min. As illustrated in Figure 6.6, the ingestion of green tea biscuits and plain biscuits with green tea drink resulted in higher average postprandial satiety than the control meal (plain biscuits with water). However, the postprandial change in satiety level was not significantly larger at any time after consumption of the test meals than consumption of the control meal. The iAUCs of satiety after the ingestion of green tea biscuits (954.7 ± 530.0 cm.min) and plain biscuits with green tea drink (900.9 ±405.0 cm.min) were not significantly higher after the ingestion of the plain biscuits (737.3 ± 591.7 cm.min), (P= 0.62).

The acceptability of the food samples for each study visit was evaluated by the participants using the 9-point hedonic scale rating from 'extremely dislike' to 'extremely like'. No difference was observed between plain biscuits and green tea biscuits; they rated the green tea biscuits as 8.1±0.6 and the plain biscuits (control) as 7.6 ± 0.8 (P= 0.14). However, there was a significant difference in acceptability between green tea biscuit and the plain biscuits with green tea drink as 8.1±0.6 and 6.9±1.1 respectively (P= 0.004). No difference was reported between the acceptability of plain biscuits and plain biscuits with green tea drink (P= 0.10).

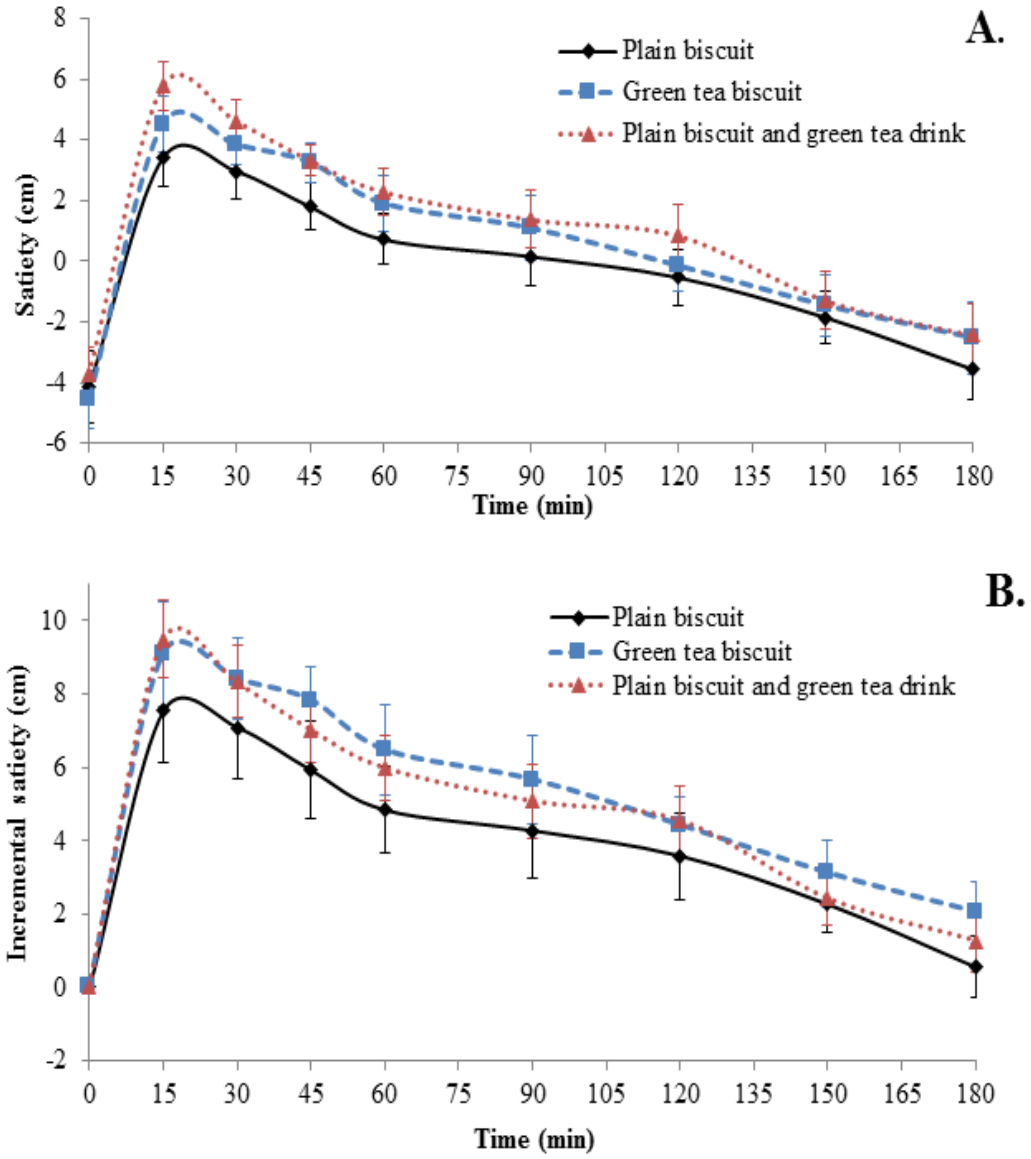


Figure 6.6: A. The mean satiety response curves and B. the mean incremental satiety response curves after ingestion of food samples over 180 minutes. (-◆-) represents the satiety response curve of plain biscuits, (-■-) represents the satiety response curve of green tea biscuits, and (..▲..) represents satiety response curve of plain biscuits consumed with green tea drink. Data expressed as the amount of blood glucose in mmol/L and the error bars represent the SEM (n=10 participants).

6.3 Discussion

Three food samples with similar carbohydrate amount were used in the study to determine the effect of MGTP incorporated into biscuit products or consumed as a drink with plain biscuits on the glucose and, triglyceride responses, and satiety levels. Brouns *et al.* (2005) suggested that selecting healthy subjects would reduce the variation within the subjects. Hence, a health-screening questionnaire was used to screen the healthy subjects with no long-term health problems such as diabetes or cardiovascular disease. During the study session, the participants were ensured to remain in a provided room; however, a number of participants had to attend lab duties, which may affect the result. Nine measurements were required for blood glucose and four measurements were needed for blood triglycerides over the 180 minutes. Hence, capillary blood was selected to measure blood glucose and triglycerides. According to FAO (1998) and Wolever (2006), capillary blood is the preferred method of sampling because capillary blood samples provide more consistent response. The consistent results are obtained because the capillary blood glucose concentration has been shown to have a lower coefficient of variation than the concentration of glucose in venous blood, which is more affected by insulin secretion after ingestion of a meal.

Even though participants may feel uncomfortable by taking blood several times, the capillary blood is preferable and a convenient method whereas venous blood is less preferably by the participants and requires special nursing and medical attention. The strenuous exercise prior the test day could increase the glucose and triglyceride uptake into the muscles on the next day (Malkova *et al.*, 2000). Participants were asked to fast 10-12 hours overnight, to refrain from strenuous exercise for 24 hours and to refrain from drinking alcohol prior to the study session in order to obtain a more consistent baseline result.

6.3.1 Glucose response

As mentioned in the introduction (chapter 1), green tea has shown to have anti-glycemic and diabetic effects in long-term interventions and prospective studies (Yang *et al.*, 2014; Stote and Baer, 2008; Cabrera *et al.*, 2006). However, there is limited information regarding the acute effect of green tea incorporated in food products with high sugar and high-fat content on glucose response.

In Vivo and *in vitro* studies suggest the potential mechanism which green tea and EGCG acutely reduce postprandial blood glucose levels is to inhibit the activity of pancreatic α-amylase (Forester *et al.*, 2012; Chen *et al.*, 2009). *In vitro* study, Forester *et al.* (2012) found that EGCG noncompetitively inhibited pancreatic amylase activity by 34%. Moreover, Tsuneki *et al.* (2004) showed that glucose tolerance was significantly (P<0.05) improved with tea administration compared with hot water administration during a glucose challenge in healthy humans. The authors speculated that the observed acute effects of green tea on blood glucose levels were mainly due to the promotion of insulin action in peripheral tissues, such as skeletal muscles and adipocytes via modification of serum protein (Tsuneki *et al.*, 2004). Lochocka *et al.*, (2015) also found that a single dose of green tea extract taken with a test meal significantly decreased starch digestion and absorption compared with the placebo treatment (P = 0.003). The study used CO_2 starch breath test, which measures the isotope ratio of $^{12}CO_2$/ $^{13}CO_2$ of breath samples. The starch digestion and absorption was measured based on cumulative percentage ^{13}C dose recovery (Lochocka *et al.*, 2015). They found that green tea extract (257.6 mg of catechins) decreases starch digestion and absorption in humans when consumed with a test meal (Lochocka *et al.*, 2015). The cumulative ^{13}C dose recovery was significantly lower for the green tea extract group than the placebo (median 11.4% vs. 16.1%; P=0.003). The green tea extract was used in powder form; 4 g of powder contained 257.6 mg of catechins, enclosed in a starch wafer, whereas the placebo was an empty starch wafer.

Due to previous studies on the anti-diabetic effect of green tea, the hypothesis in this study was that both green tea biscuits and plain biscuits consumed with a green tea drink would have lower glucose response and iAUC than the control meal (plain biscuits with water).

However, we observed no significant difference in glucose levels and iAUC between plain biscuits (control), green tea biscuits and plain biscuits consumed with a green tea drink. The glucose response of all test food samples peaked at 30 min after the intake, and were gradually decreased to baseline over 180 min. In this study, we used MGTP to incorporate into the biscuits and for the green tea drink consumed with plain biscuits, whereas the previous studies used bagged green tea or green tea extract (Lochocka *et al.*, 2015; Josic *et al.*, 2010). The result that MGTP did not lower glucose levels is consistent with a previous study on the effect of green tea on postprandial glucose in healthy subjects (Josic *et al.*, 2010) and long-term intervention studies (Brown *et al.*, 2009; Odegaard *et al.*, 2008; Chan *et al.*, 2006; Ryu *et al.*, 2006; Fukino *et al.*, 2005). Josic *et al.* (2010) found that green tea (infused green tea contained 150.6 mg of catechins) showed no glucose or insulin-lowering effect. The trial tested the effect of green tea on postprandial glucose on participants who consumed either 300 mL of green tea or water with a meal consisting of white bread and sliced turkey (Josic *et al.*, 2010). The dose of catechins (150.6 mg of catechins) used in Josic *et al.* (2010) may not be sufficient, whereas Lochocka *et al.* (2015) found that green tea extract (257.6 mg of catechins) decreases starch digestion and absorption in humans by measuring the cumulative ^{13}C dose recovery. This study used similar dose (256.8 of catechins in green tea drink) with Lochocka *et al.* (2015) study, but the significant decrease in glucose response was not found. This could be due to the different method that used. Their study measured the ^{13}C dose recovery to estimate the starch ingestion and absorption, whereas this pilot study measured the postprandial glucose. Moreover, the sugar in biscuits can interfere the result. A study by Lee and Wolever (1998) found the glucose response from sucrose (25 g) reached the maximum concentration 30 min after intake and fell back down to baseline within 60-90 min. As shown in Figure 6.2, the average blood glucose concentration of all test meals reached maximum concentration at 30 min after consumption. The response of blood glucose 30 min after consumption may come from the sucrose in biscuits. Hence, the blood glucose of green tea biscuits and plain biscuits consumed with green tea responses were not different from the plain biscuits. However, after 120 min of ingestion, the average blood glucose of test meals was lowered than the control meal. This could be due to the effects of catechins from MGTP that inhibit the

amylase enzyme to digest starch in biscuits, resulting in lower glucose absorbed into the bloodstream.

A study in animals also suggested that EGCG acutely reduces postprandial blood glucose levels in mice when co-administered with corn starch and this may be due in part to inhibition of α-amylase (Forester *et al.*, 2012). The suggested mechanism of α-amylase suppression was the formation of a catechin and α-amylase complex by hydrogen bonding or hydrophobic interaction (Goh *et al.*, 2015; Bandyopadhyay *et al.*, 2012; Siebert *et al.*, 1996). Moreover, a study on the effect of green tea supplementation on insulin sensitivity in rats suggested that regular green tea infusion drink could increase insulin sensitivity in rats by increasing the glucose uptake and insulin binding of adipocytes (Wu *et al.*, 2004a; Wu *et al.*, 2004b). They also suggested that the amelioration of insulin resistance by green tea is associated with the increased expression of the glucose transporter IV (GLUT IV) that found in adipose tissue. Their finding contradicts with the study by Park *et al.* (2009) on the ambivalent role of gallated catechins in glucose tolerance in humans that proposed that gallated catechins groups elevated blood glucose levels by blocking glucose uptake into tissues when they enter in the circulation.

The previous findings on the effect of green tea on glucose response have been inconclusive. The difference in results might be due to the methods used, the type of green tea and amount of catechins in green tea used, the literature review on the effect of green tea on glucose response is listed in the table in chapter 1 (Table1.3). Another factor that can affect the glucose response is the high fat content in shortbread biscuits. The fat content in food can lower the glucose response by slowing down the gastric emptying process (Wolever, 2006). Hence, the insignificant difference in glucose response between control and test meals could be due to the high fat content in biscuits.

In addition, the difference between results from human and animal studies may reflect species-specific differences. The possible reason for the lack of consistent findings *in vivo* might be the individual variation in the bioavailability and metabolism of catechins in humans. The factors that affect the inter-individual variation in glucose response, such as gender, BMI and ethnicity will be discussed further.

6.3.1.1 Demographic factors and glucose response

There was no significant difference of iAUC between genders in response to plain biscuits, green tea biscuits and plain biscuits with green tea drink. However, It was shown that females have a higher average plasma glucose level than men over 2 hours (Sicree *et al.*, 2008). It is most likely due to the small sample size that our result did not show a difference. In addition, the results showed that the Asian subjects did not show any significant difference in glucose responses from the European and the Mix- ethnic (South American countries) subjects after testing the control and test foods despite the different shapes of GR curves in each group. Also, the iAUCs of the Asian were higher than the iAUCs of the European and Mixed-ethnic groups in the control meal and 2 test food meals but they were not significantly different from each other. These results were in agreement with the findings from a previous study (Pratt *et al.*, 2011), where there was no significant differences found in the glucose response between the three ethnic groups (south Asian n=10, Chinese n= 10, and European n=10). However, Kataoka *et al*. (2013) found that Chinese (n=32) had 60% greater iAUC of glucose response after rice consumption ($P < 0.001$) than Europeans (n=31).

6.3.1.2 Lifestyle factors and glucose response

Even though the overweight BMI curve was larger and the iAUC of overweight BMI was higher than the iAUC of normal BMI subjects in all test food sample, there was no significant difference in iAUC between normal BMI and overweight BMI subjects in plain biscuits, green tea biscuits and plain biscuits with green tea drink ($P = 0.66$, $P = 0.43$, $P = 0.44$ respectively). Overweight subjects tend to be insulin resistant, so higher BMI has been shown to correlate with the higher glucose response levels (Wolever, 2006). Moreover, the subjects who have active physical activities tend to have lower glucose response due to improved insulin sensitivity (Wolever and Mehling, 2000). Despite the number of subject in this pilot study being less (n=10) than two previous studies (n= 24, n=34) (Perala *et al.*, 2011;

Mettler *et al.*, 2007), the result obtained supports the findings with them in which no significant association was found between the iAUC and the BMI.

6.3.2 Triglyceride response

The secondary aim of this study was to investigate the effect of MGTP on the postprandial response of triglycerides after ingestion of plain biscuits with/without green tea drink and biscuits incorporated with MGTP on human subjects in a triple crossover design. A previous study by Unno *et al.* (2005) found that green tea consumed together with a meal (a piece of bread and 20 g of butter) lowered the postprandial triglyceride level compared to a meal with a control drink. In that study, there were 2 treatments of green tea; a moderate dose containing 224 mg of tea catechins and a high dose containing 674 mg of tea catechins. Both moderate and high dose treatments lowered the postprandial triglyceride levels and reduced the iAUC by 15.1% and 28.7% respectively. However, the moderate dose did not have a significant difference in iAUC compared with the control dose, whereas the high dose showed a significant difference in iAUC compared to the control dose.

The amount of catechins in green tea biscuits (232.5 mg of total catechins per 100 g of biscuits) and green tea drink (256.8 mg of total catechins per drink) were similar to the moderate dose of tea catechins used in Unno *et al.* (2005) study. The result in this study found that green tea biscuits lowered the iAUC by 20.2% compared with iAUC of control biscuits and plain biscuits consumed with green tea lowered the iAUC by 11.7%. Both green tea biscuits and plain biscuits consumed with green tea did not show a significant difference in iAUC compared with the control. This finding is in agreement with the previous study by Unno *et al.* (2005) which the moderate dose of catechins (224 mg of tea catechins) reduced the iAUC by 15.1% but did not give a significant difference. However, the highest mean triglyceride response of green tea drink consumed with biscuits (at 0.41 mmol/L) was higher than the control meal (at 0.29 mmol/L), but its iAUC was lower than the control meal. In the previous study, the highest peak induced by both moderate dose and high dose of green tea beverage had lower incremental triglycerides than the control meal. The difference in findings could be due to the subjects recruited and food samples. For example, Unno *et al.* (2005) recruited all male adult participants with borderline and

mild hypertriacylglycerolemia whereas our study recruited both male and female adults with normal levels of fasting triglycerides and the participants' ethnicity was varied (Asian, Europe, Mixed –South American). The test meal used in the Unno *et al*. (2005) study was lower in fat (20 g of butter with bread), whereas the test meal in our study was biscuits that contained 36 g of butter. However, the result among Asian subjects (n=4) showed that the iAUC after ingestion of green tea biscuits was significantly lowered than the iAUC of plain biscuits. Moreover, the triglyceride level at one hour after ingestion of green tea biscuits was significantly lower than the triglycerides level at one hour after ingestion of plain biscuits and plain biscuits consumed with green tea drink.

The mechanism underlying the triglycerides lowering effect of green tea is mainly focused on the inhibition of the absorption of fat from the intestine (Suzuki *et al.*, 2005). Juhel *et al.* (2000) found that green tea exhibit a potential to inhibit gastric and pancreatic lipases. The suggestion that tea catechins attenuate lipid absorption via digestive lipase inhibition may explain the triglyceride lowering effect of green tea (Raederstorff *et al.*, 2003). Armand *et al*. (1999) suggested that smaller fat droplet size facilitates fat digestion by digestive lipase. However, EGCG can enlarge fat droplets size as EGCG was claimed to have potential to form complexes with lipids and lipolytic enzymes through interfering with emulsification and micellar solubility of lipids (Kim *et al.*, 2012; Chan *et al.*, 2011; Lee *et al.*, 2009). The researcher proposed that the hydroxyl moieties of ECGC interact with the hydrophilic head group of phosphatidylcholine at the exterior of a lipid emulsion by forming hydrogen bonds. This interaction may lead to the formation of cross-links followed by coalescence of the emulsion droplets. Hence, the enlarged emulsion droplets prevented the efficient emulsification of bile salt and reducing surface area for fat digestion by lipase (Walkowiak *et al.*, 2013; Koo and Noh, 2007; Unno *et al.*, 2005; Raederstorff *et al.*, 2003). Moreover, Suzuki *et al* (2005) proposed that catechins with a gallate groups are located on the surface of the lipid emulsion and destabilize the lipid emulsion, so the lipase activity is lost. Therefore, the catechins in MGTP may limit the lipid absorption in the intestine. Furthermore, the *in vitro* study suggested that the individual tea catechin has different potential to exert the inhibitory effect on the digestive lipase. The gallated catechins such as ECG, EGCG and GCG provided a stronger inhibitory effect on the digestive lipase,

whereas non-gallated catechins such as EC and EGC had less effect (Ikeda *et al.*, 2005; Ikeda *et al.*, 2003). EGCG was the major catechin in our biscuits in this study that may have a significant role in lowering postprandial lipaemia.

To our knowledge, this is the first study that has used biscuits incorporated with MGTP to investigate the effect of green tea on postprandial triglyceride levels. Even though, the finding did not show a significant effect on postprandial triglycerides with 10 participant, the significant decrease in triglyceride response of green tea biscuits was found among Asian participants. The difference in ethnicity might have a role in physiology that contribute the significant decrease in postprandial triglycerides in Asian participants. The apolipoprotein A5 is an essential regulator of triglycerides in the circulation. The pattern of gene for apolipoprotein A5 found in Chineses is different than the Caucasians (Liu *et al.*, 2010). Another study found that the postprandial triglyceride concentrations in South Asian men was significantly higher than European men (Arjunan *et al.*, 2013). Therefore, the different ethnicity may have contributed to the varied triglyceride response that leads to an insignificant difference between control and test meals.

Moreover, the plain biscuits consumed with green tea drink did not give a reduction in postprandial triglycerides among Asian subjects. This result indicated that incorporating MGTP in biscuits may enhance the triglyceride lowering effect. The catechins in biscuits may have a better potential to fat molecules to form a larger emulsion of fat droplets, which inhibit the digestion. Hence, the effect of catechins on postprandial triglycerides may be enhanced by the food matrix of biscuit.

Increasing the level of MGTP incorporated into biscuits may increase the effect of catechins on the lipid digestion, which could affect the postprandial triglyceride level. According to Unno *et al*. (2005), green tea containing 674 mg of catechins, significantly lowered the postprandial triglycerides after a meal (bread with 20 g of butter). From the HPLC analysis, 6 g of MGTP per 100 g of flour gave approximately 232.5 mg of total catechins per 100 g of biscuits, therefore, 18 g of MGTP would be required to incorporate into biscuits to achieve levels of 690 mg of total catechins in order to compare the triglyceride-lowering effect. Moreover, the meal in Unno *et al*. (2005) study, contained

18.8 g of fat and green tea containing 674 mg of catechins consuming together with meal significantly decrease postprandial triglycerides after a meal. Their result can suggested that green tea drink required 674 mg of catechins to exert the effect with 18.8 g of fat. However, the 100 g of green tea biscuits consumed in this pilot study contained 35 g of fat which is almost twice amount of fat in Unno *et al*. (2005) study and a reduction in triglyceride response was observed, compared with control, in Asain subjects. Hence, this evidence showed that biscuit matrix might enhance the triglyceride lowering effect of MGTP.

In addition, 6 hours measurement should be conducted for triglycerides in order to monitor the change in triglyceride level after the completion of fat digestion and absorption. Our study measured the triglyceride response up to 3 hours. Normally after the fat digestion and absorption, the triglyceride level rises and returns to baseline after 6 hours (Mattes, 2001), therefore, the triglyceride response should be measured up to 6-8 hours. The previous study monitored triglyceride response for 6 hours after a meal consumption (Unno *et al*., 2005).

6.3.3 Satiety response

Another aim of this study was the effect of green tea on satiety. The hypothesis was that green tea could increase satiety because Josic *et al*. (2010) found that the increased satiety and fullness were observed by participants after the consumption of green tea. The visual analog score was used to measure the satiety by asking the participant to rate their feeling on the scale from 'extremely hungry' to extremely full'. The result did not show a significant difference in satiety levels reported by the participants, even though the average of satiety level and iAUC of green tea biscuits and plain biscuits consumed with green tea drink were higher than the control meal. The hormones that regulates satiation involves both fat and carbohydrate metabolism. For example, the CCK hormone that stimulates pancreatic lipase secretion and gall bladder contraction to release bile salt, also increase satiation by signaling via vagus nerves (Benelam, 2009). Moreover, GLP-1 that stimulates insulin production, increase the satiety by signaling via the GLP-1 receptor in brain (Benelam, 2009). Catechins may increase the satiety levels by interfering with fat and carbohydrate metabolism. For instance, catechins may inhibit lipase; as a result, more lipase enzyme would be needed to digest the ingested fat. Therefore, the intestine may

produce more CCK to signal the release the lipase and bile salt, and more CCK release means an increase in satiation. However, the increase in satiety level of green tea biscuits and plain biscuits with green tea was not significantly higher than plain biscuits. This could be due to the high sugar and fat in biscuits, which could have an effect on the satiation process. Hence, this can lead to variation in satiety level results that can lead to the insignificant different result between control and test meals. In addition, the difference could be due to the demographic effect of the participants and the amount of catechins in green tea.

In addition, the measurement of acceptability of the meal in the study provides the additional information. The acceptability of green tea biscuits was significantly higher than the acceptability of plain biscuits consumed with green tea drink. This could be due to the bitterness of green tea drink that causes unpleasantness that lowers the acceptability of the meal. On the other hand, the acceptability of green tea biscuits was not significantly different than the acceptability of plain biscuits. Even though the acceptability of plain biscuits consumed with green tea drink was the least among all food meals, the satiety score of plain biscuits and green tea drink reached the highest after 15 minutes of ingestion. However, the level was not significantly different than the other meals. Therefore, the unpleasantness produced from green tea drink did not lower the satiety levels was observed because the highest level of satiety in plain biscuits consumed with green tea. This finding is in agreement with Josic *et al.* (2010) studies, which observed an insignificant difference in acceptability between green tea meal and control meal and found an increase in satiety levels with green tea meal.

There is little information on the relationship between satiety and green tea consumption. Josic *et al*. (2010) suggested that the mechanism behind the increase in satiety might be due to postprandial change in hormones that are responsible for the satiety-promoting effect due to the green tea. The study explained one of the possible mechanism underlying the increase in satiety of a meal consumed with green tea was that green tea has been known to inhibit COMT (catechol-*o*-methyl-transferase); an enzyme that degrades norepinephrine, which is a hormone response for satiety signalling in the hypothalamus (Josic *et al.*, 2010; Rossi *et al.*, 2008; Wellman, 2000).

Other components in green tea that might influence satiety are caffeine and L-theanine. Increased satiety was found in humans having habitual caffeine or tea consumption (Westerterp-Plantenga *et al.*, 2005). This study revealed that the inhibitory effect of caffeine on appetite was related to a corticotrophin-releasing factor and the sympathoadrenal system (Westerterp-Plantenga *et al.*, 2005). Moreover, dopamine and serotonin are the important hormones regulating the appetite (Zheng *et al.*, 2004). An *in vitro* study in mice suggested that L-theanine could pass through the blood brain barrier, consequently increasing the release of dopamine and reduce serotonin concentration in the brain (Zheng *et al.*, 2004). This mechanism may influence the increase in satiety effect of green tea.

In the study by Josic *et al.* (2010), they used loose-leaf green tea and brewed it in hot water for 3 min, whereas in this study, MGTP was incorporated into biscuits and used to prepare green tea drink by mixing the MGTP with hot water. The caffeine content in green tea used in the Josic *et al.* (2010) study was higher (approximately 79.5 mg of caffeine and 150.6 mg of total catechins per 300 ml of brewed green tea), whereas green tea drink used in this study contained 19.5 mg of caffeine and 256.8 mg of total catechins, and green tea biscuits contained 18.7 mg of caffeine and 232.5 mg of total catechins. The content of catechins in green tea and green tea biscuits consumed in this study was higher and the caffeine content consumed was lower. Therefore, higher caffeine content might be the reason for the significant increase in satiety found in Josic *et al.* (2010) study. In addition, the treatment used in Josic *et al.* (2010) study and this study was not blinded, so this result needs to be considered with caution, as the result might be biased. As MGTP contributed to the green colour in the shortbread biscuits, participants' awareness could have influenced the result (Benelam, 2009; Livingstone *et al.*, 2000).

6.3.4 Limitations

This study used shortbread biscuits to as means to investigate the effect of green tea on postprandial glucose and triglyceride response, whereas other studies on the effect of green tea on glycemic response used bread as the reference meal in order to calculate the GI of

the food products. GI of food products would provide more information of simplified iAUC calculation of glucose response and enable the comparison of glucose response of other food products. Therefore, another session with bread could be carried out in order to calculate the GI of the biscuits. In order to minimise the variation of result, the criteria, such as ethnicity, age, gender and BMI should be selected. Moreover, the biscuits should be made sugar free in order to eliminate the effect of sugar on glucose response. If possible, 12 hours diet prior to every study session should be standardised to ensure similar glucose levels. It was found that after a high GI dinner, the glucose response in the morning on the test day was higher than the glucose response after a low GI dinner (Wolever *et al.*, 1988). In order to minimize the large variation of the response, the control and test meal should be repeated at least twice preferably three times. The FAO/WHO (1998) stated that 6 subjects are enough for GI studies and Brouns *et al.* (2005) suggested that 10 participants would provide a "reasonable degree of power and precision for GI measurement". Moreover, the GI is calculated by comparing the iAUC of test food with reference food, which is white bread, so the variation is minimised. However, our study aimed to compare glucose response of food samples (green tea biscuits) with the control sample (plain biscuits), hence larger sample size (20-30 participants) with limited BMI status would have improved the statistical power.

Even though, this pilot study did not show the significant difference in glucose, triglyceride and satiety response between test meals, the result shows the trend that increasing MGTP incorporated in biscuits may show the difference. It would be worth to conduct another human study to confirm the effect of MGTP in biscuits on glucose, triglyceride and satiety response. In order to minimise the variation of result, the criteria, such as ethnicity, age, gender and BMI should be selected. Moreover, the biscuits should be made sugar free in order to eliminate the effect of sugar on glucose response.

6.4 Conclusion

In a summary, both the addition of MGTP (6 g per 100 g of flour) in shortbread biscuits and matcha tea drink (3g dissolved in warm water) consumed with plain shortbread biscuits did not significantly lower plasma glucose, triglyceride and satiety level. These could be due to the large inter- and intra- individual variation of the response. In terms of glucose response, BMI, gender and ethnic groups of the subjects did not affect the glucose response and its iAUC. In terms of triglyceride response, among Asian groups, iAUC of green tea biscuits was significantly lowered compared to the plain biscuit.

The result suggested that higher level of MGTP incorporated may show the hypoglycaemia and hypolipidemic effects. A higher amount of green tea added in biscuits or consumed with biscuits should be tested in order to confirm the result. Because biscuits contained a high content of fat and carbohydrate, the MGTP level (6g per 100 g of flour) used to incorporate in biscuits might not be sufficient to exert the effects.

7 Chapter seven: General discussion

7.1 Conclusion and general discussion

With HPLC-PDA analysis, the catechins found in MGTP and GTEP were catechins, EC, EGC, EGCG, GCG, ECG as well as caffeine. MGTP was found to contain a higher content of most of the catechins (EC, EGC, EGCG and ECG) than GTEP. Whereas, GTEP was found to contain gallic acids and a higher caffeine and GCG content than MGTP.

There was no significant difference in the content of major catechins (EGCG, EGC, ECG and EC) in prolonged extraction times. The total polyphenol content from Folin-Ciocalteu assay and HPLC analysis agreed that 5 min extracting time yielded the highest average catechins extracted from MGTP. In addition, no difference in total catechins and major catechins content was found after 3 months and 5 months storage comparing with the content of catechins after the package was opened. The result showed that the catechins in MGTP were relatively stable. The total catechins found in MGTP ranged from 75-165 µg/mg of powder. From the results, it can be concluded that MGTP is a good source of catechins, which are the compounds that have been found to provide the health benefits. MGTP was therefore selected to incorporate into biscuit recipes to create a new flavour to biscuit products with potential health effects. Furthermore, HPLC-PDA analysis for L-theanine in MGTP was developed to provide a simple and reliable method for quantification. L-theanine content found in MGTP was 3.8 µg/mg of powder.

The stability of catechins in MGTP incorporated into two types of biscuits; shortbread and biscuits with and without the addition of an alkalinizing agent (sodium bicarbonate) was studied. Based on the result of catechins remaining in biscuits after baking, it was revealed that catechins in MGTP were relatively heat resistant with only approximately 20 % loss of total catechins during baking at 180 ^{o}C. During 1 month storage of shortbread biscuits, 10-20% loss of total catechins was found. The EGC was the least stable with approximately 30-50% loss that contributed to the major loss of total catechins during storage. Stability of catechins depends on pH of food products, processing temperature, storage temperature, air access and moisture content. These factors affect the degradation of catechins by initiating the reactions such as oxidation, epimerization, and polymerization. Epimerization between EGCG and GCG occurred during biscuit baking, as the result found an increase in GCG and the remaining level of GCG exceeded 100% in baked shortbread and biscuits with and

without sodium bicarbonate. The result also showed that the amount of sodium bicarbonate used for biscuits (0.5 g per 100 g of flour) did not have a significant effect on the stability of catechins after baking. However, over 3 months storage of biscuits, the content of EGC and EGCG biscuits with the addition of sodium bicarbonate was significantly reduced and the catechins remaining after 3 months storage was lower than the biscuits without the addition of sodium bicarbonate. Therefore, the pH of the food matrix influenced the stability of catechins in biscuits during storage. L-theanine in biscuits was determined in shortbread dough and biscuits. The remaining percentage of L-theanine after baking was 82-97% and after 1 month storage was 92-97%.

The shortbread biscuit was chosen for further study in sensory evaluation because biscuits with addition of sodium bicarbonate may lead to a higher decrease the catechins content during storage. Shortbread biscuits were incorporated with three levels of MGTP at 2, 4, 6 g per 100 g of flour and formulated with three levels of sugar at the levels of 25, 30, 35 g per 100 g of flour. Therefore, nine formulations of biscuits were prepared with four biscuits' formulations repeated. In total 13 biscuits were evaluated with acceptability test. A 9-hedonic point scale with the sensory attributes including appearance, aroma, colour, texture, sweetness, bitterness, and overall acceptability was used. Sensory evaluations were conducted in 2 sessions; 6 biscuits were tested in the first session with 54 participants and 7 biscuits were tested in the second session with 46 participants. Statistical analysis was conducted using ANOVA and RSM to present the acceptability data and analyse the regression coefficients that form the mathematical model, which described the relationship between dependent variables, which were levels of MGTP, and sugar. Hardness was significantly increased as the level of MGTP incorporated increased. The biscuits with 2 g of MGTP and 25 g of sugar per 100 g of flour gained the highest overall acceptability, whereas the biscuits that with 6 g of MGTP and 25 g of sugar per 100 g of flour received the lowest overall acceptability. This indicated that consumers preferred the biscuits with low content of green tea. The colour, appearance and bitterness acceptability was significantly affected by the increase of MGTP. The result suggested that familiarity of the products could increase the acceptability of biscuits, as it was found that the Asian participants gave a higher average overall acceptability score to green tea biscuits than other ethnic groups. Green tea products are widely known in Asian countries, so Asian

participants are more familiar with green tea products than other ethnic groups. The result confirmed that familiarity could increase the acceptability of products. The result from chapter 6 found that participants rated the acceptability of green tea biscuits (with 6 g of MGTP per 100 g of flour) higher (8.1±0.6) than the plain biscuits. This confirmed that familiarity could increase the acceptability of biscuits because participants, who attended the pilot study, were aware about the MGTP before applying for the study. Therefore, this could lead to a way to increase the acceptability of biscuits by promoting the health benefit of MGTP.

The aim of chapter 6 was to investigate the effect of MGTP, either incorporated into biscuits or consumed as a drink with plain biscuits, on the glucose, triglyceride, and satiety responses in healthy human subjects. To our knowledge, there are limited studies on the effect of green tea on glucose, triglyceride, and satiety responses. Typically, glucose response has been studied with green tea drink consumed with bread, which is a common reference food meal. This study is the first to investigate the effect of MGTP that incorporated in biscuits on the glucose, triglyceride, and satiety response. In addition, the study compares the glucose and triglyceride responses with different backgrounds such as gender, ethnic group, and BMI status. Based on the result, there was no difference in response, despite participants' different background, which could be due to a small number of participants apart from for triglycerides. Therefore, this reveals that the MGTP level that consumers may find acceptable in shortbread biscuits would not show an acute effect on lowering glucose and satiety levels. A higher amount of MGTP added in biscuits or consumed with biscuits should be tested to confirm the effects, because biscuits contained a high content of fat and carbohydrate, the MGTP level used to incorporate in biscuits might not have been be sufficient to exert the effects. However, among Asian groups, iAUC of green tea biscuits for triglyceride response was significantly lowered than the plain biscuits and plain biscuits consumed with green tea. Therefore, the result suggested that incorporating MGTP in biscuits might enhance the triglyceride lowering effect.

The advantage of addition of MGTP to food is to increase polyphenol consumption daily, fibre and chlorophyll, especially EGCG and L-theanine, which is only catechin and amino acid, found in green tea. Catechins may have potential to inhibit carbohydrate and fat absorption in bakery products that contained high level of these components. Therefore,

MGTP incorporated to food should be promoted to increase the nutritional value to food. Stability study of catechins in biscuits during baking showed that MGTP have a potential to be a functional ingredient that can be added to other bakery products, such as puff pastry and cake. Because catechins in MGTP is relatively stable during baking with approximately 20% loss, adding MGTP to bakery products may provide health benefits to the bakery food that usually contains high amount of carbohydrate and fat. The disadvantage of adding MGTP in food products is that the food component may lead to poor catechins absorption. A study on the interaction of milk α and β - caseins with catechins found that catechins bind with casein by both hydrophilic and hydrophobic interactions led to protein unfolding (Hasni *et al.*, 2011). Due to these, formation between catechins and milk caseins may inhibit the absorption of catechins. Catechins-casein interaction is more hydrophobic than hydrophilic (Dubeau *et al.*, 2010). Hence, the effect of milk may enhance catechins to be unabsorbed. The main component in biscuit is butter, which is derived from milk, so the milk protein may form the catechins-casein complex and delay the absorption of catechins. Moreover, the lipid-protein micelles of milk also enhance the binding of catechins at the surface of the micelles (Hasni *et al.*, 2011). Therefore, this interaction could be another mechanism of catechins that inhibit the digestion of lipid in small intestine. In addition, He *et al.* (2007) found that catechins solution (0.05 mg/mL of catechins) exert the inhibitory effect on α-amylase and lipase at the ratio of 61% and 54% because catechins can bind and precipitate proteins, resulting to a suggestion that catechins have a potential to denature the digestive enzymes (He *et al.*, 2007). With addition of milk, the absorption and bioavailability of catechins could be poor, due to the formation of catechins with protein and lipid in food. However, the presence of catechins in small intestine could have beneficial effect on delaying and inhibiting the digestion and absorption of carbohydrate and lipid. Incorporating catechins in food could be the solution in weight management and controlling diet for patient with diabetes type 2 and cardiovascular diseases.

Another way to increase the amount of catechins incorporating to food products and improve stability is encapsulation. Paximada *et al.* (2017) found that encapsulation of EGCG into nanoparticles with an emulsion of whey protein isolate, bacterial cellulose and extra virgin olive oil protect EGCG from moisture, heating and dissolution condition, hence increasing stability of catechins.

No difference was seen in glucose response between the test meals and control in this study, possibly due to large inter- and intra- individual variation. The weakness of the pilot study was the small number of subjects and repetition of samples tested. A number of test repetitions for reference food and test food sample of glucose and triglyceride measurement can reduce potential intra-individual variation and improve the precision of the results. For glucose measurement, it is recommended to use the mean of at least two sessions of the reference food for each subject. The BMI status, ethnicity, gender of participants may influence the glucose and triglyceride responses, so the human study should include the specific criteria of BMI status, ethnicity, gender of participants for recruitment. Recruit participants with borderline hyperglycemia and hyperlipidemia to observe the change more clearly because subjects with elevated fasting triglyceride levels was observed to display exaggerated and prolonged postprandial triglyceride response (Tiihonen *et al.*, 2015).

In order to minimize the varied result from sugar and fat content in biscuits, the biscuits with no sugar and low fat content should be used to investigate the effect of MGTP on glucose response and satiety level. The glycemic index of test meal should be calculated by using white bread as the reference meal. Wolever *et al.* (1985) suggested that glucose response is subjective to each subject response while the glycemic index is subjective to the food. When glucose response of food is expressed as a glycemic index (GI), the human variation was reduced because 50% of within–individual variation is eliminated (Wolever *et al.*, 1985), as GI was calculated by comparing glucose response of test food and reference within each subject. Hence, white bread needs to be used as a reference food meal in order to calculate the glycemic index of the test food.

It was found that green tea drink containing 674 mg of catechins significantly lowered the triglyceride after consumption of 18.8 g of fat load (Unno *et al.*, 2005). Moreover,Venables *et al.* (2008) found that 890 mg of catechins lowered the glucose response compared to a control. The increase in content of MGTP incorporated into biscuits to 700-1000 mg of catechins per 100 g of biscuits (per 50 g of carbohydrate) may excel the effect. Therefore, it is worth to conduct a larger scale of human study investigating an acute effect of MGTP on glucose and triglyceride and satiety level, by including the listed criteria above to minimize the variation. In addition, when fat content in biscuits is lowered, this may increase the triglycerides lowering effect of MGTP. Because the green tea biscuits (with 232.5 g of

catechins) consumed in the pilot study contained 35 g of fat, and triglyceride response of green tea biscuits was lowered than control in Asian subjects. There is a possibility that the lower fat content to 18 g in biscuits may show the reduction in postprandial triglycerides, if the level of MGTP cannot be increased due to the decrease in acceptability.

The increased level of MGTP will lower the acceptability of appearance, colour and bitterness of the biscuits. The increase in familiarity of biscuits and masking the colour of biscuits will possibly increase the acceptability of biscuits. The RSM result suggested level of sugar in shortbread with MGTP to gain highest sweetness acceptability, was at 30 g per 100 g of flour, which equals 15.5 g of sugar per 100 g of shortbread biscuits. This level can be a guide to develop sugar-free biscuits to serve the health benefits. Even though high level of MGTP incorporated in biscuits will contribute to more bitterness taste that can decrease the acceptability of biscuits, there is an opportunity to increase the acceptability by promoting the health benefits of MGTP. For example, a dark chocolate with a high cocoa mass percentage provides high content of polyphenol and more bitterness than milk chocolate. It is regarded as healthy products and can be sold in high price in market. Consumers, who are health conscious, can develop the taste for bitter products by regarding it is good for health. In USA, it was reported that MGTP sale increased more than 50% in 2014 and it was predicted that the sale would continue to grow 25% annually between 2015 and 2018 (Crawford, 2015). Many food companies in USA have begun to offer products with MGTP, such as Matcha latte by Starbuck company. This evidence shows that biscuits with MGTP can have an opportunity to put in a market, especially if it was promoted as products.

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Appendices

Appendix A: Calibration curves

A.1 Calibration curve of standard for Folin-Ciocalteu assay and FRAP assay

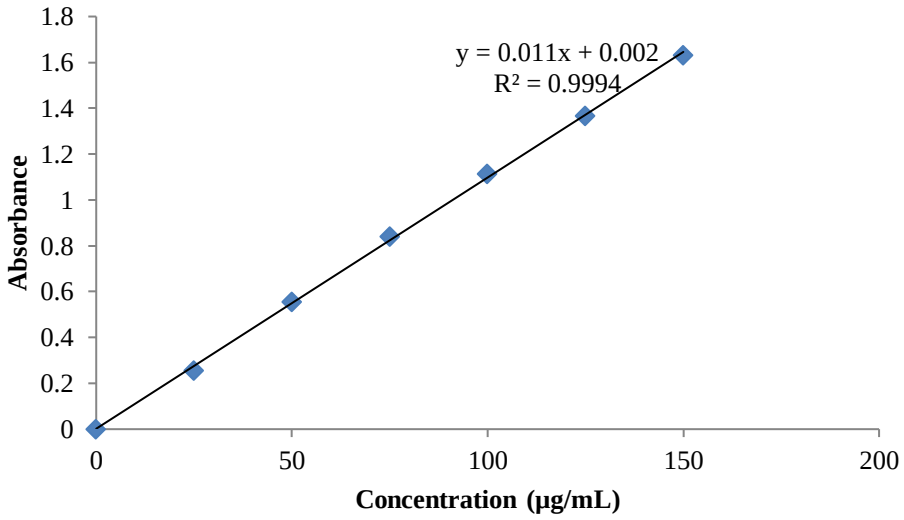


Figure 1 Example calibration curve of gallic acid for Folin-Ciocalteu assay. The calibration was constructed from fresh on every experiment day

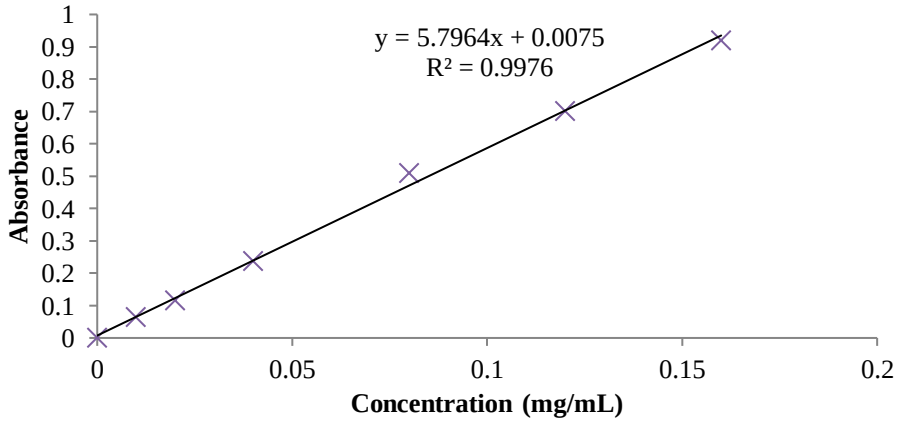


Figure 2 standard curve of Trolox (mg/mL) for quantify the antioxidant capacity expressed as mg Trolox equivalent

A.2 Calibration curve of catechins and caffeine standard for HPLC analysis

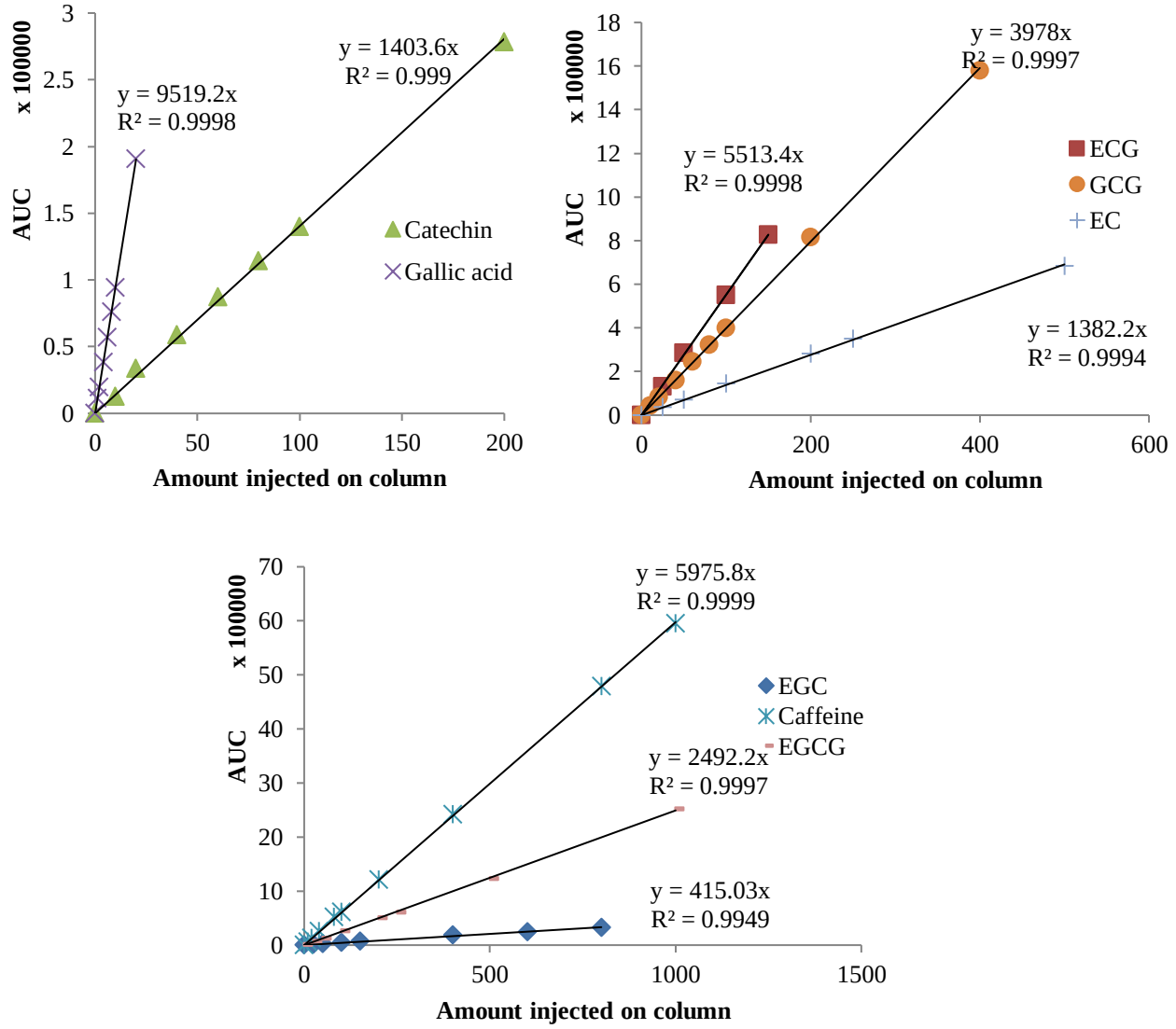


Figure 3 Calibration curves of standards in quantification of green tea powder

A.3 L-Theanine calibration curve and quantification

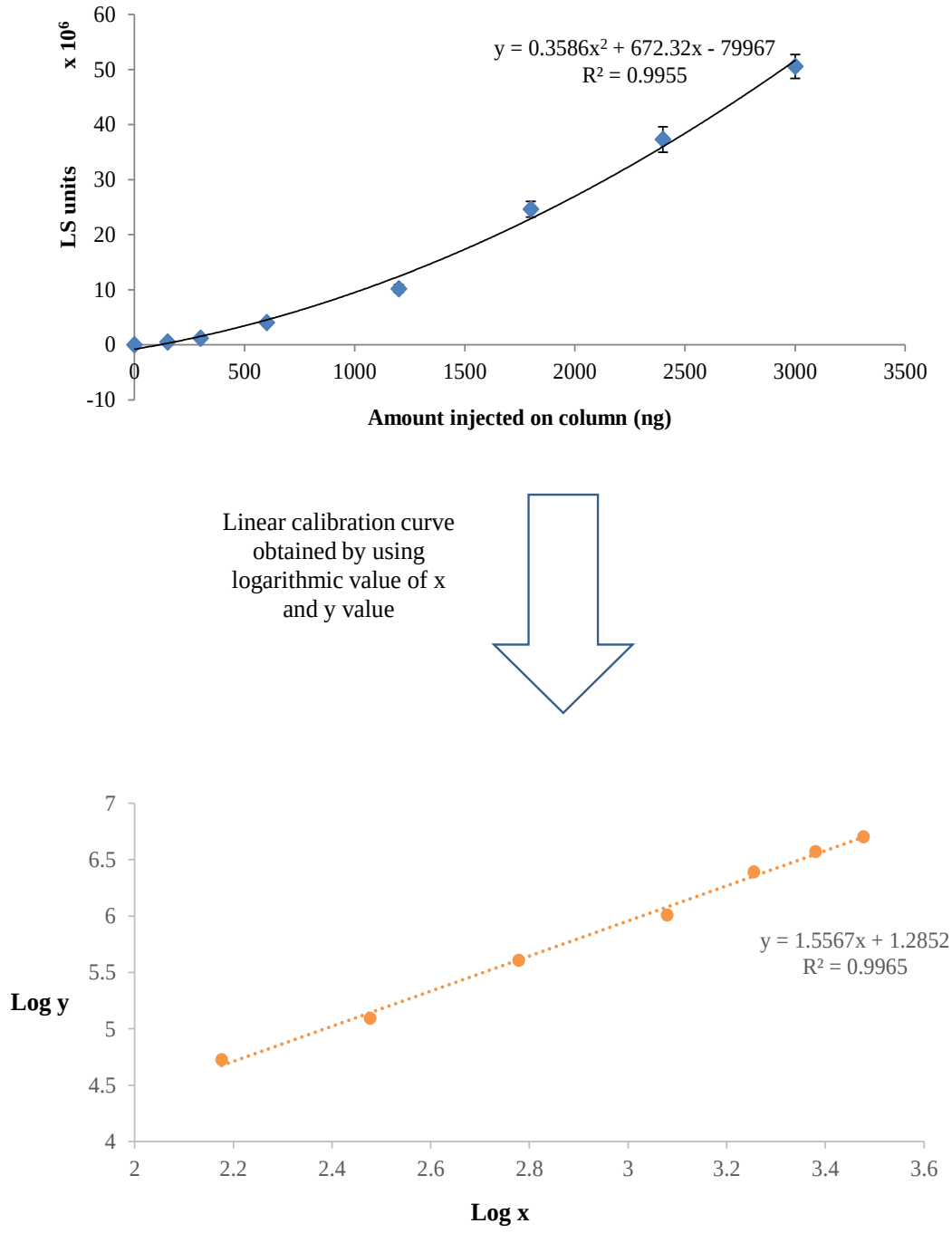


Figure 4: L-theanine standard curve which peaks were detected by ELSD, polynomial curve was obtained. The logarithmic values for y and x produced linear curve that was used to calculate L-theanine content.

The calibration curve obtained with ELSD was polynomial. Due to the various light scattering mechanisms and particle size distribution, the non-linear standard curve of the target compound occurred. The ELSD response according to empirical model as shown in equation below (Stolywho *et al.*, 1983).

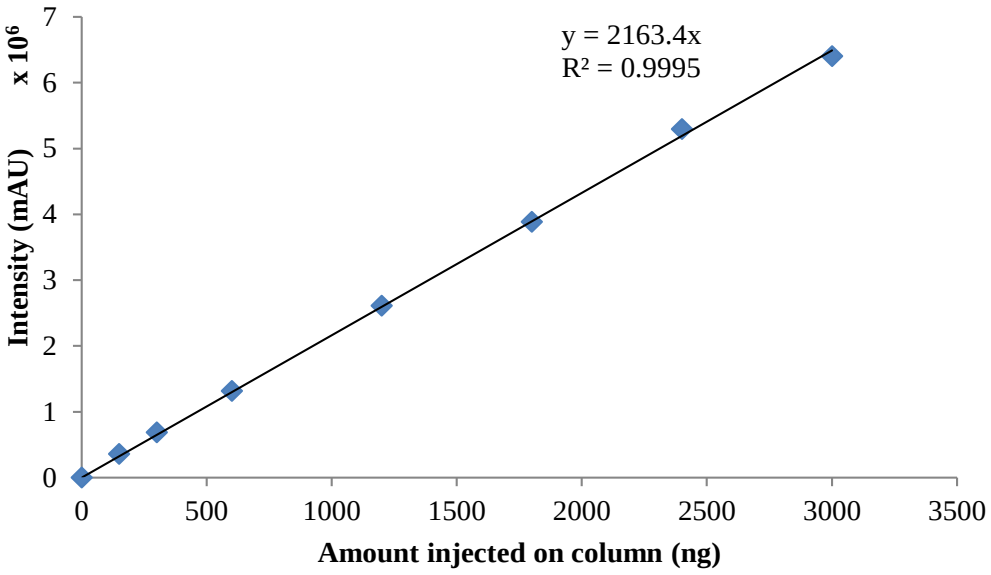
$$Y = ax^b ,$$

Where y represents the obtained peak area, x is the amount of analyte injected, b is the slope of the response line, and a is the response factor (intercept). The logarithmic values for y and x create a linear standard curve as in the equation below (Young and Dolan, 2003).

$$\text{Log } y = a + b \log x$$

For the samples that were detected with ELSD, the calibration curve constructed with logarithmic values of y and x, is used to calculate L-theanine content. The integrated peak area of each sample was calculated to logarithmic values before calculation.

$$\log x = \left\{\frac{(\log A_{sample} - intercept}{slope}\right\}$$



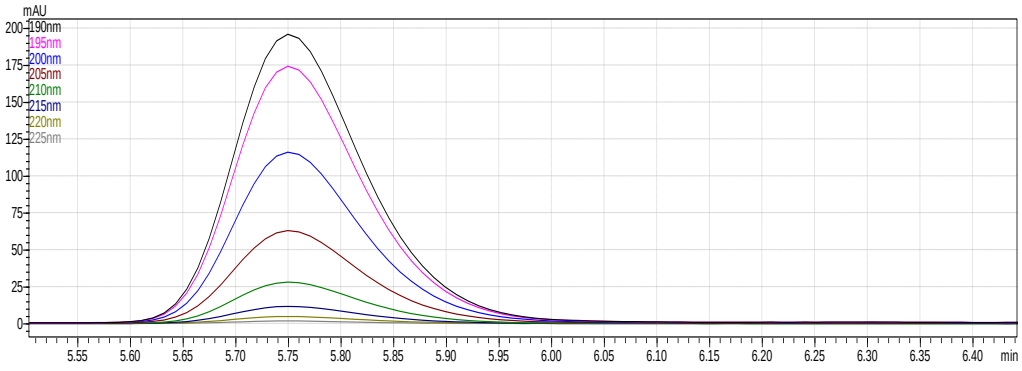


Figure 5: Calibration curves of L-theanine standards using PDA detector at 200 nm and comparison of L-theanine absorbance in different wavelengths (from 190 to 225nm).

The L-theanine calibration curve was constructed by plotting the amount of L-theanine injected in HPLC column against the intensity of the L-theanine standard peak. The calibration curves obtained from PDA detector were forced through zero (Appendix A, Figure 5). The calibration curve equations and coefficient of determination were obtained. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the y-intercept of the regression line (standard error of estimate) and slope using the calibration curve data. Due to the change of HPLC machine in L-theanine analysis from UFLC Shimadzu couples with ELSD and PDA detectors to a conventional HPLC Shimadzu coupled with UV-vis detector, the calibration curve was constructed when the HPLC condition was changed. Therefore, the calibration curves were constructed for each detector and HPLC elution used for L-theanine determination. The LOD and LOQ of each calibration curves were reported in Table 1.

Table 1: the linearity study of calibration curves used to determine L-theanine content with different HPLC elution and detector.

HPLC Machine	HPLC elution	Detector	Average retention time	%CV of retention time	Range (µg/mL)	Slope	R^2	LOD (µg/mL)	LOQ (µg/mL)
UFLC, Shimadzu coupled with PDA and ELSD	Isocratic	PDA	6.13	0.0085	5-100	2163.4	0.9995	8.07	26.89
	Isocratic	ELSD	6.06	0.51	5-100	1.5567	0.996	0.32	1.08
Conversional HPLC, coupled with UV-vis detector)	Binary	UV-vis	5.53	0.0055	5-100	2048.5	0.9982	12.42	41.41
	Isocratic	UV-Vis	5.73	0.0052	5-100	2127.3	0.999	8.44	28.13

The slope was obtained from the calibration curve plotted by logarithmic value of x (amount injected on the column) and y (the peak intensity), LOD and LOQ were calculated from the linear regression, and therefore the polynomial curve from ELSD was not suitable to calculate the LOD and LOQ. The calculated value of LOD and LOQ were very low compared to other methods.

A.3.1 Comparison of between ELSD and PDA detector used for L-theanine determination

The result (Figure 6 Appendix A) showed that the ELSD and PDA detector did not have a significant difference in determining the L-theanine content in different extraction time. The calibration curve obtained from ELSD detectors was polynomial curve, which was not suitable for quantification. The logarithmic values of x and y values were required to obtain the linear calibration curve. Due the previous reason, the PDA was chosen to detect the L-theanine for the next experiment because the detector produced linear calibration curve, which directly can be used to calculate the L-theanine content. Moreover, the ELSD and PDA did not have any difference on the L-theanine determination. This experiment was conducted on UFLC, (Shimadzu) coupled with both PDA and ELSD. When PDA detector had been selected to determine L-theanine, the later experiments were conducted with a conventional HPLC, Shimadzu (SPD-20A series), coupled with UV-vis detector. Furthermore, the 15 min extraction time with ELSD detector gave the highest content of L-theanine, which was 3.6 μg/mg of green tea powder. Samples that analysed in this experiment, were all pre-treated with PVPP. The next experiment was to determine the effect of PVPP pre-treatment on L-theanine determination with isocratic and binary HPLC elution.

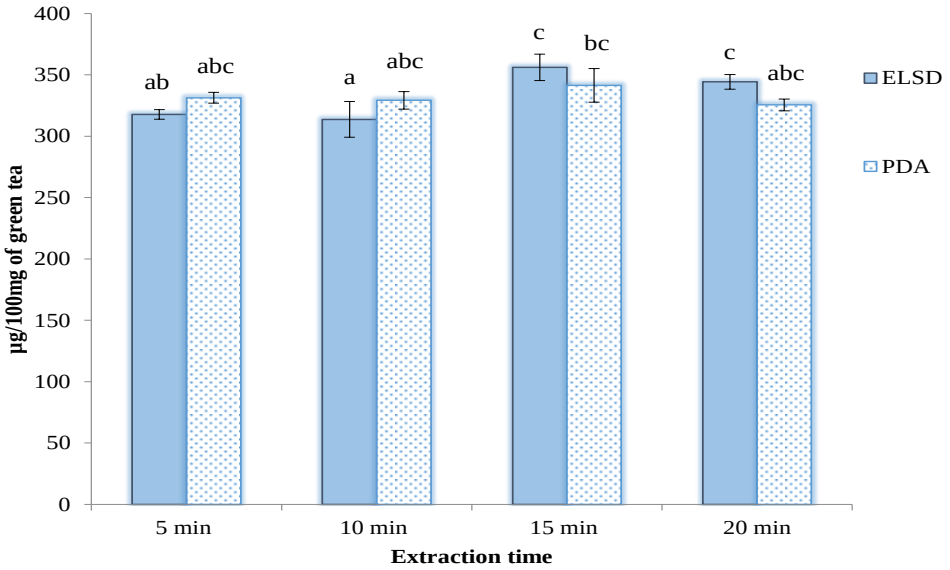


Figure 6: The effect of the detectors in L-theanine determination with HPLC isocratic elution with water at different extraction time. All of the samples were all treated with PVPP prior to HPLC injection, a,b,c indicating the difference ($p < 0.05$) of L-theanine content with extraction time and detector, using ANOVA, following with Tukey's-b post-hoc test and the error bars represent the standard deviation (n=3).

A.3.2 Comparison of PVPP pre-treatment with isocratic and binary elution of HPLC

As shown in Figure7, the untreated samples were significantly higher than the PVPP treated samples in all the extraction time with both isocratic and binary elution. There was no difference in L-theanine content determined in isocratic and binary elution among the pre-treated with PVPP samples in every extraction time. The untreated samples with PVPP can cause the high pressure during the isocratic run due to the accumulation of polyphenols in the column. Therefore, the isocratic run with 100% H_2O should be used for the samples that are treated with PVPP prior the injection to HPLC. The binary HPLC method with untreated samples with PVPP prior injection gave significantly higher L-theanine content than other methods in every extraction time point. There was no significant difference in L-theanine content with different extraction time. Based on this result, the HPLC binary elution without PVPP treatment on samples was chosen to determine L-theanine content.

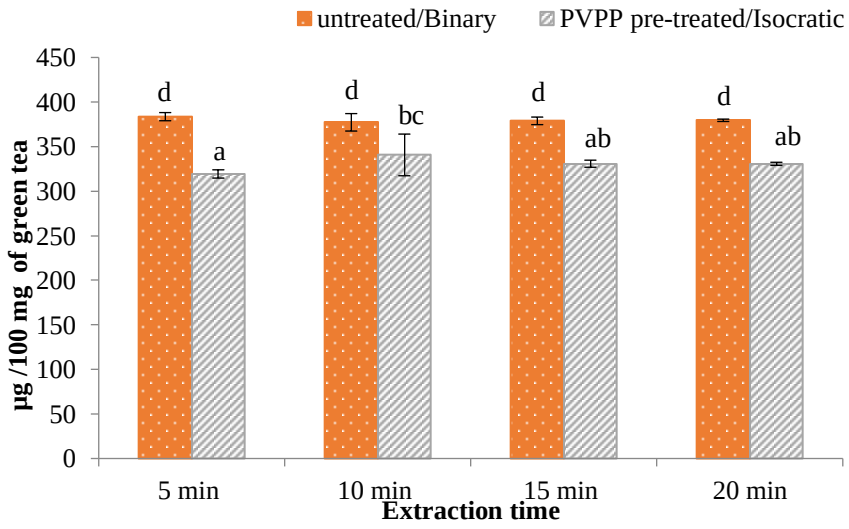


Figure 7: The effect of PVPP pre-treatment in L-theanine determination with different extraction time and HPLC isocratic and binary elution, a,b,c,d,e indicating difference ($P < 0.05$) of L-theanine content with extraction time, PVPP pre-treated and untreated and HPLC elution, using ANOVA, following with Tukey's-b post-hoc test and the error bars represent the standard deviation (n=3)

Appendix B: Ethics approval for sensory evaluation

Performance, Governance and Operations
Research & Innovation Service
Charles Thackrah Building
101 Clarendon Road
Leeds LS2 9LJ Tel: 0113 343 4873
Email: ResearchEthics@leeds.ac.uk

UNIVERSITY OF LEEDS

Benjapor Phongnarisorn
School of Food Science and Nutrition
University of Leeds
Leeds, LS2 9JT

MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC)
University of Leeds

30 July 2014

Dear Benjapor

Title of study **Sensory evaluation of shortbreads and biscuits incorporated with green tea powder**
Ethics reference **MEEC 13-026**

I am pleased to inform you that the application listed above has been reviewed by the MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC) and I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
MEEC 13-026 Ethical_Review_Form_sensory__16072014.docx	1	17/07/14
MEEC 13-026 Examples of questionnaires in sensory booth 16-07-2014.docx	1	17/07/14
MEEC 13-026 Participant information sheet 16-07-2014 (1).docx	1	17/07/14
MEEC 13-026 poster 16-07-2014.pub	1	17/07/14
MEEC 13-026 Pre_study_questionnaires 16-07-2014.docx	1	17/07/14
MEEC 13-026 sample recruiting email 16-07-2014.docx	1	17/07/14
MEEC 13-026 Sensory risk_assessment_form 16-07-2014_resubmit.doc	2	17/07/14
MEEC 13-026 sign_copy_of_the_application.pdf	1	17/07/14

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval, including changes to recruitment methodology. All changes must receive ethical approval prior to implementation. The amendment form is available at http://ris.leeds.ac.uk/EthicsAmendment.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited. There is a checklist listing examples of documents to be kept which is available at http://ris.leeds.ac.uk/EthicsAudits.

We welcome feedback on your experience of the ethical review process and suggestions for improvement. Please email any comments to ResearchEthics@leeds.ac.uk.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator, Research & Innovation Service
On behalf of Professor Gary Williamson, Chair, MEEC FREC
CC: Student's supervisor(s)

B.1 Poster


TITLE
AIM
Sensory Evaluation of shortbread and biscuit incorporated with green tea powder
We want to understand the effect of green tea powder on and sensory characteristics and acceptability of shortbreads and bis-
Fancy some biscuits ?
You are being invited to take part in our research project.
What you need to do?
you will be asked to visit the research study 2 times to undertake the sensory panel. You will be given a short questionnaire related to the frequency of consumption of biscuits and shortbread. Then, you will be given 6 shortbreads or biscuits to evaluate for appearance, smell, taste, texture and general acceptability using a 9 point scale. The entire test should not take more than 30 minutes in each visit.
FURTHER IN
FORMATION
Please contact: Benjapor (Nanny)
mt09bp@leeds.ac.uk or find me in the G.08 at school of Food Science and Nutrition, University of Leeds
Thank you for reading, You are welcome to join
UNIVERSITY OF LEEDS

B.2 Participants' information sheets and consent from

Participant's information sheets

Research project title: Sensory Evaluation of shortbread and biscuit incorporated with green tea powder

You are being invited to take part in a research project. Before you decide, please take time to read the information sheet and make sure you understand why the research is being done and what it will involve. You can discuss or ask us if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the purpose of the study?

Matcha green tea powder (MGTP) is dried, ground green tea leaves. Green tea is rich source of flavanols, such as catechins. Catechins found in Matcha green tea powder are natural antioxidants that have been found to have antidiabetic, antihyperglycemic, anticarcinogenic and antiarteriosclerotic effects. The addition of green tea powder to bakery products, such as biscuits, may turn the biscuits into a functional food. In order to create a functional food. Matcha green tea powder is used as ingredients to produce shortbread and biscuits. This approach will allow us to understand the effect of matcha green tea powder on the texture and sensory characteristics of shortbreads and biscuits.

The aim of the study is to

I. to evaluate the consumer acceptability on different level green tea powder incorporated in short bread and biscuit and effect of sugar content in biscuit on the consumer acceptability
II. to develop the formulation of short bread and biscuit with fat and sugar replacement in order to create healthier shortbread and biscuits

Why have I been asked to participate?

You have been invited to participate in the study because we are looking for healthy adults who are able to give their opinion about biscuit and shortbread with green tea powder. You should not participate in this study if:

- You are allergic to any type of food
- You are ill or suffer from any underlying health condition that can affect your ability to taste, smell, chew, digest or excrete of food
- You are taking medication (apart from the contraceptive pill)
- You are pregnant or lactating

What will I have to do if I take part in the study?

If you decide to take part, you will be asked to come in to the research centre in person. The researcher will explain the study in detail and you will be asked to sign a consent form to confirm that you agree to take part in the study. You will also be given a copy of this information sheet and the consent form for you to keep. You will also have the opportunity to ask questions.

Following signing of the consent form, you will be asked to visit the research study 2 times to undertake the sensory panel. You will be given a short questionnaire related to the frequency of consumption of biscuits and shortbreads. Then, you will be given 6 shortbreads or biscuits to evaluate for appearance, smell, taste, texture and general acceptability using a 9 point scale. The sensory questionnaire has 10 questions per food sample. You will be asked to evaluate one sample after another, and to rinse your mouth between samples to clean your palate.

The entire test should not take more than 30 minutes in each visit.

What are the advantages and disadvantages of taking part in the study?

There is no monetary reward for taking part in the study. However, the information we obtain from the study will allow us to evaluate the acceptable level of green tea powder incorporated in biscuits and shortbreads by a sample of the population.

Can I withdraw from this study at any time?

Yes, you can withdraw from the study at any time without giving any reason. However, we kindly request that you let the research team know.

Will the information collected be kept confidential?

Yes, all the information provided will be kept confidential. The information collected about all the participants will be number coded and therefore cannot be identified by any other person apart from the researcher. Information about all the participants will be kept safe in a locked location at the University of Leeds, and will be destroyed 5 years after the end of the study.

The anonymised findings may be published in a scientific journal or presented at scientific meetings. If you are interested in receiving information about the findings of this study, please let us know and we will send you a copy of the research findings.

If you would like more information or have any questions or concerns about the study, please contact

Nanny Benjapor Phongnarisorn
Study coordinator
School of Food Science & Nutrition
Faculty of Mathematics & Physical Sciences
University of Leeds
Email address: mt09bp@leeds.ac.uk

Thank you for taking the time to read this information.

CONSENT FORM

Project Title: Sensory Evaluation of shortbread and biscuit incorporated with green tea powder

Please read the following information and mark with tick (√) if appropriate:

1. I confirm that I have read and understood the information provided on the information sheet for the above study. I have had the opportunity to consider the information and ask questions. ( )

2. I understand that my participation is entirely voluntary and that I am free to withdraw at any time without giving any reason ( )

3. I understand that all the information about me collected as part of this study will be kept securely and that my personal details will not be available to anyone outside the research team. ( )

4. I agree to take part in the above study. ( )

Name of Participant: Date: Signature:

Name of Researcher: Date: Signature:

When completed: one copy for participant, one copy to secure research file.

Appendix C: Ethic approval for pilot study on the effect of MGTP

Performance, Governance and Operations
Research & Innovation Service
Charles Thackrah Building
101 Clarendon Road
Leeds LS2 9LJ Tel: 0113 343 4873
Email: ResearchEthics@leeds.ac.uk

UNIVERSITY OF LEEDS

Benjapor Phongnarisorn
School of Food Science and Nutrition
University of Leeds
Leeds, LS2 9JT

MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC)
University of Leeds

14 September 2016

Dear Benjapor

Title of study **Effect of green tea powder on postprandial glucose, triglyceride and satiety in healthy subjects (a pilot study: randomized control trial)**
Ethics reference **MEEC 14-040**

I am pleased to inform you that the application listed above has been reviewed by the MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC) and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
MEEC 14-040 signed_ethics_application.pdf	1	06/07/15
MEEC 14-040 Ethical_Review_Form_Glycaemic response to biscuit incorporated with green tea powder and effect on satiety in healthy subjects_30072015.docx	2	30/07/15
MEEC 14-040 risk_assessment_form_06072015.docx	1	06/07/15
MEEC 14-040 Appendix1-9_edited_30072015.pdf	2	30/07/15

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval, including changes to recruitment methodology. All changes must receive ethical approval prior to implementation. The amendment form is available at http://ris.leeds.ac.uk/EthicsAmendment.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited. There is a checklist listing examples of documents to be kept which is available at http://ris.leeds.ac.uk/EthicsAudits.

We welcome feedback on your experience of the ethical review process and suggestions for improvement. Please email any comments to ResearchEthics@leeds.ac.uk.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator, Research & Innovation Service
On behalf of Professor Gary Williamson, Chair, MEEC FREC
CC: Student's supervisor(s)

C.1 Consent form

Appendix2

Consent form

Project Title: Effect of green tea powder on postprandial glucose, triglyceride and satiety in healthy subjects (a pilot study: randomized control trial)

Please read the following information and mark with tick (√) if appropriate:

1. I confirm that I have read and understood the information provided on the information sheet for the above study. I have had the opportunity to consider the information and ask questions. ( )

2. I understand that my participation is entirely voluntary and that I am free to withdraw at any time without giving any reason ( )

3. I understand that all the information about me collected as part of this study will be kept securely and that my personal details will not be available to anyone outside the research team. ( )

4. I understand that any information I give may be included in published documents but my identity will be protected by the use of pseudonyms ( )

5. I agree to take part in the above study. ( )

Name of Participant: Date: Signature:

Name of Researcher: Date: Signature:

When completed: one copy for participant, one copy to secure research file.

C.2 Meal randomisation table

Table of test meal randomization

Test food for each study visit is randomized using Latin-squares design.

1= Plain short bread biscuit + water

2= Green tea short bread biscuit + water

3= Plain short bread biscuit + Green tea drink

4= Plain short bread biscuit + water (replicated sample)

15 Participants	1st visit	2nd visit	3rd visit	4th visit
1	4	2	3	1
2	3	4	1	2
3	1	3	2	4
4	2	1	4	3
5	4	1	2	3
6	3	2	1	4
7	1	3	4	2
8	2	4	3	1
9	1	2	4	3
10	4	1	3	2
11	2	3	1	4
12	3	4	2	1
13	2	4	1	3
14	4	3	2	1
15	3	1	4	2

C.3 Health screening questionnaire

Health Screening Questionnaire

Date: / / Participants Code.....

Please provide brief information about yourself by tick at the appropriate answer (s) or provide additional information where necessary.

1. Gender:	○ Male	○ Female
2. Are you pregnant?	○ Yes	○ No
3. Are you lactating?	○ Yes	○ No

4. Have you ever been told by a doctor or other health professional that you have any of the following:

a) Prediabetes	○ Yes (specify).....	○ No	○ Do not know
b) Diabetes mellitus	○ Yes (specify).....	○ No	○ Do not know
c) High blood pressure	○ Yes (specify).....	○ No	○ Do not know
	○ Yes (specify).....	○ No	○ Do not know
d) Food allergies or food tolerance	○ Yes (specify).....	○ No	○ Do not know
e) Cancer			
f) Cardiovascular diseases	○ Yes (specify).....	○ No	○ Do not know
g) Digestive problems	○ Yes (specify).....	○ No	○ Do not know

5. Currently, do you take any medication?

○ Yes	○ No

Please list the name of any medicine, you are taking?

.....

.....

.....

6. Do you have any condition that affect your ability to taste, smell, chew, digest or excrete food?

○ Yes(specify).....	○ No

7. Do you smoke?

○ Yes	○ No

8. Is there any reason that you consider yourself to be unable to fully participate in the study?

.....

.....

....

Thank you for your time

C.4 Pre-study questionnaires and blood measurement record form

Pre-study questionnaires and Blood measurement record

Participant code: ..... Date.....

Age:	Gender :
Height:	Weight:

Please Tick at the appropriate answer (s) or provide additional information where necessary.

1. ethnic background

○ White - British	○ Other Black Background	○ Mixed – White and Black African
○ White - Irish	○ Asian or Asian British - Indian	○ Mixed – White and Asian
○ White – Scottish	○ Asian or Asian British - Pakistani	○ Other Mixed Background
○ Irish Traveller	○ Asian or Asian British – Bangladeshi	○ Other Ethnic Background
○ Other White Background	○ Chinese	○ Information Refused
○ Black or Black British – Caribbean	○ Asian Other	
○ Black or Black British – African	○ Mixed – White and Black Caribbean	

2. Do you drink alcohol?

○ Yes	○ No

3. Frequency of alcohol drinking (if applicable)? .....

4. Are you currently on a special of diet?

○ Yes	○ No

5. If you answered with yes, please specify the type of diet?

○ Weight loss diet:	○ Vegetarian/ Vegan
○ High protein /Atkins	○ Low salt
○ Low fat	○ Weight gain
○ Low carbohydrate	○ Gluten-free
	○ Low cholesterol

6. Do you do any vigorous-intensity sports, fitness, or recreational activities that cause large increases in breathing or heart rate like running or basketball for at least 10 minutes continuously per day?

○ Yes (specify).....	○ No	○ Do not know

7. Do you do any moderate-intensity sports, fitness, or recreational activities that cause a small increase in breathing or heart rate such as yoga, brisk walking, bicycling, swimming, or golf for at least 10 minutes continuously per day?

○ Yes (specify).....	○ No	○ Do not know

8. Do you consider yourself to be in good health today?

○ Yes	○ No

9. How do you come to school today?

○ By walking	○ By Bicycle

○ By any transportation other than bicycle Please specify.....

10. Are you taking any medication before coming to school?

○ Yes	○ No

○ If yes, what is it? .....

11. Do you feel stress today?

○ Yes	○ No

24- Hours Food Consumption Recall

Food Intake	Time	Description	Amount
Breakfast			
Snack			
Lunch			
Snack			
Dinner			

Blood glucose and triglyceride

measurement record

Participant code: ..... Date.....

Timetable for the blood collection

Time	0 Min	15 Min	30 Min	45 Min	60 Min	90 Min	120 Min	150 Min	180 Min
Glucose									
Triglyceride									

Satiety questionnaires and acceptability test

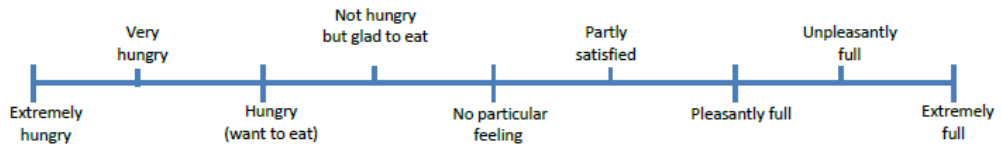
Participant Code:	**Date:**

The satiety score test questionnaires will be asked at before meal and will be repeated at 15, 30, 45, 60, 90 120, 150 and 180 minutes after start of test meal

Before test meal

How hungry to you feel right now?

Please mark on the scale line below, you may mark between the phases, if you wish



Acceptability questions

How do you like the test food? Circle the appropriate number to match your feeling

1 Dislike extremely
2 Dislike very much
3 Dislike moderately
4 Dislike slightly
5 Neither like or dislike
6 Like slightly
7 Like moderately
8 Like very much
9 Like extremely