STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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DECLARATION OF AUTHORSHIP

I hereby certify that this thesis is submitted in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a signed statement from each co-author; and endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

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LIST OF ABBREVIATIONS

°C                Degree Celsius
µL               Microliter(s)
ANOVA            Analysis of Variance
C                Catechin
CG               Catechin Gallate
CVD              Cardiovascular Disease
EC               Epicatechin
ECG              Epicatechin Gallate
EGC              Epigallocatechin
EGCG             Epigallocatechin Gallate
et al.           and others
g                gram (s)
GC               Gallocatechin
GCG              Gallocatechin Gallate
HPLC             High Performance Liquid Chromatography
IS               Internal Standard
MAE              Microwave Assisted Extraction
MAP              Modified Atmosphere Packaging
min              Minute (s)
MIPs             Molecularly Imprinted Polymers
mL               Millilitre (s)
mM               millimolar
POD              Peroxidase
PPO              Polyphenol oxidase
RP-HPLC          Revered-phase HPLC
RSM              Response Surface Methodology
sec              Second (s)
SFE-CO2          Supercritical Fluid Extraction with Carbon dioxide
SPE              Solid Phase Extraction
SWE              Subcritical Water Extraction
UAE              Ultrasound Assisted Extraction
UHPE             Ultrahigh Pressure Extraction
UV-Vis           Ultraviolet – Visible
v/v              Volume by Volume
w/v              Weight by Volume
w/w              Weight by Weight
ABSTRACT

Background

Green tea is a rich source of the strong antioxidant compounds, the catechins, and the unique amino acid, theanine, which have been linked with health benefits such as prevention of certain types of cancers and cardiovascular diseases, decrease in obesity and improvement of the immune system. However, epidemiological studies suggest that the volume of green tea required to obtain health benefits is rather large, ranging from 5-10 cups a day. Therefore, it is questionable whether individuals, especially in western countries where they are not used to drinking green tea, can consume a large enough quantity of green tea to obtain the levels of the green tea bioactive compounds needed for health benefits.

Therefore, extraction of the catechins and theanine from green tea to provide concentrated preparations for use as food supplements or as additives for functional foods has been considered as a way to increase the consumption of these green tea bioactive compounds. In addition, green tea extracts and powders can be utilised in various foods to prolong their shelf-life. Green tea also contains a high level of caffeine, which can work as a mild central nervous stimulant. However, caffeine can cause some negative effects in some people and therefore, its removal from green tea products needs to be addressed. Furthermore, as a consequence of the concerns relative to the use of organic solvents, which are usually used for extractions of plant materials in the food industry, water is the only solvent which should be used.

Another way of increasing the intake of the beneficial green tea components is to ensure that they are well extracted when people prepare their green tea themselves. A low extraction of the compounds could be one of the reasons why large amounts of the beverage appear to be needed to obtain the health benefits.

Hypothesis and Aims

The current study hypothesised that the aqueous extraction of the three bioactive components, the catechins, theanine and caffeine, from loose leaf green tea or green tea in tea bags, could be improved and that aqueous extractions could be used to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders.

The overall aims were to 1) improve the aqueous extraction of the three main bioactive components, catechins, theanine and caffeine from loose leaf green tea, 2) to prepare decaffeinated,
nor
mal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves using water as the only solvent for the extractions and freeze drying and spray drying to dry the aqueous extracts, and 3) to improve the extraction of the three green tea bioactive components form green tea in teabags using water and the microwave oven.

**Results**

The results showed that the extraction of the catechins from loose leaf green tea could be improved by brewing ground green tea ($\leq 1$ mm) twice: once at $80^\circ$C for 30min with a water-to-tea ratio of 12:1 mL/g and once at $80^\circ$C for 30min with a water-to-tea ratio of 8:1 mL/g. The extraction of the theanine from loose leaf green tea could be also improved by brewing ground green tea (0.5-1 mm) at $80^\circ$C for 30min with a water-to-tea ratio of 20:1 mL/g. Water was also found to be effective for decaffeinating freshly harvested young (apical bud to fourth leaf on the growing shoot) and old tea leaves (the fifth to tenth leaves down the stem). Blanching the young tea leaves at $100^\circ$C for 4 min at a water-to-tea ratio of 20:1 mL/g removed 83% of the caffeine while retaining 94% of the catechins whereas blanching the old tea leaves at $100^\circ$C for 10 min at a water-to-tea ratio of 20:1 mL/g removed 80% of the caffeine while retaining 83% of the catechins.

Three types of green tea powders: decaffeinated, normal caffeine and caffeine-enriched green tea powders were also prepared by brewing, filtering, concentrating extracts and then either freeze drying or spray drying them into powders. Both freeze drying and spray drying were found to be suitable for drying the green tea aqueous extracts. However, in terms of cost-effectiveness, spray drying was considered as a method of choice and its optimal conditions were found to be $180^\circ$C for the inlet temperature and $115^\circ$C for the outlet temperature. These green tea powders had catechin levels of 174-197 mg/g and theanine levels of 7-22 mg/g. The caffeine levels were 6.1-7.3 mg/g for decaffeinated powder, 21.3-21.8 mg/g for normal caffeine powder and 94.8 mg/g for caffeine-enriched powder. In addition, these green tea powders had excellent physical properties such as high water solubility ($\geq 96\%$) and low moisture content ($<2.5\%$).

Finally, the results indicated that brewing teabags for 3 min at room temperature in 200 mL of boiled water, as suggested by the manufacturers, was not efficient as only 62% of the catechins, 76% of the caffeine and 80% of the theanine were extracted from the teabags. However, the extraction of these three bioactive components could be improved by first brewing the teabags in freshly boiled water for 0.5 min at room temperature followed by irradiation for 1 min in a microwave oven. This method improved the extraction of the catechins, caffeine and theanine by 34%, 29% and 14%, respectively, in comparison with the common brewing method of 3 min in 200 mL of boiled water.
Conclusions

In conclusion, the hypothesis was supported and the aims were achieved. The aqueous extraction of the three main bioactive components, the catechins, theanine and caffeine from green tea was optimised and improved. In addition, using water as the only solvent, this study developed methods to prepare decaffeinated dried green tea and decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves. Finally, this study developed a method using the microwave oven to improve the extraction of the three green tea bioactive components from green tea in teabags.
PART 1: OVERVIEW
1.1. BACKGROUND

Tea is made from the fresh leaves of Camelia sinensis and different types of teas can be produced from different C. sinensis varieties and processes. Tea is reported to have been first discovered in China by the emperor Shen Nung in 2737 B.C. (Cheng, 2006), where it was first used as a herbal beverage and then gradually introduced to other countries and now, it is the most popular drink in the world after water (Cheng, 2006; Scharbert et al., 2004).

There are six different types of processed teas, which can be produced from the fresh leaves of C. sinensis, usually using the apical bud and the next four to five leaves of the growing shoot. These are green, black, oolong, yellow, pu-erh and white tea. Black tea is the most consumed tea in the world accounting for 78% of total tea consumption followed by green tea at 20% with the rest accounting for only 2% (Cheng, 2006; Wan et al., 2009).

Green tea differs from the other teas based on the way it is processed. As soon as possible after the leaves are harvested, the oxidative enzymes are quickly inactivated using heat to minimise the oxidation of the leaf constituents. Oxidation of the tea leaves is often referred to as fermentation and therefore, green tea is known as non-fermented tea (Cheng, 2006; Wan et al., 2009). The inactivated tea leaves are then put through further steps, including rolling and drying to produce green tea.

For the manufacture of black, oolong and white teas, the fresh tea leaves are first withered to stimulate oxidation before they are rolled or cut and then dried (black and oolong teas) or directly dried (white tea) to obtain the final tea products (Graham, 1992). These so-called fermented teas differ in the extent of oxidation which varies from close to 100% for black tea to roughly 50% for oolong to minimal for white tea. The green tea production process is also different to that of yellow and pu-erh teas, where the fresh tea leaves are first rolled and dried to some extent but then slowly dried to allow for some but minimal oxidation (yellow tea) or put through microbial fermentation (pu-erh tea) before the final drying step (Wan et al., 2009).

Due to the heat-inactivation step, green tea is an abundant source of the naturally-occurring strong antioxidants called the catechins, which are almost all oxidised in black tea. The catechins account for about 30% of the dry weight of the material extracted from the leaves (extractable solids) when the leaves are brewed in hot water to make the beverage. There are eight main individual catechins classified into two groups: epistructured catechins and non-epistructured catechins. The epistructured catechins in order of abundance in the green tea beverage are: epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and
epicatechin (EC). Although in lower quantities, these four catechins also exist in the non-
epistrostructured conformation and are then called: gallocatechin gallate (GCG), gallocatechin (GC),
catechin gallate (CG) and catechin (C) (Balentine et al., 1998; Graham, 1992; Hara, 2001).

The other important phytochemicals in green tea, which unlike the catechins are not affected
much by the oxidation step used to make other teas, are the amino acid theanine and the alkaloid
caffeine. Theanine accounts for about 3% of the dry weight of the extracted solids in the green tea
beverage. It is a unique amino acid in nature and has so far only been found to exist in the tea plant
and in the mushroom Xerocomus badius. Caffeine accounts for 3-5% of the dry weight of the
extracted solids in the green tea beverage. It is known as a “mild stimulant” and as such it is the
most widely consumed stimulant in the world (Chou & Benowitz, 1994; Chu & Juneja, 1999).

All three of the major phytochemicals, the catechins, theanine and caffeine, have been found to
significantly contribute to the quality of green tea. For example, the catechins and caffeine possess
astringent and bitter tastes whereas theanine gives a brothy, sweet and umami taste to the green tea
(Hara, 2001). More importantly, these tea components, have also been linked with various effects
on human health (Khan & Mukhtar, 2007).

The catechins are well known as antioxidants and their antioxidative properties have been
found to be stronger than those of vitamins C, vitamin E, and β-carotene (Graham, 1992; Hara,
2001; Sharangi, 2009). In vivo and in vitro studies have linked the catechins with prevention of
certain cancers (Khan & Mukhtar, 2007; Wheeler & Wheeler, 2004), prevention of cardiovascular
disease (Hara, 2001), reduction in body weight, blood levels of testosterone, insulin, insulin-like
growth factor I, glucose, cholesterol and triglycerides (Kao et al., 2006), prevention of diet-induced
obesity (Klaus et al., 2005) and reduction of the risks for microbial diseases, Parkinson’s diseases,
Alzheimer’s disease and stroke (Zaveri, 2006).

Theanine has also been found to be associated with prevention of certain cancers including
cancers of the lung, breast, colon, liver and prostate (Lu et al., 2004; Takagi, 2010), increasing
relaxation and improving learning ability (Miyagawa et al., 2008; Zheng et al., 2004), providing
effective prophylaxis and treatment for Alzheimer's disease and regulating blood pressure
(Terashima et al., 1999) and promoting weight loss and improving the immune system (Cooper et
al., 2005).

In addition, theanine is a potential adjuvant agent for anti-cancer treatments; it has been found
to reduce the adverse effects of the cancer treatment drug, doxorubicin, by providing protection
against damage caused by doxorubicin to normal tissue (Sugiyama & Sadzuka, 2004). It also acts as a biochemical modulator to improve the therapeutic efficacy of doxorubicin by suppressing the efflux of the drug from cancer cells, thereby increasing the effective doxorubicin concentration in the tumour (Sadzuka et al., 2001).

Caffeine has been linked with various human health benefits such as enhancement of cognitive functioning, improvement of neuromuscular coordination, elevation of mood and relief of anxiety (Glade, 2010), stimulation of the central nervous system and of the cardiac muscle (Griffiths & Woodson, 1998). Therefore, caffeine has been used as an additive for soft drinks and energy drinks. Caffeine has also been added to pharmaceuticals to improve analgesic effects (Chou & Benowitz, 1994; Kumar & Ravishankar, 2009).

On the other hand, there has also been an increasing concern in relation to the side effects of caffeine on human health. High consumption of caffeine is thought to be associated with arrhythmia, tachycardia, vomiting, convulsions, coma or even death (Kerrigan & Lindsey, 2005). Furthermore, consumption of caffeine has been found to cause irritation of the gastrointestinal tract and sleeplessness in certain people (Chu & Juneja, 1997).

The numerous epidemiological studies, in human populations drinking green tea, which report associations between tea consumption and health benefits, support the in vitro and in vivo studies, which provide plausible mechanisms of action. However, these epidemiological studies suggest that the volume of green tea required to obtain health benefits is rather large with a range of 5-10 cups a day (Wu et al., 2003; Zaveri, 2006; Zhang et al., 2007). Therefore, it is questionable whether individuals, especially in western countries where they are not used to drinking green tea, can consume a large enough quantity of green tea to obtain the levels of the green tea bioactive compounds needed for health benefits.

Therefore, extraction of the catechins and theanine from green tea to provide concentrated preparations for use as food supplements or as additives for functional foods has been considered as a way to increase the consumption of these green tea bioactive compounds for people who would want the health benefits but do not want or are unable to drink enough of the green tea beverage. In addition, extracted solutions or dried powders containing high levels of catechins have also been promoted as being able to improve the quality and prolong the shelf-life of foods (Hara, 2001).

Traditionally, organic solvents such as methanol, ethyl acetate, chloroform, benzene, dimethyl chloride and hexane have been used for extracting bioactive compounds from green tea and other
plant sources. According to the regulator, Food Standards Australia New Zealand (FSANZ), several organic solvents can be used for extractions in the food industry, as long as the solvent residues in the final food products meet the standards set by FSANZ for the particular solvent in Standard 1.3.3 (FSANZ, 2012). However, organic solvent residues in food products are increasingly considered unsuitable and not acceptable by consumers who are concerned about the possible impact of these solvents on their health (Wong et al., 2009; Shi et al., 2005). Organic solvents are also increasingly considered unsuitable solvents for use in the food industry because of worker safety issues and because of their potential environmental impacts.

As a consequence of the concerns relative to organic solvents, water is becoming the solvent of choice for the extraction of plant materials. Water is more readily available, relatively cheaper, more environmentally friendly, non-flammable, non-toxic and safer for workers compared to organic solvents, even solvents such as ethanol and methanol. However, water is unfortunately less efficient at extracting catechins and theanine, and less effective for the further purification of these bioactive compounds in comparison with some organic solvents (Shi et al., 2005).

Water often gives low extraction yields due to its high polarity at room temperature. However, hot or boiling water can to some extent overcome the polarity of water and increase the efficiency of extraction to levels resembling that of some of the organic solvents. However, it is also necessary to look at all the possible factors, which could have an impact on the extraction, in order to optimise the yield of the catechins and theanine when water is used to extract these bioactive compounds from green tea.

As mentioned above, because of the concern in relation to the side effects of caffeine on human health, there is also an increasing demand for decaffeinated green tea products. The most effective way to remove caffeine from green tea is by extraction with organic solvents such as chloroform and methylene chloride. However, the use of these organic solvents for decaffeination is now restricted around the world including by the US Food and Drug Administration (FDA) because of their inherent toxicities and environmental impacts (FDA, 2012; FSANZ, 2012; Ramalakshmi & Raghavan, 1999). Therefore, the use of water, a safer and more environmental friendly solvent, needs to be investigated to determine whether it can be used effectively for the decaffeination of green tea.

In addition to providing concentrated green tea extracts and powders for use as food supplements and food additives to help increase the consumption of the green tea bioactive
compounds, it is also important to determine whether these compounds are efficiently extracted when people make their own green tea beverages. Increasingly, green tea is available in tea bags, and therefore, the impact of household brewing methods on the efficiency by which the green tea constituents are extracted from teabags needs to be evaluated. Improving the extraction of the green tea bioactive components in this way may also help people obtain the levels of the green tea bioactive compounds needed for health benefits.
1.2. SYNOPIS OF LITERATURE REVIEW PAPERS

In this thesis, the literature on green tea and its constituents, the catechins, theanine and caffeine, is reviewed and presented in a series of five review papers, including three which are already published, one which is currently accepted for publication (in press) and one which has been submitted for publication.

The first review paper, entitled “Epidemiological evidence linking tea consumption to human health: A review” (Paper I), summarises the latest epidemiological evidence linking tea consumption to human health, outlines the roles of the different tea components and their link to human health, and discusses the major factors, which can affect the availability of the tea components in a cup of tea.

This review paper aimed to answer the common questions that arise about tea consumption including: whether all teas are the same, why drinking tea is linked with health benefits, how do the different ways of preparing tea impact on the availability of the tea components, how much and how long a person should consume tea to obtain health benefits and whether there are any negative health effects associated with drinking tea.

The second review paper, entitled “Isolation of green tea catechins and their utilisation in the food industry” (Paper II), indicated that, although tea consumption has been found to promote human health, only drinking tea may not provide a sufficient level of catechins to achieve health benefits. Thus, the utilisation of catechins in food supplements and additives is an alternative way to supplement catechin consumption. Furthermore, the addition of catechins in foods can prolong the shelf-life of the foods because the catechins can prevent lipid oxidation and they can also improve the colour and flavour of foods. Therefore, this review paper outlined several methods for the isolation of catechins from green tea, discussed the challenges involved, and reviewed the utilisation of the catechins in the food industry.

The third review paper, entitled “Extraction and isolation of catechins from tea” (Paper III), further revealed that the tea catechins were widely used in a variety of food products, neutraceuticals, pharmaceuticals, and cosmetics for either enhancing product shelf-life or for enhancing human health. Thus, this paper revealed that there was an increasing demand for catechins and then went on to review in more detail than Paper II, the various methods that have been proposed for effectively extracting them from green tea.
It was found that the catechins have been extracted and isolated in numerous ways involving several steps and these included: processing of the tea leaves, extraction of the catechins from green tea into solvents, separation of the catechins from other extracted components and drying of the preparations to obtain catechin extracts in powder form. Therefore, this review paper outlined the physical and chemical properties of the tea catechins and reviewed the various extraction methods. It also emphasised that water, which is safe and environmentally friendly, should be the solvent of choice for extracting and isolating the catechins from green tea. Therefore, this review served as the basis from which to further develop and improve the extraction and isolation of the catechins from green tea using the safe and environmentally friendly solvent, water.

The fourth review paper, entitled “L-Theanine: properties, synthesis and isolation from tea” (Paper IV), showed that theanine is a non-protein amino acid that occurs naturally in the tea plant, is easily extracted into the tea beverage and that, especially for Asians, it contributes to the favourable umami taste in green tea. It is also associated with beneficial effects such as the enhancement of relaxation and the improvement of concentration and learning ability and health benefits including the prevention of certain cancers and cardiovascular disease, the promotion of weight loss and an enhanced performance of the immune system.

Thus, there has been a significant rise in the demand for theanine. While theanine has been chemically and biologically synthesised, techniques to isolate theanine from its most abundant natural source, tea, remain an important area of research. Therefore, this review paper summarised the properties and health benefits of theanine and reviewed its synthesis and its isolation from tea. The review also emphasised the use of the safe and environmentally friendly solvent, water, and discussed the future prospects for the isolation of theanine from green tea.

Finally, the fifth review paper, entitled “Caffeine in green tea: its removal and isolation” (Paper V), illustrated that caffeine can act as a central nervous stimulant. It can cause negative effects in some people and this has led to a demand for decaffeinated green tea. On the other hand, caffeine is also widely used as an additive in the beverage and pharmaceutical industries and therefore, there is a potential market for the caffeine extracted during the decaffeination of green tea.

Therefore, this review paper outlined the physical and chemical properties of caffeine, reviewed the numerous decaffeination methods to produce decaffeinated green tea and decaffeinated green tea extracts and powders as well as discussed their advantages and limitations. Again, the use of water as a safe and environmentally friendly solvent was emphasised.
1.3. LITERATURE REVIEW PAPERS

The literature review for the current thesis is based on the following five review articles, which are referred to in the text by their Roman numerals as follows:


1.3.5. Paper V: Quan V. Vuong and Paul D. Roach. Caffeine in green tea: its removal and isolation. Submitted to Food Research International on 30 June 2012.
1.3.1. Epidemiological evidence linking tea consumption to human health: A review

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Epidemiological evidence linking tea consumption to human health: A review

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Abstract

Tea has been widely consumed around the world for thousands of years and drinking tea is a daily habit for people of all ages. Tea is a major source of flavanoids, which have become well known as antioxidants. Tea also contains caffeine and theanine, which have been found to associate with health benefits. Many animal and epidemiological studies have been conducted to investigate the link between tea consumption and human health. However, common questions that arise about tea consumption include: whether all teas are the same, why drinking tea is linked with health benefits, how do the different ways of tea preparation impact on availability of tea components, how much and how long a person should consume tea to obtain health benefits and whether there is any negative health effect associated with drinking tea. To answer these questions, this paper outlines the tea components and their link to human health, discusses major factors affecting availability of tea components in a tea cup and reviews the latest epidemiological evidence linking tea consumption to human health.

Keywords antioxidant, caffeine, flavanoids, human health, tea consumption, theanine.
INTRODUCTION

Tea (*Camellia sinensis*) was first discovered as a drink and medicine in China around 2737 B.C. Since then tea has been introduced to other countries and is now consumed in every country. Tea has become the second beverage to water in terms of worldwide consumption (Gööck, 1990; Scharbert et al., 2004). Presently, tea has been cultivated in six continents (Scharbert et al., 2004; Vuong et al., 2011b). World tea production in 2006 reached a record of 3.64 million tonnes, of which China, India and Kenya were the top three biggest producing countries (Vuong et al., 2011c). The world tea production has been continuously increasing; of which black tea production has been projected to grow at 1.9% annually to reach 3.14 million tons by 2017, whereas, the green tea production has been projected to grow at annual rate of 3.8% annually to achieve about 1.57 million tons for the same period (Vuong et al., 2011c).

Tea can be prepared by either brewing fresh or dried tea leaves in hot water. Brewing dried tea leaves is the most popular method to date to prepare a cup of tea (Gööck, 1990; Scharbert et al., 2004). Dried tea is produced from the fresh tea leaf, which contains chlorophyll, carbohydrates, enzymes, protein, caffeine, theanine, flavonoids and other substances. Flavonoids are the most abundant components in the tea leaf, they are comprised of six catechins and their derivatives (Chu and Juneja, 1997). Carbohydrates and protein also account for a large amount of tea components; however, these components are almost insoluble. Other components are present in small quantities such as caffeine, theanine, volatiles but they are mostly soluble (Chu and Juneja, 1997). The composition of fresh tea leaf varies with the variety, climate conditions, season, position of leaf, soil, cultivation methods and age of leaf (Balentine et al., 1998; Graham, 1992).

The sensory quality comprising of taste, color and flavor is important parameter of tea beverage and is contributed by flavonoids, caffeine, theanine, and aromatic volatile.
components (Ninomiya et al., 1997). Among these components, catechins and caffeine contribute the astringency and bitterness, whereas theaflavins and thearubigins contribute color and the astringent taste (Graham, 1992). Theanine is responsible for the brothy, sweet and umami taste (savory taste) of the tea beverage (Wan et al., 2009b). Aromatic volatile components also supplement flavor to the tea infusion (Kato and Shibamoto, 2009).

Numerous studies have investigated the roles of tea constituents in human health and found that the major tea constituents including flavonoids, caffeine and theanine are linked to the health benefits such as prevention of cancers and cardiovascular diseases, reduction the risks of obesity and diabetes, and improvement of immune system (Basu et al., 2010; Graham, 1992; Khan and Mukhtar, 2007). Several epidemiology studies have reported the association between tea consumption and health benefits (Wu et al., 2003; Zaveri, 2006; Zhang et al., 2007). However, the volume of tea required for obtaining health benefits is an area of speculation. This paper outlines the roles of tea constituents in human health, discusses various types of tea and the ways of tea preparation, which generally affect the availability of the tea constituents, and then reviews the recent epidemiological evidences on the link of tea consumption and the health benefits.

TEA CONSTITUENTS AND THEIR LINK TO HUMAN HEALTH

Flavanoids

Flavanoids are the most abundant components in tea and comprise of catechins, theaflavins, thearubigins, keamfaron and quercertin. Keamfaron and quercertin only account for a small amount; whereas, catechins, theaflavins and thearubegins present in a large quantity in tea (Balentine et al., 1998). Flavanois have been found to inhibit carcinogenesis in humans (Lin, 2009) and the mechanisms are shown in Figure 1.
Catechins

The catechins have the general structure of C6–C3–C6 with two aromatic rings and several hydroxyl groups (Vuong et al., 2010). Catechins can be classified into two groups including epistructured catechins and non-epistructured catechins. Epistucture catechins are major catechins in tea and they comprise of Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC); whereas, non-epistructured catechins only account for small composition in tea including gallocatechin gallate (GCG), gallocatechin (GC), catechin gallate (CG) and catechin (C) (Vuong et al., 2010).

Catechins are rich in green tea and they not only contribute to the beverage quality but also promote health benefits to the consumers. In tea beverage, catechins possess the bitter, astringent and slight sweet tastes (Balentine et al., 1998). In the body, catechins act as antioxidants because they can donate hydrogens, trap peroxyl radicals and thus suppress radical chain reactions and terminate lipid peroxidation (Terao et al., 1994). Their scavenging ability is thought to be more powerful than vitamin C, vitamin E, or β-carotene (Vuong et al., 2010). In addition, catechins have been found to chelate transition metal ions, modulate oxidant and antioxidant enzymes, and affect gene expression (Vuong et al., 2010). However, catechins are unstable and are sensitive to oxidation by enzymes, moisture content, acid and heat (Balentine et al., 1998; Vuong et al., 2010). In the presence of the enzymes polyphenol oxidase and peroxidase, catechins are easily oxidized to form theaflavins and thearubegins, the reason why the content of catechins is lower in black tea as compared to green tea or oolong tea (Graham, 1992). Catechins are readily degraded when storing dried tea with high moisture content (more than 10%) (Robertson, 1993), brewing tea in alkaline conditions or at high temperature (above 80°C) (Vuong et al., 2010). Therefore, care is needed during storage and preparation process of tea to minimize degradation of the tea catechins.
Catechins have been linked with cancer prevention. Catechins were found to delay the onset of skin tumor genesis, reduce the cumulative number of skin tumors and increase the tumor-free survival of rats (Roy et al., 2009). Catechins were also found to prevent nitrosamine 4-(methylnitrosamino)-l-(3-pyridyl)-l butanone -induced lung tumorigenesis and inhibit fumonisin B1 promotion in rat liver utilizing diethylnitrosamine as the cancer initiator (Marnewick et al., 2009; Xu et al., 1992). Furthermore, catechins were reported to inhibit cyclin-dependent kinases (cdks) and induce cdk inhibitors p21 and p27 in breast and prostate cancer cells (Gupta et al., 2003; Park and Dong, 2003).

Catechins have been found to lower the level of cholesterol and prevent platelet clumping, increase oxidative stress and dysfunction of the endothelium, all of which are related to the development of coronary artery disease (Sharangi, 2009). Catechins have been associated with reduction of body weight, blood levels of testosterone, insulin, insulin-like growth factor I, glucose, cholesterol and triglyceride (Kao et al., 2000). Catechins were also found to prevent diet-induce obesity by decreasing energy absorption and increasing fat oxidation (Klaus et al., 2005). In addition, catechins may play a role in preventing microbial diseases, parkinson’s diseases, alzheimer’s diseases and stroke (Khan and Mukhtar, 2007; Zaveri, 2006).

Catechins have shown synergism with vitamin E, vitamin C, and some organic acids such as citric, malic and tartaric (Graham, 1992; Hara, 2001); and also have potential in protecting the deterioration of β-carotene, a vitamin A precursor (Hara, 2001). However, catechins can easily form a complex with other components to reduce their availability. For example, catechins can interact with caffeine to form precipitate through π-π interaction and lower their availability (Ishizu et al., 2009). The amount of precipitation is critical to catechin availability and varies with extracting temperatures, precipitate is high when tea is brewed at
temperature of over 90°C and low when tea is brewed at temperature of 50°C (Liang et al., 2002).

Catechins can also interact with proteins (Vuong et al., 2010), and may inhibit the absorption of food proteins into the body. In addition, catechins can interact with enzymes such as lipoxygenase, α-amylase, pepsin, trypsin and lipase and in doing so may inhibit their activities in the body (Sekiya et al., 1984). Catechins also interfere with the emulsification, digestion, and micellar solubilization of lipids (Koo and Noh, 2006). In addition, catechins can bind and form a complex with iron, thus tend to prevent iron absorption in the body (Zijp et al., 2000). Despite these limitations catechins have shown important antioxidant properties.

**Theaflavins and thearubigins**

Unlike catechins structured as monomers, theaflavins (TF) and thearubigins (TR) are structured of dimers and polymers, respectively (Halder et al., 2006; Zijp et al., 2000) (Figure 2). TF and TR are rich in black tea (Vuong et al., 2010). Typically, black tea contains 10% flavonols, 25% catechins, 30% TF and 45% TR (Zijp et al., 2000). TF and TR are astringent compounds which contribute to the color and taste of the black tea beverage (Halder et al., 2006) and their levels have been found to correlate with black tea quality (Balentine et al., 1998). It is questioned that whether there is any difference in the anti-oxidative properties between TF and TR in comparison with catechins. Studies showed that TF and TR in black tea have the same anti-oxidative properties in comparison with catechins in green tea (Leung et al., 2001; Yoshino et al., 1994). Several studies linked TF and TR with health benefits. For example, both TF and TR were reported to inhibit lipid peroxidation *in vivo* (Yoshino et al., 1994). In addition, TF and TR were found to have significant anticlastogenic effects in human lymphocytes. However, TF was found to have more protective effects than TR (Halder et al., 2006).
Caffeine

There are three methyl xanthines including caffeine, theophylline, and theobromine in tea. However, theophylline and theobromine are only found in small quantities (0.2-0.4 % and 0.02 % (w/w), respectively) (Chu and Juneja, 1997). Caffeine accounts for a large quantity with its maximum level of about 5% (w/w) (Chu and Juneja, 1997). It is interesting to note that the level of caffeine in tea is higher than that of coffee beans, which actually have only 1.5% (w/w) of caffeine (Chu and Juneja, 1997). Caffeine is a trimethyl derivative of purine 2,6-diol and its level in tea is also influenced by several factors such as season, age of the leaf, variety, and processing methods (Balentine et al., 1998). Caffeine is responsible for the briskness of the black tea beverage because it associates with theaflavins to give the characteristic black tea flavor (Balentine et al., 1998).

After ingestion in the body, caffeine undergoes demethylation to form paraxanthine, theobromine and theophylline (Figure 3), which are then broken down in the liver by additional demethylations and oxidation to urates (Heckman et al., 2010; Safranow and Machoy, 2005). Caffeine has been found to link with enhancement of cognitive functioning, improvement of neuromuscular coordination, and elevation of mood and relieves anxiety (Glade, 2010). Caffeine was found to associate with stimulation of the central nervous system and cardiac muscle. Caffeine was also found to elevate plasma free fatty acids and glucose as well as increase peripheral vascular resistance (Griffiths and Woodson, 1998; Harbowy and Balentine, 1997). In addition, caffeine was reported to increase cerebrovascular resistance and increase gastric and other secretions as well as relax smooth muscle (Griffiths and Woodson, 1998; Harbowy and Balentine, 1997). However, it should be noted that caffeine may cause irritation of the gastrointestinal tract and sleeplessness in certain people (Chu and Juneja, 1997). Caffeine may react as a feeble base with acids to form salts, which are very readily hydrolysed (Stanley et al., 1998). Caffeine may also form the precipitate
(cream formation) with theaflavins and ester-forms of the catechins, thus to reduce their availability (Chao and Chiang, 1999).

Theanine

Theanine is a unique amino acid in nature because, with the exception of being found in the mushroom *Xerocomus badius*, it’s occurrence appears to be limited to the *C.* genus, mostly the tea plants *C. sinensis var. Sinensis* and *C. scinenisis var. assamica* and some closely related species such as *C. japonica* and *C. sasanqua* (Deng et al., 2008; Juneja et al., 1999). In tea, theanine accounts for about 50% of the free amino acids, which are involved in producing the distinctive aroma of tea. Theanine has been closely linked with tea’s umami taste, the sweet and brothy taste of the tea liquor (Balentine et al., 1998; Juneja et al., 1999). Theanine has been found to correlate highly with tea quality and price (Chu, 1997).

Theanine constitutes between 1 and 3% (w/w) of the dry weight of tea; however, the level of theanine varies in accordance with various factors including growing location and method of cultivation, tea grade and variety and time of harvest (Balentine et al., 1998; Chu, 1997, Vuong et al., 2011a). Theanine varies with different types of tea. Green tea contains lower or similar levels of theanine as compared to oolong and black teas (Ekborg-Ott et al., 1997). Theanine has been reported to facilitate the generation of alpha brain waves, which are associated with a relaxed but alert mental state and to promote the release of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), which in turn regulates dopamine and serotonin levels in the brain, thus theanine is linked with relaxation and improved learning ability (Cooper et al., 2005; Mason, 2001).

Recent studies have found evidence linking theanine to cancer prevention. Theanine was found to link with the inhibition of the *in vivo* and *in vitro* growth of human non-small cell lung cancer and leukemia cell lines (Liu et al., 2009). Theanine was also found to induce
apoptosis in four cancer cell lines including breast, colon, hepatoma, and prostate (Friedman et al., 2007). In the normal cells, theanine was found to convert to glutamate, thus to increase the intracellular glutamate level and lead to increased intracellular glutathione. Therefore, theanine was thought to inhibit doxorubicin-induced toxicity (Figure 4) (Wan et al., 2009b). In addition, theanine was associated with reducing the adverse effects of doxorubicin reactions, providing protection against tissue damage and acting as a biochemical modulator to improve therapeutic efficacy (Sugiyama and Sadzuka, 2004). Theanine has also found to link with providing effective prophylaxis and treatment for Alzheimer's disease, regulating blood pressure, promoting the weight loss and improving the immune system (Di et al., 2010; Rogers et al., 2007; Takagi et al., 2010; Yokogoshi and Kobayashi, 1998).

Other tea constituents

Tea contains a high content of carbohydrates which accounts for about 40% of dry weight and one third of it is cellulosic fibre. However, most of the carbohydrates are insoluble and therefore are not present in tea beverage after brewing (Balentine et al., 1998; Chu and Juneja, 1997). Although there is only a small amount of starch in the tea leaf, however it contributes to the quality of tea. The synthesis of starch occurs at dawn and at sunset and thus leaf harvested in the morning normally contains more starch and is considered of better quality than tea picked later on in the same day (Chu and Juneja, 1997). Tea also contains several vitamins, of which vitamin C is dominant with about 280 mg per 100 g dried green tea. Green tea has more vitamin C than oolong and black tea because vitamin C is decomposed during the fermentation process (Chu and Juneja, 1997). Tea also contains enzymes like polyphenol oxidase and peroxidase and some inorganic elements such as calcium, potassium, magnesium and copper. These enzymes and inorganic elements influence the quality of the tea infusion (Balentine et al., 1998). In addition, tea contains
some carotenoids and a large number of volatile substances, which contribute to its flavour
(Balentine et al., 1998). Tea also contains chlorophyll, which is high in fresh leaves.
However, dried green, oolong and black teas contain less chlorophyll because it is degraded
during withering and drying (Jiang, 2009).

MAJOR FACTORS AFFECTING AVAILABILITY OF TEA CONSTITUENTS IN A
TEA CUP.

There are two major factors which directly affect the availability of tea constituents in a tea
cup including the types of tea and the way of preparing a tea cup. This section describes
differences among various types of tea and how the different ways of making tea influence
the availability of tea components in a tea cup, thus is consequently related to promotion of
human health.

Tea types

There are eight different types of tea, which can be produced from same fresh tea leaves
(Cheng, 2006) (Figure 5). However, for each type of tea the biochemical quality and sensory
can vary due to the variety, climatic conditions, nutrition of the tea plant, cultivation method
and the type of leaf (Vuong et al., 2010). For example, *Thea Camellia var. assamica* has
higher catechins than *Thea Camellia var. sinensis* (Chu, 1997). The level of catechins in tea
leaves is higher when tea is grown in an area with more sunlight or higher temperature (Hara,
2001). Summer leaves have higher levels of catechins than those in spring leaves (Hara,
2001; Lin et al., 1996). Tea, which is fertilized with potassium, has higher catechins and
theanine in the leaves than those without fertilization (Venkatesan et al., 2004). Tea grown in
shade has higher content of theanine and lower level of catechins than the tea exposing to
high levels of sunlight (Hara, 2001). Tea produced from coarse leaves has lower catechins
than tea produced from young leaves (apical bud and the two youngest leaves) because the
young leaves are richer (2.7-fold) in catechins than old leaves (from the tenth to the fifth leaf)
(Chu, 1997; Hara, 2001).

Green, oolong and black teas are the major types of tea (Vuong et al., 2010). In terms of
world tea consumption, black tea accounts for 78% of total consumed tea in the world, while
20% is green tea and less than 2% is oolong tea (Cheng, 2006). Green tea is known as non-
fermented tea; whereas oolong tea and black tea refer to semi-fermented tea and fermented
tea, in which aerobic oxidation of the tea polyphenols, called catechins, is partially and fully
promoted and the catechins are enzymatically catalyzed to form theaflavins and thearubigins.
Therefore, green tea has highest content of catechins followed by oolong tea and black tea
has the lowest content of catechins (Vuong et al., 2011b). In contrast, black tea has higher
level of theaflavins and thearubigins than oolong tea, and green tea has the lowest level of
these components (Vuong et al., 2011b).

Green tea is mostly consumed in Asian countries and North Africa (Mukhtar and
Ahmad, 2000). Green tea contains up to 30% (w/w) catechins of its dry weight. It also
contains high amount of caffeine and theanine, which account for up to 5% and 3 % (w/w)
dry weight, respectively (Balentine et al., 1998; Graham, 1992). When brewing with boiling
water, green tea produces a green-yellow solution with a fresh and grassy flavor (Wan et al.,
2009a). Green tea is more astringent than black and oolong tea (Balentine et al., 1998). There
is a strong association between sensory quality and tea composition. Green tea with
acceptable sensory quality normally contains a higher amount of theanine, which produces
brothy, umamy taste. Lower amounts of caffeine and catechins are associated with bitter and
astringent tastes (Chu and Juneja, 1997).

Unlike green tea, black tea is mostly consumed in Western countries and some Asian
countries (Mukhtar and Ahmad, 2000). Black tea has a low content of catechins with about

URL: http://mc.manuscriptcentral.com/bfsn  Email: fergc@foodsci.umass.edu
9% (w/w) dry weight; however, it comprises up to 23% (w/w) dry weight of theaflavins and thearubigins (Balentine et al., 1998; Graham, 1992). When brewing in boiling water, black tea produces an orange-red solution with distinct fragrance and flavor (Wan et al., 2009a).

Theaflavins are considered to be the major compounds which are associated with color and taste of the black tea solution (Graham, 1992). Oolong tea is mainly consumed in Asian countries such as China and Taiwan (Mukhtar and Ahmad, 2000). As it is a partially fermented tea, the chemical composition and the sensory quality are somewhat intermediate between those of green and black teas (Balentine et al., 1998). Oolong tea contains up to 20% (w/w) catechins of its dry matter. Unlike black tea or green tea, oolong tea has a unique combination of the freshness of green tea and the fragrance of black tea (Wan et al., 2009a).

The ways of tea preparation

Quality of a tea serving varies with quantity of tea, volume and temperature of water, length of brewing time, application of agitation, and additional ingredients such as sugar, lime and milk (Astill et al., 2001). Preparation of tea differs between countries and individuals within a country. In Asia, green tea is generally prepared by brewing dried tea with boiling water in a tea pot. The first infusion is normally discarded and the subsequent infusions are consumed (Su et al., 2006). Recently, green tea has been ground and packed in the filter bags (2-4 g/bag) and tea liquor has been prepared by infusing a tea bag in a tea pot or a cup/mug for a period of time (3 min). This method of preparation has become popular, even in the Western countries, because of its convenience. Oolong tea is generally prepared by infusing tea in a tea pot following by stirring and steeping for a period of time (3 to 5 min) (Su et al., 2007). In some parts of China, oolong tea is prepared by first quick washing with hot water, then brewing with hot water for a certain of time (3 min) (Su et al., 2006).
Black tea is prepared by infusing a quantity of tea (in a tea bag) with boiling water in a pot or a cup/mug with a period of less than 3 min. Black tea is usually consumed when it is hot, with or without milk and/or sugar (Astill et al., 2001). In India, Pakistan, and some Middle Eastern countries, black tea is prepared by boiling a quantity of tea in a pan with water for several minutes before consumption (Astill et al., 2001).

The weight of tea in a tea bag varies from 1.5 to 3.25 g. Package instruction for brewing time is 3 min with the recommended volume of water varying between 200 and 235 ml (Astill et al., 2001). Previous studies have demonstrated that conditions for effectively extracting catechins and caffeine from tea with water were: 80°C after 20min, 90°C after 15min and 95°C after 10min of brewing (Bond et al., 2003; Perva-Uzunalić et al., 2006). For efficient extraction of theanine, a recent study found that most theanine was extracted after brewing tea in water at 80°C after 5 min (Keenan et al., 2010). It appears that household preparation of tea is not efficient for optimal extraction of tea bioactive components and this is the reason why a large volume of tea (several cups) was required for obtaining health benefits.

Recent studies have found that microwave assisted extraction of bioactive components was very effective (Pan et al., 2003; Spigno and Faveria, 2009), because it disrupts the structure of the cells and thus more tea components are extracted into the beverage (Vuong et al., 2010). It appears that the use of microwaves for 30s to 1min after infusing tea in the hot water may help to improve extraction efficiency of tea components. However, further study is needed to provide conclusive evidence.

It is common in Western countries to consume black tea with lime, milk or sugar (Gööck, 1990). There is no evidence showing an interaction between tea and sugar constituents, however care is needed when adding sugar because of the correlation between sugar consumption and atherosclerosis, heart problems, dermatosis, neoplasms, obesity,
acidosis, hypoglycaemia and dental caries (Gyntelberg et al., 2009; Wright et al., 2007). On the contrary, addition of lime is thought to benefit human health because it not only additionally provides vitamin C but also helps protect tea components from degradation, tea components are more stable at low pH (Vuong et al., 2010).

It is questionable whether addition of milk reduces activity of flavonoids in the tea solution. Findings from a study of nine healthy volunteers showed that the addition of milk reduced antioxidant power (Langley-Evans, 2000), another study of sixteen healthy female volunteers also found that addition of milk prevented vascular protective effect of black tea (Lorenz et al., 2007). Evidence on the contrary showed a slightly larger study of 21 healthy volunteers found that addition of milk into tea did not affect its antioxidant properties (Leenen et al., 2000). Other studies (n= 9 and n=18) also found that addition of milk into tea did not affect its antioxidant power (Hollman et al., 2001; Reddy et al., 2005). Therefore, it can be tentatively concluded that the addition of milk is unlikely to decrease tea antioxidant power.

TEA CONSUMPTION AND HUMAN HEALTH

Many animal studies have linked tea components with various health benefits as mentioned previously. However, the impacts of these tea components might not be the same in human body because of species differences in the bioavailability and actions of these active components involved (Chen et al., 1997). The bioavailability between individual tea components might be also different in humans. For example, Yang et al. (1998) found that EC and EGC have higher bioavailability than that of EGCG. Therefore, it is complex in determining the mechanism as well as association between tea consumption and health benefits.
Many epidemiological studies have been conducted to investigate the link between tea consumption and human health. This section reviews results from previous epidemiological studies published over the last 10 years to provide information relating to the link between tea consumption and health benefits.

Cancers

Numerous epidemiological studies have investigated the association between tea consumption and prevention of cancers. Findings were inconsistent and contradictory (Table 1). Findings from studies have shown that consumption of green tea (3 cups/day or more) significantly lowered the risk of lung cancer among non-smoking women, but not for smoking women (Zhong et al., 2001). Intake of tea was found not to be associated with prevention of lung cancer among smokers for both men and women (Baker et al., 2005). Further results from studies reported that daily tea consumption was not significantly associated with reduction of the lung cancer risk (Bonner et al., 2005; Li et al., 2008). Overall the results show that it is likely that consumption of tea might be linked to prevention of lung cancer for healthy people but not for the smokers. Further epidemiological study in this area is needed.

Squamous cell carcinomas (SCC) is a carcinomatous cancer that occurs in many different organs. A study found that regular drinking tea (at least 1 cup/day for more than 1 month) was associated with a significantly lower risk of SCC (Rees et al., 2007). It is important to note that tea should not be drunk when it is hot (more than 60°C) because drinking hot tea was found to strongly associate with a higher risk of oesophageal SCC cancer development (Islami et al., 2009).

In vivo studies have linked tea flavanoids with prevention of liver cancer. Conversely an epidemiological study found that frequent drinking tea did not decrease the risk of
hepatocellular carcinoma (Montella et al., 2007). Another study found no association between regular tea intake and prevention of liver risks or hepatitis C and B virus status (Inoue et al., 2009). Contrary evidence from a larger population based study (n = 41761, 9 years follow up) found that daily consumption of 5 cups or more could significantly lower the risks of liver cancer (Ui et al., 2009). A meta-analysis conducted recently by Sing et al. (2011) provided more evidence for the link between tea consumption and prevention of the development of primary liver cancer.

Findings have shown that consumption of more than 3 cups of tea a day for at least 3 months could significantly lower the risk of breast cancer (Shrubsole et al., 2009; Zhang et al., 2007). However, findings of a study conducted in the US have shown an association between reduction of the breast cancer risks and green tea consumption, but not for black tea intake (Wu et al., 2003). Another study confirmed that black tea consumption did not reduce the risk of breast cancer and drinking green tea could lower the risk of breast cancer among women with high-activity angiotensin-converting enzyme (ACE) but not for those with low-activity ACE (Yuan et al., 2005). A study conducted in the Netherlands found that regular drinking tea has no link with the reduction of breast cancer (Pathy et al., 2010). The results from studies in this area have not been consistent but there is an association between green tea consumption and prevention of breast cancer.

Several reviews summarize findings from previous in vivo studies, which reported that tea catechins could prevent pancreatic, bladder, gastrointestinal and prostate cancers (Khan and Mukhtar, 2007; Sharangi, 2009; Zaveri, 2006). Recent epidemiological studies found that regular tea intake has no association with reducing the risks of pancreatic cancer (Lin et al., 2008; Luo et al., 2007). No link was found between drinking tea and decreasing the risks of bladder cancer (Bianchi et al., 2000; Woolcott et al., 2002). Studies showed that tea consumption was significantly associated with reduction in the risks of gastrointestinal and
prostate cancers (Bettuzzi et al., 2006; Jian et al., 2004; Kurahashi et al., 2008; Zhang et al., 2006). Zhang et al. (2006) revealed that consumption of at least 1 cup of tea daily for at least 6 months significantly reduced biliary tract cancer. Jian et al. (2004) reported that prostate cancer risk decreased with increasing frequency, duration and quantity of green tea intake. Green tea consumption was also found to limit an antineoplastic activity, as defined by a decline in prostate specific antigen levels, among patients with androgen independent prostate carcinoma (Jatoi et al., 2003).

Overall, results from epidemiological studies were inconsistent and conflicting. In most of these studies, the data on the tea intake was obtained from the dietary questionnaires to recall the information (Table 1). This is a major limitation of the epidemiological studies inferring the relationship of the tea intake and cancers. The inevitable question that needs to be answered is how much of tea should a person drink daily and for how long to prevent certain types of cancer? In most studies the tea consumption was recorded in milliliter or cups per day, week or month for a certain time. Therefore, there are many biases which influence the results and conclusions drawn. For example, the volume of the tea cup was varied among the studies. Important parameters such as the type of tea, quantity of tea, volume of water, temperature of water and the length of brewing time, all of which directly affect the availability of the tea constituents and antioxidant power of the tea beverage have been neglected. Therefore, consumption of five cups might provide lower levels of tea constituents compared to three cups. It is recommended that future epidemiological studies should consider these issues when designing the methods to minimize bias of the results.

Coronary cardiovascular disease (CVD)

A population based-control study in Japan of patients with significant coronary stenosis (n = 109, 6 months) found that consumption of at least 6 cups of tea a day could lower
incidence of coronary artery disease (Sano et al., 2004). Another larger study in Japan (n = 13,
916, 1 year) showed that daily consumption of at least 10 cups of tea significantly decreased
levels of serum total cholesterol in both men and women (Tokunaga et al., 2002). Daily
consumption of 3 cups of tea or more could reduce the risks of CVD mortality (Gans et al.,
2010). The similar findings were observed in a large study (n = 40530, 11 years), where the
volume of tea required was higher with at least 5 cups a day (Kuriyama et al., 2006). The
volume of tea intake for obtaining benefits is clearly inconsistent.

Overall, tea consumption has been linked with reducing the risks of CVD, but it should
be noted that tea might interact with cardiovascular medication (Izzo et al., 2005). One study
showed that tea intake could antagonize the effect of warfarin, which produces
anticoagulation by inhibiting production of the vitamin K-dependent clotting factors and thus
drinking tea might reduce the patient’s degree of anticoagulation (Taylor and Wilt, 1999).
Another clinical trial found that tea consumption of at least 3 cups a day may augment drug
therapy (Bahorun et al., 2010).

Obesity and diabetes

Tea is thought to be associated with prevention of obesity and diabetes. Although the
mechanism for this association is complex bioactive components of tea are thought to play a
role in modulation of energy balance, endocrine systems, food intake, lipid and carbohydrate
metabolism, the redox status, and activities of different types of cells (Kao et al., 2006). In
addition, tea components may affect the sympathetic nervous system activity, increase energy
expenditure and promote the oxidation of fat (Rains et al., 2011). However, findings from
recent studies are conflicting. A study on obese Chinese women (BMI ≥ 28 kg/m²) with
polycystic ovary syndrome (PCOS) (n = 34, 3 months) reported that daily consumption of 2
capsules containing 90 mg EGCG (equivalent to 1.5 cups of tea) did not significantly reduce
body weight and did not alter the glucose or lipid metabolism (Chan et al., 2006). On the
contrary, a study on obese people in Thailand (BMI > 25 kg/m²) (n = 60, 3 months) found that
consumption of a 250 mg green tea capsule after breakfast, lunch, and dinner could reduce
body weight (Auvichayapat et al., 2008). A study in Oklahoma (n = 41, 2 months) with
metabolic syndrome (MeS) found that daily consumption of 4 cups (928 mg catechins) of tea
or 2 capsules (870 mg catechins) significantly decreased body weight and BMI and lowered
lipid peroxidation (Basu et al., 2010). The positive affect of tea intake and obesity was
obtained when higher amount of tea extract was taken daily by the participants (750 mg, 870
mg/4 cups) in comparison with low amount (180 mg/1.5 cups) in the study which found no
link between tea intake and obesity.

In a large study on African American women (n = 46,906, 12 years), results showed
that tea consumption was not associated with reducing the risk of diabetes (Boggs et al.,
2010). Results from another study (n = 36,908, 5 years) also revealed that green tea intake did
not associate with prevention of type 2 diabetes, however, daily consumption of 1 cup of
black tea showed a reduction of 14% in the risk of diabetes in Asian men and Singaporean
women (Odegaard et al., 2008). On the contrary, results from a study in Japan (n = 17,413)
aged 40 to 65 years and had no history of type 2 diabetes for 5 years, showed that there was
no association between consumption of black or oolong teas and diabetes. Daily consumption
of at least 6 cups of green tea was associated with a reduced risk for type 2 diabetes (Iso et
al., 2006). A meta analysis also revealed that tea consumption of more than 4 cups per day
(RR, 0.8; 95% CI, 0.7–0.93) may play a role in the prevention of type 2 diabetes (Jing et al.,
2009). Consumption of 1,500 ml (6 cups) of oolong tea a day significantly lowered
concentrations of plasma glucose and fructosamine, thus drinking oolong tea might be an
effective adjunct to oral hypoglycemic drugs in the treatment of type 2 diabetes (Hosoda et
al., 2003). Again from the findings it is difficult to draw a conclusion on what kind of tea,
how much and how long a person should consume tea to obtain the health benefits. However, epidemiological evidence showed that tea consumption was likely to prevent the risk of obesity and diabetes. It should be noted that consumption of tea with sugar is associated with weight-control and diabetes (Gyntelberg et al., 2009), thus the amount of sugar added in tea should be minimized.

Other impacts of tea intake on health

In animal studies, tea bioactive components have been found to improve the immune system, oral health and inflammatory processes (Hamer, 2007). Bioactive components have been found to prevent microbial diseases and oral health (Khan and Mukhtar, 2007; Zaveri, 2006), but there is no epidemiological evidence in human studies to confirm these health benefits.

Tea intake has been reported to improve bone health. Daily consumption of two to three cups of tea has been found to associate with higher spinal bone mineral density and daily intake of four cups or more was found to increase total body bone mineral density (Chen et al., 2003). In addition, bone mineral density was found higher in the old women who drank tea in comparison with whom did not drink tea. The inference is that tea consumption might protect against osteoporosis in older women (Hegarty et al., 2000). Tea has been found to affect the mood and cognitive performance in humans. Intake of tea caffeine (250mg) could increase alertness, jitteriness and blood pressure, whereas, intake of theanine (200mg) was found to antagonise the effect of caffeine on blood pressure but did not significantly affect jitteriness, alertness or other aspects of mood (Rogers et al., 2008). Results from another study found that intake of 2 cups of black tea (400ml) for 24 hours could positively affect human mood (Scott et al., 2004).
Tea consumption has been found to associate with various positive health impacts; however, it is questionable whether there is any negative impact of consuming tea. Tea flavanoids interact with proteins and major digestive enzymes such as α-amylase, pepsin, trypsin and lipase (He et al., 2007; Sekiya et al., 1984), thus it is thought that tea intake may inhibit absorption and digestibility of the dietary proteins and foods but there is limited epidemiological evidence confirming this link. Results from a study showed that addition of the tea extract to the diet did not change protein digestibility of weaning male rats (Chang et al., 1994). Another study also found that polyphenols in tea waste incorporated in the diet did not affect the food digestibility of goats (Kondo et al., 2004). Therefore, the absorption and digestibility of the dietary proteins and foods might not be affected by the tea consumption. Further animal and human studies are needed to investigate this association.

Iron deficient and iron deficiency anemia are major problem, especially for young women with approximately 2 billion and 1 billion people worldwide affected, respectively (Thankachan et al., 2008). Tea polyphenols were found to bind with iron (Zijp et al., 2000) and therefore tea consumption might decrease the iron absorption and increase risk of iron deficiency especially in vulnerable group. However, findings from one study found that consumption of decaffeinated black tea and green tea (4 cups/day) during or immediately following the meals did not affect on the absorption of iron and other minerals such as calcium, copper, magnesium and zinc among young adults (Prystai et al., 1999). Another study also found that tea intake had no association with the risks of iron depletion among healthy adults (Mennen et al., 2007). Tea intake was also not found to be related to iron status in vulnerable group who is at risk of iron deficiency (Hogenkamp et al., 2008). Conversely findings from a study revealed that intake of black tea at 1 or 2 cups/day decreased iron absorption by 49% (P < 0.05) or 66% (P < 0.01), respectively (Thankachan et al., 2008). Further study defining the link between tea intake and iron absorption is necessary. To
minimize the negative impact of tea intake and iron absorption: tea should not be consumed during meals (Johnson, 2006).

CONCLUSIONS

Tea is a rich source of bioactive constituents such as flavanoids, caffeine and theanine which have been found to link with various health benefits such as prevention of cancers, CVD, obesities and diabetes. However, availability of these components in a tea cup varies and depends on the types of tea and the ways of preparation. Eight major types of tea can be produced from the fresh leaves of *Camellia Sinensis* and these teas are different in terms of biochemical quality and sensory attributes. Preparing tea with milk or lime with/without sugar does not affect the antioxidant properties of the tea constituents. However, amount of sugar addition should be controlled if large volume of tea is consumed daily.

Results from epidemiological studies were inconsistent and thus it is difficult to conclude how much of tea a day and how long a person should consume tea to obtain the health benefits. Most studies confirmed that there is no harm in drinking too much tea and tea consumption has associated positive health impacts, and yet, drinking several cups of tea daily can keep the doctor away.

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Table and Figure Legends

Table 1 The link between tea consumption and human health.

Figure 1 Biochemical mechanisms of anticarcinogenesis by tea flavanoids (Lin, 2009).

Figure 2 Chemical structure of theaflavins and thearubigins. R = Galloyl Group (Zijp et al., 2000).

Figure 3 Metabolism of caffeine in humans (Safranow and Machoy, 2005). CYP 450: Cytochrome P450; XO: Xanthine oxidoreductase; NAT 2: N-acetyltransferase 2; MX, DMX, and TMX: mono-, di-, and trimethylxanthines; MUA, DMU, and TMU: mono-, di-, and trimethyluric acids; AFMU: 5-acetylamino-6-formylamino-3-methyluracil.

Figure 4 Mechanism of theanine to protect normal tissue from doxorubicin (DOX) toxicity via the changing glutathione (GSH) level (Wan et al., 2009b).

Figure 5 Processing techniques of dried teas (Balentine et al., 1998; Wan et al., 2009a)
**Table 1** The link between tea consumption and human health

<table>
<thead>
<tr>
<th>Disease</th>
<th>Subjects/population and location</th>
<th>Method for information of tea intake</th>
<th>Volume of tea consumed</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>Study for 2 years; 649 women with primary lung cancer and 675 controls aged 35–69 years; Shanghai, China Study for 7 years; n = 41440 aged 40–79 years, Northeastern Japan</td>
<td>In-person interview Food frequency questionnaire (FFQ).</td>
<td>≥ 3 cups/week for at least 1 year ≥ 5 cups/day</td>
<td>Green tea intake lowered the risk of lung cancer among nonsmoking women (Odds ratios (OR) = 0.65; 95% CI = 0.45–0.93) Green tea intake was not associated with reduction of lung cancer (The multivariable-adjusted HRs as compared to ≤ 1 cup/day: 1.17 (95% CI: 0.85–1.61)</td>
<td>(Zhong et al., 2001) (Li et al., 2008)</td>
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<tr>
<td>Skin cancer</td>
<td>Study 1: 2 years; study 2: 3 years; 770 with basal cell (BCC), 696 with squamous cell carcinomas (SCC) and 715 controls subjects aged 25–74 years, New Hampshire Study on 300 participants with incidence of oesophageal SCC and 571 controls; Golestan province, Iran</td>
<td>In-person interview In-person interview</td>
<td>≥ 2 cups/day ≥ 1 L/day (4 cups)</td>
<td>Tea intake was associated with a significantly lower risk of SCC (OR = 0.65; 95% CI 0.44–0.96), but not with BCC (OR = 0.79; 95% CI 0.63-0.98) Hot tea (60-64°C) and very hot tea (≥65°C) intake was associated with an increased risk of oesophageal cancer (OR = 2.07; 95% CI 1.28-3.35; and OR = 8.16; 95% CI 3.93-16.9, respectively)</td>
<td>(Rees et al., 2007) (Islami et al., 2009)</td>
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<tr>
<td>Liver cancer</td>
<td>Hospital-based study for 3.5 years; 185 incidents of hepatocellular carcinoma (HCC) and 412 controls aged 43–84 years, Italy Study for 9 years; n = 41761 aged 40–79 years, Northeastern Japan</td>
<td>FFQ FFQ</td>
<td>≥ 1 cup/week ≥ 5 cups/day</td>
<td>No association was found between tea intake and reduction of HCC risk (OR = 1.43; 95% CI 0.76-2.66) Drinking green tea had a significantly lower risk of liver cancer among men (HRs = 0.63; 95% CI 0.41–0.98), and women (HRs = 0.50; 95% CI 0.27–0.90)</td>
<td>(Montella et al., 2007) (Ui et al., 2009)</td>
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<td>Disease</td>
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<td>Gastrointestinal tract cancer</td>
<td>Study for 4 years; 627 with biliary tract cancer, 1,037 with biliary stones and 959 controls, Shanghai, China</td>
<td>In-person interview</td>
<td>≥ 3 cups/week for at least 6 months</td>
<td>Tea intake significantly reduced risks of biliary stones (OR = 0.73; 95% CI 0.54-0.98) and gallbladder cancer (OR = 0.56; 95% CI 0.38-0.83)</td>
<td>(Zhang et al., 2006)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Study for 5 years; 297 breast cancer and 665 controls aged 45-74 years, Singapore</td>
<td>24 h food recalls</td>
<td>≥ 1 cups/week</td>
<td>There was no association between intake of black tea and breast cancer. Intake of green tea was not associated with decrease of breast cancer among women with low-activity ACE but was strongly associated for women with high-activity ACE (OR = 0.29; 95% CI 0.10-0.79)</td>
<td>(Yuan et al., 2005)</td>
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<td></td>
<td>Study for 9.6 years; n = 27323 participants, Netherlands. Study for 3 years; 501 breast cancer and 594 controls aged 25-74 years, Los Angeles County</td>
<td>FFQ</td>
<td>≥ 1 cups/day</td>
<td>No association between tea intake and breast cancer in women</td>
<td>(Pathy et al., 2010)</td>
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<td></td>
<td>Study for 1 year; n = 1009 aged 20-087 years, Southeast China;</td>
<td>FFQ</td>
<td>≥ 2 cups/day</td>
<td>Risk of breast cancer was not statistically associated with black tea intake but with green tea consumption (OR = 0.53; 95% CI 0.35-0.78)</td>
<td>(Wu et al., 2003)</td>
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<tr>
<td>Pancreatic cancer</td>
<td>Study for 11 years; n = 102137, Japan</td>
<td>FFQ</td>
<td>≥ 1 cup/day</td>
<td>Tea intake was not associated with reducing the risks of pancreatic cancer</td>
<td>(Luo et al., 2007)</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Study for 2 years; 927 bladder cancer and 2118 controls, Canada</td>
<td>Telephone interview</td>
<td>≥ 1 cup/day</td>
<td>Tea intake had no association with bladder cancer</td>
<td>(Bianchi et al., 2000)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Study for 1 year; 130 prostate cancer and 274 controls, Hangzhou, China</td>
<td>In-person interview</td>
<td>≥ 3 cups/day</td>
<td>Green tea intake lowered the risks of prostate cancer (OR = 0.27; 95% CI 0.15-0.48)</td>
<td>(Jian et al., 2004)</td>
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1.3.2. Isolation of green tea catechins and their utilisation in the food industry

Quan V. Vuong, Costas E. Stathopoulos, John B. Golding, Minh H. Nguyen and Paul D. Roach

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Isolation of Green Tea Catechins and Their Utilization in the Food Industry

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Tea is a rich source of catechins, which are well-known antioxidants. Tea consumption has been found to promote human health; however, only drinking tea may not provide a sufficient level of catechins to achieve health benefits. Thus, the utilization of catechins in foods is an alternative way to supplement catechin consumption. Furthermore, catechins can prevent lipid oxidation and improve color and flavour of foods; hence, addition of catechins can also prolong the shelflife of foods. Therefore, catechins have recently been isolated from green tea for utilization in food products to enhance their shelflife and health benefits. This article outlines several methods for the isolation of catechins from green tea, discusses the challenges involved, and reviews the utilisation of catechins in the food industry.

Keywords tea, catechins, isolation, utilisation, tea extract

Introduction

Tea is said to have first been discovered as a medicinal beverage in 2737 B.C. in China and now has become a very popular beverage, second only to water in worldwide consumption. World tea production in 2007 reached a record 3.95 million tonnes and the trend is continuously increasing with an annual growth rate of 1.9% for black tea and 3.8% for green tea and is projected to reach a production of 3.14 million tonnes of black tea and 1.57 million tonnes of green tea by 2017. Tea is a rich source of catechins, which account for about 30% of its dry weight. However, the content of catechins in tea varies with the location of cultivation, variety, nutrition of the tea plant, time of year, and processing conditions.

Tea catechins are well known as antioxidants and their antioxidative properties were found to be stronger than those of vitamins C, vitamin E, and β-carotene. Numerous in vivo and epidemiology studies have shown that tea catechins are linked to the prevention of certain types of cancer such as skin, lung, liver, pancreatic, gastrointestinal, breast, and...
prostate cancers.\(^{(6,7)}\) In addition, tea catechins are proposed to prevent cardiovascular diseases (CVD), microbial diseases, diabetes, and obesity.\(^{(8)}\) However, studies have revealed that the health benefits are only achieved when tea is consumed in sufficient amounts. For example, although one intervention trial showed that two cups of green tea lowered low-density lipoprotein (LDL) cholesterol, a major risk factor for CVD, by 11%,\(^{(9)}\) epidemiological studies indicate that links with beneficial effects on CVD are often only seen when five cups or more of green tea are consumed daily.\(^{(10)}\)

It is questionable whether individuals can consume a large enough quantity of tea to obtain the levels of catechins needed for health benefits. Furthermore, consumption of tea, which contains caffeine, may cause irritation of the gastrointestinal tract and sleeplessness in certain people.\(^{(11)}\) Therefore, utilisation of tea extracts in foods has been considered as an alternative way to provide the health benefits from tea catechins. In addition, utilisation of catechins in foods can prolong the shelflife and improve the quality of foods.\(^{(5)}\) However, the major properties of catechins, production of tea extracts, and the challenges encountered when using tea extracts in foods have not been well described previously. This article aims to describe the major properties of catechins and the production processes for preparing catechin extracts to outline the challenges that may be encountered when using tea extracts in foods, as well as to review the utilisation of catechins in the food industry.

**Major Properties of Tea Catechins**

*Structure and Synthesis of Catechins*

Catechins are comprised of a central 3-carbon unit, which is connected to two phenolic nuclei (two aromatic rings) with several hydroxyl groups.\(^{(5,12)}\) Tea catechins are classified into two groups: epistructured catechins and nonepistructured catechins (Fig. 1).\(^{(13)}\) Epicatechins are the major catechins in tea, and epigallocatechin gallate (EGCG) accounts for the highest content, followed in decreasing order by epigallocatechin

![Figure 1. Structure of catechins. EGCG = epigallocatechin gallate; EGC = epigallocatechin; ECG = epicatechin gallate = EC: epicatechin; GCG = galallocatechin gallate; GC = galallocatechin; CG = catechin gallate; C = catechin.](image-url)
Isolation of Green Tea Catechins

![Figure 2. Biosynthesis of catechins in tea plant.](image)

(EGC), epicatechin gallate (ECG), and epicatechin (EC). In contrast, nonester-structured catechins, including galloallocatechin gallate (GCG), galloallocatechin (GC), catechin gallate (CG), and catechin (C), are only present in small quantities in tea.\(^\text{[12]}\)

Catechins are synthesised in the leaves of the *Camellia sinensis* plant through the acetic–malonic acid and shikimic–cinamic acid metabolic pathways (Fig. 2). Chalcone and gallic acid are produced from the shikimic acid pathway, which then produce the different catechins.\(^\text{[11]}\) There is some variation in the content of individual catechins in the fresh tea leaf; the composition depends on the location of cultivation, variety, nutrition of the tea plant, time of year, and type of leaves (coarse or young leaves). A typical catechin profile in an extract from green tea leaf is comprised of 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC.\(^\text{[4]}\)

**Physical and Chemical Properties of Catechins**

The physical and chemical properties of catechins can be utilised for effectively isolating catechins from tea and fortifying them in foods (Tables 1 and 2). Catechins are colourless but they are bitter and astringent in taste.\(^\text{[14]}\) Individual catechins have different tastes; for example, EGCG and ECG possess a bitter and astringent taste, whereas EGC and EC are bitter, with a sweet aftertaste.\(^\text{[15]}\) Catechins can react with caffeine and proteins to form precipitates, which makes the solution look hazy; this process is referred to as **cream formation**.\(^\text{[16,17]}\) Catechins also react with enzymes such as lipoxygenase, α-amylase, pepsin, trypsin, and lipase to form precipitates and consequently inhibit the activity of these enzymes.\(^\text{[18]}\) Catechins containing an ester bond, such as EGCG and ECG, have a greater ability to form precipitates with the enzymes compared to the non-ester bond–containing catechins.\(^\text{[18]}\) Precipitation of catechins with caffeine or protein can be used in the isolation of catechins for their application in foods.

Catechins have a marked iron-binding ability because the galloyl group in their structure tends to bind with iron in foods, thus inhibiting its absorption in the body.\(^\text{[19]}\) Tea catechins are sensitive and unstable to oxidising enzymes, heat, and high pH.\(^\text{[1]}\) In the presence of the enzymes polyphenol oxidase (PPO) and peroxidase (POD), catechins are oxidised to form theaflavins and thearubegins.\(^\text{[1]}\) The optimum conditions for activity of PPO and POD are at a temperature of 40°C and a pH of 5.5.\(^\text{[20]}\) Thus, adjustment of the temperature and pH can reduce the activities of these enzymes.\(^\text{[21]}\) Caution

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\(^{[1]}\) The reference number in square brackets corresponds to the document's internal citation format. For a full list of references, please consult the document's bibliography or the provided bibliography.
Table 1
Major physical properties of catechins

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>EGC</th>
<th>ECG</th>
<th>EGCG</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C_{15}H_{14}O_{6}</td>
<td>C_{15}H_{14}O_{7}</td>
<td>C_{22}H_{18}O_{10}</td>
<td>C_{22}H_{18}O_{11}</td>
<td>(12)</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>290</td>
<td>306</td>
<td>442</td>
<td>458</td>
<td>(12)</td>
</tr>
<tr>
<td>Max. absorption (nm)</td>
<td>280</td>
<td>269</td>
<td>280</td>
<td>273</td>
<td>(12)</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>218</td>
<td>218</td>
<td>253</td>
<td>254</td>
<td>(19)</td>
</tr>
<tr>
<td>Solubilisation</td>
<td>Time</td>
<td>Time/solvent</td>
<td>Time/ temperature</td>
<td>Time/ temperature/solvent</td>
<td>(87, 88)</td>
</tr>
<tr>
<td>Crystallisation</td>
<td>From water</td>
<td>From water</td>
<td>From water</td>
<td>From water</td>
<td>(87)</td>
</tr>
<tr>
<td>Optical rotation (a)20</td>
<td>−59.0° (c 1.0 in acetone)</td>
<td>−67.5° (c 1.0 in ethanol)</td>
<td>−179° (c 0.28 in ethanol)</td>
<td>−179° (c 0.28 in ethanol)</td>
<td>(87)</td>
</tr>
<tr>
<td>Purification</td>
<td>–</td>
<td>On Sephadex LH-20 elution with water; followed by chloroform : ethanol 7:1 (v/v)</td>
<td>On Sephadex LH-20 elution with acetone increased from 10 to 25%</td>
<td>On Sephadex LH-20 elution with acetone increased from 10 to 25%</td>
<td>(87)</td>
</tr>
</tbody>
</table>

Note: EC = epicatechin; EGC = epigallocatechin; ECG = epicatechin gallate; EGCG = epigallocatechin gallate.

Table 2
Different physical and chemical properties between catechins, caffeine, and theanine(12,16,17,19,28,89)

<table>
<thead>
<tr>
<th></th>
<th>Catechins</th>
<th>Caffeine</th>
<th>Theanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold water</td>
<td>Less soluble</td>
<td>Less soluble</td>
<td>Soluble</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Soluble</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Ether</td>
<td>Soluble</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Insoluble</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Iron</td>
<td>Binding</td>
<td>Nonbinding</td>
<td>Nonbinding</td>
</tr>
<tr>
<td>Proteins</td>
<td>Precipitate</td>
<td>Not precipitated</td>
<td>Not precipitated</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Precipitate</td>
<td>Not precipitated</td>
<td>Not precipitated</td>
</tr>
</tbody>
</table>
is also needed when adjusting temperature because at high temperatures (>95°C) the epistructured catechins tend to epimerise to nonepistructured catechins.\(^{(22)}\) In addition, though catechins are very stable in acidic solutions (pH < 4), their stability progressively decreases as the pH increases from 4 to 8, and catechins become extremely unstable in alkaline solutions above pH 8.\(^{(23)}\) These properties can be employed for stabilising catechins when they are applied in foods.

Catechins have strong antioxidative properties because they contain hydroxyl groups in their structure and thus can scavenge reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite, which play crucial roles in carcinogenesis.\(^{(24)}\) The hydroxyl radical (HO\(^-\)) scavenging ability decreases in the order of ECG, EC, EGCG, and ECG.\(^{(25)}\) In addition, catechins can trap peroxyl radicals, thus suppressing free radical chain reactions and terminating lipid peroxidation.\(^{(26)}\) The effectiveness of the four major catechins in preventing lipid peroxidation decreases in the order of EGCG, ECG, EGC, and EC.\(^{(25)}\) This characteristic is important when using catechins in foods, especially those with high fat or oil content.

### Preparation of Catechins from Green Tea

Tea contains a range of soluble substances such as catechins, caffeine, theanine, chlorophyll, organic acids, and vitamins (Table 3).\(^{(14)}\) Catechins account for a large amount compared to other soluble substances; however, separation from those substances is complicated because they are not significantly different in solubility or molecular size.\(^{(27)}\) Recently, several methods have been developed to isolate catechins from teas for utilisation in nutraceuticals, pharmaceuticals, and cosmetic products.\(^{(27–29)}\) Because green tea contains a higher level of catechins compared to other teas such as black tea or oolong tea,\(^{(1)}\) most catechins have been isolated from green tea in the form of crude tea extracts, catechin mixtures, and individual catechins (Fig. 3). The term *crude tea extracts* refers to extracts containing not only catechins but also other tea components such as caffeine and theanine; *catechin mixture extracts* contain high-purity catechin mixtures, and *individual catechin extracts* contain a high concentration of either EGCG, ECG, EGC, or EC.

### Table 3

<table>
<thead>
<tr>
<th>Components</th>
<th>% of Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechins</td>
<td>30</td>
</tr>
<tr>
<td>Theanine</td>
<td>3</td>
</tr>
<tr>
<td>Amino acids</td>
<td>4</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>11</td>
</tr>
<tr>
<td>Proteins</td>
<td>15</td>
</tr>
<tr>
<td>Organic acids</td>
<td>2</td>
</tr>
<tr>
<td>Lipids</td>
<td>3</td>
</tr>
<tr>
<td>Minerals</td>
<td>10</td>
</tr>
<tr>
<td>Chlorophyll and other pigments</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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Preparation of Crude Tea Extracts

Crude tea extracts have been prepared from green tea through three main steps: extracting soluble substances from tea leaf into tea infusions, concentrating the tea solution, and finally drying it to obtain the products in powder form. Kumar and Rajapaksha\(^{(30)}\) developed a process to produce a crude tea extract from tea by first extracting tea with boiling aqueous ethanol (70% v/v) for 5 minutes and then concentrating the solution on a rotavapor at 40°C before partitioning it with an equal volume of light petroleum. The aqueous phase was further partitioned with ethyl acetate to form an upper layer, which was then concentrated on a rotavapor (<40°C) and finally freeze-dried to obtain a powdered crude tea extract. The drawback to this extraction process is that petroleum and ethyl acetate were used and their residue could affect the health of consumers. However, this process is easy to scale up for industrial production.

In another method, Li et al.\(^{(31)}\) produced crude tea extracts from green tea leaf by using an ultrafiltration membrane. Tea leaf was first ground and extracted by hot water and then centrifuged to remove extracted leaf. This step was followed by filtration with a cellulose acetate–titanium composite ultrafiltration membrane to obtain an ultrafiltrate, which after drying contained 40% (w/w) total catechins. The ultrafiltrate was then loaded onto a column filled with a resin and eluted with a mixture of ethanol : ethyl acetate : water (3:1:1, v/v/v) at a flow rate of 1.75 mL/min to collect an extract, which after drying contained...
90% (w/w) total catechins with less than 4% (w/w) caffeine. This method produced a tea extract with a high purity of catechins; however, the membrane filtration system is difficult and expensive to scale up to industrial production.

Bazinet et al. (32) also produced a crude extract using a two-step extraction procedure. Tea was first brewed in water at 50°C for 10 minutes. The extracted leaf was then separated and re-extracted with water at 80°C for 10 minutes. The infusion from each step was finally freeze-dried for 24 hours to obtain the tea extract in powder form. The extract from the first step was rich in EGC (78.9% w/w of total catechins), and the extract of the second step was rich in EGCG (47.6% w/w of total catechins). The purity of catechins obtained from this method was lower compared to the method of Li et al. (33); however, this method uses water for the extraction process rather than potentially harmful organic solvents like ethyl acetate.

Because the resulting crude tea extracts still contain other tea compounds such as caffeine and chlorophyll, they might cause challenges when they are used in foods. For example, crude tea extracts appear pale yellow in colour, and this may affect the appearance of the foods they are used in. (30) In addition, the caffeine remaining in the extracts may contribute a bitter taste to food products. Caffeine can also react with the catechins through π-π interactions to form cream or haze when the extracts are redissolved in hot water and cooled to low temperatures. (16,33)

Preparation of Catechin Mixtures

Because tea leaf has a high content of caffeine (up to 5% of dry weight), which is even more than in the coffee bean (1.5% of dry weight), (11) the most important step during the preparation of catechin mixtures is to eliminate caffeine. Several methods have been used to do this, including organic solvent extraction, synthetic resin absorption, and supercritical fluid extraction.

Copeland et al. (34) prepared a catechin mixture by first decaffeinating the tea infusion using chloroform and then re-adding caffeine to precipitate the catechins. The caffeine was then removed from the mixture using chloroform. The mixture was further partitioned with ethyl hexanoate and propyl acetate and finally freeze-dried to obtain a catechin mixture that was rich in EGCG. Jin et al. (35) also produced a mixture of catechins by using chloroform to eliminate caffeine from the tea solution. The catechins were then isolated using ethyl acetate before evaporating the solvent to obtain a catechin mixture extract free of caffeine. Recently, Dong et al. (36) prepared catechins using ethyl acetate to extract the catechins and washed the ethyl acetate phase with a citric acid solution to remove 79% of the caffeine and obtained a product containing 69% (w/w) catechins and 4% (w/w) caffeine.

Generally, organic solvents such as chloroform, ethyl acetate, ethyl hexanoate, and propyl acetate are very effective for the extraction of catechins and decaffeination, but they can affect human health if residues are left in the final product; consequently, products prepared with organic solvents may not be accepted by consumers. (28,37) Furthermore, although organic solvent extraction processes are able to efficiently scale up for industrial production, the use of organic solvents presents serious occupational health and safety issues due to the volatility and flammability of the solvents and poses difficult disposal and environmental challenges.

Synthetic resin absorbents such as macroporous polymeric adsorbents and polyamides have also been used to prepare green tea catechins. Zhao et al. (38) used N-vinyl-2-pyrrolidinone, ethylene glycol dimethacrylate, and triallyl isocyanurate to selectively adsorb catechins from mixtures containing caffeine and then used ethanol to wash the resin to obtain a mixture that contained 98% catechins and 2% caffeine. Lu et al. (39) used
a column packed with polyacrylamide-co-ethylene glycol dimethacrylate to separate the catechins and applied ethanol (80%) for washing the column to collect catechins. In this method, the recovery of EGC, EC, EGCG, and ECG in the eluting mixture was 92, 97, 96, and 96% (w/w), respectively, whereas only 1.5% (w/w) of the caffeine remained. In another method, Bailey et al.\(^{(40)}\) prepared catechins from green tea using a polyamide CC6 column. The tea infusion was loaded onto the column, which was first washed with water to elute the caffeine and then washed with ethanol (95%) to obtain a mixture of catechins (70% catechins including 30% EGCG with less than 1% of caffeine).

The use of synthetic resin absorbents to prepare catechins can help avoid the utilization of undesirable organic solvents such as chloroform, and catechin mixtures of higher purity can also be produced. The methods are also applicable on an industrial scale and do not suffer as much from occupational health and safety issues (OH&S) and difficult environmental challenges compared to organic solvent extraction processes. Ethanol is often used and is safer than organic solvents such as chloroform; it is classified as Class 3 by the U.S. Food and Drug Administration (FDA) and therefore is more acceptable as a small residue in foods.\(^{(41)}\) However, it still has some OH&S issues because in pure form it is somewhat flammable and toxic and can be an environmental waste problem. The scale-up of the absorbent methods can also be expensive.\(^{(28)}\)

Supercritical fluid extraction with carbon dioxide (SFE-CO\(_2\)) is an alternative method for removing caffeine to produce catechin mixtures.\(^{(42)}\) The main advantage of this method is that it combines the characteristics of gases and liquids for extraction; CO\(_2\) can diffuse through solids like a gas and dissolve materials like a liquid. The process is very fast because of the low viscosity and high diffusion rate associated with the gas phase of the solvent substance.\(^{(43)}\) However, the temperature, pressure, and other solvents must also be well controlled and optimised to obtain high extraction efficiencies for caffeine while leaving the remaining constituents in the mixture.\(^{(28)}\) Park et al.\(^{(44)}\) found that temperature, pressure, and cosolvents had a significant impact on the extraction of catechins during the removal of caffeine from green tea using SFE-CO\(_2\). They removed over 97% of the caffeine by extraction with 95% (v/v) ethanol at 7 g of tea per 100 g of CO\(_2\) at 300 bar and 70\(^\circ\)C for 120 minutes. However, this treatment also had the unwanted effect of extracting over 37% of the EGCG from the tea along with most of the caffeine.

Supercritical fluid extraction is appropriate for large-scale production. However, high extraction efficiencies for the catechins are not always possible\(^{(44)}\) and it is relatively costly to set up the system.\(^{(28)}\)

### Isolation of Individual Catechins

Isolation of individual catechins from their mixture is a challenge because their structures and molecular weights are very similar.\(^{(27)}\) Countercurrent chromatography and column chromatography have been used to isolate individual catechins from their mixtures. Countercurrent chromatography has been done using two different solvent mixtures flowing in opposite directions, and column chromatography includes a number of solid support phases that include silica gel, alumina, and Sephadex.\(^{(45)}\) Based on the different affinities of the individual catechins for the column material, they are separated by differentially eluting the catechins with various solvents.

Amarowicz and Shahidi\(^{(46)}\) separated individual catechins from green tea by first producing catechin mixtures by countercurrent chromatography with water : chloroform (1:1, v/v) and water : ethyl acetate (1:1, v/v) as the solvent systems and then isolated the individual catechins using Sephadex LH-20 eluted with ethanol. Cao et al.\(^{(47)}\) separated the
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individual catechins EGCG, GCG, and ECG from green tea by using countercurrent chromatography with two solvent mixtures, (1) ethyl acetate : ethanol : water and (2) hexane : ethyl acetate : water. In another study, Kang et al.\(^{(48)}\) isolated EGCG from green tea by initially eliminating caffeine with chloroform and then using two preparative C18 high-pressure liquid chromatography (HPLC) columns eluted with solvent containing acetic acid : acetonitrile : water to obtain EGCG with a purity of more than 98%.

In general, extraction and purification methods have successfully been used to produce individual catechin preparations of high purity. These methods can be scaled up for commercial production, but because they require specialised equipment, they can be costly to set up. Furthermore, because of their health, safety, and environmental issues, the use of organic solvents such as chloroform, hexane, and ethyl acetate need to be minimised. Therefore, the use of safer alternative solvents and methods need to be further examined. Water is obviously the safest solvent and should be the solvent of choice. However, water is unfortunately less efficient at extracting catechins and less useful for further processing of the extracts.\(^{(49)}\)

In conclusion, several methods have been developed for isolating catechins from green tea. However, unsafe organic solvents have been used during the isolation processes, and residues may remain in the extracts, which will not be acceptable for consumers. Therefore, safe alternative solvents need to be further examined for application in the isolation processes. In addition, the cost-effectiveness of the isolation processes needs to be monitored to minimise the cost of tea catechins and ensure their wider utilisation in the food industry.

Challenges of the Utilisation of Catechins in Foods

As discussed previously, addition of catechins in food has the potential to improve the shelflife of food products and supply antioxidants to consumers.\(^{(29)}\) Generally, higher concentrations of catechins have more potency for the prevention of lipid oxidation in foods\(^{(50)}\); however, catechins possess a very astringent and bitter taste.\(^{(14)}\) Therefore, adding a high concentration of catechins could make the taste of foods bitter and astringent, and this may not be accepted by consumers because recent studies have shown that a bitter taste is one of the main reasons for the rejection of many food products.\(^{(51)}\) Thus, it is a challenge to identify the balance between the dose of catechins added and the taste of the fortified products in order to obtain both consumer acceptability and the benefits from the bioactivities of the catechins.

The stability of catechins in food is also an issue. Catechins fortified in foods can be lost due to degradation and epimerisation during thermal processing\(^{(52)}\); catechins may be degraded to form phloroglucinol carboxylic acid and protocatechuic acid\(^{(53)}\) (Fig. 4), and epestructured catechins can be epimerised to non epestructured catechins at high temperatures\(^{(54)}\) (Fig. 5). Wang et al.\(^{(52)}\) studied the stability of EGCG used in the fortification of bread and found that EGCG underwent thermal degradation and epimerisation simultaneously during bread baking. Chen et al.\(^{(55)}\) examined the stability of catechins in tea drinks under various processing conditions and found that catechins were degraded by 20% when heated at 98°C for 7 hours. Therefore, precautions are necessary when catechins are added to foods that are then thermally processed or cooked.

Catechins have also been found to be very unstable in alkaline solutions.\(^{(23)}\) Thus, application of catechins to some foods, especially foods in liquid form, having high pH values could lead to degradation of the catechins during processing and storage. In order to maintain the catechins in these foods, acids may have to be added to reduce their pH.
Figure 4. Degradation of catechins.\(^\text{[106]}\)

Figure 5. Epimerisation of epistructured catechins to respective nonepistructured catechins.

\[
\begin{align*}
\text{Epistructured catechins} & \quad \text{Non-epistructured catechins} \\
\text{EGCG: } R1=\text{OH, } R2=\text{X} & \quad \text{GCG: } R1=\text{OH, } R2=\text{X} \\
\text{EGC: } R1=\text{OH, } R2=\text{H} & \quad \text{GC: } R1=\text{OH, } R2=\text{H} \\
\text{ECG: } R1=\text{H, } R2=\text{X} & \quad \text{CG: } R1=\text{H, } R2=\text{X} \\
\text{EC: } R1=R2=\text{H} & \quad \text{C: } R1=R2=\text{H}
\end{align*}
\]

However, it should be noted that different acids have different impacts on the stability of catechins as well. For instance, addition of ascorbic acid was found to significantly increase the stability of catechins, whereas the addition of citric acid only had a minor effect.\(^\text{[56]}\) Another challenge therefore is to identify suitable acids for the stabilisation of catechins in foods.

A different challenge is encountered when using catechins in beverages that are rich in proteins, because catechins can bind with proteins, including several enzymes, to form sediments that make the solution look hazy.\(^\text{[17]}\) In this case, the precipitation can be minimised by pH adjustment because precipitation does not occur at low or high pH.\(^\text{[18]}\) For example, precipitation of the enzyme soybean lipoxygenase occurs mostly in the pH range from 4 to 7 and not when the pH is less than 3 or more than 8.\(^\text{[57]}\) In addition, catechins can bind with iron and inhibit iron absorption in the body. This factor needs to be considered when using catechins in foods that are rich in iron, such as cocoa, liver, kidney, dried fish, sesame seed, and shellfish.\(^\text{[19]}\)
Utilisation of Tea Catechins in the Food Industry

In the food industry, lipid oxidation is a major problem because it causes the development of undesirable rancidity and potentially toxic reaction products. Meats and meat products usually have a high lipid content, ranging from 4.5 to 11%, and thus they are susceptible to lipid oxidation. Fish is even more susceptible to lipid oxidation than meat and meat products because fats in fish tissues are composed of highly unsaturated fatty acids. To prevent lipid oxidation, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ) have been commonly used as preservatives in the food industry because they are inexpensive and effective at inhibiting lipid oxidation. However, these synthetic antioxidants have been found to be potentially toxic at high concentrations and, thus, natural antioxidants have been sought as alternatives for the prevention of lipid peroxidation.

Tea extracts containing catechins have been found to have a high potency for the prevention of lipid peroxidation in foods. Catechins, especially EGCG, are more than 20 times more effective at preventing lipid peroxidation than \(\alpha\)-tocopherol (vitamin E) and more than 4 times more effective than BHA. A mechanism by which catechins can prevent lipid peroxidation involves the catechins' ability to scavenge free radicals. This scavenging reaction of catechins allows them to trap superoxide anions or hydroxyl radicals and thus suppress and terminate the free radical chain reaction that occurs during lipid peroxidation.

Another mechanism by which catechins can inhibit lipid oxidation involves their metal ion-chelating properties. Catechins have been found to effectively chelate iron and copper ions, both of which are catalysts for the initiation and propagation of the lipid peroxidation chain reaction.

In addition, catechins can prevent the growth of bacteria; for example, crude catechin extracts have been found to inhibit the growth of certain pathogenic species of Clostridium and Bacillus. The catechins EGCG and GCG were found to be the most effective in preventing the growth of bacteria such as C. botulinum, C. butyricum, B. cereus, and Escherichia coli O157:H7.

Catechins also have a synergistic effect with other antioxidants such as vitamin E, vitamin C, trolox, and some organic acids such as citric, malic, uric, and tartaric acid and they can protect against the deterioration of \(\beta\)-carotene, a vitamin A precursor used as food colorant. Furthermore, catechins can inhibit the formation of mutagens, which can be formed during the broiling or frying of meats and can cause an increase in the risk of cancers such as breast and colon cancers.

Therefore, there is substantial scope for catechins to be used as potential antioxidant, antibacterial, and antimutagen ingredients in the food industry. In fact, catechins have recently been effectively used in a number of foods to improve quality and prolong shelflife.

Utilisation of Tea Catechins in Meat and Meat Products

The meat of each species has different levels of fatty acids and iron; thus, its susceptibility to lipid oxidation also varies. For example, beef has been found to be the most susceptible to lipid oxidation; it is followed in decreasing order of susceptibility by duck, ostrich, pork, and chicken. The green tea catechins have been found to effectively prevent lipid oxidation when they were applied to some meats and meat products. Application of tea catechins (300 mg/kg) in minced muscle of fresh red meat (beef and pork) and poultry (chicken, duck, and ostrich) significantly \((P < 0.05)\) reduced lipid oxidation for all species.
Table 4
Utilisation of tea extracts in foods

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Foods</th>
<th>Effects</th>
<th>Refs.</th>
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</thead>
<tbody>
<tr>
<td><strong>Crude tea extracts</strong></td>
<td></td>
<td>--------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cooked ground beef, chicken and pork</td>
<td>Inhibit growth of <em>Clostridium perfringens</em> spores</td>
<td>(90)</td>
<td></td>
</tr>
<tr>
<td>Sun flower oil and lard</td>
<td>Prevent lipid oxidation</td>
<td></td>
<td>(91)</td>
</tr>
<tr>
<td>Turkish dry-fermented sausage</td>
<td>Reduce putrescine formation</td>
<td></td>
<td>(92)</td>
</tr>
<tr>
<td>White pollack liver oil</td>
<td>Inhibit lipid oxidation</td>
<td></td>
<td>(93)</td>
</tr>
<tr>
<td>Bonito fillets</td>
<td>Improve quality</td>
<td></td>
<td>(94)</td>
</tr>
<tr>
<td>Fresh-cut lettuce</td>
<td>Maintain ascorbic acid and carotenoid; prolong the shelflife</td>
<td></td>
<td>(95)</td>
</tr>
<tr>
<td>Fresh pork sausages</td>
<td>Inhibit lipid oxidation and improve flavour</td>
<td></td>
<td>(72)</td>
</tr>
<tr>
<td>Raw and cooked pork patties</td>
<td>Inhibit lipid oxidation and improve colour</td>
<td></td>
<td>(96)</td>
</tr>
<tr>
<td>Fish oil, soybean oil, and lard for frying noodles</td>
<td>Retard lipid oxidation</td>
<td></td>
<td>(77)</td>
</tr>
<tr>
<td>Blue sprat</td>
<td>Retard lipid oxidation</td>
<td></td>
<td>(97)</td>
</tr>
<tr>
<td>Bread</td>
<td>Provide antioxidants</td>
<td></td>
<td>(85)</td>
</tr>
<tr>
<td><strong>Catechin mixtures</strong></td>
<td></td>
<td>--------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cooked red meat, poultry and fish patties</td>
<td>Inhibit oxidation</td>
<td></td>
<td>(58)</td>
</tr>
<tr>
<td>Seal blubber oil and menhaden oil</td>
<td>Inhibit lipid oxidation</td>
<td></td>
<td>(98)</td>
</tr>
<tr>
<td>Fresh and frozen pork patties</td>
<td>Retard lipid oxidation</td>
<td></td>
<td>(99)</td>
</tr>
<tr>
<td>Sponge cakes</td>
<td>Greater antioxidant properties</td>
<td></td>
<td>(100)</td>
</tr>
<tr>
<td>Beef, pork, chicken, duck, ostrich, whiting, and mackerel</td>
<td>Reduce lipid oxidation</td>
<td></td>
<td>(70)</td>
</tr>
<tr>
<td>Beef and chicken</td>
<td>Inhibit lipid oxidation and cause discoloration</td>
<td></td>
<td>(71)</td>
</tr>
<tr>
<td>Minced beef patties</td>
<td>Inhibit lipid oxidation and improve colour retention</td>
<td></td>
<td>(73)</td>
</tr>
<tr>
<td>Raw sulphite beef</td>
<td>Inhibit lipid oxidation, improve colour retention, and delay onset of rancid flavour</td>
<td></td>
<td>(74)</td>
</tr>
<tr>
<td>Salmon fillets and dried horse mackerel</td>
<td>Extend the shelflife and improve flavour</td>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>Cooked mackerel</td>
<td>Prevent lipid oxidation</td>
<td></td>
<td>(79)</td>
</tr>
<tr>
<td>Silver carp</td>
<td>Delay spoilage</td>
<td></td>
<td>(78)</td>
</tr>
<tr>
<td>Canola oil</td>
<td>Inhibit oxidation</td>
<td></td>
<td>(83)</td>
</tr>
<tr>
<td>Rice starch</td>
<td>Retard retrogradation</td>
<td></td>
<td>(84)</td>
</tr>
<tr>
<td><strong>Individual catechins</strong></td>
<td></td>
<td>--------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>Inhibit metalloproteinases</td>
<td></td>
<td>(81)</td>
</tr>
<tr>
<td>White shrimp (<em>Litopenaeus vannamei</em>)</td>
<td>Retard melanosis, lipid oxidation, and bacterial growth</td>
<td></td>
<td>(101)</td>
</tr>
</tbody>
</table>

(Continued)
Isolation of Green Tea Catechins

Table 4
(Continued)

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Foods</th>
<th>Effects</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken breast meat</td>
<td>Inhibit lipid oxidation</td>
<td>(102)</td>
<td></td>
</tr>
<tr>
<td>Menhaden oil-in-water emulsion</td>
<td>Inhibit lipid oxidation</td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>and mackerel mince</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edible <em>Gelidium corneum</em> used</td>
<td>Inhibit the growth of *Escherichia</td>
<td>(104)</td>
<td></td>
</tr>
<tr>
<td>for packing sausage</td>
<td><em>coli</em> and <em>Listeria monocytogenes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Big-eye snapper surimi</td>
<td>Increase the gel strength</td>
<td>(105)</td>
<td></td>
</tr>
<tr>
<td>Apple juice</td>
<td>Antibacterial</td>
<td>(86)</td>
<td></td>
</tr>
</tbody>
</table>

meats during refrigerated storage. Similarly, utilisation of catechins (200 or 400 mg/kg) also significantly \( (P < 0.01) \) inhibited lipid oxidation in cooked or raw beef patties packaged under modified atmosphere packaging (MAP) conditions \( (80\% \text{O}_2:20\% \text{CO}_2) \) and stored refrigerated for 7 days at \( 4^\circ\text{C} \).\(^{71}\)

In addition, application of catechins (200 mg/kg) was found to significantly prevent lipid oxidation of fresh pork sausages packed in an atmosphere of \( 80\% \text{O}_2 \) and \( 20\% \text{CO}_2 \) and stored at \( 2^\circ\text{C} \) in the dark for 20 days.\(^{72}\) Another study also revealed that fortification with catechins (200 mg/kg) minimised lipid oxidation of minced beef patties packed under aerobic MAP conditions with \( 80\% \text{O}_2 \) and \( 20\% \text{CO}_2 \) during refrigerated storage.\(^{73}\) Similar results were reported when catechins (300 mg/kg) were added to raw sulphite beef patties packed under aerobic condition during refrigerated storage.\(^{74}\)

Therefore, green tea catechins have been shown to be effective in their ability to prevent lipid oxidation in many meats and meat products. The catechins are thought to minimise the lipid oxidation in meats by chelating iron, which is the major active catalyst for oxidative rancidity in meat.\(^{58}\) However, the ability of the catechins to trap superoxide, hydroxyl, and peroxyl radicals and suppress free radical chain reactions undoubtedly also plays an important role in preventing and terminating lipid oxidation in meat products.\(^{24}\)

Catechins have also been found to have an impact on the colour of meat and meat products kept under different storage conditions. However, contradicting results have been presented. Several studies have reported that the addition of catechins did not prevent the discolouration of meat and meat products. For example, Martínez \textit{et al.}\(^{72}\) found that addition of catechins (200 mg/kg) did not prevent colour loss in fresh pork sausages packed in an atmosphere of \( 80\% \text{O}_2 \) and \( 20\% \text{CO}_2 \) and stored at \( 2^\circ\text{C} \) in the dark for 20 days. Similarly, Mitsumoto \textit{et al.}\(^{71}\) revealed that catechin addition (200 or 400 mg/kg) did not prevent, and may have caused, discolouration of cooked beef and chicken meat patties held in a refrigerated \( (4^\circ\text{C}) \) display cabinet under MAP conditions \( (80\% \text{O}_2:20\% \text{CO}_2) \) for 7 days.

However, other studies have found that catechins could protect or improve the colour of meat and meat products. Tang \textit{et al.}\(^{73}\) reported that the addition of catechins (200 mg/kg) improved the colour stability of minced beef patties packed under aerobic MAP condition, with \( 80\% \text{O}_2 \) and \( 20\% \text{CO}_2 \) during refrigerated storage for 7 days. The use of catechins (300 mg/kg) in combination with sulphite has also been found to delay the discolouration of raw sulphite beef patties packed under aerobic condition during refrigerated storage for 9 days.\(^{74}\) Similar results have also been reported for the addition of catechins at 0.1% (w/w) in pork patties packaged in oxygen-permeable polyethylene and stored for 15 days at \( 4^\circ\text{C} \).\(^{96}\) Finally, a recent study has indicated that the stabilisation of
meat colour is more effective when a mixture of catechins (3–19%), carnosine (78–94%), and α-tocopherol (0–12%) is used. (75)

The reasons for the contradictory findings on the effect of catechins on meat colour remain unclear. Mitsumoto et al. (71) proposed that the added catechins might bind with the iron component of meat muscle myoglobin and thus cause the discolouration of the cooked beef and chicken meat patties during refrigerated storage. On the other hand, Tang et al. (73) assumed that the addition of catechins delayed discolouration in minced meat patties during refrigerated storage because they maintained oxymyoglobin, which imparts a bright red colour to meats and meat products, and delayed the formation of metmyoglobin, which presents as dull and brown in colour. Thus, further studies are needed to identify the mechanisms by which catechins have an effect on colour retention in meats and meat products. Differences in the various experiments, such as the meats used, the methods and conditions used to add the catechins to the meats and meat products, and the techniques used to evaluate the retention of colour, also need to be investigated.

Notwithstanding their variable effects on colouration, catechins have also been found to improve the flavour of various meat and meat products. For example, the addition of 0.2% (w/w) catechins in hamburgers was found to effectively improve their characteristic inferior sensory flavour when cooked. (5) Similarly, utilisation of catechins (200 mg/kg) in fresh pork sausage significantly inhibited its off-odour formation during storage. (72) Finally, fortifying pork patties and cooked beef patties with catechins added at 0.1% (w/w) and 300 mg/kg, respectively, significantly delayed the onset of rancid flavours during storage. (74, 96) The potency of the catechins at protecting the flavour of the meats in these studies is most likely explained by their strong antioxidant properties preventing the development of rancid and undesirable flavours that can be caused by lipid peroxidation during cooking or storage. (5, 74)

The previous findings have revealed that catechins have a high potential for use with many kinds of meats and meat products to improve their quality and prolong their shelflife and to provide additional antioxidants to consumers. However, future studies need to consider the stability of the catechins added to these foods during processing, storage, and cooking. Caution is also needed when using catechins in meats and meat products rich in iron (59) because catechins can bind with iron to reduce its absorption in the body. (19)

Utilisation of Tea Catechins in Fish and Fish Products

In fish and fish products, oxidative deterioration is one of the major causes leading to quality degradation and off-flavour development. (76) The green tea catechins have been added to various fish and fish products to prevent oxidative deterioration (Table 4). For example, when applied in salmon fillets at a concentration of 0.5% (w/w), a commercial catechin preparation was found to extend the shelflife of the fillets from 3 days to one week. (5) This product was also applied to dried horse mackerel and it was found to significantly lengthen the retention of flavour compared to untreated control samples. (5)

When the catechins were added at varying concentrations in other fish and fish products such as silver carp at 0.2% (w/w), whiting, and mackerel patties at 300 mg/kg and fish oil at more than 250 ppm, lipid peroxidation was significantly delayed in these fish and fish products during storage. (70, 77, 78) The potency of the catechins in preventing lipid peroxidation was also higher than for tocopherol, BHA, BHT, and TBHQ. (77, 79)

Fish and fish products are very susceptible to lipid peroxidation because they have a high content of polyunsaturated fatty acids, which are responsible for the initial development of oxidation as initiated by the enzymes lipoxygenase and peroxidase. (80)
Furthermore, proteinases such as serine proteinase, metalloproteinase, and cysteine proteinase, as well as bacteria such as \textit{Vibrionaceae}, \textit{Pseudomonas}, \textit{Shewanella}, and \textit{Photobacterium}, also deteriorate the flavour, texture, and taste of fish and fish products during storage.\textsuperscript{(81,82)} Catechins, especially EGCG, have been found to effectively inhibit lipoxygenase, peroxidase, proteinases, and bacterial growth; therefore, they have a high potency for inhibiting lipid peroxidation and protecting the flavour of fish and fish products.\textsuperscript{(80–82)}

Therefore, catechins have a high potential for application in a wide range of fish and fish products in order to enhance the shelflife and health benefits of these food products. However, future studies need to consider the stability of the added catechins during the thermal processing and cooking of fish and fish products.

\textbf{Utilisation of Tea Catechins in Plant Food Products}

Recently, green tea catechins have been added to some plant food products such as vegetable oils, cakes, starch, crackers, bread, and juice to enhance the shelflife and health benefits of these products\textsuperscript{(29)} (Table 4). Chen and Chan\textsuperscript{(83)} applied catechins at a level of 200 ppm in canola oil and found that the catechins significantly prevented lipid oxidation. This study also found that the stability of the catechins in oil was significantly greater than that of BHT when the oil was heated at 95°C. Similarly, Koketsu and Satoh\textsuperscript{(77)} added tea catechins at levels of 40 and 60 ppm in soybean oil and found that the catechins exhibited antioxidative activity, whereas addition of tocopherol at a level of 200 ppm did not result in any antioxidative activity in the soybean oil.

Wang \textit{et al.}\textsuperscript{(29)} reported that the addition of catechins in traditional Chinese cakes effectively prolonged the shelflife and improved the flavour of the cakes. Koketsu and Satoh\textsuperscript{(77)} determined the impact of adding green tea catechins to lard used for frying noodles on the oxidative stability of the resulting fried noodles. The authors reported that the catechins added to the lard significantly improved the oxidative stability of the fried noodles. In addition, Wu \textit{et al.}\textsuperscript{(84)} found that catechins significantly retarded the retrogradation of rice starch and concluded that catechins could be potentially used in many rice products to enhance both their quality and nutrition. Wang and Zhou\textsuperscript{(85)} used green tea catechins in breads and found that the catechins were stable dough for up to 9 weeks under frozen storage conditions at $-20^\circ$C, although there was a 10–20% loss during baking. In addition, catechins have been added to apple juice to effectively protect it from spoilage by bacteria like \textit{Escherichia coli} 0157:H7 and \textit{Salmonella typhimurium.}\textsuperscript{(86)}

In conclusion, tea catechins can potentially be used in a wide range of plant food products to prolong their shelflife and to provide antioxidant supplementation to consumers. However, it should be noted that the catechins may be lost under various conditions such as baking, cooking, frying, or microwaving. In addition, the catechins can be degraded if they are added to plant products that contain high levels of PPO and POD because these enzymes oxidise them to form theaflavins and thearubegins, a reaction related to the browning of apples and potatoes when they are cut.\textsuperscript{(1)}

\textbf{Conclusion and Future Considerations}

Tea catechins have been found to be associated with health benefits, and utilisation of tea catechins in foods is an alternative way to promote human health. Tea catechins have been isolated from tea in the forms of crude extracts, catechin mixtures, and individual catechins.
Ultrafiltration membranes, column chromatography (including preparative HPLC), and high-speed countercurrent chromatography have great potential for the isolation of catechins from green tea. However, unsafe chemicals such as chloroform, ethyl acetate, and hexane are used in these processes, often to remove caffeine. Thus, there is the potential for chemical residues to remain in the tea extracts and adversely affect human health. At the very least, consumers will be wary of products prepared using such unsafe chemicals.

To avoid organic chemical residues, water should be the extraction solvent of choice for isolating catechins from green tea. However, because water has often been found to be less efficient, the yield and the purity of products may need to be improved for its use to be commercially viable. Optimisation of the extraction conditions, such as temperature, length of extraction time, tea particle size, ratio of tea to water, and extraction-assisting methods (microwave, ultrasound, or continuous extraction methods), needs to be fully investigated in order to address the deficiencies of water as a solvent. The cost-effectiveness of the production processes using water should also be monitored to minimise the cost of the catechin extracts.

Supercritical fluid extraction is also worth investigating further because it makes use of liquid CO2 as the extraction solvent and the risk of having residual chemicals in the extract is alleviated. Alternatively, methods using synthetic resin adsorbents, which make use of water and ethanol as the main solvents, could also be acceptable. Although ethanol has safety issues, it is considerably safer to use than other organic solvents such as chloroform, ethyl acetate, and hexane. However, the cost of scaling up these systems for industrial production needs to be well evaluated for commercial viability to be ensured.

The use of catechins in foods also has its challenges; heat, enzymes, and high pH need to be well controlled because catechins can be unstable under certain conditions. Furthermore, to improve the effective utilisation of catechins in the food industry, future studies need to address the balance between the dose of catechins and the taste of the final products and the tendency of catechins to bind iron and proteins when they are added to protein- or iron-rich foods.

Nonetheless, tea catechins offer considerable promise as food additives. They have been found to be very effective at preventing lipid peroxidation, growth of bacteria, and formation of mutagens. In addition, tea catechins can act synergistically with vitamin E, vitamin C, and some organic acids such as citric, malic, and tartaric acid. Hence, addition of catechins can prolong the shelflife and improve the colour and flavour of foods. Therefore, tea catechins are an exciting novel potential additive for the food industry.

Acknowledgment

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References


1.3.3. Extraction and isolation of catechins from tea

Quan V. Vuong, John B. Golding, Minh H. Nguyen and Paul D. Roach

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Review

Extraction and isolation of catechins from tea

Tea is a major source of catechins, which have become well known for their antioxidant potential. Numerous human, animal, and in vitro studies have linked tea catechins with prevention of certain types of cancers, reduction of the risks for obesity, diabetes, and cardiovascular disease, and improvement of the immune system. Tea catechins are widely used in various nutraceuticals, pharmaceuticals, and cosmetics for either enhancing product shelf-life or for enhancing human health. Thus, the demand for catechins has increased considerably. Catechins have been extracted and isolated from tea leaves by numerous methods through several steps including: treatment of the tea leaves, extraction of catechins from teas into solvents, isolation of catechins from other extracted components, and drying the preparations to obtain catechin extracts in a powder form. This paper outlines the physical and chemical properties of the tea catechins and reviews the extraction steps of the various extraction methods, as a basis to improve and further develop the extraction and isolation of the tea catechins.

Keywords: Catechins / Extraction / Isolation / Separation / Tea
DOI 10.1002/jssc.201000438

1 Introduction

Tea (Camellia sinensis) is native to the southern regions of China and parts of India, Laos, Thailand, Vietnam, and Myanmar [1]. Tea is said to have first been discovered as a drink and medicine in China around 2737 BC. It was then introduced to Japan during the early 13th century and to Europe in the 16th century, then to America, Africa and other regions of the world [1–3]. Tea is presently cultivated in over 30 countries around the world and the tea beverage is second only to water in terms of worldwide consumption [4].

Based on the oxidation of the polyphenols in the tea leaves during the fermentation process, tea has been classified into three types: green tea, black tea, and oolong tea [4]. Green tea refers to non-fermented tea, in which the oxidation of the tea polyphenols, called catechins, is prevented by quickly heating the leaves after harvest to inactivate the main oxidising enzyme, polyphenol oxidase (PPO), and thus, most of the catechins are preserved during the processing. Black tea refers to fully fermented tea and oolong tea is semi-fermented tea. In these teas, aerobic oxidation of the tea leaf polyphenolics is allowed to occur and the catechins are enzymatically catalysed to form theaflavins and thearubigins. For black tea, the fermentation reaction is promoted to maximize the oxidation of the catechins but for oolong tea, it is usually stopped half-way before completion [1, 4].

Green tea is a rich source of catechins, which account for up to 30% of the leaf dry weight [4]. A typical composition of catechins (polyphenolics) and other components in the green tea is shown in Table 1. As catechins can donate hydrogens from the hydroxyl groups in their structure, they have been found to have excellent antioxidant activities, expressed through their free radical scavenging ability being more powerful than vitamin C, vitamin E, or β-carotene [5–7]. They have also been shown to chelate transition metal ions, modulate oxidant and antioxidant enzymes, and affect gene expression [5].

The catechins are receiving considerable interest for their potential benefits on human health. The recent in vivo and epidemiology studies have linked the green tea catechins with the prevention of some skin and liver cancers [8–11]. Other studies have linked the catechins with a reduced development of lung, gastric, and breast cancers [12–14]. In addition, green tea and its catechins have been linked with reductions in cardiovascular disease, dental decay, obesity, diabetes, and an improvement in the immune system [15–20].

Abbreviations: CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin gallate; ECGC, epigallocatechin gallate; GCG, gallocatechin gallate; MAE, microwave assisted extraction; PPO, polyphenol oxidase; RP-HPLC, reversed-phase high-performance liquid chromatography; SFE-CO₂, supercritical fluid extraction with carbon dioxide; SPE, solid phase extraction; SWE, subcritical water extraction; UAE, ultrasound assisted extraction; UHPE, ultrahigh pressure extraction.
In the food industry, the catechins have proven useful because of their high potency for preventing lipid peroxidation of oil-containing foods; they scavenge free radicals and stop the auto-oxidative degradation of lipids [21]. The catechins, especially epigallocatechin gallate (EGCG), have been found to have more than 20 times the relative potency of vitamin E for preventing lipid peroxidation and more than 4 times the relative potency of butylated hydroxyanisole, which is often added in food to preserve fat or oil [22]. In addition, the catechins can inhibit the formation of mutagens, normally formed during broiling or frying of meats, which have been shown to increase the risk of developing cancers such as breast and colon cancers [23].

In the Asian food industry, the green tea catechins have been used in a wide range of commercial products as functional food ingredients to enhance both the shelf-life of products and to provide added health benefits for consumers [22]. For example, the tea catechins have been added to cereals, cakes, biscuits, ice cream, and other dairy products, confectionary products, instant noodles, fried snacks, sausages, and soft drinks [22, 24, 25].

In the pharmaceutical industry, the tea catechins have been used in toothpastes, mouthwashes, and breath fresheners to improve oral health [25]. In addition, they have been used as supplement tablets or in drinks to enhance consumer health [22]. They have also been added to air filters in “antiinfluenza” masks for protection from airborne viruses [22]. In the cosmetics industry, the tea catechins have been used in various products, such as shampoos, moisturising creams, perfumes, and sunscreens with the aim of providing soothing effects on the skin as well as protecting the skin from free radical damage [25].

### Table 1. Composition of major components in tea leaf [1, 4]

<table>
<thead>
<tr>
<th>Components</th>
<th>% of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>36</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3</td>
</tr>
<tr>
<td>Theanine</td>
<td>1.5</td>
</tr>
<tr>
<td>Protein</td>
<td>15</td>
</tr>
<tr>
<td>Lignin</td>
<td>6.5</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>25</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>0.5</td>
</tr>
<tr>
<td>Organic acids</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

#### 2 Physical and chemical properties of catechins

##### 2.1 Structure of catechins

The catechins have the general structure of C6–C3–C6 with two aromatic rings and several hydroxyl groups (Fig. 1). The catechins are classified into two groups; free catechins and esterified catechins. The free catechins are catechin, gallocatechin, epicatechin (EC), epigallocatechin (EGC), whereas the esterified catechins are EGCG, epicatechin gallate (ECG), gallocatechin gallate (GCG), and catechin gallate (CG). The esterified catechins contribute substantial astringency and a bitter taste, whereas the free catechins are much less astringent and have a slightly sweet taste [22]. Among the catechins, EGCG, ECG, EGC, and EC are the four major catechins found in green tea; EGCG is normally present at the highest concentration, followed by EGC, ECG, and EC [26].

The catechins are synthesised in the leaves of the *C. sinensis* plant through the acetic-malonic acid and shikimic–cinamic acid metabolic pathways. Chalcone and gallic acid are produced from the shikimic acid pathway from which these different catechins are produced [26]. There is some variation in the content of the individual catechins in fresh tea leaf; the composition depends on the location of cultivation, variety, nutrition of the tea plant, time of year, and the type of leaves (coarse or young leaves). A typical catechin profile in an extract from green tea leaves comprises 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC [27].

#### 2.2 Physical properties of tea catechins

Catechins are colourless and are soluble in water and polar organic solvents [1, 6]. However, the solubility of the individual catechins varies and depends on the extraction temperature and duration and on the type of solvent used (Table 2). Labbé et al. [28] showed that the solubilisation of EC and EGC depended on the extraction duration, whereas the solubilisation of EGCG and ECG depended on both extraction duration and temperature. Hu et al. [29] also reported that while the impact of solvent type, extraction duration, and temperature on the solubilisation of EC and ECG was not significant, these factors were important for the solubilisation of EGCG and EGC.
In comparison with other important components in tea such as theanine and caffeine, the solubilisation of catechins is slower because the catechins have a higher molecular mass than theanine (174) and caffeine (194) [30]. However, the melting points of the catechins are similar to the melting points of theanine (218°C) and caffeine (238°C) [30, 32]. The absorption of ultraviolet light by the catechins, EGCG, EGC, ECG, and EC, was found to be maximum at the wavelengths of 210 nm and from 269 to 280 nm [30,33]. This characteristic is used in the identification and quantification of catechins.

### 2.3 Chemical properties of catechins

#### 2.3.1 Stability of catechins

The tea catechins are sensitive to oxidation by enzymes, acid, and heat [4]. In the presence of the enzyme PPO, the catechins are easily oxidised to form theaflavins and thearubigins (Fig. 2). During the manufacture of oolong tea or black tea, PPO is stimulated to activate the oxidation of the catechins; therefore, the content of catechins is lower in black and oolong teas compared to green tea for which PPO is inactivated immediately after harvest [4]. The optimal conditions for PPO activity in fresh tea leaves are at pH 5.5 and at a temperature of 40°C. Therefore, controlling pH and temperature can minimize oxidation of the catechins [35].

The catechins are very stable in acidic solutions (pH less than 4), but their stability progressively decreases as the pH is increased from 4 to 8. In contrast, they are extremely unstable in alkaline solutions (pH above 8) [36]. Therefore, acids are normally added to increase the stability of catechins in solution but the effect of different acids on the stability of catechins in solution varies. For example, the addition of ascorbic acid has been shown to significantly increase the stability of the catechins whereas the addition of citric acid has been shown to have little effect on the stability of the catechins [37].

The normal epistructure of catechins has also been found to be unstable in that the ECs tend to epimerize to non-epistructured catechins at temperatures above 80°C (Fig. 3) [37]. In addition, the catechins have been found to epimerize faster in tap water than in purified water [38]. However, the catechins have been shown to epimerize at a much slower rate in solutions with a pH of less than 6. Thus, EDTA, which gives a pH of 5.5 in water, has been added to decrease the epimerisation of the tea catechins [39]. In addition, the epimerisation of catechins can be minimised by extracting dry tea with 50% v/v ethanol or with water at or below 80°C [40].

#### 2.3.2 Protein interaction

The catechins can react with proteins to form a sediment, which makes the solution look hazy. This process is referred to as “cream” formation [41]. The catechins are also known to interact with enzymes, such as lipoxygenase, α-amylase, pepsin, trypsin, and lipase to form precipitates and consequently inhibit the activity of these enzymes [42]. The catechins that have an ester bond, EGCG and ECG, also have a greater ability to form precipitates with these enzymes than EC and EGC [42].

The precipitation is also influenced by the pH of the solution. Studies have shown that precipitation of the soybean lipoxygenase occurs mostly in the pH range from 4 to 7 and not when the pH is less than 3 or more than 8 [42, 43]. The use of proteins to form precipitates can be of some practical use to isolate the catechins from tea infusions.

#### 2.3.3 Caffeine interaction

Catechins also interact with caffeine to form a “cream” or “haze” but this precipitate is only obvious when the tea

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**Table 2. Physical characteristics of the major catechins**

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>EGC</th>
<th>ECG</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol. formula</td>
<td>C_{15}H_{14}O_{6}</td>
<td>C_{15}H_{14}O_{7}</td>
<td>C_{22}H_{18}O_{10}</td>
<td>C_{22}H_{18}O_{11}</td>
</tr>
<tr>
<td>Mol. weight</td>
<td>290</td>
<td>306</td>
<td>442</td>
<td>458</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>280</td>
<td>269</td>
<td>280</td>
<td>273</td>
</tr>
<tr>
<td>Solubilization</td>
<td>Time dependence</td>
<td>Time/solvent dependence</td>
<td>Time/temperature dependence</td>
<td>Time/temperature/solvent dependence</td>
</tr>
<tr>
<td>Taste</td>
<td>Bitter with a sweet after taste</td>
<td>Bitter with a sweet after taste</td>
<td>Bitter and astringent</td>
<td>Bitter and astringent</td>
</tr>
</tbody>
</table>

The abbreviations are EC: epicatechin; EGC: epigallocatechin; ECG: epicatechin gallate; EGCG: epigallocatechin gallate.
infusion is cooled down to temperatures below 10°C [44]. The driving force for the formation of this precipitate is thought to be mainly π–π interactions between the A, B’ rings of the catechins and the six-membered ring of caffeine (Fig. 4) [45]. However, it has been shown that the interaction of the different individual catechins with caffeine also varies. The gallated catechins, EGCG, and ECG, have a stronger caffeine-creaming ability than the un gallated catechins, EC, and EGC [46].

The formation of cream in tea depends on the brewing temperature, the pH of the solution and the presence of minerals such as calcium, magnesium, and aluminium. For example, cream formation from tea brewed at 50°C is lower than from tea brewed at 90°C [46]. Cream formation from tea brewed at high pH is also lower than from tea brewed at low pH. Furthermore, the cream tends to dissolve and the colour of the tea darkens when the pH of tea with this cream is raised to make it alkaline. However, this phenomenon is most likely not directly related to the formation of catechin–caffeine complexes. It can be better explained by the ability of low pH in either releasing more solids from the leaves into the infusion or stimulating the catechins to interact with polysaccharides and nucleophilic groups on proteins in the tea infusions [46, 47]. Furthermore, the addition of calcium has been found to strongly promote the formation of creaming and this is most likely due to its neutralising effect on the charges that stabilize small colloidal particles [48].

3 Identification and quantification of the tea catechins

There are several methods for identifying and quantifying the tea catechins [6, 49]. These methods can be employed for determining the concentration, the yield, and purity of catechins in final products. The identification and quantification of catechins has been largely facilitated by chromatographic techniques, such as HPLC and CE using various detectors such as UV, electrochemical, and MS detectors to analyse the individual catechins [6]. Alternatively, near-infrared reflectance spectroscopy, high-speed countercurrent chromatography, TLC, and GC have also been used to identify and quantify the catechins [49, 50].

HPLC is currently the most common method employed for determination of the tea catechins, as it enables good separation and can be combined with an array of detectors [6, 50]. A reverse-phase C18 column is now generally employed for HPLC and the mobile phases used generally contain an aqueous methanol or acetonitrile solution with the addition of acids such as orthophosphoric, formic, or acetic acid and column conditioners such as tetrahydrofuran [6, 49]. As mentioned in Section 2.2, catechins have maximum absorbance wavelengths of 210 and 269–280 nm [30, 33] and, therefore, UV and diode array detectors have been widely used to determine the individual catechins. A typical HPLC chromatogram of a tea sample is presented in Fig. 5.

4 Extraction and isolation of tea catechins

Tea leaves do not only contain catechins, but they also have a range of other compounds such as chlorophyll, carbohydrates, enzymes, protein, caffeine, theanine, and other substances (Table 1). Among these compounds, catechins, carbohydrates, and protein are the most abundant components; however, most of the proteins and carbohydrates are not able to get out of the leaf structure or are not soluble in
water or polar organic solvents while other components, such as caffeine, theanine, and chlorophyll, which are only present in small quantities, are generally soluble in water [26]. The catechins are soluble in water. They are also soluble in polar organic solvents, such as ethanol, methanol, and acetone; however, other tea components such as caffeine and chlorophyll are also soluble in these organic solvents. Therefore, to obtain pure catechins from tea, they need to be separated from the other tea components, which are also dissolved in the solvents used to brew the tea.

Numerous methods have been developed for the separation and isolation of tea catechins. The extraction process is conducted through several steps, which fall into several basic categories: starting material treatments, extraction of tea constituents from tea leaves into infusions, concentration of the extracted constituents, separation and isolation of tea catechins from other impurities, and drying of catechins to obtain an extract powder which can be used in industry (Fig. 6).

4.1 Starting material

The tea catechins are susceptible to oxidation and therefore improper treatment or storage can lead to their loss [1]. Fresh tea leaves contain high levels of moisture, ranging from 65 to 78% w/w on a wet basis, and high levels and activities of oxidative enzymes, especially PPO [1, 51]. Therefore, after harvest, the fresh tea leaf needs to be quickly treated to prevent any oxidation of the catechins. For most extraction methods, the fresh tea leaf is first heated, either by steaming, microwaving, blanching or heating in an oven, to stop the oxidation of the catechins by PPO [32, 52]. However, for some extraction methods, the tea leaf is directly extracted with solvent immediately after harvest or first crushed into a fine paste and then quickly extracted with solvent without going through the heating step [53, 54].

For teas that are dried, the moisture content should be lowered and maintained at less than 10% w/w; a higher moisture content can significantly reduce the tea quality and the catechin content [55]. Exposure to light can also accelerate photooxidation of lipids and non-enzymatic browning reactions involving catechins, which can lead to a loss of quality and catechins during storage [56]. Therefore, dried teas need to be packaged in bags well sealed from moisture and light and stored at room temperature or lower to minimize degradation of the tea catechins [28, 57].

To increase the efficiency of extracting the catechins, dried tea can also be ground into small particles. Small tea pieces shorten the path the solvent needs to travel to extract the catechins thereby reducing the time needed for efficient extraction. However, it should be noted that the extraction of catechins from tea with small particle sizes is only quicker, compared to large particle sizes, when the solution is well agitated [58]. When the solvent is forced through the packed bed of leaves by external agitation, the small size particles have a greater surface area in contact with the solvent and thus infuse more efficiently than larger particles.

4.2 Extraction of tea constituents into infusions

The aim for this process is to maximize the solubilisation of the catechins into a solvent and thereby maximize their efficiency of extraction from the tea. The extraction efficiency for the catechins is very important because it directly affects the productivity and the cost of the extraction process. However, the safety of the workers and the consumers and the environmental impacts also have to be taken into account.

4.2.1 Impact of various factors on extraction efficiency

The extraction efficiency for the catechins depends on several factors, such as the type of solvent used, the temperature at which the extraction is done, the ratio of solvent to tea, the pH of the mixture, and the particle size of the tea. Thus, the extraction efficiency can be improved by optimising each of these factors during the extraction process.

The solvents commonly used for the extraction of catechins from tea are water, polar organic solvents, and aqueous organic solvent mixtures and each solvent system has both advantages and disadvantages. Water is an inexpensive and safe solvent, which does not leave any residues behind. However, Perva-Uzunalić et al. [59] reported that the extraction of catechins from tea using water alone resulted in the lowest extraction efficiency in comparison with polar organic solvents, such as acetone, acetonitrile, ethanol, or methanol alone or as aqueous organic solvent mixtures. On the other hand, extraction using organic solvents may also
leave chemical residues or contaminating substances, which are often not acceptable [57, 60].

The extraction efficiency of tea constituents also depends on the extraction temperature. High extraction temperatures improve extraction efficiencies because the heat renders the cell walls more permeable to solvents and components and increases the solubility and diffusion coefficients of the tea components to be extracted. An increased extraction temperature can also decrease the viscosity of some solvents and thereby facilitate the extrac-

Figure 6. Separation and isolation of tea catechins.
tion process [61]. However, excessive extraction tempera-
tures above 80°C can add to the costs and can also cause
degradation of the catechins by promoting the change in
epistructured catechins to non-epistructured catechins [37].

The ratio of solvent to tea can also significantly affect
the efficiency of catechin extractions. Perva-Uzunalić et al.
[59] indicated that the extraction yields, as well as the
efficiency for caffeine and the major catechins,
increased with increasing amounts of solvent. Similarly,
Pinelo et al. [62] reported that the higher the solvent to solid
ratio, the higher is the amount of extracted solid obtained.
Undoubtedly, the force driving the mass transfer of consti-
tuents from a solid is greater when the solid is placed in a
high volume of solvent.

The pH of the extraction solution also strongly affects
the extraction efficiency of the catechins. For example, at a
pH of less than 7.0, the extraction efficiency was greater for
low tea concentrations than for higher tea concentrations
[57]. However, when the pH of the solution was more than
7.2, the extraction efficiency at the higher tea concentrations
was greater than for the lower tea concentrations [57].
Furthermore, as stated previously, the epimerisation of the
tea catechins is also much lower if the solutions have a pH
of 6 or lower. Thus, the addition of EDTA, which results in a
pH of 5.5 in water, has been found to decrease the
epimerisation [38]. However, because EDTA is a divalent
cation chelator, the observed decrease may also result from
these cations being no longer available to act as catalysts in
the epimerisation process.

The particle size of the tea sample can also affect the
extraction efficiency of the catechins. As mentioned in
Section 4.1, reducing the particle size improves the extraction
efficiency because it increases the contact surface area
between the tea and the solvent. However, vigorous agitation
of the small particles should be applied during the extraction
to increase the contact of the tea with the solvent; without
agitation, the tea particles can settle to the bottom of the
container and not effectively interact with the solvent [58].

4.2.2 Methods to extract components from tea

Tea catechins and other components have been extracted
using several methods including cold and hot water
extraction, organic solvent extraction, microwave-assisted
extraction (MAE), and other extraction techniques (Table 3).
Each method has its own advantages and disadvantages, and
each method suits different requirements and applications.

4.2.2.1 Water extraction

Tea has been extracted with water using a wide range of
temperatures to obtain high extraction efficiencies of
catechins to meet particular purposes. Cold water extraction
is normally conducted at temperatures ranging from
4–25°C. Lin et al. [63] extracted the tea components with
cold water at a temperature of 4°C and showed that the yield
of the extract was significantly lower than for hot water
extraction at a temperature of 90°C. They also reported that
the cold water extract was more effective at scavenging 1,1-
diphenyl-2-picrylhydrazyl free radicals and at chelating
ferrous ions than the hot water extract [63]. In contrast,
and somewhat contradictory, the antioxidant activity
measured using the conjugated diene method was higher
for the hot water than for the cold water extract [63].

Table 3. Conditions used for extraction of tea constituents

<table>
<thead>
<tr>
<th>Tea type</th>
<th>Method of extraction</th>
<th>Solvent</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Ratio of solvent/tea (mL/g)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tea</td>
<td>Cold water</td>
<td>Water</td>
<td>4°C</td>
<td>1440</td>
<td>50:1; 17:1; or 10:1</td>
<td>[63]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Hot water</td>
<td>Water</td>
<td>50°C</td>
<td>240</td>
<td>100:1</td>
<td>[92]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Hot water</td>
<td>Water</td>
<td>80°C</td>
<td>60</td>
<td>10:1</td>
<td>[93]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Hot water</td>
<td>Water</td>
<td>80 or 95°C</td>
<td>20 or 10</td>
<td>40:1</td>
<td>[59]</td>
</tr>
<tr>
<td>Black tea</td>
<td>Hot water</td>
<td>Water</td>
<td>95°C</td>
<td>15</td>
<td>16:1</td>
<td>[41]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Hot water</td>
<td>Water</td>
<td>90–100°C</td>
<td>10 min</td>
<td>10:1</td>
<td>[88]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Hot water</td>
<td>Water</td>
<td>100°C</td>
<td>10</td>
<td>14:1</td>
<td>[78]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Organic solvent</td>
<td>Acetone (50% v/v)</td>
<td>Boiling point</td>
<td>120</td>
<td>20:1</td>
<td>[59]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Organic solvent</td>
<td>Methanol (100% v/v)</td>
<td>Boiling point</td>
<td>120</td>
<td>20:1</td>
<td>[59]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Organic solvent</td>
<td>Ethanol (80% v/v)</td>
<td>Boiling point</td>
<td>120</td>
<td>20:1</td>
<td>[59]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Organic solvent</td>
<td>Acetonitrile (50% v/v)</td>
<td>Boiling point</td>
<td>120 min</td>
<td>20:1</td>
<td>[59]</td>
</tr>
<tr>
<td>Green tea</td>
<td>MAE (600 W)</td>
<td>Water</td>
<td>80 and 100°C</td>
<td>60</td>
<td>20:1</td>
<td>[69]</td>
</tr>
<tr>
<td>Green tea</td>
<td>MAE (700 W), initial 45 s on, then 10 s off, followed by 3 s on, then 10 s off</td>
<td>Ethanol, acetone, or methanol (50% v/v)</td>
<td>Pre-leaching at room temperature and MAE from 85–90°C and 4 min for pre-leaching</td>
<td>90 min for pre-leaching</td>
<td>20:1</td>
<td>[67]</td>
</tr>
<tr>
<td>Black tea</td>
<td>SWE</td>
<td>Water</td>
<td>175°C</td>
<td>180</td>
<td>72:1</td>
<td>[72]</td>
</tr>
<tr>
<td>Green tea</td>
<td>UAE (40 kHz, 250 W)</td>
<td>Water</td>
<td>60°C</td>
<td>40 min</td>
<td>100:1</td>
<td>[75]</td>
</tr>
<tr>
<td>Green tea</td>
<td>UHPE at 400 MPa</td>
<td>Ethanol (50% v/v)</td>
<td>Room temperature</td>
<td>15</td>
<td>20:1</td>
<td>[76]</td>
</tr>
</tbody>
</table>

The abbreviation ultrasounds are MAE: microwave assisted extraction; SWE: subcritical water extraction; UAE: ultrasound assisted extraction; UHPE: ultrahigh pressure extraction.
Table 4. Properties of polar organic solvents commonly used for tea extraction [65]

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Polarity index</th>
<th>Refractive index @20 °C</th>
<th>UV (nm) cutoff @ 1 Au</th>
<th>Boiling point, °C</th>
<th>Viscosity (cPoise)</th>
<th>Solubility in water % w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>6.2</td>
<td>1.372</td>
<td>230</td>
<td>118</td>
<td>1.26</td>
<td>100</td>
</tr>
<tr>
<td>Acetone</td>
<td>5.1</td>
<td>1.359</td>
<td>330</td>
<td>56</td>
<td>0.32</td>
<td>100</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5.8</td>
<td>1.344</td>
<td>190</td>
<td>82</td>
<td>0.37</td>
<td>100</td>
</tr>
<tr>
<td>Alcohol</td>
<td>5.2</td>
<td>1.360</td>
<td>210</td>
<td>78</td>
<td>1.20</td>
<td>100</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>4.4</td>
<td>1.372</td>
<td>260</td>
<td>77</td>
<td>0.45</td>
<td>8.7</td>
</tr>
<tr>
<td>Methanol</td>
<td>5.1</td>
<td>1.329</td>
<td>205</td>
<td>65</td>
<td>0.60</td>
<td>100</td>
</tr>
</tbody>
</table>

The hot water extraction is carried out at temperatures between 50 and 100 °C. Perva-Uzumalić et al. [59] extracted tea components with hot water at different temperatures for varying lengths of time and reported that the maximum yield of extract was obtained at a temperature of 80 °C with a 20-min extraction time or alternatively at 95 °C for 10 min. The yield of the extract was found to decrease when the extraction was performed at temperatures lower than 80 °C or higher than 95 °C [59]. However, despite higher yields with hot water extraction and the extracts possessing greater antioxidant activity [63], care still has to be taken in order to prevent any catechin degradation during hot water extraction, especially epimerisation [36].

A combination of cold and hot extraction has also been used. In this combination, the tea is first extracted with water at low temperatures from 0 to 50 °C, the water is removed and the leaves are then re-extracted with water at higher temperatures from 50 to 100 °C [64]. This protocol has been shown to provide a better extraction efficiency for catechins compared with either single cold or hot water extractions [64]. In addition, the combination extraction method improved the stability of the extracts and reduced the denaturation of the tea catechins [64]. It should be noted that the extraction efficiency can also be improved by the adjustment of other factors, such as pH, particle size, and the length of extraction as discussed above (Section 4.2.1), when using water alone as a solvent for extraction.

4.2.2.2 Organic solvent extraction

Several polar organic solvents have been used to extract catechins from tea (Table 4) [65]. Extractions with polar organic solvents have the advantage of having higher extraction efficiencies than extractions with water only [66]. Perva-Uzumalić et al. [59] showed that the highest extraction efficiency for tea catechins was achieved by using 25 or 50% v/v aqueous acetone followed by 50% v/v acetonitrile, 80% v/v ethanol, and 100% v/v methanol. Such polar organic solvent extractions can also efficiently extract tea constituents without having undesirable epimerisation of the major catechins occurring. They also have the advantage of requiring less energy for evaporating the solvents after the extraction [57]. However, organic solvents can leave unwanted residues in the final extracts if the solvents are not pure or are not appropriately removed and such extracts may not be acceptable to consumers. Therefore, the use of organic solvents for extractions needs to be considered carefully in respect to their intended final use.

4.2.2.3 Microwave-assisted extraction

The relatively new extraction technique MAE makes use of a microwave oven to quickly heat the solvent and extract the catechins instead of the more traditional direct heating methods. Several studies have shown that MAE has some advantages over the conventional extraction methods including shorter extraction times, the use of less solvent, and generally higher extraction rates. The microwave treatment can disrupt the structure of the cells and aids the extraction process due to a rapid increase in the internal cell temperature and pressure. When the cells are disrupted, the tea constituents can be more quickly extracted into the solvent [67–69]. However, MAE is more difficult to scale up for industrial applications than the traditional heating methods [70].

Pan et al. [67] studied the optimal conditions for MAE and found that dry tea should be preleached with 50% v/v ethanol at room temperature for 90 min at a ratio of solvent to tea of 20:1 (mL/g). The tea was then extracted using a microwave oven with the temperature controlled between 80 and 100 °C for 30 min and the solvent to tea ratio at 20:1 (mL/g). Under these conditions, Nkhili et al. [69] extracted tea using a 600 W microwave oven with the temperature controlled between 80 and 100 °C for 30 min and the solvent to tea ratio at 20:1 (mL/g). Under these conditions, Nkhili et al. [69] reported that the extraction efficiency for the tea catechins was higher than for the conventional heating methods. In general, the MAE technique has been shown to be an efficient extraction method for catechins and has been promoted as a promising new method for the extraction of tea constituents [70].

4.2.2.4 Other extraction techniques

Recently, subcritical water extraction (SWE) has been used as an alternative method for the effective extraction of natural bioactive compounds [71]. For example, SWE has been employed to extract caffeine from tea or tea waste [72, 73]. The SWE technique uses hot water at temperatures
ranging from 100 to 374°C under high pressures from 10 to 60 bar [71]. The technique has some advantages, compared to the conventional extraction methods, including the simplicity of the extraction system, and the higher extraction rates achieved [71]. However, the tea catechins may well degrade at the high temperatures used in SWE [39, 40] and therefore research is required to optimise the conditions for efficiently extracting the catechins from tea using this technique.

Ultrasound-assisted extraction (UAE) is another alternative method used effectively for the extraction of natural bioactive components [74]. The UAE technique has been found to be more effective at extracting bioactive components, than the conventional extraction methods, because it provides a greater penetration of the solvent into the cellular matrix and thus improves the mass transfer of the constituents into the solvent [74]. The UAE method has another advantage over SWE in that it is conducted at lower extraction temperatures [75] thereby reducing the risk of catechin degradation. To extract catechins from green tea, UAE has been applied with an input power of 250 W, giving an extraction temperature of 60°C for 40 min [75]. The results showed that the amount of extracted catechins was higher with UAE than with a conventional extraction method [75].

Another method, the ultrahigh pressure technique is a potential alternative. It uses very high extraction pressures from 100 to 800 MPa to extract bioactives for their use in the food industry [76]. Jun [77] found that ultrahigh pressure extraction (UHPE) at 500 MPa was more effective at extracting caffeine from green tea than other extraction methods, such as room temperature, ultrasonic, and heat reflux extraction. In comparison with the conventional extraction techniques, UHPE has several advantages such as high extraction yields, short extraction times, and low energy consumption [77]. For example, Jun et al. [76] extracted catechins from green tea using the ultrahigh pressure at 400 MPa for 15 min and showed that this provided yields of catechins similar to the extraction with aqueous 50% v/v ethanol for 2 h. The ultrahigh pressure technique is a promising method for effectively extracting catechins from tea. However, further studies are needed to optimise the conditions for extracting the catechins from tea.

4.3 Concentration of the extracted constituents

The aims of concentrating tea extracts are to partly remove the solvents to obtain tea catechin preparations with higher concentrations, to obtain dry crude catechin extracts or to concentrate the catechins before subjecting them to further isolation processes (Fig. 6).

4.3.1 Separation of spent tea leaves from infusions

Depending on the particle size of the tea insoluble material (solid) and the differences in density between the solid and the liquid, this separation step is mainly carried out using filtration or centrifugation.

4.3.1.1 Filtration

There are many kinds of filters that can be used for the separation of the spent (extracted) tea leaves from the liquid infusion. For example, cotton wool has been used as a filter to separate the spent leaves from the tea infusion [78]. In addition, glass wool, hydrophilic membranes, and filter paper have also been used for separating the spent tea leaves from the extract [79–81]. Even very small particles can be removed with filters; filters with pore sizes less than 0.45 μm or less than 0.22 μm are usually used prior to HPLC analysis to ensure the columns do not get clogged. However, the disadvantage of filtration is that small particles can block filters, which results in low liquid permeation rates [65]. Also, the smaller the pore size, the more expensive they are.

4.3.1.2 Centrifugation

Centrifugation has also been widely used to separate the spent leaves from the tea infusions after brewing. A range of speeds and lengths of centrifugation have been reported [82, 83]. In a typical method, Baldermann et al. [82] separated the insoluble material by sedimentation centrifugation at 6000 × g for 10 min. Centrifugation is also influenced by the particle size of the insoluble material [65]. Generally, the smaller the particle size, the more speed and time are needed to sediment them. This means that, in the commercial setting, the costs of separation by centrifugation increase rapidly as the particle size decreases, even more so than it does for filtration. The solid–liquid density difference is also an important factor for the centrifugation method but not for filtration [65].

4.3.2 Concentration of tea infusions

After removal of the insoluble tea material, the tea infusions, containing the soluble substances, are then concentrated by lowering the volume of the extraction solvent usually using an evaporator [84]. The concentrated tea infusions are then further dried using either a stream of inert gas, a rotary evaporator, a vacuum centrifuge, a vacuum dryer with or without heat, or a freeze-dryer to obtain crude tea catech extracts in powder form [85].

Sinija et al. [54] developed a method to make a soluble tea powder by brewing tea and then centrifuging at 10 000 rpm for 20 min to remove the solids. This extract was finally dried by either vacuum drying at 50°C and 400 mmHg for 16–19 h or air drying at 50°C for 12–16 h. In another method, Bazinet et al. [86] produced a crude tea extract by freeze drying their tea infusion for 24 h. However, such catechin extracts are not pure because they contain a substantial amount of other tea compounds such as caffeine, chlorophyll, and theanine.
4.4 Separation and isolation of catechins

The aim of the separation and isolation steps is to remove the impurities from the catechins and to purify the individual catechins from the catechin mixture, respectively. However, the separation and isolation processes are difficult, especially in larger commercial quantities, because the impurities and individual catechins have very similar structures and therefore very similar physical properties \[87\]. The tea catechins have been separated and purified using various methods, such as: caffeine precipitation, membrane separation, resin addition, solid-phase extraction (SPE), column chromatography, and supercritical fluid extraction (SFE) (Fig. 6).

4.4.1 Caffeine precipitation

As described in Section 2.3.3, catechins can interact with caffeine to form a precipitate (Fig. 4). This property was used by Copeland \textit{et al.} \[78\] to develop a method for isolating EGCG from green tea. In this method, caffeine was added to a green tea infusion (2 \times 350 mL water, 25 g leaf tea) at 70°C to give a final concentration of 30 mM caffeine. The solution was cooled for 2 h and chilled to 4°C for a further hour and then centrifuged at 31,000 \times g for 20 min to provide a pellet of material, which was then resuspended in distilled water.

A decaffeination step was then carried out by using chloroform (4 equal volumes) at 60°C. The decaffeinated fraction was then partitioned with ethyl hexanoate to obtain an EGCG-rich aqueous fraction. Following this, the EGCG rich aqueous fraction was partitioned with propyl acetate. The solvent was then evaporated and the residue was dissolved in water and finally freeze-dried to yield a powder. This method yielded 400 mg of 80% pure EGCG in powder form from 25 g of dry green tea \[78\]. However, this method uses chloroform to decaffeinate the extract and ethyl hexanoate and propyl acetate in the purification process, solvents that are unsafe for human consumption.

4.4.2 Membrane separation

Membranes have been used to separate catechins from other compounds based on their respective sizes. Ramar ethinam \textit{et al.} \[88\] used a filtration membrane to increase the concentration of catechins from a tea infusion. They infused 1 kg of dry green tea in 10 L of water at 90–100°C for 10 min. The infusion was then filtered through a 140-mesh (0.037 mm) sieve to remove insoluble material and then forced through an ultrafiltration membrane made of polyether sulphone material with a pore size of 0.014 μm and an area of 0.2 m², under 1000 kPa of pressure to obtain a retentate containing a high content of catechins \[88\].

Nwuha \[89\] employed G-10 and G-20 membranes for isolation of catechins from a crude tea extract (Polyphenon-60). The tea extract was dissolved in 80% v/v ethanol and then filtered through a G-10 or G-20 membrane, which was held by a porous Teflon layer in the form of an O-ring. A magnetic stirrer at 450 rpm and 5 MPa of pressure were used to promote the filtration and a filtrate with a high level of catechins and a low level of caffeine was obtained (Fig. 7). In another method, a cellulose acetate-titanium composite ultra filtration membrane was used to separate polyphenols from tea to obtain a product containing more than 40% w/w total polyphenols \[83\].

The main advantage of using membranes for separation is that the catechins can be fractionated from impurities such as polymeric substances or lipids and that the catechins can be concentrated during the process. However, the disadvantages of this method are that the catechins are generally obtained in low purity and that the membranes can often foul during filtration, resulting in low permeate flux rates \[65\].

4.4.3 Resin adsorption

Synthetic resin absorbents such as macroporous polymeric adsorbents and polyamide have been used to isolate tea catechins. Zhao \textit{et al.} \[90\] used a macroporous polymeric adsorbent made from N-vinyl-2-pyrrolidinone, ethylene glycol dimethacrylate, and triallyl isocyanurate to selectively adsorb catechins from mixtures with caffeine. As a result of the high adsorption capacity of the resin for the tea catechins and its low adsorption capacity for caffeine, the product eluted from the resin using ethanol contained 98% w/w catechins and 2% w/w caffeine \[90\].

Polyamide has also been used for the separation of catechins from caffeine in tea infusion. Bailey \textit{et al.} \[91\] isolated catechins from green tea by loading a tea infusion onto a Polyamide CC6 column to absorb the catechins. Owing to the weak affinity of caffeine for the column material, the caffeine could be rinsed off the column using 4–5 column volumes of water. The column-bound catechins were then washed off using 95% v/v food grade ethanol. The resulting fraction contained less than 1% w/w caffeine but was comprised of 70% w/w total catechins including 30% w/w EGCG.
The utilisation of synthetic resin absorbents to purify catechins can help avoid the use of undesirable solvents such as chloroform, acetone, ethyl hexanoate, and propyl acetate. However, the catechins are still obtained as mixtures with low purity and therefore, other methods are required to improve the purity of the catechin preparations.

4.4.4 Column chromatography

Column chromatography such as preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and high-speed counter current chromatography have been widely used in the separation of natural products including tea catechins [92, 93]. Based on the differing retention times of the different tea components in these systems, the individual catechins can be isolated and purified.

Kang et al. [92] isolated EGCG having a purity of more than 98% w/w by using two analytical C18 HPLC columns: (i) 4.6 × 250 mm, 15 µm and (ii) 3.9 × 300 mm, 10 µm. Caffeine was initially eliminated from the tea infusion by chloroform extraction and the catechins were also extracted from the water layer using ethyl acetate prior to the HPLC chromatography. The same mobile phase of 0.1% v/v acetic acid in water/acetone (87:13, v:v) was used at a flow rate of 1.0 mL/min to first separate the EGCG from most of the other substances using column 1 and then to further purify EGCG from the other catechins using column 2 [92].

Amarowicz et al. [93] used a semi-preparative RP-HPLC column (10 × 250 mm, 7 µm) to separate and purify individual catechins from a tea extract. Caffeine was initially removed from the tea infusion using a chloroform extraction. The catechins in the infusion were then separated into six fractions using silica gel column chromatography and chloroform/methanol/water (65:35:10, v:v:v) elute the six fractions from the column. To isolate EC and EGC, fractions 2, 3, and 4 were put through the RP-HPLC column with an isocratic water/dimethylformamide/methanol/acetetic acid (157:40:2:1, v:v:v:v) solvent system at a flow rate of 4 mL/min to differentially elute the individual catechins. To isolate EGC, ECG, and EGCG, fractions 5 and 6 were put through the RP-HPLC column with an isocratic solvent system at a flow rate of 3 mL/min [93].

Cao et al. [94] also developed a method to isolate and purify the three catechins EGCG, GCG, and ECG from tea by employing counter current chromatography. Two sets of solvent systems, composed of (i) ethyl acetate, ethanol, and water and (ii) hexane, ethyl acetate, and water, were used for separating the catechins. In the first step, EGCG was purified using the first solvent system, ethyl acetate/ethanol/water, in a stepwise elution fashion from 25:1:25 to 10:1:10 (v:v:v). The unseparated catechins were then concentrated and subjected to the second step of the separation procedure using the second solvent system composed of hexane/ethyl acetate/water at 1:4:5 (v:v:v). The resulting eluents were pure fractions of GCG and ECG, and along with the EGCG fraction from the first step, they were collected and freeze-dried in the dark. Starting from 1 g of a polyphenol mixture, Cao et al. [94] reported a yield of 275 mg of EGCG, 140 mg of GCG, and 130 mg of ECG.

In general, these extraction and purification methods are successful but the use of solvents such as chloroform, ethyl acetate, and hexane should be avoided if at all possible when products are for human consumption. Therefore, methods, in which safe alternative solvents can be used, need to be further examined.

4.4.5 Solid-phase extraction

SPE is an alternative to liquid–liquid extraction for the separation, purification, and concentration of the components from solutions, including tea. Tian et al. [95] used an ionic liquid-modified polymer as an SPE material for the isolation of caffeine and theophylline from green tea and showed that it provided a high recovery of caffeine and theophylline (89.9 and 89.6% w/w, respectively) but the purity was not high. Ding et al. [96] used a molecularly imprinted SPE template to obtain a recovery of 85% w/w fairly pure EC after isolation from green tea.

Since the first experimental trials, using activated carbon filters for SPE, were conducted 50 years ago, SPE has been widely used for the separation and isolation of analytes from many different solutions [97]. However, the materials and methodology used in SPE need to be further studied in order to improve its effectiveness for the isolation of catechins from tea.

4.4.6 Supercritical fluid extraction

SFE with carbon dioxide (SFE-CO2) has been used to separate tea components, such as catechins and caffeine [98]. The main advantage of this method is that it combines the characteristics of gases and liquids for extraction, thus the solvent can diffuse through solids like a gas and dissolve materials like a liquid. The process is very fast because of the low viscosities and high diffusion rates associated with the gas phase of the solvent substance [61]. In addition, using SFE-CO2 for extraction can minimise the degradation of catechins due to the absence of air and light during the extraction process as compared to other extraction methods. Furthermore, the use of CO2 in SFE does not leave any residues and, therefore, the products are more likely to be safe for consumers [61].

SFE-CO2 has been used as an alternative method for removing caffeine to produce decaffeinated tea and tea powders [99]. However, the temperature, pressure, and other solvents must be well controlled and optimised to obtain high extraction efficiencies for caffeine without affecting the recovery of catechins [60]. Park et al. [98] demonstrated that the extraction conditions such as temperature, pressure and co-solvents have a significant impact on the extraction of catechins during the removal of caffeine from tea using SFE-CO2. They showed that an extraction with 95% v/v ethanol at 7 g of tea per 100 g of CO2,
at 300 bar and 70°C for 120 min, extracted over 97% of the caffeine. However, this treatment also had the unwanted effect of extracting over 37% of the EGCG from the tea. Therefore, future research is needed to optimise the SFE-CO₂ conditions required to effectively separate caffeine from the catechins in tea.

4.5 Drying process

The aim of the final step of drying is to enhance the stability of the catechins while minimising the packaging requirements and reducing transport weight. Basically, drying is a process of removing moisture through the simultaneous transfer of heat and moisture. The drying processes have a significant influence on the cost and quality of the final product [100]. As catechins are heat and oxygen sensitive, the selection of the drying method for dehydration of the final catechin product is important. The optimal drying method must also consume less energy while maintaining the natural physical and biochemical quality of the catechins. The methods which have been applied to the drying of tea catechins include spray drying, rotary evaporation, vacuum drying, and freeze-drying [51, 53, 54, 86, 94]. However, the impact of the various drying methods on the quality of the final catechin products as well as on the overall production costs has not been clearly compared and reported. Therefore, further studies are needed to identify the optimal methods and conditions for drying the catechin extracts. In addition, the cost effectiveness of the various drying methods also needs to be evaluated in order to maximise commercial viability.

5 Concluding remarks and future consideration

The tea catechins process significant antioxidant properties, which are linked to beneficial human health effects, such as prevention of cancers, cardiovascular disease, improvement of the immune system, and prevention of obesity and diabetes. However, only drinking tea may not provide a sufficient level of catechins to achieve these health benefits. Therefore, catechins have been isolated from tea to supplement the consumption of catechins by the broader community in order to maximise their impact on health outcomes. Several methods have been developed to extract and isolate catechins from tea. However, further research is needed to develop new methods or improve the existing methods, which have limitations.

Membrane separation, caffeine precipitation, SPE, and column chromatography (including HPLC) have been employed to isolate catechins from tea. However, undesirable organic solvents such as chloroform, ethyl acetate, acetonitrile, and hexane have been used in these methods, thus presenting the potential for chemical residues in the final extracts, which could affect consumer’s health. To overcome this limitation, water should be used as the solvent of choice during the extraction and isolation of catechins from tea. Water is a cheap and safe solvent; however, it is less effective at extracting catechins compared with polar organic solvents. Therefore, extraction conditions such as temperature, length of extraction time, particle size, pH of the solution, and the ratio of water to tea need to be further studied in order to optimise the extraction efficiency of catechins from tea. In addition, using water with promising novel extraction techniques such as MAE, UAE, SWE, and UHPE should also be thoroughly investigated.

Extraction of catechins from tea using resin adsorbents has also shown great potential. This process usually uses water and ethanol as solvents. Although ethanol is an organic solvent, it is considered to be “safer” than other organic solvents such as chloroform, hexane, and ethyl acetate. However, further research is still needed to improve the effectiveness of this technique and to determine whether it can be scaled up for industrial application.

Similarly, SFE-CO₂ is a potential method for isolating tea catechins. It is fast, safe and has a high extraction efficiency. However, the extraction conditions, such as temperature, pressure, and the type of solvent need to be further investigated to optimise the extraction efficiency for the catechins. The cost of setting up this system for industrial production also needs to be evaluated to ensure commercial viability.

In conclusion, the demand for catechins has considerably increased due to their exciting potential for use as novel bioactive ingredients in the pharmaceutical, cosmetic and food industries. The world tea production is continuously increasing, so therefore, there is an abundant source of starting material for the isolation of catechins. However, in order to take full advantage of the increasing demand, investment will be needed for the development of safe, sustainable, and environmentally friendly methods for the isolation of the catechins from tea. It is hoped that this review provides some insight into the production of safe and healthy catechin products.

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The authors have declared no conflict of interest.

6 References

1.3.4. L-Theanine: properties, synthesis and isolation from tea

Quan V. Vuong, Michael C. Bowyer and Paul D. Roach

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L-Theanine: properties, synthesis and isolation from tea

Quan V Vuong,∗ Michael C Bowyer and Paul D Roach

Abstract
Theanine is a non-protein amino acid that occurs naturally in the tea plant (Camellia sinensis) and contributes to the favourable taste of tea. It is also associated with effects such as the enhancement of relaxation and the improvement of concentration and learning ability. It is also linked with health benefits including the prevention of certain cancers and cardiovascular disease, the promotion of weight loss and enhanced performance of the immune system. Thus, there has been a significant rise in the demand for theanine. While theanine has been chemically and biologically synthesised, techniques to isolate theanine from natural sources remain an important area of research. In this review article, the properties and health benefits of theanine are summarised and the synthesis and isolation of theanine are reviewed and discussed. Future perspectives for the isolation of theanine from natural sources are also outlined.

Keywords: health benefits; isolation; properties; synthesis; tea; theanine

INTRODUCTION
Theanine is an abundant non-protein derived amino acid that was first isolated from green tea leaves in the late 1940s by Sakato.1 Theanine has been named as 2-amino-4-(ethylcarbamoyl) butyric acid by the International Union of Pure and Applied Chemistry (IUPAC). However, it has also been referred to as γ-glutamylethylamide, 5-N-ethylglutamine, γ-glutamyl-L-ethylamide, γ-ethylamino-L-glutamic acid and γ-L-glutamylamide.2–5 Similar to other amino acids in nature, theanine is a chiral species and occurs in nature predominantly as the L-(S) enantiomer (Fig. 1), while synthetic theanine is normally prepared as a racemic mixture of L- and D-forms.2

Theanine is considered to be a unique amino acid in nature because, with the exception of being found in the basidiomycete mushroom Xerocomus badius, its occurrence appears to be limited to the Camellia genus, mostly the tea-producing plants C. sinensis var. sinensis and C. sinensis var. assamica and some closely related species such as C. japonica and C. sasanqua.6 In the leaves of the tea plant species, theanine accounts for about 500 g kg⁻¹ of the free amino acids. Many of these amino acids are involved in producing the distinctive aroma and taste of tea and theanine has been linked with giving tea its distinctive umami taste.7 Because of its contribution to taste, the theanine content in tea leaves correlates highly with tea quality and price; the teas with a high content of theanine are normally evaluated as having a higher quality and thus command a higher price.8

Theanine occurs in the cotyledons, shoots and roots of the tea plant seedling and it is biosynthesised from glutamic acid and ethylamine via the enzyme theanine synthetase (Fig. 2).4 In mature plants, biosynthesis occurs mostly in the roots, from where it is transferred, via the phloem, through the stem to the growing shoots where it subsequently accumulates in the developing leaves. In the leaves, theanine can be hydrolysed back to its parent constituents through exposure to sunlight and heat. The ethylamine liberated as a result of this reaction is then utilised as a precursor in the synthesis of catechins.9 Consequently, tea growing in climatic conditions of reduced sunlight has been shown to develop higher concentrations of theanine and lower amounts of catechins.9 Trials to boost theanine levels in tea through controlled exposure to sunlight (e.g. with the use of shade cloth) have also been successful.6

Theanine constitutes between 10 and 30 g kg⁻¹ of the weight of dry tea leaf.2 However, the level of theanine varies in accordance with a variety of factors, including growing location and method of cultivation, tea grade and variety and time of harvest. As mentioned previously, tea growing in shaded areas or in areas of reduced exposure to direct sunlight generally contains higher levels of theanine.10 Tea variety is also important, with the C. sinensis var. sinensis known to contain higher concentrations of theanine compared to C. sinensis var. assamica.8 Also, tea harvested in early summer possesses a higher theanine content compared with tea harvested in late summer.8 The latter may explain why theanine levels differ significantly between grades of tea from the same species and growing location. For example, Ceylon black tea (grade Pekoe) from Sri Lanka has a higher theanine content than Ceylon black tea (grade Broken) from the same growing region.11 Post-harvest processing has little impact on theanine as green (or non-fermented) tea contains similar levels of theanine when compared to oolong (semi-fermented) and black (fermented) teas.11

Several in vivo and epidemiological studies have shown that theanine consumption can enhance health and well-being by influencing factors such as stress levels, improvements in learning...
ability, prevention of cancers and vascular diseases, promotion of weight loss and enhancement of immune response. However, in these studies the beneficial effects were observed with daily consumption of pure L-theanine of at least 50 mg, an amount equivalent to a minimum of three cups of tea (Table 1). Furthermore, many of these studies were done using between 150 and 250 mg L-theanine, doses which would not be easily achieved even by the most avid tea drinkers; these doses would only be achieved when drinking between nine and 15 cups of tea per day. Moreover, for some people, the caffeine present in tea can cause gastrointestinal tract irritation or sleeplessness thus limiting their tea consumption and making it very difficult for them to obtain the doses of L-theanine linked with beneficial effects.

Therefore, based on the doses of pure theanine used in the studies showing beneficial effects (Table 1), there is a perceived demand for L-theanine as a supplement or food ingredient. This has spawned exploration for efficient and economical extraction techniques for the isolation of natural theanine and investigation into efficient synthetic and biosynthetic means of producing the amino acid. These approaches have become central to expanding the commercial availability of theanine for inclusion in supplements and in processed foods and beverages.

Figure 1. Chemical structure of L-theanine.

HEALTH BENEFITS OF THEANINE

After oral administration, theanine is quickly absorbed into the bloodstream through intestinal absorption, from whence it is transported to the major organs of the body, including the brain. Theanine reaches a maximum concentration in the blood between 30 min and 2 h after administration. The amino acid is cleared by excretion into urine but it is also catabolised via breakdown by amide hydrolysis, yielding glutamic acid and ethylamine, with ethylamine then excreted from the body in the urine.

Ingestion of theanine has been reported to facilitate the generation of alpha brain waves, which are associated with a relaxed but alert mental state. In addition, ingestion of theanine is reported to promote the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), which in turn regulates dopamine and serotonin levels in the brain. Thus, theanine consumption has been closely associated with relaxation and improved learning ability (Table 1).

A recent study found that ingestion of 50 mg of L-theanine dissolved in 100 mL of water could elicit a significant effect on the general state of mental alertness or arousal in subjects by increasing alpha-wave brain activity. Another study also found a link between theanine consumption (200 mg) and the reduction of anxiety. Other studies have shown that consumption of theanine in combination with caffeine could further improve concentration and learning ability. For example, the intake of a combination of 250 mg L-theanine and 150 mg caffeine was found to enhance rapid simple reaction time, fast numeric working memory reaction time and improve verification accuracy during reading tasks. A separate study found that consuming a combination of 100 mg L-theanine and 50 mg caffeine improved both speed and accuracy performance during attention-switching tasks performed 60 min after ingestion and reduced susceptibility to distracting information in memory tasks at 60 and 90 min following ingestion.

Moreover, it is thought that theanine may provide effective prophylaxis and treatment for Alzheimer’s disease as it has been reported to exert neuroprotective effects through inhibition of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors and its related pathways in a transgenic neuronal cell model. Theanine has also recently been linked to cancer prevention. Liu et al. found that theanine was linked to the inhibition of the in vivo and ex vivo growth of human non-small cell lung cancer and leukaemia cell lines. In another study, Friedman et al. found that theanine intake was associated with the induction of apoptosis in four cancer cell lines of breast, colon, hepatoma and prostate origin.

In addition to enhanced antitumour activity, theanine can reduce the adverse effects of the cancer treatment drug, doxorubicin by providing protection against damage caused by doxorubicin to normal tissue. It also acts as a biochemical modulator to improve the therapeutic efficacy of doxorubicin by suppressing the efflux of the drug from cancer cells, thereby increasing the effective doxorubicin concentration in the tumour.

Recent studies have also found that theanine was linked with regulation of blood pressure, promotion of weight loss and improvement of the immune system. Injection of L-theanine at a dose rate of 2 g kg⁻¹ was found to significantly reduce blood pressure in spontaneously hypertensive rats. In humans, consumption of a single dose of 200 mg of theanine was also found to reduce blood pressure and, more importantly, theanine was found to antagonise the negative effect of caffeine increasing blood pressure, when the latter was consumed as a single 250 mg dose.

In addition, co-administration of L-theanine and L-cystine was reported to enhance antigen-specific immunoglobulin G (IgG) production, partly through augmentation of glutathione (GSH) levels and T-helper cell (Th2)-mediated responses. Similarly, co-treatment of L-theanine with L-cystine was found to improve the immune response via an increase in GSH production, which significantly prevented weight loss associated with infection in aged mice.

In summary, findings from several studies have revealed that theanine not only contributes to a favourable flavour in tea but also provides significant health and cognitive benefits. No studies

Figure 2. Biosynthesis and decomposition of L-theanine in the tea plant.
L-Theanine from tea

**Table 1.** Beneficial effects of theanine

<table>
<thead>
<tr>
<th>Impact on</th>
<th>Report of beneficial effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxation effects</td>
<td>Intake of 200 mg of L-theanine dissolved in 100 mL of water generated α-brain waves in female volunteers aged from 18 to 22 years.</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Ingestion of 50 mg of L-theanine produced greater and increased α-activity in young participants.</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Administration of 200 mg of L-theanine dissolved in 100 mL of water resulted in the generation of α-brain waves in female university students.</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Ingestion of 200 mg of L-theanine had some relaxing effects under resting conditions in male and female university students.</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>L-theanine intake resulted in a reduction in heart rate and salivary immunoglobulin A responses to an acute stress task in male undergraduate students.</td>
<td>18</td>
</tr>
<tr>
<td>Improvement in learning ability</td>
<td>Intake of a combination of 150 mg of caffeine and 250 mg of L-theanine improved reaction time, working memory and sentence verification accuracy in participants aged 18–34 years.</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Ingestion of a combination of 100 mg of L-theanine and 50 mg of caffeine enhanced speed and accuracy of performance in the attention-switching task.</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Ingestion of 250 mg of L-theanine enhanced the difference between the effects of visual versus auditory stimuli on the α-wave activity over the parieto-occipital scalp.</td>
<td>21</td>
</tr>
<tr>
<td>Cancer prevention</td>
<td>Theanine was found to inhibit the in vivo and ex vivo growth of human non-small cell lung cancer A549 and leukaemia K562 cell lines.</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Theanine treatment at 400 µg mL⁻¹ was found to induce cell death of four cancer cell lines: breast (MCF-7), colon (HT-29), hepatoma (liver) (HepG2), and prostate (PC-3).</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Theanine (40 mg mL⁻¹ at a dose of 1 mL 100 g⁻¹ of rat) could inhibit invasion of the receptor-mediated cancer cell line AH 109A.</td>
<td>24</td>
</tr>
<tr>
<td>Prevention of vascular diseases</td>
<td>Theanine injection (200 g kg⁻¹) reduced blood pressure in spontaneously hypertensive rats.</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Theanine consumption (200 mg) antagonised the effect of caffeine (250 mg) on blood pressure in healthy adult participants.</td>
<td>26</td>
</tr>
<tr>
<td>Improvement of immune system</td>
<td>Co-administration of L-theanine (8 g kg⁻¹) and L-cystine (20 g kg⁻¹) enhanced antigen-specific IgG production partly through augmentation of GSH levels and Th2-mediated responses.</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Co-administration of L-cystine (700 mg day⁻¹) and L-theanine (280 mg day⁻¹) before vaccination enhanced the immune response to influenza vaccine in elderly people with low serum total protein or haemoglobin.</td>
<td>28</td>
</tr>
<tr>
<td>Effects on weight</td>
<td>Co-treatment of L-theanine and L-cystine to aged mice improved immune response and prevented the weight loss associated with infection.</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Theanine (30 g kg⁻¹ diet) and caffeine (50 g kg⁻¹ diet) were responsible for the suppressive effect of a green tea powder on body weight increases and fat accumulation in mice.</td>
<td>30</td>
</tr>
</tbody>
</table>

Benefits of theanine are associated with theanine toxicity in human or animal models have been reported. However, the US Food and Drug Administration (FDA) recommends that the total daily consumption of theanine should not exceed 1200 mg.

Because of its contribution to favourable flavour and health benefits, theanine has great potential for utilisation as a food ingredient or as a dietary supplement. Future studies should therefore further explore the utilisation of theanine as a food additive and the potential impacts of prolonged theanine consumption on human health.

**PHYSICAL AND CHEMICAL PROPERTIES OF THEANINE**

The major physical properties of theanine are described in Table 2. Theanine, like the protein-based amino acids, exists as a zwitterionic species and is a colourless crystalline solid (needles, melting point 214–216 °C). Studies on the buffering capacity of green tea extracts suggest the pKₐ of the theanine amino group to be 8.9. The pKₐ of the carboxyl unit was not formally quantified due to interference from other acidic species. However, comparisons with close structural analogues such as glutamine suggest the value lies in the range 2.1–2.5.

Theanine is stable under acidic conditions but undergoes base hydrolysis to yield glutamic acid and ethylamine. During infusion, theanine does not react chemically with any of the other tea components. This is in contrast to catechins, which can precipitate from solution as a result of π stacking interactions with caffeine, or can react with proteins and enzymes such as lipoygenase, α-amylase, pepsin, trypsin and lipase.

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C₇H₁₄N₂O₃</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>174.2 g mol⁻¹</td>
</tr>
<tr>
<td>Melting point</td>
<td>217–218 °C</td>
</tr>
<tr>
<td>Appearance</td>
<td>Crystallises in needle form</td>
</tr>
<tr>
<td>Colour</td>
<td>Colourless</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water and insoluble in ethanol, methanol, chloroform and ether</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable in acidic and unstable in alkaline conditions</td>
</tr>
<tr>
<td>Taste</td>
<td>Umami taste with little or no aftertaste</td>
</tr>
</tbody>
</table>
Of the tea components, theanine exhibits a higher water solubility (385 g L\(^{-1}\) at 0 °C, 556 g L\(^{-1}\) at 100 °C) than caffeine (21.7 g L\(^{-1}\)) and the catechins (e.g. epigallocatechin gallate, 5 g L\(^{-1}\)); this permits a very effective diffusion of theanine from tea during hot-water infusions. The relative insolubility of theanine in organic solvents such as methanol and chloroform (Table 2) allows for its easy separation from caffeine and the catechins, which possess a molecular rather than a zwitterionic structure.

Theanine has a complex umami taste. It also exhibits a synergism with the common umami flavouring agents monosodium glutamate and the purine nucleoside inosine 5′-monophosphate, which leads to an enhancement of the umami taste experience. The term umami is a Japanese-derived expression and it is classified as the fifth taste after sweet, salt, bitter and sour.

Most of the typical umami substances are divided into two groups: \(\alpha\)-amino acids, usually represented by monosodium glutamate and 5′-ribonucleotides and their derivatives, usually represented by inosine 5′-monophosphate or disodium 5′-guanylate.

**Analytical Methods for Theanine**

Several analytical methods have been developed for identification and quantification of theanine, which can be employed for determining theanine concentration, production yield or theanine purity in final products. Analysis of theanine has been mainly facilitated by chromatographic techniques such as high-performance liquid chromatography (HPLC), capillary electrophoresis and micellar electrokinetic chromatography. Peng et al. developed a method to determine theanine and other tea components by HPLC using an amide-C16 column coupled with a UV detector (210 nm and 280 nm). Syu et al. developed an improved detection method to quantify theanine by derivatising with the chromophoric labelling reagent dabsyl chloride (4-dimethylaminoazobenzene-4'-sulfonyl chloride) to form dabsyl-theanine, which was subsequently analysed by reverse phase HPLC (C18 column, 425 nm).

Chen et al. used capillary electrophoresis conducted with an uncoated fused-silica capillary column coupled with a diode array detector (214 nm) for determination of the theanine concentration. Li et al. used micellar electrokinetic capillary chromatography, which was conducted on an open tubular fused-silica capillary column coupled to a UV detector set at 360 nm. Hsiao et al. also used micellar electrokinetic capillary chromatography for quantification of theanine, although with a slightly different system and analysis conditions. The system used an untreated fused-silica capillary column and a built-in photodiode array detector set at 200, 205, 220, 266 and 280 nm. In addition, theanine has also been determined using isotachophoretic anion-exchange chromatography with integrated pulsed amperometric detection or enzymatic flow injection analysis.

**Chemical Synthesis and Biosynthesis of Theanine**

Chemical synthesis of theanine

Theanine was first chemically synthesised in 1942 by Lichtenstein by treating pyrrolidone-5-carboxylic acid with aqueous ethylamine for 20 days at 37 °C. A number of other synthetic approaches have since been developed including a large-scale production method involving the reaction of \(\gamma\)-benzyl glutamate in the presence of trityl chloride and ethylamine (339 g kg\(^{-1}\)) and a two-step approach involving initial dehydration of L-glutamic acid to L-pyrrolidone carboxylic acid followed by ring opening in the presence of ethylamine to yield theanine (374 g kg\(^{-1}\)).

More recently, theanine was produced in four steps starting from commercially available \(N\)-phthaloyl-L-glutamic acid, which was dehydrated to the corresponding cyclic anhydride by reaction with acetic anhydride and then the ring was opened by reaction with ethylamine. Subsequent deprotection of the amine unit with hydrazine hydrate gave theanine with a 700 g kg\(^{-1}\) overall yield.

Chemical synthesis of theanine offers a simple, convenient and cost-effective alternative to direct extraction of the amino acid from natural sources or preparation by biosynthetic methods. However, limitations exist for the use of synthetic theanine as a food supplement or ingredient because of increasing consumer resistance to the inclusion of so-called ‘non-natural’ additives in the diet. In addition, theanine produced by some processes requires protection and de-blocking procedures for its reactive groups,

Biosynthesis of theanine

In the tea plant, theanine is biosynthesised from glutamic acid and ethylamine by the enzyme theanine synthetase. The enzyme is very labile and cannot be used to produce the amino acid in commercial quantities. Therefore, other methods for the enzymatic synthesis of theanine have been developed using bacterial enzymes such as glutaminase, glutamine synthetase and \(\gamma\)-glutamyltranspeptidase.

A glutaminase from *Pseudomonas nitroreducens* has been used which can simultaneously hydrolyse glutamine to ammonia...
and glutamic acid and catalyse the reaction of glutamine with ethylamine to form theanine.\(^6\) However, the use of glutamine in preference to glutamic acid as the starting material in this process suffers from the disadvantage of being more expensive, more time consuming and from glutamine being less stable.

The enzyme glutamine synthetase and related enzymes, originating from *Escherichia coli*, \(^6\) *Pseudomonas taioreldens*, \(^6\) *Methylovorus mays*, \(^6\) and *Bacillus subtilis*, \(^6\) have been utilised for the synthesis of theanine from glutamic acid and ethylamine. Unlike the glutaminase biosynthetic pathway discussed previously, this glutamic acid transformation, while using a cheaper and more stable starting material, requires a continuous supply of ATP to drive the reaction.\(^6\) Another non-ATP-dependent biosynthetic pathway, involving \(\gamma\)-glutamy1transpeptidase from *E. coli* and glutamine as the starting substrate, has also been successfully developed but the reaction requires a high concentration of ethylamine to drive the conversion of glutamine to theanine.\(^5\,6\,4\,5\,6\)

Despite some limitations, theanine biosynthesis shows great potential as an industrial-scale preparation method. Enzymes are also stereospecific and offer the advantage of producing the naturally occurring L-(S) enantiomer of theanine (Fig. 1) thereby overcoming a drawback that synthetic production techniques have in producing a racemic mixture of L- and D-forms.\(^2\) However, future studies need to focus on method optimisation parameters including product purity, yield maximisation and the reduction of production costs associated with scale-up procedures.

Table 3. Biosynthesis of theanine using bacterial enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Brief description</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaminase</td>
<td>The glutaminase isolated from <em>Pseudomonas nitroreducens</em> IFO 12 694 was incubated for 7 h at 30°C and pH 11 to simultaneously hydrolyse glutamine to glutamic acid and to react with ethylamine to form theanine. The theanine was continuously synthesised from glutamine and ethylamine using immobilised <em>P. nitroreducens</em> IFO 12 694 cells for 12 days at 30°C and pH 9.5.</td>
<td>58, 59</td>
</tr>
<tr>
<td>Glutamine synthetase and related enzymes</td>
<td>The theanine was synthesised from glutamic acid and ethylamine using a glutamine synthetase from <em>P. taioreldens</em> Y-30 with sugar fermentation by baker's yeast cells as an ATP-regeneration system. The theanine was formed from glutamic acid and ethylamine using a (\gamma)-glutamylmethylamide synthetase from <em>Methylovorus mays</em> coupled to yeast sugar fermentation for ATP regeneration. The theanine was synthesised from glutamic acid and ethylamine using a glutamine synthetase from <em>Bacillus subtilis</em> coupled with an alcoholic fermentation system.</td>
<td>61, 62, 63</td>
</tr>
<tr>
<td>(\gamma)-Glutamyl-transpeptidase</td>
<td>The theanine was synthesised from glutamic acid and ethylamine using (\gamma)-glutamyltranspeptidase from <em>Escherichia coli</em> k-12 SH642 at pH 10 and 37°C over 2 h. The (\gamma)-glutamyltranspeptidase from <em>E. coli</em> k-12 MG1655 catalysed the reaction between glutamic acid (\gamma)-methyl ester and ethylamine to form theanine at pH 10 and 45°C over 8 h.</td>
<td>64, 65</td>
</tr>
</tbody>
</table>

Figure 4. Biosynthesis of L-theanine using bacterial enzyme systems.\(^5\).

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>360</td>
</tr>
<tr>
<td>Caffeine</td>
<td>30</td>
</tr>
<tr>
<td>Theanine</td>
<td>15–20</td>
</tr>
<tr>
<td>Protein</td>
<td>150</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>5</td>
</tr>
<tr>
<td>Organic acids</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4. Composition of soluble components in dry tea leaf

ISOLATION OF NATURAL THEANINE FROM TEA

Extraction methods for theanine from tea

There has been an increase in demand for natural foodstuffs and food additives perceived to impart therapeutic benefits. Similarly, the stress-relieving and other beneficial properties reported for theanine (Table 1) has led to a rising demand for naturally sourced extracts of the amino acid.\(^2\)\(^4\) Therefore, significant research has been directed towards optimising theanine extraction from tea leaves. However, isolation of theanine in high purity presents a considerable challenge due to the presence of other soluble substances, such as caffeine and polyphenols, in significant concentrations (Table 4).

Several attempts have been made to isolate theanine from tea but these methods still have limitations. For example, Zhang *et al.* \(^6\) isolated theanine from green tea by using preparative HPLC. The catechins were initially extracted from green tea with ethyl acetate. The theanine was then extracted from the tea with water.
at 50 °C and purified using a 732 cation exchange column to yield an extract containing 500 g kg⁻¹ theanine. Final purification was achieved using a preparative C18 reversed-phase HPLC column with a formic acid mobile phase buffered to pH 3.0. While the final theanine purity from the procedure was high (980 g kg⁻¹), the low overall yield (2.53g day⁻¹) coupled with high production costs and limited scale up potential make this method unappealing as an industrial purification method.

Lachová et al.⁶⁷ used molecularly imprinted polymer (MIP) technology to selectively isolate theanine from green tea extracts. Two separate MIP formulations were prepared from Nylon-6 dissolved in formic acid using phase inversion techniques. The polymers were then washed with acetic acid solution to remove the template from the generated imprint cavities. The selectivity of theanine rebinding to the MIP was then evaluated by solid phase extraction. While the theanine recovery proved satisfactory (880 g kg⁻¹), the analyte purity was not stated and appeared to be low.⁶⁷

A number of patents have been developed covering theanine extraction. Ekamayake and Li⁶⁸ successfully developed a theanine extraction method which avoids the use of organic solvents. A hot-water extract of tea leaves was passed through a preparative column packed with a polyamide solid phase. Collection of theanine rich fractions was then undertaken. While cost-effective, the procedure yielded theanine contaminated with impurities such as saccharides, polyphenols and caffeine. Another patent by Baudouin⁶⁹ reported purifying theanine by extracting black tea with water, then filtering and drying the extract to obtain a theanine-rich solid. The extract was then redissolved and purified by fractionation using cation exchange chromatography with Diaion UBKS50. However, the final theanine purity was relatively low (440 g kg⁻¹) and the production procedure was complicated and long.

In summary, several attempts have been made to isolate natural theanine from tea. While variable in success, all methods involve laborious and time consuming protocols that give relatively low yields of theanine. None of the methods outlined offer a reliable and cost-effective procedure for commercial-scale harvesting of high purity theanine. Therefore, there is a need for further investigation of extraction and purification methods for the isolation of theanine from tea.

Future perspectives for isolating theanine from tea

The starting material for the isolation of natural theanine is abundant because world tea production is high and increasing at an annual growth rate of 2%.⁷⁰ Like other bioactive compounds in plants, theanine can potentially be extracted and isolated using several steps, which fall into several basic categories: starting material treatments, extraction of tea constituents from tea leaves into solvents, separation of theanine from other soluble components and drying of the theanine to obtain a powder (Fig. 5).

As mentioned previously, the theanine content in tea varies in accordance with the growing location, the method of cultivation, the tea grade, the tea variety and the time of harvest.⁶¹,¹⁰,¹¹ In order to obtain a high yield of theanine, teas possessing a high natural theanine content should be selected as the starting material for extraction and isolation. To accelerate theanine release and thereby improve extraction efficiency, dried tea should be finely ground.⁷¹ However, it should be noted that the extraction of constituents from tea ground to small particle sizes is only quicker, compared to large particle sizes, when the solution is well agitated.⁷²

Before separating it from the other components in tea, the theanine and other components are extracted into solvents. The extraction process can be efficiently conducted by employing novel extraction techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), ultrahigh pressure extraction (UHPE) and subcritical water extraction (SWE).⁷² In comparison with conventional extraction techniques, these novel extraction techniques possess advantages such as shorter extraction times, reduced solvent consumption, higher extraction yields and better quality final extracts.⁷²

However, the conditions for each extraction technique need to be further studied to optimise the extraction of theanine. For example, MAE is a promising method for the extraction of tea constituents but the conditions for MAE, such as solvent character, volume of solvent, pre-leaching time, MAE time and microwave power, are important and have to be optimised to achieve a high extraction efficiency.⁷³ Similarly, SFE is also an effective extraction method for plant bioactives. However, temperature, pressure and solvent character must be carefully controlled to optimise extraction efficiencies without affecting the recovery rate.⁷⁸

Once in solution, theanine can potentially be separated from the other tea components, such as caffeine and catechins, by employing column chromatography, membrane separation, resin adsorbency and solid phase extraction techniques. Column chromatography, such as preparative reverse-phase HPLC and high-speed counter current chromatography, has been widely used for the separation of natural products.⁷⁴,⁷⁵ However, safe solvent systems for extracting bioactive compounds and for use as mobile phases in chromatographic separation procedures need to be developed to replace undesirable solvents such as chloroform, ethyl acetate and acetone, which are unsafe because of their known impacts on human health.

Membrane technology can potentially be used to separate theanine from other compounds based on their molecular size. The disadvantage of this approach is that the membranes can often foul during operation, resulting in low permeate flux rates.⁷⁶ Synthetic resin adsorbents such as macroporous polymeric and polyamides have been successfully used to isolate caffeine and catechins in tea.⁷⁷,⁷⁸ The potential therefore exists for these materials to be utilised for the isolation of theanine. Alternatively, solid phase extraction (SPE) has been widely used for the separation and isolation of analytes from many different solutions, and may therefore find potential application in theanine isolation.⁷⁹ However, the materials and methodology used in SPE need to be further studied in order to improve their effectiveness and capacity for the isolation of significant quantities of theanine.

Once isolated in solution, theanine powder preparations can then be obtained through drying processes; theanine is stable in solid form and can be transported and stored for long periods without loss of quality.⁸⁰ However, further studies are required to identify the optimal drying processes and conditions to minimise energy consumption and production costs.

CONCLUSIONS

Theanine is a unique amino acid predominately found in tea where it contributes significantly to the taste quality of the infusion. Theanine has also been associated with benefits on human function
and health such as enhancement of relaxation, improvement of learning ability, prevention of cancer and cardiovascular disease, promotion of weight loss and improvement of the immune system. Due to its favourable contribution to taste and health benefits, there is a perceived increasing demand for its use in dietary supplements, food additives and functional foods in recent years. Many efforts have been made to synthesise and produce theanine to meet this demand. Synthetic and biosynthetic methods for theanine preparation have been reported, with procedures varying in complexity, yield and product purity.

Interest in more-natural health additives has also seen an increased demand for naturally sourced theanine. Isolation of theanine from tea has been accomplished. However, the methods used are generally complex, time consuming and costly. Therefore, further research is needed to develop safe, sustainable, environmentally friendly and cost-effective methods for the isolation of theanine from tea in high yield and purity.

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1.3.5. Caffeine in green tea: its removal and isolation

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Caffeine in Green Tea: Its Removal and Isolation

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Abstract

The world production and consumption of green tea is increasing. Although known as a rich source of the strong antioxidants, the catechins, green tea also contains high levels of caffeine. Being a central nervous stimulant, caffeine can cause negative effects in some people and this has led to a demand for decaffeinated green tea. On the other hand, caffeine is also widely used as an additive in the beverage and pharmaceutical industries and therefore, there is a potential market for the caffeine extracted during the decaffeination of green tea. In this article, the numerous decaffeination methods developed to produce decaffeinated green tea and decaffeinated green tea extracts and powders are reviewed and their advantages and limitations are discussed. Undoubtedly, future studies will need to focus not only on the productivity, production costs and the quality of the products, but also on the safety, human health and environmental impact implications of the processes used and the products produced. Priority will need to be given to the development of safe, sustainable, and environmentally friendly methods for the decaffeination of green teas and on the isolation of caffeine from these teas.

Keywords: Decaffeination; Decaffeinated tea; Caffeine; Isolation; Green tea.
1. Introduction

Caffeine is considered a “stimulant” and it is the most widely consumed stimulant in the world (Chou & Benowitz, 1994). Approximately 75% of the consumed caffeine is taken in the form of coffee followed by tea (16%) and the rest from other sources (Chou & Benowitz, 1994). Caffeine levels in coffee (80-135mg/serving of brewed coffee) are usually higher than in tea (20-40mg/serving of tea). However, it is interesting to note that the level of caffeine in coffee beans (≤ 3% of dry weight) is often lower than that contained in green tea (2-5% of dry weight) (Chou & Benowitz, 1994; Chu & Juneja, 1997). Obviously, the difference in the levels of caffeine between the two beverages is simply because more coffee beans (5-10g) are used to prepare a serving of coffee compared to the amount of tea (1-3g) used to prepare a serving of tea.

Studies have linked caffeine consumption with various human health benefits such as enhancement of cognitive functioning, improvement of neuromuscular coordination, elevation of mood and relief of anxiety (Glade, 2010), stimulation of the central nervous system and the cardiac muscle (Griffiths & Woodson, 1998). Therefore, caffeine has been used as an additive for soft drinks and energy drinks. Caffeine has also been added to pharmaceuticals to improve analgesic effects (Chou & Benowitz, 1994; Kumar & Ravishankar, 2009). A recent review article states that 74% of total caffeine production is utilized for beverages while 25% of total caffeine goes to pharmaceutical preparation and the rest for other purposes (Kumar & Ravishankar, 2009).

Due to the substantial potential for its utilisation in the food and pharmaceutical industries, numerous attempts have been made to produce caffeine powder and several methods have been developed to isolate caffeine, mainly from coffee. However, with the demand for naturally sourced caffeine extracts for use in foodstuffs increasing (Vuong, Bowyer, & Roach,
there is a need to find other sources, besides coffee, for the isolation of caffeine. Green tea is such a potential alternative source for producing commercial quantities of caffeine because it contains high levels of caffeine and its production is increasing around the world at the annual rate of 3.8%; it is projected to reach 1.57 million tons by 2017 (Vuong, Stathopulos, Nguyen, Golding, & Roach, 2011).

On the other hand, there has also been an increasing concern in relation to the side effects of caffeine on human health. High consumption of caffeine is thought to be associated with arrhythmia, tachycardia, vomiting, convulsions, coma or even death (Kerrigan & Lindsey, 2005). Furthermore, consumption of caffeine has been found to cause irritation of the gastrointestinal tract and sleeplessness in certain cases (Chu & Juneja, 1997).

Therefore, there is also an increasing demand for decaffeinated versions of the common beverages, from those who would like to consume these beverages without the potential harmful effects of caffeine. In response to this demand, decaffeinated versions of coffee have been widely available for some time now. Similarly, there is a demand for decaffeinated versions of black and green tea and therefore, there is an increasing interest in the methods used to extract the caffeine from coffee and whether they can be applied for the decaffeination of these teas. However, because of an increasing environmental awareness among consumers, methods that are environmentally friendly and sustainable are also more likely to be the most acceptable techniques for the decaffeination of tea.

2. Green tea and caffeine

Tea (Camellia sinensis) is the most widely consumed beverage in the world after water (Vuong, Golding, Nguyen, & Roach, 2010). It was first used as a herbal beverage in China, from where it
was gradually introduced to other countries and presently, it is becoming a popular drink on all continents of the world (Cheng, 2006; Vuong, 2012).

At first, fresh tea leaves were used to prepare the tea beverage. Subsequently, fresh tea leaves were used to make different types of processed teas for trading purposes. Presently, six different types of processed tea are produced from fresh tea leaves, usually using the apical bud and the next four to five leaves on the growing shoots. These are green, black, oolong, yellow, pu-erh and white teas (Vuong, 2012). Of these, black tea is the most consumed tea in the world, accounting for 78% of total tea consumption. Black tea is followed by green tea with 20% consumption, while the rest comprise only 2% of the world’s tea consumption (Cheng, 2006; Vuong, 2012).

Green tea is produced by first quickly inactivating the oxidative enzymes in the fresh leaves under heat, then rolling the leaves and finally drying to obtain dried green tea. Green tea is a rich source of bioactive components such as caffeine, catechins, and theanine (Vuong, Golding, Stathopoulos, Nguyen, & Roach, 2011; Vuong, Stathopoulos, Golding, Nguyen, & Roach, 2011). In fresh tea leaves, caffeine accounts for 14-28 mg/g of tea leaf; however, it is higher in dried green tea with a range from 20 to 50 mg/g of dried green tea (Lin, Tsai, S., Tsay, & Lin, 2003; Vuong, Nguyen, Golding, & Roach, 2011). The levels of caffeine vary because of different factors such as the tea plant species or variety or the type of leaf harvested. For example, the level of caffeine in *C. Sinensis* is higher than in *C. ptilophylla* or in *C. assamica Var. kucha* (Yang, Ye, Xu, & Jiang, 2007). Similarly, fresh tea leaves harvested from growing shoots also contain higher amounts of caffeine than those harvested from more mature (older) parts of the plant.
Due to the high levels of caffeine in green tea, some people may not want to consume regular green tea because they want to avoid caffeine and for these people, decaffeinated versions may be preferable. To satisfy this potential market, the methods used to remove caffeine need to be tested to determine whether acceptable decaffeinated green teas can be produced. For example, green tea is characterised by its high content of epicatechins and therefore, the effect of the decaffeination methods on the epicatechins needs to be considered. Furthermore, as noted above, increasingly, methods that are environmentally friendly and sustainable are also more likely to be better accepted by the consumers of decaffeinated green tea.

At the same time, the caffeine extraction methods also need to be tested to see if acceptable caffeine preparations can be produced from green tea for use in the food and pharmaceutical industries thereby adding commercial value to the decaffeination process. For example, can the extracted caffeine be separated from the other major green tea constituents, the catechins and the amino acid theanine.

Therefore, this paper first outlines the properties of caffeine and the methods used for its extraction and measurement. The different techniques used for decaffeination and their use so far for the decaffeination of green tea are then examined.

3. Properties of caffeine

Caffeine is a trimethyl derivative of purine 2,6-diol. In powder form, caffeine is colorless and odorless with a slightly bitter taste (Chu & Juneja, 1997). Caffeine sublimes at 180°C and melts at 136°C under pressure (Tarka & Hurst, 1998). The solubility of caffeine varies depending on the type of solvent and the temperature, as shown in Table 1. At room temperature, caffeine dissolves best in chloroform (18% w/v). In water, the solubility of caffeine is approximately 2%
(w/v) at room temperature. However, caffeine dissolves well in boiling water, with its solubility increasing to 70% (w/v) at 100°C (Ramalakshmi & Raghavan, 1999).

In aqueous solution, caffeine can be crystallised as a monohydrate (Tarka & Hurst, 1998). However, it can associate to form dimers and polymers and it also associates with purines and pyrimidines, either the free bases or their nucleosides. Caffeine also forms complexes with chogenic acid, isoeugenol, coumarin, indole-acetic acid, and anthoxyanidin. This formation of complexes can modify the physiological effects of caffeine by affecting its solubility, absorption and bioavailability. For example, the solubility of caffeine is increased in the presence of alkali benzoates, cinnamates, citrates, and salicylates (Tarka & Hurst, 1998). In addition, caffeine also reacts as a feeble base with acids to form salts, which are very readily hydrolysed (Tarka & Hurst, 1998).

In green tea, there are three methyl xanthines: caffeine, theophylline and theobromine. Caffeine is the major substance (2-5% of dry weight) with theophylline and theobromine only accounting for 0.2-0.4 % and 0.02 % of dry weight, respectively (Chu & Juneja, 1997). Caffeine is synthesized in the tea leaves from adenosine, which is a major product of RNA metabolism throughout the life of the tea plant (Figure 1), but it also occurs during the withering stage of other teas such as black and oolong teas (Astill, Birch, Dacombe, Humphrey, & Martin, 2001; Balentine, Harbowy, & Graham, 1998).

During the extraction of green tea with boiling water, caffeine dissolves faster than the catechins but slower than theanine. The water solubility of caffeine is 21.7 g/L of boiling water whereas the water solubility of the major green tea catechin, epigallocatechin gallate (EGCG), is 5g/L and that of theanine is 556 g/L (Liang et al., 2007; Vuong et al., 2010). Variation in the water solubility can be explained by differences in the molecular mass of caffeine, EGCG and
The molecular mass of caffeine is 194.2 g/mole whereas the molecular masses of EGCG and theanine are 458.4 g/mole and 174.2 g/mole, respectively.

The extraction of caffeine from green tea is low when the tea is extracted using cold water or pure organic solvents such as acetone, methanol, ethanol or acetonitrile. In contrast, caffeine is well extracted using boiling water or water-organic solvent mixtures. For example, caffeine is found to only be extracted at very low levels with pure methanol or ethanol but its extraction can be significantly increased in 70% (v/v) methanol or 50% (v/v) ethanol and water mixtures (Jun, 2009; Wong et al., 2009).

In the resulting green tea extracts using boiled water (the green tea beverage), caffeine can bind with the catechins through π–π interactions (Charlton et al., 2000; Ishizu, Tsutsumi, & Sato, 2009). These complexes of caffeine and catechins can come out of solution and result in “cream formation” in the green tea extract when it is cooled down to below 10°C (Vuong et al., 2010). It should be noted that this “cream formation” depends on several factors, including the brewing temperature, the pH of the brewing solution and the presence of minerals such as calcium, magnesium and aluminum; a high brewing temperature, a low pH of the brewing solution and the presence of minerals all increase “cream formation” (Liang, Lu, & Zhang, 2002; Liang & Xu, 2001). This “cream formation” property of caffeine can also be used to isolate caffeine and the catechins from the other components in green tea.

4. Measurement of caffeine

The maximum spectrophotometric absorption of caffeine is in the ultraviolet range of 250-280nm (Tarka & Hurst, 1998). Therefore, several methods have been developed to determine the level of caffeine in samples using this absorption range. The most popular and effective method
is High Performance Liquid Chromatography (HPLC). The HPLC system is usually equipped with a reverse phase C$_{18}$ column and the detection wavelength ranges from 273-280nm (Bispo et al., 2002; Hartley, Smith, & Cookman, 1985; He, Lv, Zhou, & Shi, 2010). This HPLC method is the gold standard; however, it requires expensive equipment and organic solvents and it is time consuming. The traditional and less costly UV-VIS spectrophotometer can also be used to quantify caffeine at the same UV wavelengths after a specific extraction process (Alpdoğan, Karanina, & Sungur, 2002; Atomssa & Gholap, 2011; Ishler, Finucane, & Borker, 1948). This spectrophotometric method is fast, simple and does not require expensive equipment and organic solvents in comparison with the HPLC method (Alpdoğan et al., 2002).

Caffeine can also be determined using an oscillating chemical reaction in a continuous-flow stirred tank reactor (Gao, Ren, Yang, Liu, & Yang, 2003) but this method is complicated and requires strict control of factors like temperature, stirring rate and injection point. The micro-volume liquid–liquid flow-extraction system with one-capillary spectroscopy is also effective at determining the level of caffeine in samples (Carlsson & Karlberg, 2000). However, ultimately, the method chosen for the quantification of caffeine in samples mainly depends on the availability of the equipment needed for that particular technique.

5. Decaffeination of green tea

Typically, a 250 mL cup of green tea contains 10 – 45mg of caffeine and the level can vary depending on the amount of tea, the volume of boiled water used, the brewing temperature and the length of brewing time (Astill et al., 2001; Kerrigan & Lindsey, 2005; Vuong, Tan, Stathopoulos, & Roach, 2012). In the human body, the low level of caffeine achieved by drinking such beverages mostly works as a mild central nervous stimulant. However, even at low
quantities, caffeine may cause irritation of the gastrointestinal tract, fatigue and sleep deprivation in some people (Chu & Juneja, 1997). Plus, when taken in large quantities (> 5g), caffeine can be severely toxic with grave effects including arrhythmia, tachycardia, vomiting and convulsions and it can even cause coma and death (Kerrigan & Lindsey, 2005).

Therefore, because of the adverse effects associated with caffeine, decaffeination is a big industry and the demand for decaffeinated teas, including green tea, is increasing from health conscious consumers (Kumar & Ravishankar, 2009). Many attempts have been made to produce decaffeinated teas to meet the market demand. Presently, there are two main types of decaffeinated teas; decaffeinated tea leaves and decaffeinated tea powder. For teas or tea powders to be labeled as “decaffeinated”, the maximum caffeine levels are limited to 4 mg/g of dry weight for tea leaves and 10mg/g for tea powders (Ye et al., 2007).

In the following section, the principles behind the various decaffeination methods and the advantages and limitations of these methods for the production of decaffeinated green tea leaves or decaffeinated green tea powders will be covered.

5.1. Decaffeination using organic solvents

Caffeine was first isolated in the late nineteenth century and, up to the mid-1970s, all the decaffeination processes were conducted using organic solvents such as benzene, chloroform, petroleum ether, methylene chloride, trichloroethylene, carbon tetrachloride, acetone, methanol and ethanol and strong bases and acids such as ammonium hydroxide and sulfuric acid (Ramalakshmi & Raghavan, 1999). The advantage of using organic solvents for decaffeination was the relatively low operating costs. However, due to the health concerns related to the use of
organic solvents, their use for decaffeination has significantly decreased in recent years (Kumar & Ravishankar, 2009).

Organic solvent decaffeination is generally done by first softening the tea leaves in water and then mixing in the organic solvent of choice. Caffeine is about ten times more soluble in organic solvents than in water at room temperature (Senol & Aydin, 2006) and therefore, most of the caffeine is extracted from the leaves into the organic phase, but the other major components of green tea, the catechins and theanine remain in the leaves, which are then dried to obtain decaffeinated green tea.

Methylene chloride was known as the best and most popular solvent used for decaffeination until the mid-1970s. However, use of this solvent is presently limited because it has been identified as a contributor to the depletion of the ozone layer and also because it can leave toxic residues behind in the decaffeinated products (FDA, 2012; Ramalakshmi & Raghavan, 1999). Similarly, the use of other organic solvents such as chloroform, benzene, trichloroethylene, carbon tetrachloride and acetone in the decaffeination process is now restricted by the US Food and Drug Administration (US FDA) because of their inherent toxicity (FDA, 2012).

Ethyl acetate, also known as acetic acid ethyl ester, is widely used for the extraction of caffeine and polyphenols from teas and coffee for the preparation of tea and coffee extracts and powders (Kumar & Ravishankar, 2009). Ethyl acetate is an ester and is a clear, volatile, flammable liquid, with a fruity flavor and a pleasant taste when diluted (Ramalakshmi & Raghavan, 1999). As it is found in many fruits, such as apples, peaches, and pears and is completely digestible, it has been used in a wide range of foods such as salad dressings, fruit
desserts and has been approved for decaffeination by the US FDA since 1982 (Kumar & Ravishankar, 2009; Ramalakshmi & Raghavan, 1999).

Ethyl acetate is considered to be the solvent of choice in comparison with the other organic solvents, which can be used for preparing tea and coffee extracts and powders including decaffeinated products (Kumar & Ravishankar, 2009). After the caffeine and polyphenols are extracted into the ethyl acetate, a 1% (w/v) citric acid aqueous solution can be used to efficiently extract the caffeine from the organic solvent. Using ethyl acetate followed by the citric acid aqueous solution in this manner, is more effective at producing green tea extracts and powders with high levels of catechins and low levels of caffeine in comparison with the use of other organic solvents such as n-butanol, n-hexane and chloroform to produce decaffeinated green tea extracts and powders enriched in catechins. (Dong, Ye, Lu, Zheng, & Liang, 2011).

5.2. Decaffeination using carbon dioxide

Due to the increase in consumer concerns toward chemical residues in decaffeinated products, supercritical fluid extraction with carbon dioxide (SFE-CO₂) has been proposed and used as a selective and commercially viable method for the removal of caffeine from teas (Vuong et al., 2010). The carbon dioxide decaffeination method was discovered by Kurt Zosel and patented in the early 1970s (Ramalakshmi & Raghavan, 1999). Since then, research on decaffeination using SFE-CO₂ has been intensive and the resulting methods have been covered by several patents (Margolis & Chiovini, 1981; Theissing, Saamer, & Korner, 1991) and described in many publications (Huang, Wu, Chiu, Lai, & Chang, 2007; Kim, Kim, & Oh, 2007; Park, Choi, et al., 2007; Park, Im, & Kim, 2012; Park, Lee, et al., 2007).
The decaffeination of green tea using SFE-CO$_2$ is usually done by first grinding the tea leaves into small particle sizes (<1mm in diameter). The ground tea leaves are then soaked in a co-solvent (e.g. 95:1, ethanol:water), to enhance the extraction of the caffeine (Park, Lee, et al., 2007), and are loaded into an extraction vessel that is sealed. Liquid carbon dioxide is pumped in at a designated pressure and the back pressure is continuously monitored. The liquid carbon dioxide is then heated and pumped into the extraction vessel to extract the caffeine from the ground tea leaves (Park, Choi, et al., 2007; Park et al., 2012). The caffeine, dissolved in the supercritical carbon dioxide, is separated from the carbon dioxide and collected by a reduction in pressure which occurs in the separator sections of the SFE-CO$_2$ extractor, (Park et al., 2012; Park, Lee, et al., 2007). A typical decaffeination process using SFE-CO$_2$ is shown in Figure 2.

It should be noted that the outcomes of the decaffeination process using SFE-CO$_2$ are influenced by various factors including the tea particle size, the co-solvent used to soak the tea in and the temperature, pressure and flow rate of the CO$_2$ (Park et al., 2012; Park, Lee, et al., 2007). These factors need to be well monitored and controlled in order to obtain high decaffeination efficiencies. The main limitation of the SFE-CO$_2$ decaffeination method is the high setup costs. However, this is outweighed by several advantages such as it being a fast process with no toxic residues, less degradation of the tea catechins and a high retention of the tea flavours (Ramalakshmi & Raghavan, 1999; Vuong et al., 2010).

5.3. Decaffeination using water

Water is a safe and environment-friendly solvent and it is relatively inexpensive and accessible in comparison with organic solvents (Vuong, Golding, et al., 2011). Therefore, it is usually considered as a solvent of choice for any separation or isolation process. Water was initially used
for the decaffeination of coffee in 1938 by the Coffex Company (Ramalakshmi & Raghavan, 1999). Since then, two types of water extraction methods have been developed to decaffeinate coffee beans - the Swiss and the French water decaffeination techniques (Figure 3). It is acknowledged that the advantage of the Swiss water process is that it produces decaffeinated coffee beans with a richer flavour whereas the advantage of the French water process is that it results in decaffeinated coffee beans with higher solids and less moisture (Ramalakshmi & Raghavan, 1999).

Several attempts have been made to use water for the extraction of caffeine from the tea leaves to produce decaffeinated green tea (Liang et al., 2007; Vuong, Golding, Nguyen, & Roach, 2012). The decaffeination process is similar to the initial steps for the water decaffeination of coffee (Figure 4). Water decaffeination is done by first blanching freshly harvested green tea leaves in boiling water for a short period of time. As the water solubility of caffeine is higher than the solubility of the tea catechins (Vuong et al., 2010), most of the caffeine can quickly be extracted into the boiling water whereas the catechins mostly remain behind in the tea leaves. The leaves are then quickly removed from the boiling water, containing the caffeine, and are then dried to obtain decaffeinated dried green tea.

The water decaffeination process is influenced by the water temperature, the length of the blanching time, the ratio of water-to-tea and the type of fresh tea leaves used (Liang et al., 2007; Vuong et al., 2012). The optimal conditions for decaffeinating young fresh green tea leaves (the bud and up to four leaves) are 100°C, blanching time of 3 min and a water-to-tea ratio of 20:1 mL/g. With these optimal conditions, 83% of the caffeine can be removed and 95% of the catechins retained in the decaffeinated green tea leaves (Liang et al., 2007). However, for decaffeinating more mature, old tea leaves, from the fourth to the tenth leaves down the stem,
Vuong et al. (2012) found that the optimal blanching time at 100°C and the water-to-tea at 20:1 mL/g, was longer (10 min) than for the young tea leaves. Under these conditions, 80% of the caffeine was removed while 85% of the catechins were retained in the decaffeinated green tea (Vuong et al., 2012).

The water decaffeination process is inexpensive and is without the risk of organic solvent residues being left behind in the decaffeinated product. However, this method may cause the loss of flavour and of theanine, a valuable amino acid unique to tea in the plant world (Vuong, Bowyer, & Roach, 2011). However, as seen in Figure 3 for coffee, flavour and other solids can be recovered using the Swiss and the French water decaffeination processes. Therefore, although not looked into yet, similar methods may be able to be developed to retain the flavour and theanine components of green tea, which are potentially lost during the decaffeination of tea using boiling water.

5.4. Decaffeination using absorbents

Absorbents have been widely studied for the separation of bioactive components because they are thought to have the advantages of high efficiency and low toxicity and of being a relatively uncomplicated process (Vuong et al., 2010). Absorbents, which can bind caffeine through hydrophobic interactions, have been used.

In a recent study (Ma et al., 2012), various copolymers of triallyl isocyanurate (TAIC) and vinyl acetate (VA), referred to as poly(TAIC-co-VA), were synthesized by free radical suspension copolymerization of TAIC and VA with n-heptane and butyl acetate as porogenic agents and α,α′-azobis (isobutyronitrile) (AIBN) as the initiator. The copolymers were treated with organic solvents and then dried to form macroporous polymeric adsorbents with different
hydrophilic and hydrophobic affinities. Green tea powders with different caffeine to catechin ratios were dissolved in water and mixed with the different copolymers. In this fashion, it was found that the copolymer with 20% TAIC in its structure could remove most of the caffeine from the green tea extracts through hydrophobic interactions while leaving behind >95% of the catechins (Ma et al., 2012). Poly(acrylamide-co-ethylene glycol dimethylacrylate) and polyvinylpolypyrrolidone have also been found to be useful absorbents for the decaffeination of green tea (Dong et al., 2011; Lu et al., 2010).

Another absorbent method, using molecular imprinted polymers (MIPs), has received considerable interest because MIPs have the features of being easy and inexpensive to synthesise, having high selectivity and can be made to work under different conditions, especially under extreme pH values and with organic solvents (Jin & Row, 2007).

The MIPs are synthesised by complexing a template molecule (i.e. the analyte of interest) with appropriate functional monomers capable of forming polymers, which surround the template molecule through interactions via covalent or non-covalent bonds (Farrington, Magner, & Regan, 2006; Theodoridis et al., 2006). The most common bonding is non-covalent via hydrogen bonding, electrostatic attraction or hydrophobic interaction, which leads to the template molecule being surrounded by the polymer as it is rigidly cross-linked into a polymer matrix monolith using an appropriate cross-linker (Theodoridis et al., 2006). In this way, multiple casts of the template molecule are imprinted within the polymer matrix. The template molecule is then removed by washing with appropriate solvents and the resultant imprinted polymer has the ability to selectively adsorb the template molecule from solution in preference to other molecules through a lock and key interaction mechanism (Farrington et al., 2006; Theodoridis et al., 2006). Finally, the targeted molecule can be eluted from the polymer and
thereby purified several fold from the original solution through what is essentially affinity chromatography.

In a recent study, a MIP was synthesised, to remove caffeine from green tea, by using methacrylic acid (MAA) as the monomer, ethylene glycol dimethacrylate (EGDMA) as the crosslinker, $\alpha,\alpha'$-Azobis (isobutyronitrile) (AIBN) as the initiator and caffeine as the template molecule. The resulting MIP was packed in a cartridge, which was washed with, 90:10 methanol:acetic acid and then methanol before green tea was passed through it for removal of its caffeine. After elution with 90:10 methanol:acetic acid, the main component adsorbed on the MIP was found to be caffeine; the purity of the eluted caffeine was 85%. Therefore, MIPs have been shown to have potential for the separation of caffeine from green tea (Jin & Row, 2007).

In general, these absorbent methods can be effective at separating caffeine from the other green tea components and could potentially be used for industrial production of decaffeinated green tea extracts and powders and for the purification of caffeine from green tea. However, studies on the use of absorbents for these purposes are limited. Therefore, further studies are needed to investigate the usefulness of absorbents for the decaffeination of green tea and the isolation of caffeine from green tea.

5.5. Decaffeination using microorganisms

Since the first report on decaffeination by microorganism in 1971 (Kurtzman & Schwimmer, 1971), numerous studies have been done to investigate the use of microorganisms for removal of caffeine from beverages. Certain microorganisms can use caffeine as their source of carbon and nitrogen for growth and thus, they can be used to reduce the level of caffeine in the cafffeinated samples (Gokulakrishnan, Chandraraj, Sathyanarayana, & Gummadi, 2005). The decaffeination
process is generally done by incubating the microorganisms in the caffeinated solutions under aerobic conditions until most of the caffeine is metabolised and then removing the cells by filtration or centrifugation to obtain the low caffeine solutions (Ramalakshmi & Raghavan, 1999).

Studies show that the bacterial strains, which are able to be used for decaffeination, mainly belong to the *Pseudomonas* and *Serratia* genera (Gokulakrishnan et al., 2005). For example, *Pseudomonas alcaligenes* CFR 1708, *Pseudomonas* sp. NCIM 5235, and *Serratia marcescens* were isolated from soils in tea and coffee plantations and shown to be effective at degrading caffeine in solutions (Babu, Patra, Thakur, Karanth, & Varadaraj, 2006; Dash & Gummadi, 2006; Mazzafera, Olsson, & Sandberg, 1996). Interestingly, a *Bacillus* strain was also found to reduce the level of caffeine in tea leaves; a suspension of *Bacillus licheniformis*, sprayed on the surface of tea leaves, was shown to reduce the level of caffeine in the leaves, which could be used for producing decaffeinated green tea (Ramarethinam & Rajalakshmi, 2004).

In general, microorganisms can potentially be used for the production of decaffeinated green tea. However, these methods have some limitations; they are difficult to scale up for industrial production and require strict control of the conditions, which include the type of bacteria, the initial level of caffeine, the incubating temperature and the length of incubation, in order for the decaffeination process to be effective. Therefore, further studies are needed to optimize the use of microorganisms for the decaffeination of green tea.
5.6. Other decaffeination methods

Other attempts have been made to decrease the caffeine in green teas. Miyagishima et al. (2011) introduced a simple method to produce low-caffeine green tea by delaying the leaf picking period and shortening the post-harvest leaf-rolling process. This reduced the caffeine content while retaining high levels of the catechins, theanine, and vitamin C in the resulting green tea.

This method has potential because no extra processing steps are needed and it is safe because no chemicals are involved. However, although the green tea produced was low in caffeine (12 mg/g) it was not low enough to be called decaffeinated, which is less than 4 mg/g (Ye et al., 2007).

Furthermore, other factors would need to be considered such as the quality and yield of the green tea produced from the late season autumn picking. Such late harvest green teas are usually considered to be of very low quality and the yield is also low because the plants are entering into their dormant period. The effect of the short rolling process on the appearance and quality of the green tea also needs to be considered.

A combination of microwave irradiation for 6 min at 350 W and ice water extraction under vacuum for 2.5 h at 0.1 MPa was also found to remove 88% of the caffeine from green tea while retaining 64% of the catechins in the tea (Lou et al., 2012). This method is safe and also has potential for industrial production. However, the loss of the catechins is relatively high (36%) and further studies are required to optimise the conditions for use of the microwave-enhanced vacuum ice water extraction method for the decaffeination of green tea.

Activated carbon (AC) has also been found to be useful for the decaffeination of green tea. Ye et al. (2007) found that treatment of a green tea extract with AC for four hours could remove 80% of the caffeine while 68% of the catechins were retained in the tea. This method does not require expensive and complicated equipment but it is difficult to scale up for industrial
production and the energy or solvent requirements for the regeneration of the activated carbon,
either by thermal, solvent or electrochemical techniques, can be fairly costly.

Ultrasonic devices have also been found to help in the removal of caffeine from green tea (Tang, Li, LV, & Jiang, 2010). This method is influenced by the power of the ultrasound machine, the extraction pressure, the length of the extraction time, the moisture content of the tea and the temperature used (Tang et al., 2010). Finally, lignocellulose prepared from sawdust has also been found to have potential for the decaffeination of green tea extracts (Sakanaka, 2003).

However, this method is difficult to scale up for industrial production.

6. Purification of caffeine from green tea

Caffeine has been used in numerous beverages such as soft drinks and high energy drinks (Reissig, Strain, & Griffiths, 2009). In addition, caffeine has been used in pharmaceutical preparations as an adjuvant to drugs such as paracetamol (Dash & Gummadi, 2006). Therefore, there is a need for isolating caffeine as a commercial product for use in the beverage and pharmaceutical industries. Presently, the commercially available caffeine is usually prepared from coffee (Dash & Gummadi, 2006). It is conventionally isolated by extracting caffeine from the coffee beans into an aqueous solution and then using a two-phase extraction system with organic solvents such as chloroform or methylene chloride or employing various adsorbents to isolate the caffeine from the other soluble substances (Gokulakrishnan et al., 2005). Supercritical fluid extraction with CO$_2$ has also been used to isolate caffeine (Gokulakrishnan et al., 2005).

Several methods have also been used to isolate caffeine from black teas. As for its extraction from coffee, caffeine is generally isolated by first extracting the black tea components into an aqueous solution and then adding organic solvents, including chloroform and methylene
chloride, to separate the caffeine from the other solubilised tea components. The organic solvent is then evaporated to produce a crude caffeine preparation from which the caffeine is further purified (Gürü & İçen, 2004; Landgrebe, 1993; Pavia, Lampman, & Kriz, 1976; Senol & Aydin, 2006; Tarka & Hurst, 1998).

However, the use of these organic solvents is now becoming more and more restricted because of their deleterious effects on human health, including their carcinogenic properties (Senol & Aydin, 2006). In an attempt to reduce the use of chloroform, (Senol & Aydin, 2006) isolated caffeine from black tea waste by solid-liquid extraction using water and chloroform in a pilot plant battery type extractor. However, water was found to have a low extraction efficiency for caffeine - about two times less than chloroform (Senol & Aydin, 2006). This was a pilot plant study and the system could be easily scaled up for industrial production. However, the use of chloroform would need to be restricted because of its known toxicity, even though water has low extraction efficiency for caffeine.

In comparison with chloroform and methylene chloride, isopropanol is a more suitable solvent for the isolation of caffeine because it is less toxic and also inexpensive. Furthermore, it is more stable (less flammable) under normal storage conditions and caffeine has a high solubility in it (Murray & Hansen, 1995). In a classic method designed for use in undergraduate teaching laboratories, caffeine is isolated by first brewing black tea teabags in boiled water and adding NaCl to the resulting tea. To precipitate the tannins, Ca(OH)₂ is added and after filtration, isopropanol is used to extract the caffeine from the aqueous phase. After evaporating the isopropanol phase, the crude caffeine preparation is solubilised in acetone and then further purified by recrystallization or by sublimation (Murray & Hansen, 1995). However, the crude caffeine obtained after evaporation of the isopropanol phase is still highly contaminated with
tannins and NaCl; this can be improved by washing the isopropanol phase with 10% aqueous NaOH and subsequently drying with anhydrous sodium sulfate prior to evaporation (Hampp, 1996).

However, as discussed in Section 4.1, ethyl acetate is considered to be the solvent of choice in comparison with the other organic solvents for the extraction of caffeine and it has been approved for decaffeination by the US FDA since 1982 (Kumar & Ravishankar, 2009; Ramalakshmi & Raghavan, 1999).

Published methods on the extraction and purification of caffeine from green tea are very limited in comparison with coffee and black tea. Numerous methods have been developed to isolate the catechins from green tea (Vuong et al., 2010) but caffeine is mostly neglected as a waste product during these isolation processes. However, as shown in Figure 5, purified caffeine could be produced from the extraction processes used to make decaffeinated green tea extracts and powders in order to add value to the production of the decaffeinated green teas as well as to provide caffeine for use in the beverage and pharmaceutical industries.

7. Conclusions and future considerations

Numerous methods have been used to remove caffeine and to produce decaffeinated green tea. Organic solvents such as chloroform, methylene chloride, isopropanol and ethyl acetate have been effectively used. However, consumers have increasing health concerns about the use of organic solvents in food processing and therefore, alternatives have been studied.

Supercritical fluid extraction with carbon dioxide has shown potential for the decaffeination of green tea. The process is fast, leaves no toxic residue, produces less degradation of the catechins and less of the flavor is lost. However, it is costly to set up on an
industrial scale. Decaffeination using absorbents, including activated carbon, has also shown promise. Absorbent processes have high efficiencies and low toxicity if organic solvents are not used but they are difficult to scale up for large production. Microorganisms have been found that can reduce caffeine in green tea extracts but this biotechnological method requires strictly controlled conditions and which increases the difficulties and the costs.

The use of water as the only solvent used in the decaffeination process has also been studied because it is without inherent health risks. Simply blanching the green tea in boiling water for 3 to 10min has been found to work quite well and the method is relatively inexpensive and relatively easy to scale up to industrial production. However, as for the decaffeination of coffee, aromas and flavours and some of the important bioactive components of green tea, the catechins and theanine, are also partially lost during the blanching process. Other methods such as delaying the picking of the leaves and shortening the rolling process and the use of the microwave-enhanced vacuum ice-water extraction method also have shown promise for the production of decaffeinated green tea.

Caffeine is associated with some positive health effects and therefore, caffeine will continue to be used as an additive for various beverages and as an adjuvant for several drugs. Therefore, the development of safe, sustainable, and environmentally friendly methods for the purification of the caffeine from green tea will also be required in the future.

In summary, there are growing needs for producing decaffeinated green tea and for purifying caffeine from green tea and therefore, these are areas of interest for future studies. These studies will need to focus not only on the productivity, production costs and the quality of the products, but also on the safety and human health implications of the processes and the products and on the impact of the production processes on the environment. In essence, priority
will need to be given to the development of safe, sustainable, and environmentally friendly methods for the decaffeination of green teas and on the isolation of caffeine from these teas.

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The authors have declared no conflict of interest.
Figure Captions

Figure 1. Biosynthesis of caffeine in tea.

Figure 2. The SFE-CO$_2$ extraction system (Park, Lee, et al., 2007). 1 - CO$_2$ cylinder; 2 - electronic balance; 3 - chiller; 4 - CO$_2$ pump; 5 - controller; 6 - heating bath; 7 - circulation pump; 8 - extraction vessel; 9 - separator 1; 10 - separator 2; V-1 - valve 1; V-2 - valve 2; BPR - back pressure regulator; dotted lines - water; solid lines - CO$_2$.

Figure 3. The Swiss and French water decaffeination processes for coffee.

Figure 4. Water decaffeination process for green tea.

Figure 5. Proposed process for the isolation of caffeine from green tea.
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Vuong, Q. V., Tan, S. P., Stathopoulos, C. E., & Roach, P. D. (2012). Improved extraction of green tea components from teabags using the microwave oven. *Journal of Food Composition and Analysis, 26*. In Press. DOI: [http://dx.doi.org/10.1016/j.jfca.2012.06.001](http://dx.doi.org/10.1016/j.jfca.2012.06.001)


Figure 1
Figure 2
Swiss water process

Soaking in boiled water

Decaffeinated beans

Partial drying

Filtrating using active carbon to absorb caffeine

Partial evaporating

Flavours

Re-mixing

Drying

Decaffeinated coffee beans

French water process

Soaking in boiled water

Water with caffeine, solids, flavours

Filtering using natural filter to absorb caffeine

Partial drying

Re-mixing

Drying

Decaffeinated coffee beans
Fresh green tea leaves

Blanching in boiling water

Caffeinated water  Decaffeinated tea leaves

Drying

Decaffeinated tea leaves
Tea leaves

Solvent isolation → Crude caffeine

Supercritical fluid extraction

Absorbent isolation → Tea polyphenols

Further purification → Drying → Caffeine
Table 1. Solubility of caffeine in various solvents and temperatures. Adapted from Tarka and Hurst (Tarka & Hurst, 1998)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temperature (°C)</th>
<th>Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>25</td>
<td>2.2</td>
</tr>
<tr>
<td>Water</td>
<td>100</td>
<td>66.7</td>
</tr>
<tr>
<td>Alcohol</td>
<td>25</td>
<td>1.2</td>
</tr>
<tr>
<td>Alcohol</td>
<td>600</td>
<td>4.5</td>
</tr>
<tr>
<td>Ether</td>
<td>25</td>
<td>0.3</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>25</td>
<td>2.5</td>
</tr>
<tr>
<td>Chloroform</td>
<td>25</td>
<td>18.0</td>
</tr>
<tr>
<td>Acetone</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>Benzene</td>
<td>25</td>
<td>1.0</td>
</tr>
</tbody>
</table>
1.4. EXPERIMENTAL RATIONALE

As shown in the background section (Section 1.1.) and the five review papers, green tea is a major source of the catechins and it is unique in the plant world as a natural source of the amino acid theanine. Numerous in vivo and in vitro studies have found a positive link between the catechins and theanine and health benefits such as prevention of cancers and cardiovascular diseases, reduction in the risk of obesity and diabetes, and improvement of the immune system.

However, several epidemiological studies have shown that large volumes of green tea (5-10 cups a day) may be required to obtain these health benefits and this is not easy to achieve for many tea drinkers. Therefore, extraction of the catechins and theanine from green tea has been considered in order to provide concentrated preparations (extracts and powders) for use as food supplements or as additives for functional foods and thereby increase the consumption of these green tea bioactive compounds.

Green tea also contains caffeine, which affects certain people, but it is also widely used in the beverage industry as an additive. Therefore, it is important to develop methods to prepare decaffeinated green tea powder for people who want to avoid caffeine and but also to prepare caffeine-enriched preparations for utilisation in the food industry.

Consumers and the food industry are increasingly becoming health-conscious and therefore, the organic solvents traditionally used for extracting plant materials including decaffeination are no longer acceptable. Therefore, to prepare extracts and powders from green tea enriched in catechins and theanine and for the decaffeination of green tea and preparation of caffeine-enriched extracts and powders, it is important to use a safe and eco-friendly solvent such as water.

To use water as efficiently as possible, it is important to optimise the conditions used to extract green tea. The literature review papers showed that several extraction parameters such as temperature, length of extraction, ratio of water-to-tea, tea particle size, pH and the number of times the same tea sample is extracted could affect the efficiency with which the catechins, theanine and caffeine are extracted from green tea. The conditions for using water for the decaffeination of green tea also need to be optimised. In addition, the drying processes and conditions used to prepare green tea powders from the extracts are important as they can significantly affect the recovery rate and the quality of the tea powders. In this study, the two most popular drying methods, freeze drying and spray drying, will be investigated for drying the tea extracts.
Finally, improving the extraction of the green tea bioactive components, when people make their own tea, may also help people obtain the levels of the green tea bioactive compounds needed for health benefits. With the increasing availability of green tea in teabags, the impact of household brewing methods on the efficiency by which the green tea constituents are extracted from teabags needs to be evaluated.

1.5. HYPOTHESIS, AIMS AND OBJECTIVES

Importantly, in this study, no organic solvents were used. Only water, the safe, environment-friendly, inexpensive and accessible solvent was used for the extraction of the green tea constituents.

The hypothesis was that the aqueous extraction of the three bioactive components, the catechins, theanine and caffeine, from loose leaf green tea or green tea in tea bags, could be improved and that aqueous extractions could be used to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders.

Therefore, the overall aims of the study were:

- to improve the aqueous extraction of the three main bioactive components, the catechins, theanine and caffeine from loose leaf green tea,
- to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves using water as the only solvent for the extractions and freeze drying and spray drying to dry the aqueous extracts,
- to improve the extraction of the three green tea bioactive components from green tea in teabags using water and the microwave oven.
Therefore, the **specific objectives** of the study were:

1. **Paper VI:** To comprehensively investigate the impact of six factors (temperature, length of the extraction time, ratio of water-to-tea, tea particle size, pH and the number of times the same sample was extracted) on the yield of catechins extracted from green tea using water,

2. **Paper VII:** To comprehensively investigate the impact of four factors (temperature, length of the extraction time, ratio of water-to-tea and tea particle size) on the yield of theanine extracted from green tea using water and to use the RSM technique to further optimise the conditions for extracting theanine,

3. **Paper VIII:** To comprehensively investigate the effect of the pH of the aqueous brewing solution, on the yield of the catechins, caffeine, theanine and total solids extracted from green tea and the on the important tea phenomenon called “cream formation”.

4. **Paper IX:** To determine whether old green tea leaves (the fifth to the tenth leaves down the stem) could be used as an underutilised source of catechins and whether these otherwise wasted old tea leaves could be used to prepare decaffeinated green tea powders using hot water as the solvent and freeze drying and spray drying to dry the aqueous extract.

5. **Paper X:** To optimise the decaffeination of young tea leaves (the apical bud and the first four leaves down the stem) using hot water as the solvent and the spray drying procedure for producing decaffeinated and high caffeine green tea powders.

6. **Paper XI:** To investigated the impact of the typical household teabag brewing conditions (the volume of boiled water used and the length of the brewing time) on the extraction of the catechins, theanine and caffeine from green tea and to determine whether MAE could improve the extraction of these bioactives.

The first three papers (VI-VIII) relate to the extraction of the three green tea bioactive compounds under laboratory conditions.

The next two papers (IX and X) relate to the techniques which can be used for the preparation of green tea powders from young and old tea leaves; such powders could be used as food supplements or additives in functional foods to increase the consumption of the green tea bioactive compounds.

The final paper (XI) relates to the extraction of the green tea bioactive compounds under household conditions and therefore, it is more relevant to increasing the intake of the green tea bioactive compounds by increasing their concentrations in the green tea beverage.
PART 2: RESULTS
2.1. SYNOPSIS OF RESEARCH RESULT PAPERS

In this thesis, the results are presented in a series of six papers, including three which are already published, one which is currently accepted (in press) and two which have been submitted for publication.

The first research paper, entitled “Optimizing conditions for the extraction of catechins from green tea using hot water” (Paper VI) examined the impact of six different factors on the yield of catechins extracted from green tea using hot water. How best to minimise the amount of water used for the extraction (the efficiency of water use) was also investigated. The parameters studied were: temperature, length of the extraction time, ratio of water-to-tea, tea particle size, pH and the number of times the same sample was extracted.

The results showed that all six of the factors had an impact on the yield of catechins extracted from green tea using hot water and two had an impact on the efficiency of water use. The best temperature and time combination for catechin extraction was at 80°C for 30 min. The yield of catechins was also optimal with a tea particle size of 1 mm, a brewing solution pH < 6 and a water-to-tea ratio at 50:1 (mL/g). In terms of efficient use of water in a single extraction, a water-to-tea ratio of 20:1 (mL/g) gave the best results. At the water-to-tea ratio of 20:1 mL/g, the highest yield of catechins per gram of green tea was achieved by extracting the same sample of green tea twice. However, for the most efficient use of water, the best extraction was found to be once at a water-to-tea ratio of 12:1 (mL/g) and once at a water-to-tea ratio of 8:1 (mL/g).

The second research paper, entitled “Optimum conditions for the water extraction of L-theanine from green tea” (Paper VII) examined the effects of four different extraction conditions on the yield of theanine extracted from green tea using water. The parameters studied were: temperature, length of the extraction time, ratio of water-to-tea and the tea particle size. The yield of theanine extracted from green tea using water was further optimised using the response surface methodology (RSM).

The results showed that the yield of theanine was increased by increasing the temperature and the length of extraction time and reached a plateau at 80°C and 30 min, respectively. The ratio of water-to-tea also affected the yield of theanine; the higher ratios of water-to-tea resulted in higher yields of theanine. However, taking into account both theanine yield and the efficient use of water (cost-effectiveness), the results suggested that the optimum was a water-to-tea ratio of 20:1 mL/g. The yield of theanine was also optimum with tea particle sizes between 0.5–1 mm.
However, the results showed that the temperature and the length of extraction had more impact on the yield of theanine extracted than the ratio of water-to-tea and the tea particle size. Therefore, RSM was used to design and predict a model for further optimising the conditions to extract theanine from green tea using temperature and the length of extraction as the two most influential factors. The model predicted that the optimum conditions for extracting theanine from green tea were 79.4°C for 29.5 min, values which were not very different from the 80°C and 30 min previously found. Therefore, the optimal conditions for extracting theanine from dried green tea with water were found to be 80°C for 30 min with a tea particle size of 0.5–1 mm and a water-to-tea ratio of 20:1 mL/g.

The third research paper, entitled “Effects of aqueous brewing solution pH on extraction of the major green tea constituents” (Paper VIII) examined the impact of the pH of the aqueous brewing solution on the extraction and stability of the catechins, theanine and caffeine. The impact of the pH on the amount of total solids extracted (extractable solids) from green tea and the formation of a precipitate upon cooling the extract solution, which is referred to as “tea cream formation” was also investigated. Furthermore, the impact of pH on the four epistructured catechins and on the four non-epistructured catechins was determined.

The results showed that the pH of the aqueous brewing solution significantly affected the extraction and stability of the catechins but less so the extraction of theanine and caffeine. The epistructured catechins were stable under acidic conditions but epimerised or degraded at pH ≥ 6. The extractable solids contained more epistructured catechins at pH 3-5.3 than at more basic pH and more non-epistructured catechins at pH 6-7 than at more acidic pH. More tea cream was obtained at pH ≤ 2 and pH ≥ 8 than at the pH in between but the concentration of catechins and theanine was low in these fractions. Therefore, to maximise the extraction of the epistructured catechins and to minimise their epimerisation and degradation, and to maximise the extraction of caffeine and theanine, the results showed that the pH should be maintained between 3-5.3 during the aqueous brewing process.

The fourth research paper, entitled “Production of caffeinated and decaffeinated green tea catechin powders from underutilised old tea leaves” (Paper IX) examined the conditions for using water for the decaffeination of freshly harvested but old (mature) tea leaves, from the fifth to the tenth leaf position down the stems, leaves which are not usually used for making green tea and are mostly cut and discarded at the end of the growing season. To determine the optimal conditions for decaffeination, the old green tea leaves were blanched in water for 5 to 25 min at 100°C and a
water-to-tea ratio of 20:1 mL/g. Green tea extracts (normal and decaffeinated) were then prepared and dried using freeze drying and spray drying to obtain green tea powders. The physical properties and composition of the tea powders were then determined.

The results showed that the optimal conditions for decaffeination of the fresh old tea leaves were 100°C for 10 min at a ratio of water-to-tea of 20:1 mL/g. This decaffeination process removed 80% of the caffeine while retaining 85% of the catechins in the leaves. The results also showed that both freeze drying and spray drying were suitable for preparing powders from the aqueous extracts of the normal and decaffeinated green teas. Freeze drying yielded 100% of the extractable solids and 100% of the catechins while spray drying gave 20–25% lower yields.

However, due to the high cost and the length of time needed for freeze drying, the study suggested that spray drying was a more suitable method for the preparation of caffeinated and decaffeinated green tea powders. Both caffeinated and decaffeinated green tea powders had acceptable physical properties and chemical compositions. In addition, the decaffeinated green tea powder had a lower content of caffeine (6.1-6.4 mg/g) than the maximum permitted for classification as a decaffeinated tea powder (10mg/g).

The fifth research paper, entitled “Preparation of decaffeinated and high caffeine powders from green tea” (Paper X) examined the conditions for the decaffeination of young tea leaves (the bud and the next four to five leaves on the growing shoot). The fresh young tea leaves were blanched for 1 to 15 min in water at 100°C and at a water-to-tea ratio of 20:1 m/g. The decaffeinated leaves were then extracted and the extract and the water used for blanching (containing the extracted caffeine) were filtered, concentrated and spray-dried to obtain a decaffeinated green tea powder and a caffeine-enriched green tea powder, respectively. To optimise the conditions for spray drying, inlet temperatures ranging from 170-220°C and outlet temperatures ranging from 70-115°C were tested. The physical properties and composition of the tea powders were also determined.

The results showed that the optimal conditions for decaffeination of the fresh young tea leaves were 100°C for 4 min at a water-to-tea ratio of 20:1mL/ g. With these decaffeination conditions, 83% of the caffeine could be removed from the leaves while 94% of the catechins were retained. The spray drying conditions which gave the highest yield of green tea powder and the highest concentrations of the naturally occurring epistructured catechins, were found to be 180°C for the inlet temperature and 115°C for the outlet temperature. Using these optimal conditions, a decaffeinated green tea powder (7 mg/g) and a caffeine-enriched green tea powder (95mg/g) were
produced. These two green tea powders also had acceptable physical properties and chemical compositions.

Finally, the sixth research paper, entitled “Improved extraction of green tea components from teabags using the microwave oven” (Paper VI) investigated the impact of typical household teabag brewing conditions on the extraction of the green tea constituents and whether their extraction could be improved using a microwave oven. To determine the impact of typical household brewing conditions, teabags were brewed in boiled water and left at room temperature for 3 min as suggested by the manufacturers. To improve the extraction using microwave assisted extraction (MAE), two types of MAE were applied: cold MAE, where a teabag was put in 200 mL of room temperature water and placed in a microwave oven for 30 sec to 120 sec, and hot MAE, where a teabag was put in 200 mL of boiled water and kept at room temperature for 0.5, 1, 2, 3 or 4 min and then placed in a microwave oven for irradiation times of 30, 45 or 60 sec.

The results showed that the short brewing time of 3 min suggested by the manufacturers was not long enough to extract all of the catechins, caffeine and theanine as only 62% of the catechins, 76% of the caffeine and 80% of the theanine in the green tea could be extracted under these conditions. The findings illustrated that irradiating the teabags in water at room temperature was not effective at extracting the green tea components and the procedure did not improve on the extraction of the catechins, caffeine and theanine compared to the typical household brewing conditions with freshly boiled water.

However, first brewing a teabag in 200 mL of freshly boiled water for 0.5 min before irradiation for 1 min in a microwave oven (hot MAE), the extraction of the catechins and caffeine was improved to 80% and 92%, respectively, although the extraction of theanine was not significantly affected. Therefore, the hot MAE technique could help maximise the extraction of the catechins for those who consume green tea for the potential health benefits of the catechins.
2.2. RESEARCH RESULT PAPERS

The results for the current thesis are based on the following six research result papers, which are referred to in the text by their Roman numerals as follows:


2.2.1. Optimizing conditions for the extraction of catechins from green tea using hot water

Quan V. Vuong, John B. Golding, Costas E. Stathopulos, Minh H. Nguyen and Paul D. Roach

This research paper was published in the Journal of Separation Science, 2011, 34, 3099-3106.
Research Article

Optimizing conditions for the extraction of catechins from green tea using hot water

Six different factors involved in the extraction of catechins from green tea using water were examined for their impact on the yield of catechins and on the efficiency of water use. The best temperature and time combination for catechin extraction was at 80 °C for 30 min. The yield of catechins was also optimal with a tea particle size of 1 mm, a brewing solution pH < 6 and a tea-to-water ratio at 50:1 (mL/g). In terms of efficient use of water in a single extraction, a water-to-tea ratio of 20:1 (mL/g) gave the best results; 2.5 times less water was used per gram of green tea. At the water-to-tea ratio of 20:1 mL/g, the highest yield of catechins per gram of green tea was achieved by extracting the same sample of green tea twice. However, for the most efficient use of water, the best extraction was found to be once at a water-to-tea ratio of 12:1 (mL/g) and once at a water-to-tea ratio of 8:1 (mL/g). Therefore, all six of the factors investigated had an impact on the yield of catechins extracted from green tea using water and two had an impact on the efficiency of water use.

Keywords: Extraction conditions / Extraction yield / Tea catechins / Water extraction
DOI 10.1002/jssc.201000863

1 Introduction

Green tea is a rich source of catechins, antioxidants which account for about 30% of its dry weight [1, 2]. Numerous animal, in vitro and epidemiology studies have linked green tea catechins with various human health benefits such as prevention of some cancers, cardiovascular diseases, dental decay, obesity, diabetes, and improvement in the immune system [3–5]. They are also strong antioxidants and have been used to improve the shelf-life of food products [2].

The catechins can be classified into two groups based on their structure: epistructured catechins and non-epistructured catechins. The epistructured catechins are comprised of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC); whereas the non-epistructured catechins are gallocatechin gallate (GCG), catechin gallate (CG), gallatechin (GC) and catechin (C) [1].
have been used in some of the previous studies [1, 2, 7]. The present study also investigated the efficiency with which the water was used and took this into account as an important criterion in the process of extracting the catechins from green tea.

2 Materials and methods

2.1 Materials

Green tea of the Shan variety (Camellia sinensis var. pubilimba) [10] from the Thai Nguyen region, was obtained from the Vietnam Tea (Hanoi, Vietnam). The dried green tea was ground by using a commercial blender (John Morris Scientific, USA) and then sorted into six different particle sizes by passing through a series of EFL 2000 stainless steel sieves (Endecotts, England) with diameters of 0.25, 0.5, 1, 2, 2.8, and 4 mm.

The following chemicals were used for analyses: L-tryptophan (used as an internal standard), EC, ECG, EGC, EGCG, C, CG, GC and GCG obtained from Sigma (Castle Hill, NSW, Australia); sodium hydroxide, hydrochloric acid, acetonitrile, orthophosphoric acid and tetrahydrofuran purchased from Lomb Scientific (Taren Point, NSW, Australia). Ultra-pure (type 1) de-ionised (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia, North Ryde, NSW, Australia).

2.2 Extraction of green tea

For determining the effect of the extraction temperature, 1 g of green tea was extracted with 100 mL of water at various temperatures (5–90°C) for 30 min using a temperature-controlled shaking water bath (Ratek Instruments, Boronia, VIC, Australia). The optimal temperature (80°C) was then used to determine the impact of the extraction time. For this, 1 g of green tea was extracted with 100 mL of water at 80°C for various lengths of time (5–120 min). The optimal time (30 min) and temperature (80°C) were then used to determine the influence of the water-to-tea ratio; 1 g of green tea was extracted in water at various ratios of water-to-tea (10:1–100:1, mL/g). To determine the impact of the tea particle size, the optimum temperature (80°C), time (30 min) and water-to-tea ratio (20:1, mL/g) were used to extract 5 g of ground green tea having various sizes (0.25, 0.5, 1, 2, 2.8, and 4 mm) with 100 mL of water.

To determine the impact of the pH of the extraction solution, 5 g of ground green tea (1 mm) was extracted in 100 mL of water at 80°C for 30 min with pH of the solution adjusted to 1, 2, 3, 4, 5, 6, 7, 8, and 9 and maintained at these values during the extraction using 0.1 M HCl and 0.1 M NaOH. The pH of the solution was carefully monitored and controlled during the brewing process using a lab CHEM-pH meter version 1.02 (TPS, Springwood, Brisbane, Australia), which was calibrated for 80°C. Finally, to determine the impact of how many times the same sample was extracted, 5 g of ground green tea (1 mm) was extracted using a single or multiple steps with different amounts of water at 80°C for 30 min. The pH of the brewing solutions was 5.3 and did not need adjustment.

2.3 Determination of tea catechins

Tea constituents were determined by high-performance liquid chromatography (HPLC) as described by Vuong et al. [11]. After extraction, the tea solutions were cooled to room temperature and diluted at 1:1 with 500 μM L-tryptophan (as internal standard) in DI water, then filtered through 0.45-μm cellulose syringe filters (Phenomenex Australia, Lane Cove, NSW, Australia) and transferred to brown glass vials. The extraction solutions were then injected onto a 250 × 4.6 mm Synergi 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia, Lane Cove, NSW, Australia) maintained at 35°C using a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW, Australia) with UV detection at 210 and 280 nm.

The mobile phases consisted of solvent systems A and B; solvent A was 0.2% v/v orthophosphoric acid/acetonitrile/tetrahydrofuran, 95.5:3:1.5% (v:v:v) and solvent B was 0.2% v/v orthophosphoric acid/acetonitrile/tetrahydrofuran, 73.5:25:1.5% (v:v:v). A gradient elution schedule was used: 100% A from 0 to 10 min; a linear gradient from 100% A to 100% B from 10 to 40 min; a linear gradient from 100% B to 100% A from 40 to 50 min, with a post-run re-equilibration time of 10 min with 100% A before the next injection. An autoinjector was used to inject 20 μL of the tea solution onto the HPLC column and the flow rate was 1 mL/min.

A chromatogram, representing the peaks for the individual catechins, caffeine and the internal standard, is shown in Fig. 1. The tea constituents were quantified by dividing the peak areas of the tea constituents by the peak area of the internal standard, 250 μM L-tryptophan, and determining their concentration from a standard curve of the peak area ratios for increasing concentrations of the pure tea constituent external standards, all compared to the peak area of 250 μM L-tryptophan.

2.4 Determination of extractable solids

The extractable solids from the green tea extractions were determined by the method described in a previous study [12], with some modifications. The tea solutions were filtered using Whatman number 1 filter paper (90 mm diameter) (Lomb Scientific) to remove hydrated leaves and fine suspended material. A sample of each solution was then weighed to the nearest 0.0001 g and placed in a weighing container for drying to a constant weight using a vacuum drier (Thermoline Scientific Equipment, Smithfield, NSW, Australia) set at 40°C. The dry solids (DS) in the
sample were expressed as mg of dry solids per gram of dry green tea used in the extraction, as per the following equation:

\[
DS (\text{mg/g}) = \frac{W_1 \times V_2}{W_2 \times V_1}
\]

where DS is milligram dry solids per gram of dry tea (mg/g); \(W_1\) is the weight of dry matter after drying (mg); \(W_2\) is the weight of the green tea sample extracted (g); \(V_1\) is the volume of the tea extraction solution used for drying (mL); \(V_2\) is the total volume of the tea solution after extraction (mL).

### 2.5 Statistical analysis

The one-way ANOVA and the LSD post-hoc test were conducted using the SPSS statistical software version 18.0 for Windows. Differences in the mean levels of the components in the different experiments were taken to be statistically significant at \(p < 0.05\).

### 3 Results and discussion

#### 3.1 Effect of extraction temperature

The temperature of the water was considered as one of the important factors that could affect the yield of catechins extracted from green tea [1, 2]. In theory, high extraction temperatures can increase the yield of tea catechins because the cell walls of the green tea leaves become more permeable to the solvent and to the constituents and, thus, the solubility and diffusion coefficients of the tea catechins are increased [1, 2]. However, the catechins can also be subject to degradation when the extraction is conducted at too high temperatures due mainly to the epimerization of their structure [13]. Therefore, to investigate the impact of temperature on the yield and stability of the catechins, a wide range of temperatures from 5 to 90°C was applied during the extraction of 1 g of green tea in 100 mL water for 30 min.

The results, presented in Table 1, show that the temperature had a dramatic impact on the yield of the individual catechins. The yield of each of the catechins increased as the extraction temperature was increased. However, the increase in the yields (shown in brackets) of the individual catechins when going from 5 to 90°C was found to be higher for EGCG (80), ECG (16), GCG (21), GC (10), CG (15) and C (33) than for EGC (5) and EC (5). This finding was in agreement with the results of previous studies [14, 15], which also showed that the extraction temperature had less of an impact on EGC and EC, compared to the other catechins.

It is interesting to note that at the 5°C extraction temperature, 20% w/w of EGC and 20% w/w of EC could be extracted and they accounted for 55 and 22% w/w of the total catechins (Table 1) extracted at this temperature, respectively. Therefore, a solution enriched in these two catechins, with EGC and EC accounting for 77% w/w of the
The values are mean ± standard deviations for triplicate extractions and, in the same column, those not sharing the same superscript letter are significantly different from each other (p<0.05).

total catechins, could be obtained by extracting green tea at 5°C. In contrast, only 1.2% w/w of the EGCG was extracted at 5°C and it accounted for only 6.5% w/w of the total catechins at this temperature. However, EGCG accounted for 43% w/w of the total catechins when the green tea was extracted at 80°C. Therefore, to obtain a solution enriched in EGCG the tea should be extracted at 80°C.

The results also showed that temperatures exceeding 80°C may impact on the stability of the epistructured catechins (EGCG, EGC, ECG, and EC). The epi-structured catechins did not significantly differ (p>0.05) between 80°C (104.6±0.4 mg/g) and 90°C (100.0±0.5 mg/g) whereas the non-epistructured catechins (GCG, GC, CG and C) continued to increase (p<0.05) as the extraction temperature was increased from 80°C (23.8±0.9 mg/g) to 90°C (26.5±2.1 mg/g). These results indicate that excessive extraction temperatures above 80°C could lead to an increased epimerization of the epistructured catechins to non-epistructured catechins. This has also been observed in previous studies [16, 17], which found that epimerization from the epistructured to the non-epistructured catechins happened predominantly when the brewing temperature was above 80°C. Therefore, care should be taken to prevent exposure of the catechins to temperatures above 80°C during hot water extractions.

Temperature also had a significant influence on the total yield of catechins extracted (Fig. 2A). The total yield of catechins significantly increased when the temperature was increased from 5 to 80°C, where it reached a plateau. Therefore, the optimal extraction temperature was chosen to be 80°C and this temperature was subsequently used for determining the effect of the other factors on the yield of catechins extracted.

### 3.2 Effect of extraction time

The length of extraction is also considered as an important factor affecting the yield of catechins extracted [1]. The longer extraction times can enable more of the tea catechins to move into the solution. However, the solubility of the individual catechins in water may differ because of variation in their structure and molecular weight. In addition, the stability of the catechins may be affected when tea is brewed for too long because of an increased chance of epimerization, oxidation and degradation, especially under higher extraction temperatures [1]. Therefore, the impact of extraction time on the extraction and stability of the catechins was investigated by brewing 1 g green tea in 100 mL of water at the optimal temperature of 80°C for various extraction times ranging from 5 to 120 min.

As shown in Table 2, the extraction time had a significant effect on the yield of the individual catechins. However, the extraction of the individual catechins varied. The yield of the individual catechins increased as the extraction time was increased from 5 to 30 min. However, the yield of the epistructured catechins (EGCG, EGC, ECG and EC) reached a plateau around 30 min whereas the non-epistructured catechins (GCG, GC, CG and C) continued to increase with longer extraction times up to 120 min. Thus, the extraction of the non-epistructured catechins was more time dependent than for the epistructured catechins. However, among the epistructured catechins, the extraction of the slightly more hydrophobic catechins, EGCG and EGC, appeared to take longer to reach a plateau than for EGCG and EC.

The impact of the extraction time on the total yield of catechins is presented in Fig. 2B, which shows that the total yield of catechins rapidly rose when the extraction time increased from 5 to 30 min and then reached a plateau when the extraction time was further increased to 120 min. These findings are in agreement with results reported in a previous study [18], which found that the maximum extraction of bioactive compounds from loose green tea was achieved when the extraction was done at 80°C for 30 min. However, these results were slightly different to those reported by Perva-Uzunalić et al. [7], who found that the maximum extraction efficiency for the catechins was achieved when green tea was extracted at 80°C for 20 min or at 95°C for 10 min. These differences in observations from different studies can be explained by the differences in the extraction methods. In the present study, the green tea was extracted using regular brewing conditions (described in
Section 2.2) whereas Uzunalić et al. [7] used a refluxing system to extract their green tea, a system that may very well have shortened the extraction time needed to achieve maximum catechin extraction.

Therefore, the findings of the present study indicated that the extraction time was also a factor of major influence on the extraction of catechins and that for the conventional brewing method used, an extraction time of 30 min was found to be optimal for extracting catechins from green tea.

### 3.3 Effect of extraction water-to-tea ratio

The ratio of water-to-tea has been considered as another important factor, which may affect the yield of extracted catechins [1]. In theory, the higher the water-to-tea ratio, the higher the yield of catechins obtained [1]. Therefore, the impact of the water-to-tea ratio on the yield of catechins was investigated by brewing green tea at the optimal temperature and time of 80°C and 30 min, respectively, in water with various ratios of water-to-tea ranging from 10:1 to 100:1 mL/g.

As shown in Fig. 3A, the total yield of extracted catechins increased sharply when the water-to-tea ratio was increased from 10:1 to 20:1 mL/g. The yield then only gradually increased and reached a plateau when the water-to-tea ratio exceeded 50:1 mL/g. In terms of the maximal extraction of catechins per gram of green tea, the best extraction efficiency was achieved with the ratio of tea-to-water at 50:1 mL/g. However, in terms of water efficiency and cost-effectiveness, the water-to-tea ratio of 20:1 mL/g gave the best results; this ratio required 2.5 times less water while still achieving approximately 85% w/w catechin extraction efficiency in comparison with extraction at the water-to-tea ratio of 50:1 mL/g. The lower volume of water needed for this extraction is preferable because not only is less water needed for the extraction but also less energy is required for heating it up. Therefore, the ratio of water-to-

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**Table 2.** Effect of extraction time on the yield of individual catechins

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>EGC</th>
<th>EGC</th>
<th>ECG</th>
<th>EC</th>
<th>GCG</th>
<th>GC</th>
<th>CG</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3.9 ± 0.6a</td>
<td>7.0 ± 0.5a</td>
<td>5.3 ± 0.4a</td>
<td>2.2 ± 0.2a</td>
<td>1.3 ± 0.2a</td>
<td>1.8 ± 0.4a</td>
<td>0.8 ± 0.1a</td>
<td>1.1 ± 0.1a</td>
</tr>
<tr>
<td>10</td>
<td>15.8 ± 2.8b</td>
<td>14.7 ± 0.7b</td>
<td>7.5 ± 0.9b</td>
<td>5.8 ± 1.8b</td>
<td>1.7 ± 0.1ab</td>
<td>4.4 ± 0.2b</td>
<td>0.9 ± 0.1a</td>
<td>2.7 ± 0.3a</td>
</tr>
<tr>
<td>15</td>
<td>36.1 ± 1.9c</td>
<td>22.0 ± 2.5c</td>
<td>7.7 ± 0.5c</td>
<td>7.7 ± 1.1c</td>
<td>2.2 ± 0.1b</td>
<td>7.3 ± 0.1c</td>
<td>1.2 ± 0.1a</td>
<td>3.7 ± 0.2a</td>
</tr>
<tr>
<td>20</td>
<td>38.5 ± 3.5c</td>
<td>25.1 ± 0.5d</td>
<td>10.0 ± 0.7c</td>
<td>8.6 ± 0.9cd</td>
<td>2.4 ± 0.2c</td>
<td>8.8 ± 0.1e</td>
<td>1.7 ± 0.2a</td>
<td>5.1 ± 0.6a</td>
</tr>
<tr>
<td>25</td>
<td>53.8 ± 2.1de</td>
<td>25.4 ± 0.5e</td>
<td>11.4 ± 0.3cd</td>
<td>9.1 ± 0.5de</td>
<td>2.4 ± 0.9c</td>
<td>9.6 ± 0.4de</td>
<td>2.3 ± 0.2d</td>
<td>5.5 ± 0.3d</td>
</tr>
<tr>
<td>30</td>
<td>55.7 ± 1.1de</td>
<td>26.5 ± 1.5e</td>
<td>12.7 ± 0.6ed</td>
<td>9.7 ± 0.4e</td>
<td>3.0 ± 0.8c</td>
<td>10.9 ± 1.3e</td>
<td>3.4 ± 0.1c</td>
<td>6.5 ± 0.1e</td>
</tr>
<tr>
<td>40</td>
<td>53.4 ± 2.2c</td>
<td>25.5 ± 1.0d</td>
<td>12.6 ± 1.2d</td>
<td>9.3 ± 0.5de</td>
<td>4.6 ± 0.1e</td>
<td>11.1 ± 0.8ef</td>
<td>3.6 ± 0.1c</td>
<td>6.6 ± 0.1e</td>
</tr>
<tr>
<td>50</td>
<td>55.7 ± 2.0de</td>
<td>26.3 ± 0.8d</td>
<td>12.9 ± 1.2c</td>
<td>9.5 ± 0.6ed</td>
<td>4.8 ± 0.2c</td>
<td>12.0 ± 1.6c</td>
<td>3.8 ± 0.1a</td>
<td>7.0 ± 0.1f</td>
</tr>
<tr>
<td>60</td>
<td>56.6 ± 1.0de</td>
<td>26.5 ± 1.2d</td>
<td>13.5 ± 1.1de</td>
<td>9.7 ± 0.8d</td>
<td>4.9 ± 0.6d</td>
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<td>3.8 ± 0.1a</td>
<td>7.1 ± 0.1f</td>
</tr>
<tr>
<td>80</td>
<td>57.1 ± 1.3d</td>
<td>26.3 ± 1.5c</td>
<td>13.4 ± 0.1de</td>
<td>9.5 ± 1.3c</td>
<td>5.1 ± 0.3de</td>
<td>13.7 ± 1.1f</td>
<td>4.2 ± 0.3b</td>
<td>7.1 ± 0.1f</td>
</tr>
<tr>
<td>100</td>
<td>57.3 ± 1.1d</td>
<td>25.6 ± 0.8d</td>
<td>13.8 ± 1.6de</td>
<td>9.3 ± 1.3de</td>
<td>5.8 ± 0.3c</td>
<td>14.6 ± 1.1f</td>
<td>4.4 ± 0.2a</td>
<td>7.1 ± 0.1f</td>
</tr>
<tr>
<td>120</td>
<td>57.6 ± 2.0d</td>
<td>25.1 ± 1.1d</td>
<td>14.0 ± 1.8de</td>
<td>9.2 ± 1.3de</td>
<td>6.4 ± 0.3c</td>
<td>15.6 ± 0.8e</td>
<td>4.4 ± 0.2a</td>
<td>7.1 ± 0.1f</td>
</tr>
</tbody>
</table>

The values are mean ± standard deviations for triplicate extractions and, in the same column, those not sharing the same superscript letter are significantly different from each other (p < 0.05).
tea of 20:1 mL/g was used for further optimizing the extraction of catechins with water at 80°C for 30 min.

### 3.4 Effect of particle size of green tea

In theory, the smaller the particle size of tea the higher the yield of catechins should be due to the higher contact surface area of the tea with the water [1]. However, the extraction of the catechins may also be impaired when brewing very small particle sizes because these small particles may settle to the bottom and, like sand, form a sediment at the bottom of the extraction container, which could reduce the flow-through of water and, therefore, the tea would not effectively interact with the water [1]. The effect of the particle size on the yield of extracted catechins was therefore determined by brewing ground tea with various particle sizes (0.25, 0.5, 1, 2, 2.8 and 4 mm) in water at the optimal temperature, extraction time and water-to-tea ratio of 80°C, 30 min and 20:1 mL/g, respectively, using a shaking system for agitation to prevent the particles from settling. The extraction was also done with unground loose tea.

The results (Fig. 3B) showed that the total yield of catechins was not affected when the tea particle size was reduced to 2 mm. However, the yield significantly increased when the particle size was further reduced to 1 mm and less. Therefore, in terms of extraction efficiency as well as in terms of energy efficiency and cost-effectiveness, the current study suggests that the best particle size for the extraction of the catechins was with a tea particle size of 1 mm. To make smaller particle sizes, excessive grinding of the tea is required, which requires more energy and therefore generates higher costs. Furthermore, it is more difficult to separate the smaller particles (<1 mm) from the tea solution after brewing, either through filtration or centrifugation. Therefore, the tea particle size of 1 mm was used for further optimizing the extraction of catechins with water at 80°C for 30 min and the ratio of water-to-tea of 20:1 mL/g.

### 3.5 Effect of pH of extraction solution

The pH of the extraction solution has been considered as another factor which may affect the solubility and stability of the catechins during the brewing process [1]. Therefore, the impact of the pH, of the extraction solution during the brewing process, on the yield and stability of the catechins was examined by brewing tea in water at the optimal temperature, time, water-to-tea ratio and particle size of 80°C, 30 min, 20:1 mL/g and 1 mm, respectively, with the pH of the brewing solution ranging from 1 to 9.

The impact of the pH of the brewing solution on the yield and stability of the catechins is shown in Fig. 4A. With extraction pH values of less than 5, the yield of catechins extracted into the tea infusion did not significantly differ. These findings were in agreement with the results reported by Kim et al. [19], which showed that the extraction of catechins was not influenced when green tea was brewed under acidic conditions (pH≤5). However, with extraction pH values higher than 5, the yield of catechins was significantly lower because of epimerization and degradation (Fig. 4A). At pH 6 and 7, the epistructured catechins were partially epimerized to non-epistructured catechins and both groups were degraded when the pH was further increased to 9. These findings were similar to results reported in previous studies [19, 20], which found that the degradation of catechins occurred when green tea was brewed under alkaline conditions. Furthermore, the epistructured catechins tended to epimerize to non-epistructure catechins when the brewing pH ranged from 6 to 7.6 [6]. Therefore, these observations indicated that the pH of the extraction solution was a factor of major influence on the extraction of catechins with a low extraction pH (<6) giving the best yield and stability of the catechines during high temperature water extraction (Fig. 4A).

The pH of the extraction solution also had a significant impact on the extraction of tea solids (Fig. 4B). More solids were extracted at the low (pH<2) and high pH values (pH>7) than at the other pH values. According to other
Total catechins solution was 5.3 and was not adjusted. The results were extraction with 100 mL of water. The pH of the extraction and twice with 20 mL of water) and compared to one ground green tea was extracted, twice (once with 60 mL and each extraction step. In experiment 2, a 5-g sample of the extracted once, twice or three times with 100 mL of water for experiment 1, a 5-g sample of the ground green tea was

3.6 Effect of multiple extraction steps

To determine the impact of multiple extraction steps on the total yield of catechins, all the above optimal extraction conditions were applied to investigate the impact of two sets of multiple extraction steps on the extraction of catechins in comparison with a single extraction step (Table 3, experiments 1 and 2).

Ground green tea (1 mm) was brewed at 80°C for 30 min at a water-to-tea ratio of 20:1 mL/g (control). In experiment 1, a 5-g sample of the ground green tea was extracted once, twice or three times with 100 mL of water for each extraction step. In experiment 2, a 5-g sample of the ground green tea was extracted, twice (once with 60 mL and once with 40 mL of water) or three times (once with 60 mL and twice with 20 mL of water) and compared to one extraction with 100 mL of water. The pH of the extraction solution was 5.3 and was not adjusted. The results were expressed in two ways: (i) in milligram of catechins per gram of dry tea (mg/g) to determine the total yield of catechins per gram of green tea extracted and (ii) in milligram of catechins per liter of water (mg/L) to determine the most efficient use of water for the extraction.

The results (Table 3) showed that a higher yield of catechins was achieved when the green tea was extracted with multiple extraction steps in comparison with a single extraction but there was no significant difference between 2 and 3 extractions with 100 mL of water. Therefore, extracting the same green tea sample twice with 100 mL of water (a water-to-tea ratio of 20:1, mL/g) each time gave the best results, especially from the point of view of efficient use of water (Table 3). These findings were in agreement with results reported by Perva-Uzunalic et al. [7].

However, this multiple-step extraction procedure required twice the volume of water, time and energy for the extraction process and the concentration of the catechins was significantly lower (679.5 mg/L) compared to when only one extraction was done (1033.2 mg/L) (Table 3). Furthermore, to produce a dry tea extract, the higher the amount of water, which needs to be evaporated, the higher the drying costs. In experiment 2, when the total amount of water used was restricted to a total of 100 mL, the results (Table 3) showed that the yield of catechins per gram of green tea could still be significantly improved with more than one extraction step, although there was no difference between two or three extractions. Furthermore, the concentration of catechins in the extraction solution was significantly higher (1204.5 mg/L) than with a single extraction (1033.2 mg/L). Thus, from the point of view of efficient use of water, the optimal extraction procedure was extracting 5 g green tea once with 60 mL (a water-to-tea ratio of 12:1, mL/g) and once with 40 mL of water (a water-to-tea ratio of 8:1, mL/g). The cost of drying such an extraction would also be less than when extracting twice with 100 mL of water.

Therefore, the current study suggests that for the maximum extraction of catechins, green tea should be

![Figure 4](image-url)
The authors have declared no conflict of interest.

Table 3. Effect of the number of extraction steps on the total yield of catechins

<table>
<thead>
<tr>
<th>Ratio of tea:water (g/mL)</th>
<th>Number of extractions</th>
<th>Total volume of water (mL)</th>
<th>Catechins (mg/g)*</th>
<th>Catechins (mg/L)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:100 (Exp. 1 and 2)(^a)</td>
<td>1</td>
<td>100</td>
<td>103.3 ± 4.9(^a)</td>
<td>1033.2 ± 49.3(^a)</td>
</tr>
<tr>
<td>5:100+100 (Exp. 1)(^a)</td>
<td>2</td>
<td>200</td>
<td>135.9 ± 9.5(^b)</td>
<td>679.5 ± 47.3(^b)</td>
</tr>
<tr>
<td>5:60+40 (Exp. 2)(^b)</td>
<td>3</td>
<td>300</td>
<td>148.6 ± 10.4(^b)</td>
<td>495.5 ± 34.7(^b)</td>
</tr>
<tr>
<td>5:60+20+20 (Exp. 2)(^b)</td>
<td>2</td>
<td>100</td>
<td>120.5 ± 6.6(^c)</td>
<td>1204.5 ± 66.4(^c)</td>
</tr>
</tbody>
</table>

The values are mean ± standard deviations for quadruplicate extractions and they are expressed in two ways: (i) in mg of catechins per gram of dry tea (mg/g)* and (ii) in mg of catechins per liter of water used in the extraction (mg/L)**. Values in the same column not having the same superscript letter are significantly different from each other (\(p<0.05\)).

a) Exp. 1 and Exp. 2 refer to experiment 1 and experiment 2, respectively. The 5:100 (g/mL) extraction was common to both experiments.

4 Concluding remarks

This study showed that the yield and stability of the catechins extracted from green tea using water as the solvent were affected by all six of the factors investigated; the extraction temperature, extraction time, water-to-tea ratio, tea particle size, extraction pH and the number of extractions were all important factors which directly affected the efficiency of the catechin extraction. Furthermore, the water-to-tea ratio and the number of extractions also affected how efficiently the water was used.

In terms of the maximal extraction of catechins per gram of green tea, the best extraction efficiency was achieved with water extraction at 80°C for 30 min, a tea particle size of 1 mm, a brewing solution pH < 6 and a tea-to-water ratio at 50:1 (mL/g). In terms of efficient use of water in a single extraction, and the consequent cost-effectiveness of any drying process, the water-to-tea ratio of 20:1 (mL/g) gave the best results. At the water-to-tea ratio of 20:1 (mL/g), the highest yield of catechins per gram of green tea was achieved by extracting the same sample of green tea twice. However, for the most efficient use of water, and for the lowest cost of any subsequent drying process, the best extraction was found to be once at a water-to-tea ratio of 12:1 (mL/g) and once at a water-to-tea ratio of 8:1 (mL/g).

Therefore, all six of the factors investigated had an impact on the yield of catechins extracted from green tea using water and two had an impact on the efficiency of water use.

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The authors have declared no conflict of interest.

5 References


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2.2.2. Optimum conditions for the water extraction of L-theanine from green tea

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Research Article
Optimum conditions for the water extraction of L-theanine from green tea

Theanine is a unique non-protein amino acid found in tea (Camellia sinensis). It contributes to the favourable umami taste of tea and is linked to various beneficial effects in humans. There is an increasing interest in theanine as an important component of tea, as an ingredient for novel functional foods and as a dietary supplement. Therefore, optimal conditions for extracting theanine from tea are required for the accurate quantification of theanine in tea and as an efficient first step for its purification. This study examined the effects of four different extraction conditions on the yield of theanine from green tea using water and applied response surface methodology to further optimise the extraction conditions. The results showed that temperature, extraction time, ratio of water-to-tea and tea particle sizes had significant impacts on the extraction yield of theanine. The optimal conditions for extracting theanine from green tea using water were found to be extraction at 80°C for 30 min with a water-to-tea ratio of 20:1 mL/g and a tea particle size of 0.5–1 mm.

Keywords: Extraction conditions / Green tea / Response surface methodology / Theanine
DOI 10.1002/jssc.201100401

1 Introduction

Theanine is a non-protein derived (free) amino acid (Fig. 1). In the plant world, it is unique to the Camellia genus and it is most prominent in the Camellia sinensis (L) O. Kuntze species, which is commonly known as the tea plant [1]. Free theanine is ubiquitously found in the cotyledons, shoots, leaves, stems, trunk and roots of the tea plant. However, it is mostly biosynthesised in the roots from glutamic acid and ethylamine by the enzyme theanine synthetase. From there, it is transferred via the phloem in the trunk and stems through to the growing shoots and leaves, where it accumulates and serves as a precursor for the biosynthesis of both caffeine and the catechins [2, 3].

In the tea leaves, theanine constitutes between 1 and 2% w/w of the dry weight and accounts for about 50% of the total free amino acids [1]. Many of these amino acids are involved in producing the distinctive aroma and taste of tea and theanine has been associated with the typical umami taste of tea [4]. Because of its contribution to taste, the theanine content in tea leaves has been found to correlate with tea quality and price; teas with high levels of theanine are normally evaluated as being of better quality and therefore can command higher prices than those with low levels of the amino acid [5].

Theanine has also been found to be associated with beneficial effects in humans, such as enhancement of relaxation and improvement of concentration and learning abilities [1, 6]. It has also been associated with the prevention of cardiovascular disease and certain cancers, the promotion of weight loss and an enhanced performance of the immune system [7–9]. Consequently, there is an increasing interest in theanine as an important component of tea, as an ingredient for novel functional foods and as a dietary supplement [1, 3, 9]. Therefore, optimal extraction of theanine from tea into solvents is a necessary step for its accurate quantification in tea and for its isolation for use in foods or as a dietary supplement.

Water is the solvent of choice for the extraction of bioactives because it is safe, inexpensive and accessible in comparison with organic solvents [10, 11]. Several factors have been found to directly affect the extraction yield of bioactive components using water. These include extraction temperature, extraction time, the ratio of water to the tea sample and the particle size of the tea [10, 11]. Several studies have reported methods for extracting theanine from fresh tea leaves and commercially available dried green tea, oolong tea and black tea [12–15]. However, although these studies have applied specific extraction conditions for temperature, time and the ratio of water to tea, the impact of these various extraction conditions on the extraction yield of theanine from tea has not been extensively studied.

In a recent study [16] the impact of these three factors, temperature, time and the ratio of water to tea, on the extraction yield of theanine was investigated. However, this
2 Materials and methods

2.1 Chemicals and materials

The following chemicals were used for analysis: \( \alpha \)-theanine, \( \alpha \)-tryptophan (used as an internal standard), caffeine, epigallocatechin, epicatechin, epigallocatechin gallate, gallo-catechin gallate and epicatechin gallate obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and acetoni-trile, orthophosphoric acid and tetrahydrofuran purchased from Lomb Scientific (Taren Point, NSW, Australia). Ultrapure (type I) de-ionised water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia, North Ryde, Australia). Green tea of the \( \textit{Shan} \) variety (\( \textit{C. sinensis} \) var. \( \textit{pubilimba} \)) [17] from the Thai Nguyen region was obtained from the Vietnam Tea Corporation (Hanoi, Vietnam). For the tea particle size experiment, the green tea was ground using a blender (John Morris Scientific, Chatswood, NSW, Australia) for 5 min. The ground tea was then stratified into five different particle sizes (less than 0.25, 0.25–0.5, 0.5–1, 1–2.8 and 2.8–4 mm) by continuously shaking for 10 min using the Endecotts EFL2000 stainless steel sieving system (Rowe Scientific, Melbourne, VIC, Australia).

2.2 Single factor extraction procedures

For determining the effect of the extraction temperature, 1 g of green tea was extracted with 100 mL of water at various temperatures (5–90°C) for 30 min using a shaking water bath (Ratek Instruments, Boronia, VIC, Australia). The optimal extraction temperature (80°C) was then used to determine the impact of the length of the extraction time; 1 g of green tea was extracted with 100 mL of water at 80°C for various lengths of extraction time (5–120 min). Similarly, the optimal length for the extraction time (30 min) and the optimum temperature (80°C) were then used to determine the influence of the ratio of water-to-tea; 1 g of green tea was extracted at 80°C for 30 min at various ratios of water-to-tea (10:1–100:1 mL/g). Finally, to determine the impact of the tea particle size, the optimum ratio of water-to-tea (20:1 mL/g) was used and 1 g of tea ground to various sizes (less than 0.25, 0.25–0.5, 0.5–1, 1–2.8 and 2.8–4 mm) was extracted at 80°C for 30 min.

2.3 Determination of theanine

After the extractions, the tea infusions were immediately placed onto ice to cool them down to 25°C. The tea infusions were then diluted 1:1 with 500 \( \mu \)M \( \alpha \)-tryptophan in de-ionised water, giving a final concentration for the internal standard of 250 \( \mu \)M. The solutions were then filtered and transferred to brown glass vials using 5 mL disposable syringes and 0.45 \( \mu \)m cellulose syringe filters (Phenomenex Australia, Lane Cove, NSW, Australia). The solvent system consisted of mobile phases A and B; mobile phase A was 0.2% orthophosphoric acid/acetonitrile/tetrahydrofuran, 95.5:3:1.5% v/v/v and mobile phase B was 0.2% orthophosphoric acid/acetonitrile/tetrahydrofuran, 73.5:25:1.5% v/v/v. A gradient elution schedule was used: 100% mobile phase A from 0–10 min; a linear gradient from 100% mobile phase A to 100% mobile phase B from 10–40 min; a linear gradient from 100% B to 100% A from 40–50 min, with a post-run re-equilibration time of 10 min with 100% A before the next injection. An autoinjector was used to inject 20 \( \mu \)L of the tea infusions onto the HPLC column and the flow rate was 1 mL/min. A representative chromatogram, showing that the theanine external standard peak was well separated from the internal standard peak as well as from the external standards for other green tea constituents, is shown in Fig. 2.

The quantification of theanine was achieved by dividing the peak area of theanine by the peak area of the internal standard, \( \alpha \)-tryptophan, and determining the concentration of theanine in the sample from a standard curve of the peak area ratios for increasing concentrations of the pure thea-nine external standard and the same concentration for the internal standard, 250 \( \mu \)M \( \alpha \)-tryptophan. The yield of thea-nine was expressed as mg of theanine extracted per gram of dried green tea (mg/g).
2.4 Data analysis

For the single factor extraction experiments, the one-way ANOVA analysis and the Fisher Least Significant Difference post hoc test were performed on the theanine extraction yields to determine whether there were significant differences (p < 0.05) at the various temperatures, lengths of extraction time, water-to-tea ratios and tea particle sizes. This was conducted using SPSS-PASW GradPack 18.0 for Windows.

2.5 RSM

Based on the single factor experimental results, the two factors with the most influence on the extraction yield of theanine, temperature (60–80°C) and extraction time (10–30 min), were used as the design variables for the RSM, as presented in Table 1. The other factors, water-to-tea ratio and particle size were kept constant at 20:1 mL/g and 0.50–1 mm, respectively. The design and the analysis of the experiments were performed using JMP software (Version 8.0). The experimental design generated by the software consisted of ten randomly ordered experimental runs, which are presented in the first three columns of Table 2. The software was also used to establish the model equation, to graph the 3-D plot and to predict the optimum values for the two response variables.

### Table 1. Factors and levels for the RSM

<table>
<thead>
<tr>
<th>(A) Temperature (°C)</th>
<th>(B) Length of extraction (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1</td>
<td>80</td>
</tr>
<tr>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
</tr>
</tbody>
</table>

The experimental data, obtained for the ten experimental runs, were fitted to the following second-order polynomial model

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j
\]

where the various \(X_i\) values are for the independent variables affecting the response \(Y\), \(k\) is the number of variables and \(\beta_0\), \(\beta_i\), \(\beta_{ii}\) and \(\beta_{ij}\) are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively [19, 20].

### Table 2. Experimental design and the yield of theanine for the RSM

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature (°C)</th>
<th>Length of extraction (min)</th>
<th>Theanine (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>10</td>
<td>23.01</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>10</td>
<td>24.18</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>20</td>
<td>25.66</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>10</td>
<td>23.08</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>30</td>
<td>24.02</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>20</td>
<td>23.81</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>30</td>
<td>25.23</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>20</td>
<td>23.15</td>
</tr>
<tr>
<td>9</td>
<td>80</td>
<td>30</td>
<td>26.48</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>20</td>
<td>23.81</td>
</tr>
</tbody>
</table>

a) The runs were randomly assigned by the JMP software.
b) The values for theanine (mg/g) are the means of triplicate runs for each respective temperature and length of extraction.

3 Results and discussion

3.1 Impact of single factors on the extraction yield of theanine

3.1.1 Effect of different temperatures on the extraction yield of theanine

Temperature plays an important role in the extraction yield of bioactive components from plant material into solvents [21]. High extraction temperatures usually increase extraction yield; the heat renders the plant cell walls more permeable to solvents and bioactives and also increases the solubility and diffusion coefficient of bioactives in the solvents [11]. In addition, high extraction temperatures can decrease the viscosity of some solvents and thereby facilitate the extraction process [21]. In this study, the extraction process was first carried out over a wide range of extraction temperatures, from 5 to 90°C, while the other extraction conditions were fixed at 30 min for the length of extraction, 100:1 mL/g for the water-to-tea ratio and unground tea was used.

The impact of temperature on the extraction yield of theanine is shown in Fig. 3. The yield of theanine increased steadily as the extraction temperature was increased from 5 to 90°C. The yield of theanine from the dried and unground...
catechins, also abundant components in green tea, which is stable at high temperatures. This is unlike the solubility in water over a range of temperatures: similar to previous report, which showed that pure theanine had high extraction temperatures. This finding is in agreement with a temperature for extracting theanine from the unground green tea was 80°C and remained the same at 90°C. Therefore, these results show that the optimum temperature for extracting theanine from the unground green tea was 80°C. However, it is interesting to note that around 50% of the extractable theanine was obtained when the tea was infused at 5°C for 30 min (Fig. 3). This indicates that the infusion rate of theanine into water was high, even at low extraction temperatures. This finding is in agreement with a previous report, which showed that pure theanine had high solubility in water over a range of temperatures: similar to those used in the present study: 1 g in 2.6 mL water at 0°C and 1 g in 1.8 mL water at 100°C [3, 9].

The high yield of theanine obtained when the extraction temperature was higher than 80°C also suggests that theanine is stable at high temperatures. This is unlike the catechins, also abundant components in green tea, which can degrade when the extraction temperature exceeds 80°C due to epimerisation or oxidation [22]. Its stability at high temperatures is an important characteristic of theanine, which can be taken advantage of, as it means theanine is unlikely to degrade when added in a variety of functional foods that may undergo thermal processes such as cooking.

As the yield of theanine was doubled by increasing the temperature from 5 to 80°C (Fig. 3), the extraction temperature was determined to be a factor of major influence for further extraction optimisation using RSM.

### 3.1.2 Impact of various extraction times on the extraction yield of theanine

The extraction time is another important factor that affects the extraction yield of bioactive components from plants into solvents [11]. Longer extraction times generally enhance higher extraction yield [10]. The impact of extraction time on the extraction yield of theanine from unground green tea into water was investigated, at 80°C and a water-to-tea ratio of 100:1 mL/g, for different extraction times ranging from 5 to 120 min. The results presented in Fig. 4 show the yield of theanine increased with extraction time and reached a plateau when the length of extraction was 30 min. When the extraction time exceeded 30 min, the yield of theanine remained stable up to 120 min (Fig. 4). Therefore, these results show that the optimum time for extracting theanine from the unground green tea was 30 min.

As the yield of theanine was quadrupled by increasing the extraction time from 5 to 30 min (Fig. 4), the extraction time was determined to be a factor of major influence for further extraction optimisation using RSM and our findings were in agreement with results in a recent study, which found that the theanine level increased when a commercial green tea was extracted up to 30 min [23].

### 3.1.3 Effect of different water-to-tea ratios on the extraction yield of theanine

The ratio of solvent-to-plant material also influences the extraction yield of bioactives from plants. In general, the higher the ratio of solvent to plant material, the higher is the extraction yield [10, 21]. However, from a cost-effectiveness point of view, the higher the solvent to plant material ratio, the higher the extraction cost will be, due to the larger volumes of solvent required. Furthermore, if a dry product is required, the expense of drying the extract will also be higher. Therefore, the ratio of water-to-tea is an important parameter for the economical extraction and isolation of theanine and thus the optimum ratio was determined by extracting green tea at 80°C for 30 min with the water-to-tea ratio ranging from 10:1 to 100:1 mL/g.

The results show that the yield of theanine rapidly increased when the ratio was increased from 10:1 to 20:1 mL/g and then only increased very slightly when the ratio was increased from 20:1 to 100:1 mL/g (Fig. 5). Therefore,
from the yield of theanine and the cost-effectiveness point of view, the optimum ratio of water-to-tea was 20:1 mL/g. However, compared to temperature, which doubled the yield, and the length of extraction, which quadrupled the yield, the water-to-tea ratio only increased the yield of theanine by one-third (Fig. 5). Therefore, the water-to-tea ratio was determined not to be a factor of major influence for further extraction optimisation using RSM and it was kept at 20:1 mL/g for this procedure.

3.1.4 Effect of particle size on the extraction yield of theanine

The particle size of the extracted material is also an important factor that can affect the extraction yield of bioactives from plants. Theoretically, reducing the particle size increases the extraction yield by increasing the contact surface area between the plant material and the solvent [21]. However, a previous study reported that higher extraction yields of caffeine and catechins from tea could only be obtained with small tea particle sizes by agitating the extraction media to prevent the small tea particles from partially settling down to the bottom of the beaker [24]. Based on this observation, in this study, the impact of various tea particle sizes on the extraction yield of theanine was determined by extracting the tea at 80 °C for 30 min for the length of extraction and unground tea was used. HPLC was used to determine the yield of theanine (mg/g) and the values are means ± standard deviations for triplicate extractions. The points with the superscript (*) are not significantly different from each other (p < 0.05).

The results show that the yield of theanine was significantly higher (p < 0.05) when the tea particle size was 0.5–1 mm compared to unground green tea, whether the water-to-tea ratio was 20:1 or 100:1 mL/g (Fig. 6). The yield of theanine was not significantly different (p > 0.05) from unground tea, whether the water-to-tea ratio was 20:1 or 100:1 mL/g, when the tea particles size was reduced to 1–2.8 mm. When the particle size was 2.8–4 mm, the theanine yield was not significantly different (p > 0.05) from unground tea extracted with the water-to-tea ratio at 20:1 mL/g but it was lower compared to unground tea extracted with the water-to-tea ratio at 100:1 mL/g (Fig. 6).

However, contrary to expectations [21, 24] when the tea particle size was further decreased to 0.25–0.5 and < 0.25 mm, the yield of theanine declined significantly (p < 0.05) compared to unground tea, whether the water-to-tea ratio was 20:1 or 100:1 and compared to the other three bigger particle-sized fractions (Fig. 6). This result is difficult to explain. However, the distribution of theanine in the various prepared tea particle fractions may have differed. The tea had stems as well as leaf material in it. As reported in a previous study [25], it is well known that theanine is often found at a higher concentration in stems than in leaves. The stems are harder to grind into small pieces than the leaves and therefore the particle sizes of the ground stem material may have been bigger than the particle sizes from the ground leaf material. Consequently, the material with the lowest particle size of < 0.5 mm may have only come from the leaves. Therefore, the theanine content may have been lower because the stem material was not represented in the two samples with particle sizes < 0.5 mm.

Although less likely, the fine tea particles may have partially settled down to the bottom of the beaker during the extraction in the shaking water bath; these small tea particles have a tendency to clump together and be harder to suspend in water [11]. Therefore, this could result in less surface contact area between these small particles of tea and the water and the extraction yield of theanine thus decreased.
Importantly, the results showed that the optimum yield of theanine was obtained when the tea particle size was 0.5–1 mm (Fig. 6). Therefore, in terms of efficiency of extraction, dried tea should be uniformly ground to a size of 0.5–1 mm before extracting theanine. However, the yield of theanine was only increased by less than 20% by grinding the green tea (Fig. 6). Therefore, the particle size was determined not to be a factor of major influence for further extraction optimisation using RSM and it was kept at 0.5–1 mm for this procedure.

3.2 RSM

3.2.1 Mathematical model, interaction and optimisation of extraction conditions for theanine

The extraction yield of theanine from green tea was further optimised by using the RSM approach. The two major influence factors: temperature at 60, 70 and 80°C (A) and the length of extraction for 10, 20, 30 min (B) were chosen as the design variables (Table 1). The other two factors, the water-to-tea ratio (20:1 mL/g) and the tea particle size (0.50–1.00 mm), were fixed for these experiments. The RSM experiment was designed with two centre points for the two factors and their three levels and the theanine extracted from the green tea (mg/g) was the response value (Tables 1 and 2).

The significance and adequacy of the model was tested and the results are presented in Table 3. The applicability of the model was adequate \( p = 0.0025 \) and the model predictor had an \( R^2 \) of 0.9725. Table 3 also shows that the two chosen factors of major influence, temperature \( p = 0.008 \) and the length of extraction \( p = 0.0013 \), both had separate significant effects on the yield of theanine. However, there was no significant \( p > 0.05 \) interaction between temperature and the length of extraction on the yield of theanine. The regression equation of the model was

\[
Y = 23.921 + 1.023A + 0.91B + 0.397A^2 + 0.147B^2 + 0.3225AB
\]

where the yield of theanine \( Y \) is expressed in terms of the two test variables, temperature \( A \) and the length of extraction \( B \).

As can be seen from the regression equation, the linear coefficients \( \beta_i \) for the A and B variables are positive and thus increases within the range of the critical values (60–80°C for temperature and 10–30 min for the length of extraction) can improve the response value (yield of theanine). In addition, the quadratic coefficients \( \beta_i^2 \) for the A and B variables in the equation are also positive, thus providing further support that the yield of theanine increases with increasing temperature and/or length of extraction within the range of the critical values. This conclusion is consistent with the findings of the single-factor experiments.

The effect of temperature and the length of extraction on the yield of theanine is graphically presented as a 3-D response surface plot in Fig. 7. These results show that increasing either temperature or the length of extraction within the range of the critical values increases the yield of theanine. Furthermore, the optimum yield of theanine, which is predicted by the model, is 26.72 ± 0.68 mg/g when the temperature and the length of extraction are 79.4°C and 29.5 min, respectively. These predicted values were very close to the values of 80°C and 30 min derived from the single-factor experiments for the temperature and the length of extraction, respectively, and the value of 26.48 mg/g obtained at the conditions of 80°C and 30 min in Table 2.

3.2.2 Validation of the predictive model

To verify the validity of the predictive model, a further experiment was conducted using the suggested values for the extraction variables although the values were slightly adjusted to 80°C for temperature and to 30 min for the

Table 3. Statistical analysis of regression equation results for RSM

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of freedom</th>
<th>F ratio</th>
<th>Probability &gt; F(0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5</td>
<td>32.02</td>
<td>0.0025*</td>
</tr>
<tr>
<td>Temperature (A)</td>
<td>1</td>
<td>82.83</td>
<td>0.0008*</td>
</tr>
<tr>
<td>Extraction length (B)</td>
<td>1</td>
<td>65.50</td>
<td>0.0013*</td>
</tr>
<tr>
<td>A^2</td>
<td>1</td>
<td>5.63</td>
<td>0.0767</td>
</tr>
<tr>
<td>B^2</td>
<td>1</td>
<td>0.67</td>
<td>0.4603</td>
</tr>
<tr>
<td>AB</td>
<td>1</td>
<td>5.48</td>
<td>0.0792</td>
</tr>
</tbody>
</table>

a) The **“*” symbol indicates a significant effect \( p < 0.05 \).
length of extraction. The tea was ground to particle size 0.5–1 mm, the water-to-tea ratio was 20:1 mL/g and the extraction media was agitated using a shaking water bath. The results showed that the obtained yield of theanine was 26.80 ± 0.42 (mg/g) (experimental value), which was entirely consistent with the value of 26.72 ± 0.68 (mg/g) predicted by the RSM model.

4 Concluding remarks

The impact of extraction conditions on the yield of theanine extracted from green tea using water was thoroughly investigated. The results showed that the temperature and the extraction time had major significant impacts on the yield of theanine extracted. The yield of theanine increased with increasing temperatures and extraction time and reached a plateau at 80 °C and 30 min, respectively. The ratio of water-to-tea also affected the yield of theanine; the higher ratios of water-to-tea resulted in higher yields of theanine. However, taking into account both theanine yield and efficient water use (cost-effectiveness), the results suggested that the optimum ratio was water-to-tea of 20:1 mL/g. The tea particle size was also found to have an impact on the yield of theanine and the optimum size was 0.5–1 mm. However, the results showed that this factor had the least impact of the four tested.

Finally, the RSM was employed to design and predict a model for further optimising the conditions for effectively extracting theanine from green tea using temperature and the extraction time as the two most influential factors. The model was found to be of high precision; it predicted that the optimum conditions for extracting theanine from green tea were 79.4 °C and 29.5 min for temperature and length of extraction, respectively. The predicted optimum conditions were further tested by an actual extraction experiment and the experimental yield of theanine was found to be very close to the predicted yield.

In conclusion, the optimal conditions for extracting theanine from dried green tea with water were found to be 80 °C for 30 min with a particle size of 0.5–1 mm and a water-to-tea ratio of 20:1 mL/g. These optimised conditions should prove useful for the accurate quantification of theanine in tea and as an efficient first step for its purification.

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The authors have declared no conflict of interest.

5 References


2.2.3. Effects of aqueous brewing solution pH on 
extraction of the major green tea constituents

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and Paul D. Roach

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Effects of aqueous brewing solution pH on the extraction of the major green tea constituents

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The pH of the aqueous brewing solution was maintained at values ranging from 1 to 9 during the green tea extraction and the effects on the tea’s extracted constituents were studied. The epistructured catechins were stable under acidic conditions but epimerized or degraded at pH ≥ 6. The extractable solids contained more epistructured catechins at pHs 3–5 but more non-epistructured catechins at pHs 6–7. More tea cream was obtained at pH 1 but the concentration of catechins, caffeine and theanine was low in this fraction. Therefore, to maximize the extraction of the epistructured catechins and to minimize their epimerization and degradation and to maximize the extraction of caffeine and theanine, the results suggest that the pH should be maintained between 3 and 5.3 during the aqueous brewing process.

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1. Introduction

Green tea refers to non-fermented tea and it is an abundant source of catechins, theanine and caffeine, which have been receiving considerable interest for their potential health benefits (Vuong, Golding, Nguyen, & Roach, 2010). Recent in vivo and epidemiological studies have linked these green tea constituents with various health benefits such as the prevention of cancers, cardiovascular diseases, dental decay, obesity, diabetes, and improvement in the immune system (Kao, Chang, Lee, & Chen, 2006; Khan & Mukhtar, 2007; Vuong, Bowyer, & Roach, 2011; Wheeler & Wheeler, 2001). These green tea constituents do not only promote human health but also have been found to be potentially useful as food preservatives because they can prevent lipid peroxidation and improve the color and flavor of some foods (Vuong, Stathopoulos, Nguyen, Golding, & Roach, 2011).

Due to their potential as health promoters and food preservatives, there is a need to maximize the extraction of these constituents from green tea in order to improve the production of green tea extracts and powders on an industrial scale. Several extraction conditions, including tea particle size, brewing temperature, length of extraction, ratio of solvent to tea, type of solvent and the number of times the same tea is extracted, have been found to directly affect the extraction efficiency of the tea constituents (Friedman, Levin, Choi, Kozukue, & Kozukue, 2006; Perva-Uzunalić et al., 2006; Vuong, Golding, Stathopoulos, Nguyen, & Roach, 2011; Yoshida, Kiso, & Goto, 1999).

The few studies conducted to determine the impact of the brewing solution pH on the extraction of the green tea constituents have found an effect. However, the range of the brewing solution pH values studied was limited to between 4 and 7 in one study (Kim, Park, Lee, & Han, 1999) and 6–8 in the other (Yoshida et al., 1999). In addition, the pH of the brewing solution in these studies was only adjusted prior to the brewing process and it was not monitored and adjusted during brewing. Therefore, it is very likely that the brewing solution pH changed during the brewing process as it was observed in a similar study with black tea (Liang & Xu, 2001). This means that, if the pH of the brewing solution was not monitored and adjusted during the brewing process, the experiments were conducted at pH values other than at those intended.

In a recent study (Vuong et al., 2011), our preliminarily results on the impact of the brewing solution pH, which was strictly controlled from 1 to 9 during the brewing process, showed that it can significantly affect the amount of total catechins and extractable solids. However, its impact on the extraction and stability of individual catechins as well as on the other two important components, caffeine and theanine, was not studied. Therefore, the present study extends and expands on our previous findings, to optimize the pH for the preparation of green tea aqueous extracts.

Therefore, the aim of this study was to investigate the effect of various strictly-controlled brewing solution pH values on the amount of the eight main catechins, caffeine and theanine extracted from green tea. Furthermore, its impact on an important tea phenomenon called cream formation was also comprehensively studied. In addition, the impact of pH on a commercially available pure EGCG was examined in an attempt to determine whether pH affected the efficiency of extraction or the stability of the catechins.
2. Materials and methods

2.1. Materials

The Shan green tea variety (*Camellia sinensis var. pubilimba*), dried by pan firing, was from the Thai Nguyen region of Vietnam and was obtained from the Vietnam Tea Corporation (Hanoi, Vietnam). The green tea was ground, using a Waring blender (John Morris Scientific Pty, Chatswood, NSW, Australia), to small particle sizes which could pass through a 1 mm sieve. The following chemicals were used for analysis: L-theanine, L-tryptophan (as an internal standard), caffeine, epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG), galloylcathechin gallate (GCC), catechin (C), gallatecatechin (GC), catechin gallate (CG) and epicatechin gallate (EGG) were obtained from Sigma Chem. Co. (Castle Hill, NSW, Australia); Teavigo™ EGCG extract was obtained from RejuvaCare (Sydney, NSW, Australia); sodium hydroxide, hydrochloric acid, acetonitrile, orthophosphoric acid and tetrahydrofuran were purchased from Lombr Scientific (Taren Point, NSW, Australia). Ultra-pure (Type 1) de-ionised (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia Pty Ltd, North Ryde, Australia).

2.2. Extraction of green tea

Five grams of ground tea was brewed with 100 mL of distilled water at 80 °C for 30 min using a shaking water bath (Ratek Instruments Pty. Ltd, Boronia, Vic, Australia). To determine the impact of pH on the tea constituents during the extraction process, the pH of the water used to brew the tea was adjusted to values of 1, 2, 3, 4, 5, 6, 7, 8 and 9. In the first experiment, the pH was monitored during the brewing process and was found to change considerably. In subsequent experiments, the pH was maintained constant at 1, 2, 3, 4, 5, 6, 7, 8 or 9 during the whole brewing process by the addition of 5 M or 0.1 M HCl and 5 M or 0.1 M NaOH; the volume added was kept to a minimum (≤0.5 mL) in order not to affect the concentration of the extracted constituents. The pH of the solution during the brewing process was closely monitored and controlled using a laboratory CHEM-pH meter version 1.02 (TPS Pty Ltd, Springwood, Brisbane, Australia), which was calibrated for 80 °C (Vuong et al., 2011).

When the pH of the water was not adjusted prior to or during brewing, the resulting green tea infusion was found to have a pH of 5.3. The extractions which were not pH adjusted were referred to as ‘control’ (abbreviated ‘Ctrl’ in figures and tables) samples.

2.3. Measurement of green tea constituents

The extracted tea constituents, theanine, caffeine and catechins were measured by high performance liquid chromatography (HPLC). The tea solutions were allowed to cool to room temperature and then diluted 1:4 with DI water and further diluted 1:1 with 500 μM L-tryptophan (as internal standard) in DI water. These solutions were filtered using a 0.45 μm cellulose syringe filter and transferred to brown glass vials using 5 mL disposable syringes. The solutions were then diluted 1:1 with 500 μM L-tryptophan (as internal standard) in DI water and then filtered and injected onto HPLC for analysis. The results were expressed as mean ± SD in mg of catechin per gram of EGCG incubated (mg/g).

A before the next injection. An auto-injector was used to inject 20 μL of the tea solution onto the HPLC column and the flow rate was 1 mL/min.

The tea constituents were then quantified by dividing the peak areas of the tea constituents by the peak area of the internal standard, L-tryptophan, and determining their concentration from a standard curve of the peak area ratios of increasing concentrations of pure constituent external standard to 250 μM L-tryptophan.

2.4. Determination of EGCG stability

Commercial Teavigo™ EGCG (RejuvaCare, Sydney, NSW, Australia) with a minimum purity of 90% was used to test the impact of pH on the stability of the catechin. A 0.5 mM EGCG solution was prepared in DI water. The pH of aliquots was adjusted to values ranging from 1 to 9 and the pH-adjusted solutions were incubated at 80 °C for 30 min, the same conditions used to brew the green tea. The solutions were then allowed to cool to room temperature, diluted 1:1 with 500 μM L-tryptophan (as internal standard) in DI water and then filtered and injected onto HPLC for analysis. The results were expressed as mean ± SD in mg of catechin per gram of EGCG incubated (mg/g).

2.5. Determination of extractable solids

The amount of extractable solids obtained after brewing was determined using the method described by Obanda, Owuora, Mang’oka, and Kavoi (2004). After brewing, the tea solution was filtered using a Whatman #1 filter paper (90 mm diameter) (Lombr Scientific, Taren Point, NSW, Australia), and then weighed to the nearest 0.0001 g. Aliquots were placed in pre-weighed containers and the solution was dried to a constant weight using a vacuum drier set at 40 °C (Thermoline Scientific Equipment, Smithfield, NSW, Australia). The weight of the dried matter was determined and the recovery of the dried extractable solids was calculated and expressed in mg per g of tea brewed as per Eq. (1):

\[
DM (mg/g) = \frac{W_1 \times V_2}{W_2 \times V_1}
\]

where DM is dry matter or extractable solids (mg/g); W1 is the weight of dry matter after drying (mg); W2 is the weight of the tea sample extracted (g); V1 is the volume of the filtered tea solution dried (mL); and V2 is the total volume of the tea solution after brewing (mL).

2.6. Determination of green tea cream formation

The amount of tea cream formed from solutions after brewing was determined using the method described by Liang, Lu, and Zhang (2002). Freshly brewed tea solution (25 mL) was quickly cooled on ice and stored at 4 °C for 24 h. The tea solution was then centrifuged at 17, 400 × g for 20 min at 4 °C using a Beckman centrifuge (Beckman Instruments Inc., Palo Alto, California, USA) to precipitate any formed tea cream. The precipitate was then dried to constant weight using a vacuum drier set at 40 °C (Thermoline Scientific Equipment, Smithfield, NSW, Australia). The weight of the dried precipitate was determined and the recovery of the dried tea cream was calculated as per Eq. (2) and expressed in mg per g of dried extractable solid (from Eq. (1)):

\[
DP (mg/g) = \frac{W_1 \times V_2}{DM \times V_1}
\]

where DP is the dried precipitate or cream (mg/g); W1 is the weight of the tea cream precipitate after drying (mg); DM is the weight of dry matter (g) obtained from the same volume of tea used to obtain the cream; V1 is the volume of the tea solution used to obtain the tea.
Fig. 1. Relationship between the pH of the water prior to brewing and the pH of the brewing solution. The pH of the DI water was adjusted to the indicated values prior to brewing green tea at 80 °C for 30 min. The pH was monitored during the brewing process but it was not adjusted. The measured pH values are expressed as mean±SD of three independent experiments and with the asterisk symbol are significantly different (P<0.05) from the unadjusted water (pH 6.4) values, which were used as control (Ctrl).

The experiment was repeated six times and the values are expressed as mean±SD in mg of constituent per gram of dry tea used for brewing (mg/g). Values with the asterisk symbol are significantly different (P<0.05) from the control value (Ctrl).

The results in all tables and figures are presented as the mean value±standard deviation of at least three independent experiments. The one-way ANOVA analysis and the Dunnett post hoc test were performed to determine whether there were significant differences (P<0.05) between samples brewed at the various pH values and the control sample. The statistical analysis was performed using the SPSS-PASW GradPack 18.0 for Windows.

3. Results and discussion

3.1. Effect of pH on the extraction and stability of green tea catechins

Firstly, the buffering capacity of green tea was investigated and the results are shown in Fig. 1. When green tea was brewed in water, which was adjusted to pH values ranging from 1 to 9 prior to brewing, the pH of the brewing solution tended to change to the pH of the control brewing solution. As seen in Fig. 1, the unadjusted pH of control DI water was 6.4 but it went down to pH 5.3 during the brewing process due to the buffering capacity of the green tea and its extracted constituents. For the water adjusted to pH values from 4 to 8 prior to brewing, the pH of the brewing solution ended up similar to the pH of the control (pH 5.3). However, although there were changes toward 5.3, the pH values of the brewing solutions adjusted to 1, 2 and 3 or to 9 prior to brewing, were significantly lower or higher than for the control, respectively (Fig. 1).

Therefore, similar to the buffering capacity of black tea found in a previous study (Liang & Xu, 2001), green tea was also observed to have a strong buffering capability. As a consequence, it was deemed necessary to monitor and control the pH of the brewing solution throughout the brewing process in order to be able to accurately determine the impact of the pH on the extractability and stability of green tea constituents in the subsequent experiments.

The effect of pH on the extraction of the green tea catechins is shown in Table 1. At the low extraction water pH values between 1 and 5, the levels of the individual and total catechins extracted were not significantly different from control—i.e. when the pH of the extraction water was not adjusted prior to or during the brewing process. These results confirmed the findings of our previous study (Vuong et al., 2011) in that the extraction of the total catechins was not significantly different when the green tea was brewed at pH values of 5 or less. However, the results extended the previous findings in showing that the extraction of the total catechins at each of these pH values also did not differ from when the pH was not adjusted prior to or during the brewing process. Furthermore, Table 1 shows that the same was observed for all eight of the individual catechins as well as for caffeine and theanine.

The findings were also similar to those found by Kim et al. (1999) and indicate that an acidic extraction water pH (≤5) does not influence the extraction efficiency of the catechins from green tea. However, the authors did not report on the extraction of the individual catechins and did not investigate pH values lower than 4. Therefore, the present study is the first to show that lowering the pH of the brewing solution to less than 4 does not decrease or increase the
The level of GCG measured was also stable under acidic conditions up to pH 4. However, the level of GCG progressively increased when the pH of the solution was increased from pH 4 to 8, obviously due to more GCG being formed from EGCG. When the pH was 9, the GCG concentration decreased and was similar to the levels measured at pHs 1 to 4. However, at pH values from 4 to 8, the increase in GCG only accounted for less than 4% of the loss in EGCG and at pH 9, GCG also decreased (Fig. 3). Therefore, epimerization to GCG, was increased when the pH of the solution was increased from pH 6 to 9. These results showed that the epistructured catechins could be more easily epimerized to their non-epistructures when the green tea was extracted in solutions having a pH from 6 to 8. However, as shown in Table 1, there was a general decrease in the levels of all the catechins (epistructured and non-epistructured) when the pH of the brewing solution was higher than 7. Therefore, in terms of absolute amounts, the optimal pH range for the non-epistructured catechins was from 6 to 7.

These observations (Fig. 2 and Table 1) confirmed our previous findings (Vuong et al., 2011) and were consistent with another study (Yoshida et al., 1999), which showed that the epistructured catechins tended to epimerise to non-epistructured catechins when green tea was extracted in solutions with pH values ranging from 6 to 7.6. Yoshida et al. (1999) also found that both the epistructured and the non-epistructured catechins became very unstable when the tea was extracted at pH values from 7 to 9. Therefore, adjustment of the brewing pH can minimize the epimerization of the natural epistructured catechins to their non-epistructured counterparts but the pH can also be adjusted to stimulate the epimerization process for the production of the non-epistructured catechins should it be desired.

The effect of pH on the stability of the catechins was further investigated by incubating 0.5 mM EGCG at 80 °C for 30 min at the same pH values as the previous brewing experiment. The findings in Fig. 3 showed that EGCG was very stable in acidic solutions having pH values less than 4 but its stability progressively decreased as the pH of the extraction solution increased from pH 4 to 8 and it was extremely unstable when the pH was 9. Although not identical, the results presented in Fig. 3 for the stability of EGCG at the different pH values are very similar to those presented in Table 1 for the extraction of EGCG and the other catechins from green tea at the same pH values. Therefore, because of this high similarity, it appears that the bulk of the differences caused by pH during the extraction process is due to instability of the catechins at the higher pH values rather than differences in the efficiency of their extraction.

As shown in Fig. 2, the pH of the brewing solution was also found to influence the epimerization of the catechins. Normally, the catechins in green tea are epistructured (EGC, EGCG, EC and ECG) but epimerization to their non-epistructured counterparts (GC, GCG, C and CG) can happen during processing and brewing (Wang & Helliwell, 2000). In the present study, the ratio of the non-epistructured catechins to the epistructured catechins was constant and similar to control when the pH of the solution was 5 or lower. However, this ratio dramatically increased when the pH of the solution was increased from pH 6 to 9. These results showed that the epistructured catechins could be more easily epimerized to their non-epistructures when the green tea was extracted in solutions having a pH from 6 to 8. However, as shown in Table 1, there was a general decrease in the levels of all the catechins (epistructured and non-epistructured) when the pH of the brewing solution was higher than 7. Therefore, in terms of absolute amounts, the optimal pH range for the non-epistructured catechins was from 6 to 7.

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As shown in Fig. 2, the pH of the brewing solution was also found to influence the epimerization of the catechins. Normally, the catechins in green tea are epistructured (EGC, EGCG, EC and ECG) but epimerization to their non-epistructured counterparts (GC, GCG, C and CG) can happen during processing and brewing (Wang & Helliwell, 2000). In the present study, the ratio of the non-epistructured catechins to the epistructured catechins was constant and similar to control when the pH of the solution was 5 or lower. However, this ratio dramatically increased when the pH of the solution was increased from pH 6 to 9. These results showed that the epistructured catechins could be more easily epimerized to their non-epistructures when the green tea was extracted in solutions having a pH from 6 to 8. However, as shown in Table 1, there was a general decrease in the levels of all the catechins (epistructured and non-epistructured) when the pH of the brewing solution was higher than 7. Therefore, in terms of absolute amounts, the optimal pH range for the non-epistructured catechins was from 6 to 7.

These observations (Fig. 2 and Table 1) confirmed our previous findings (Vuong et al., 2011) and were consistent with another study (Yoshida et al., 1999), which showed that the epistructured catechins tended to epimerise to non-epistructured catechins when green tea was extracted in solutions with pH values ranging from 6 to 7.6. Yoshida et al. (1999) also found that both the epistructured and the non-epistructured catechins became very unstable when the tea was extracted at pH values from 7 to 9. Therefore, adjustment of the brewing pH can minimize the epimerization of the natural epistructured catechins to their non-epistructured counterparts but the pH can also be adjusted to stimulate the epimerization process for the production of the non-epistructured catechins should it be desired.

The effect of pH on the stability of the catechins was further investigated by incubating 0.5 mM EGCG at 80 °C for 30 min at the same pH values as the previous brewing experiment. The findings in Fig. 3 showed that EGCG was very stable in acidic solutions having pH values less than 4 but its stability progressively decreased as the pH of the extraction solution increased from pH 4 to 8 and it was extremely unstable when the pH was 9. Although not identical, the results presented in Fig. 3 for the stability of EGCG at the different pH values are very similar to those presented in Table 1 for the extraction of EGCG and the other catechins from green tea at the same pH values. Therefore, because of this high similarity, it appears that the bulk of the differences caused by pH during the extraction process is due to instability of the catechins at the higher pH values rather than differences in the efficiency of their extraction.
not the major factor for the loss of EGCG. In other words, the loss of EGCG was due to degradation that did not lead to the stable formation of GCG.

Therefore, these findings showed that the major green tea catechin, EGCG, is stable under low pH conditions but unstable at pH values higher than 4 due to a small part to an increased epimerization to GCG in the range of pH from 4 to 8, but mostly due to degradation other than epimerization (Komatsu et al., 1993; Suzuki et al., 2003). It is also known that the risk of catechin degradation is greater at extraction temperatures higher than 80 °C (Vuong et al., 2010). Therefore, adjustment of the brewing solution to acidic conditions may help to stabilize the catechins during the extraction process at high temperatures.

3.2. Effect of pH on the extraction and stability of green tea theanine and caffeine

Theanine is an important component of tea because of its contribution to the umami taste of the tea infusion and to its health benefits (Vuong et al., 2011), and thus, it is necessary to examine the impact of the brewing solution pH on the extraction efficiency of theanine. When the green tea was brewed at pH values from 1 to 9, the concentrations of theanine were between 11.5 and 13.9 mg/g and were not significantly different from the concentration achieved with the control method (Table 1).

These results (Table 1) showed that the pH of the brewing solution did not affect the extraction efficiency of theanine. These findings were in agreement with the results of a previous study (Kim et al., 1999). However, under a very prolonged incubation of up to 336 h, Ekborg-Ott, Taylor, and Armstrong (1997) reported that there was more hydrolysis of theanine at pH 11 than at pH 3 and pH 7.

Caffeine is also an important component of green tea, and thus, the impact of the brewing solution pH on the extraction of caffeine was also investigated. The findings showed that the pH of the brewing solution did not affect the extraction efficiency of caffeine (Table 1). The levels of caffeine extracted at pH values from 1 to 9 ranged from 25.1 to 27.6 mg/g and were not significantly different from the concentration achieved with the control method. These findings differ from those of a previous study (Kim et al., 1999), which found that the concentration of caffeine increased when the pH of the extraction solution was increased from 4 to 7. However, these findings are in agreement with the results reported in a study with black tea (Spiro & Price, 1987).

3.3. Effect of pH on the components of the green tea extractable solids

The results of our previous study (Vuong et al., 2011) showed that more tea solids were extracted when the tea was brewed under acidic conditions (pHs 1 and 2) or under alkaline conditions (pHs 8 and 9). In addition, the concentration of the total catechins in the dried extractable solids was lower when the green tea was extracted at pHs 1 and 2 as well as at pHs 7–9. However, the concentration of the epistructured and the non-epistructured catechins in the extractable solids was not studied.

The current study further investigated the impact of brewing pH on the concentration of the epistructured and the non-epistructured catechins of the extractable solids. Fig. 4 shows that the concentration of the non-epistructured catechins in the dried extractable solids did not change and was not significantly different from control when the green tea was brewed at pH values from 1 to 5. In contrast, the concentration of the epistructured catechins was significantly lower at pHs 1 and 2 and higher at pH values from 3 to 5 compared to control (pH 5.3).

At pH 6, the concentration of the epistructured catechins in the extractable solids was not different than for the control whereas the concentration of the non-epistructured catechins was significantly increased. The concentration of the non-epistructured catechins was even higher at pH 7 while the concentration of the epistructured catechins was dramatically reduced to 26% of control. When the pH exceeded 7, both the epi and non-epistructured catechins were found to be at very low levels in the extractable solids, indicating that both types were likely to be unstable at these high pHs as found previously for the total catechins (Vuong et al., 2011). Therefore, this suggests that, in order to prepare green tea powders with a high level of epistructured catechins relative to the total extractable solids, the tea should be brewed at pH values ranging from 3 to 5.3. However, in order to produce green tea powders with a high concentration of non-epistructured catechins, the tea should be brewed at pH 6 or 7.

The findings (Fig. 4) also indicated that the pH of the brewing solution had a significant but more muted effect on the content of caffeine and theanine in the green tea extractable solids compared to the effects on the content of the catechins. For theanine, the levels were lower than control at pHs 1 and 2 and at pHs 8 and 9 (Fig. 4) but were not different from pHs 3 to 7. The same pattern was observed for caffeine (Fig. 4). Therefore, in order to produce green tea

![Fig. 4. Effect of brewing solution pH on the concentration of components in the extractable solids. The values are expressed as mean ± SD of six experiments in mg of component per gram of dry weight of the extractable solids (mg/g). Values with the asterisk “*” symbol are significantly different (P<0.05) from the control (Ctrl) samples at pH 5.3.](image-url)

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powders with a high theanine and caffeine content, the tea should be brewed at pH values ranging from 3 to 7.

### 3.4. Effect of pH on cream formation and on its components

The effects of the brewing solution pH on the subsequent formation of tea cream are shown in Fig. 5 and the composition of the cream is shown in Table 2. The results revealed that more cream was formed when the tea was extracted under highly acidic conditions (pH 1). In contrast, very low levels of cream were formed when the green tea was extracted at pH values from 7 to 9. This can be explained by the mechanism by which tea cream is formed. The creaming of tea is a result of an interaction between the catechins with either proteins or caffeine (Vuong et al., 2010). Therefore, with low levels of catechins in the extracts from tea brewed at pHs 1, 2 and 4 and lower at pHs 6 to 9 (Table 1), there was little cream formed (Fig. 5) and the cream had low levels of catechins at these pH values (Table 2).

However, the concentrations of catechins were also low in the cream obtained after the tea was brewed at pH 1 (Table 2). According to Liang and Xu (2001), at highly acidic pH, the catechins can be converted to ‘transient intermediate species’, which allows them to be captured by polysaccharides and nucleophilic species such as HS- and HN- on protein molecules and thus, can stimulate cream formation. Furthermore, higher amounts of polysaccharides and proteins are released from the tea at acidic pH, which results in high levels of these precipitating by themselves without the need for high concentrations of catechins (Spiro & Price, 1987).

It was also found that the relative concentration of the esterified catechins (EGCG, EGC, GCG and CG) was much higher in the tea cream compared to the un-esterified or free catechins (EGC, EC, GC and C) (Table 2). For example, under the control brewing conditions (pH 5.3), the esterified catechins accounted for 86.2±1.6% of the total catechins compared to 13.8±1.6% for the free catechins. These proportions also differed markedly from those in the tea infusion brewed at the control pH before the formation of tea cream (Table 1); in the tea infusion the esterified and free catechins accounted for 52.3±2.3% and 47.7±2.3% of the total catechins, respectively.

It can also be seen that when to the values for EGC and EC in Tables 1 and 2 are compared, the differences between the tea infusion and the tea cream were mainly due to these two catechins being at low concentrations in the tea cream. However, changing the pH of the brewing solution did not have a major impact on the relative content of the esterified and free catechins in the tea cream. Thus, this property could be exploited to separate the esterified catechins from the un-esterified catechins.

The percentages of the catechins precipitated in the tea cream and left behind in the supernatant at the different brewing pH values after separation of the tea cream by centrifugation are presented in Fig. 6. The results revealed that, at pHs 1 to 6, 35–40% of the catechins were precipitated in the tea cream while 60–65% of the catechins remained in the supernatant. At pH values from 7 to 9, the proportion of catechins measured in the cream was markedly decreased; most of the catechins (≥80%) were found in the supernatant.

The findings also indicated that the pH of the brewing solution also had a significant effect on the content of caffeine and theanine in the tea cream. For theanine, the levels in the tea cream were higher at pHs 1, 2 and 4 lower at pHs 6 to 9 than for control (Table 2).
However, for caffeine the levels in the cream were lower than control at all pH values except for pH 5, which was the same as for control (Table 2). Therefore, to efficiently extract and recover theanine in the tea cream, a pH ≤ 4 would be preferable. However, for caffeine, a pH around 5 would be optimal.

4. Conclusions

This study showed that the extraction of the catechins was significantly affected by the pH of the brewing solution; it was higher under acidic conditions (pH < 6) than under more neutral and basic conditions. This was most likely due to instability of the catechins at the higher pH values rather than to differences in the efficiency of their extraction. The extraction of caffeine and theanine was less influenced by changing the brewing solution pH. The extractable solids contained more epistructured catechins at pHs 3–5 but more non-epistructured catechins at pHs 6–7. More tea cream was also obtained when the green tea was brewed at pH 1, but the concentration of catechins, caffeine and theanine was low in this fraction. Therefore, to maximize the extraction and minimize the degradation of catechins during the brewing process, the current study suggests that the pH should be maintained at acidic conditions. In addition, to maximize the epistructured catechins relative to their non-epistructured counterparts, the green tea should be brewed at pHs between 3 and 5.3.

Conflict of interest

The authors have declared no conflict of interest.

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2.2.4. Production of caffeinated and decaffeinated green tea catechin powders from underutilised old tea leaves

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Production of cafffeinated and decaffeinated green tea catechin powders from underutilised old tea leaves

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Abstract

Only the apical bud and the top four leaves are normally used to make high quality green teas, while the older lower leaves are cut and used for mulch. The aim was to determine whether the old fifth to tenth leaves could be used to make cafffeinated and decaffeinated green tea catechin powders. The leaves were decaffeinated by blanching in water at 100°C for 10 min to remove 80% of the caffeine while retaining 85% of the catechins. The leaves were then extracted in water at 80°C and freeze drying gave 100% yields of extractable powder and catechins while spray drying gave 20–25% lower yields. Decaffeination and spray drying also increased the conversion of epistructured to non-epistructured catechins. Therefore, this study has shown that old green tea leaves, which are usually discarded, could be used as an underutilised source to make cafffeinated and decaffeinated green tea catechin powders.

1. Introduction

Green tea from the Camellia sinensis plant is a rich source of the strong antioxidant compounds called the catechins, which account for about 30% of the weight of the infusion's extractable solids when the beverage is dried (Graham, 1992; Vuong et al., 2010). Numerous epidemiological studies have linked the tea catechins with many human health benefits such as prevention of cancers, cardiovascular diseases, microbial diseases, diabetes and obesity (Khan and Mukhtar, 2007). The catechins have also been found to prevent lipid peroxidation, a major problem in the food industry, which can cause undesirable rancidity and potentially toxic reaction products (Vuong et al., 2011c).

The apical bud, the top four leaves and the stem down to the fourth leaf, are the only parts of the tea plant normally used to make green teas of the highest quality whereas the older more mature leaves from the fifth to the tenth leaf down the stem are generally cut during pruning and left on the ground as mulch for the soil. These older leaves are therefore underutilised and have been considered as waste because they cannot be used for making high quality tea (Zandi and Gordon, 1999). Nonetheless, as shown by Farhoosh et al. (2007) these old tea leaves have antioxidant properties similar to that of the new young leaves because of their content of catechins. Thus, these older tea leaves may be useful for making catechin powders for use by the food and supplements industries.

Various methods have been developed to produce green tea powders with low levels of caffeine (Copeland et al., 1998; Dong et al., 2011; Jin et al., 2006). However, the methods using water as the solvent (Bazinet et al., 2007; Sinija et al., 2007) produce powders with high levels of caffeine, which may also limit their acceptance by health-conscious consumers and their utilisation in the food industry.

Attempts have been made to produce green tea powders with low levels of caffeine (Copeland et al., 1998; Dong et al., 2011; Jin et al., 2006). However, organic solvents such as chloroform, ethyl acetate, ethyl hexanoate and propyl acetate have been used to remove the caffeine but with these methods there is always the potential for contaminating residues, which are even less likely to be acceptable to discerning consumers than the caffeine itself.

Resin and activated charcoal based methods (Lu et al., 2010; Ye et al., 2007; Zhao et al., 2007) and supercritical fluid extraction with carbon dioxide (Park et al., 2007; Kim et al., 2008), which do not need organic solvents, have also been used to produce decaffeinated tea powders. However, these methods are costly to set up on an industrial scale.
A recent study by Liang et al. (2007) found that hot water could be effectively used to remove caffeine from freshly picked young tea leaves. Therefore, hot water is promising as a solvent for decaffeination because it is safe, inexpensive and accessible in comparison with organic solvents (Vuong et al., 2011a).

Therefore, the aims of this study were to determine whether old green tea leaves (the fifth to the tenth leaves on the stem), which are usually discarded, could be used as an underutilised source of catechins and whether hot water could be used to prepare decaffeinated green tea powders from these otherwise wasted old tea leaves. Two drying methods, freeze drying and spray drying were also compared.

2. Materials and methods

2.1. Materials

The fifth to the tenth leaves and their stems were harvested from the Yabukita cultivar of the C. sinensis var. sinensis tea plant in March 2011, at the end of the Australian tea growing season, from the experimental green tea plantation on the NSW Department of Primary Industries research farm at Somersby, NSW, Australia (latitude 33°22’S; longitude 151°21’E). The leaves are referred to as ‘underutilised old tea leaves’ or ‘old tea leaves’ in this study.

The leaves and stems were immediately stored at −18 °C, to minimise the oxidation and degradation of the catechins, until they were thawed to produce caffeinated and decaffeinated green tea powders.

The chemicals used for analysis, L-theanine, L-tryptophan (used as an internal standard), caffeine, the epistructured catechins, epigallocatechin gallate (EGC), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) and the non-epistructured catechins, gallocatechin gallate (GCG), gallocatechin (GC), catechin gallate (CG) and catechin (C) were obtained from Sigma Chem. Co. (Castle Hill, NSW, Australia) and acetonitrile, orthophosphoric acid and tetrahydrofuran were purchased from Lomb Scientific (Taren Point, NSW, Australia). Ultrapure (type 1) de-ionised (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia Pvt. Ltd., North Ryde, Australia).

2.2. Methods

2.2.1. Experiment design

A schematic of the processes used to produce the caffeinated and decaffeinated green tea powders is presented in Fig. 1. To produce the caffeinated green tea powder, 500 g of stored old tea leaves and stems were thawed at room temperature for 10 min, the leaves were plucked from the stem, steamed for 30 s to inactivate the oxidation enzymes and then heated at 80 °C for 4 h in a drying oven (Andrew Thorn Ltd., Sydney, Australia) to obtain dried green tea leaves (Liang et al., 2007). To produce the decaffeinated green tea powders, 500 g of stored old leaves and stems were thawed at room temperature for 10 min, the leaves were plucked from the stem and then blanched in 10 L of water at 100 °C for 10 min to remove as much caffeine as possible and to inactivate the oxidising enzymes, instead of steaming for 30 s. The stems were not used because they were too woody.

To determine the optimal blanching time for decaffeination, the old green tea leaves were thawed at room temperature for 10 min and blanched in water for various lengths of time from 5 to 25 min at the optimal temperature and water-to-tea ratio of 100 °C and 20:1 mL/g, as reported by Liang et al. (2007) for the removal of caffeine from fresh young green tea leaves. The blanched tea leaves were then removed from the water and heated at 80 °C for 4 h in a drying oven (Andrew Thorn Ltd., Sydney, Australia) to obtain dried green tea leaves.

The dried caffeinated and decaffeinated tea leaves were put through a series of steps including grinding, brewing, filtering and drying to produce caffeinated and decaffeinated tea powders.

![Flow chart of the methods used to prepare caffeinated and decaffeinated dried green tea powders from old tea leaves. The process outlined on the left side of the diagram was used to prepare caffeinated spray dried and freeze dried green tea powders. The process outlined on the right side of the diagram was used to prepare decaffeinated spray dried and freeze dried green tea powders.](image-url)
the dried tea leaves were ground into particles < 1 mm (1 mm blender (John Morris Scientific Pvt., Chatswood, NSW, Australia) to remove fine colloidal particles and finally filtered through a 90 mm Whatman #1 filter paper (Lomb Scientific, Taren Point, NSW, Australia) before freeze drying or spray drying (Fig. 1).

For freeze drying, the tea solutions were frozen using liquid nitrogen and freeze dried using a FD3 freeze dryer (Thomas Scientific Equipment, Smithfield, NSW, Australia) set at 70 °C to a constant weight using a vacuum oven dryer (Thermoline Scientific, Smithfield, NSW, Australia) at a spray flow rate of 50 mL/min, inlet air temperature of 110 ± 2 °C, outlet air temperature of 170 °C, outlet air temperature of 110 ± 2 °C, spray flow rate of 250 mL/h and aspiration set at 100%.

In this process (Fig. 1), caffeinated and decaffeinated freeze dried and decaffeinated freeze spray dried green tea powders were produced. The four types of powders were stored at room temperature in a sealed box and kept dry in a desiccator containing silica until they were analysed.

2.2.2.1. Yield of dried tea leaves and tea powders and recovery of catechins in the tea powders. The yield of caffeinated and decaffeinated dried tea leaves was expressed as a percentage as calculated using Eq. (1):

\[
\text{Yield (\%)} = \frac{\text{DL} \times 100}{\text{WL}}
\]  

where DL is the weight (g) of dried tea leaves and WL is the wet weight (g) of the harvested old tea leaves used to make that amount of dried tea leaves.

The dry weight of extractable solids (ES) in the dried tea leaves was calculated using Eq. (2):

\[
\text{ES (mg/g)} = \frac{W_1 \times V_2}{V_1 \times W_2}
\]

where ES is the dry weight of extractable solids (mg) per g of dried tea leaves extracted (mg/g), \(W_1\) is the dry weight (mg) of the solids obtained after drying a measured volume \(V_1\) (mL) of the tea extract to a constant weight using a vacuum oven dryer (Thermoline Scientific Equipment, Smithfield, NSW, Australia) set at 70 °C, \(W_2\) is the weight (g) of the dried tea leaves used to make the tea extract and \(V_2\) is the total volume (mL) of the tea extract.

The yield of the freeze and spray dried caffeinated and decaffeinated tea powders was expressed as a percentage as calculated using Eq. (3):

\[
\text{Yield (\%)} = \frac{P \times 100}{\text{ES}}
\]

where \(P\) is the weight (g) of the dried tea powder, obtained by freeze or spray drying, and ES is as derived from Eq. (2).

The recovery of catechins in the tea powders was expressed as a percentage as determined using Eq. (4):

\[
\text{Catechin recovery (\%)} = \frac{C_1 \times 100}{C_2 \times Y}
\]

where \(C_1\) is the weight (mg) of total catechins per g of tea powder, \(C_2\) is the weight (mg) of total catechins per gram of dried tea leaves and \(Y\) is the yield of powder from dried tea leaves as per Eq. (3).

2.2.2.2. Physical properties of the dried tea leaves and the tea powders. The moisture content, water activity (a_w) and the tapped bulk density of the dried tea leaves and the tea powders were determined as described by Nadeem et al. (2011). The solubility of the tea powders was also determined as per Nadeem et al. (2011). The colour characteristics of the tea powders and of the solutions, when the powders were reconstituted in water, were measured using a CR-400 Minolta Chroma Meter (Konica Minolta Ltd., North Ryde, NSW, Australia) calibrated with a white standard tile as described by Kha et al. (2010) and Sinija et al. (2007) to determine \(L^*\) (lightness), \(a^*\) (redness and greenness) and \(b^*\) (yellowness and blueness). Chroma and hue angle (H°) were determined from \(a^*\) and \(b^*\), also as described previously (Kha et al., 2010; McLellan et al., 1995).

2.2.3. Determination of chemical composition of the dried tea leaves and tea powders. The constituents of the tea leaves and the green tea powders, comprising of the 8 individual catechins, caffeine and theanine, were determined using high performance liquid chromatography (HPLC) (Shimadzu Australia, Rydalmere, NSW, Australia) and a 250 × 4.6 mm Synergi 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW, Australia). The solvents, HPLC conditions and quantification methods were as described in previous studies (Vuong et al., 2011a,b).

The catechin content of the dried tea leaves was determined by HPLC by extracting ground tea leaves (<1 mm) twice in water at 80 °C for 30 min, once with a ratio of water-to-tea of 12:1 mL/g and once with a ratio of water-to-tea of 8:1 mL/g. To determine the catechin composition of the green tea powders by HPLC, 0.4 g of powder was dissolved in 100 mL water at 80 °C for 10 min in a shaking water bath.

2.2.4. Statistical analyses

The Student t-test was used when two treatments were compared and the one-way ANOVA with Fisher’s Least Significant Difference (LSD) post hoc test was used when comparing more than two treatments. The SPSS-PASW GradPack 18.0 for Windows program was used to do the statistical tests and significance was set at \(P < 0.05\).

3. Results

3.1. Decaffeination of the underutilised old tea leaves by blanching in hot water

The results presented in Fig. 2 show that the length of the blanching time at 100 °C at a water-to-tea ratio of 20:1 mL/g (wet tea weight) had a significant impact on the levels of caffeine and catechins that remained in the old tea leaves. The levels of both caffeine and total catechins in the tea leaves decreased with increasing blanching time. However, their removal rates were different. For the first 10 min of blanching, the removal rate of caffeine was much quicker than that of the catechins. The levels of caffeine decreased to ~20% (w/w) of its original content, while the levels of the total catechins only reduced to ~85% (w/w) of...
their original concentration in the old tea leaves. When the blanching time was further increased to 25 min, the levels of caffeine in the tea leaves did not significantly decrease whereas the levels of total catechins continued to decline to below 80% (w/w) of their original concentration in the old tea leaves.

The increasing blanching times also significantly affected the ratio of total catechins to caffeine (Fig. 2). The ratio of total catechins to caffeine significantly increased when the blanching time was increased to 10 min but it reached a plateau when the blanching time exceeded 10 min. Therefore, the optimal conditions for decaffeinating the underutilised old tea leaves were found to be a 10 min blanching time at 100°C and a water-to-tea ratio of 20:1 mL/g wet weight of old tea leaves. This caffeine extraction procedure was then followed for the rest of the study.

The yield of dried tea leaves and the physicochemical properties of the caffeinated and decaffeinated dried tea leaves are shown in Table 1. The results showed that the decaffeination process had a significant impact on the yield of dried tea leaves; the yield of decaffeinated dried tea leaves (44/100 g of old green tea leaves) was significantly lower than that of the caffeinated dried tea leaves (48/100 g of old green tea leaves). However, after grinding, the moisture content and the bulk density of the ground dried tea leaves were not significantly different between the caffeinated and decaffeinated dried tea leaves. However, the water activity of the decaffeinated dried tea leaves was significantly higher than for the caffeinated dried leaves.

The results also revealed that the decaffeination process had a significant impact on the amount of extractable solids and on the levels of the constituents in the dried tea leaves (Table 1). The total amount of solids which could be extracted with hot water (extractable solids) was found to be 24% lower for the decaffeinated dried tea leaves compared to the caffeinated tea leaves. As expected, some of this difference was due to the decaffeinated dried tea leaves having five times less caffeine than the unblanched tea leaves. The content of total catechins (17.5% less) and of the epistructured catechins (34.5% less) was also lower in the decaffeinated dried tea leaves compared to the caffeinated leaves. A similar loss (30% less) of theanine was observed with the decaffeination process.

The decaffeination process also stimulated the conversion of the epistructured catechins to their non-epistructured forms. The levels of the non-epistructure catechins were 43% higher in the decaffeinated dried tea leaves than in the unblanched tea leaves. Therefore, only 62% of the catechins in the decaffeinated leaves were epistructured compared to 78% in the caffeinated dried tea leaves.

In general, the results show that in a scaled up production process, a 100 kg of underutilised old tea leaves could be expected to yield approximately 44 kg of dried decaffeinated green tea leaves with a moisture content of less than 3.5% and a caffeine level of less than 0.15%.

3.2. Production of decaffeinated and caffeinated green tea catechin powders

3.2.1. Yield of powder and recovery of catechins

The dried tea leaves produced using the processes outlined in Fig. 1 were used for producing caffeinated and decaffeinated green tea powders. The yields for the tea powders and the recovery of catechins in the caffeinated and decaffeinated powders are shown in Fig. 3. Relative to the extractable solids (Eq. (2)) in the respective dried tea leaves (Table 1), the results showed that the yields of the decaffeinated tea powders were not significantly different to those of the caffeinated tea powders. Thus, the decaffeination process did not significantly affect the yield of green tea powders.

However, the yields of the spray dried powders were significantly lower than those of the freeze dried powders (Fig. 3); relative to the extractable solids in the dried tea leaves (Table 1), the

### Table 1

Yield and physicochemical properties of caffeinated and decaffeinated dried tea leaves.

<table>
<thead>
<tr>
<th></th>
<th>Caffeinated dried tea leaves</th>
<th>Decaffeinated dried tea leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td>47.59 ± 0.41</td>
<td>43.75 ± 0.35</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>2.69 ± 0.11</td>
<td>3.39 ± 0.76</td>
</tr>
<tr>
<td>Bulk density (g/cm³)</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Water activity (ωw)</td>
<td>0.15 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Extractable solids (mg/g)</td>
<td>255.0 ± 6.4</td>
<td>195.0 ± 1.1</td>
</tr>
<tr>
<td>Caffeine (mg/g)</td>
<td>7.36 ± 0.02</td>
<td>1.49 ± 0.01</td>
</tr>
<tr>
<td>Total catechins (mg/g)</td>
<td>59.82 ± 3.83</td>
<td>49.34 ± 2.51</td>
</tr>
<tr>
<td>Epistructured catechins (mg/g)</td>
<td>46.71 ± 2.86</td>
<td>30.58 ± 1.65</td>
</tr>
<tr>
<td>Non-epistructured catechins (mg/g)</td>
<td>13.11 ± 0.96</td>
<td>18.76 ± 0.85</td>
</tr>
<tr>
<td>Theanine (mg/g)</td>
<td>2.46 ± 0.04</td>
<td>1.72 ± 0.01</td>
</tr>
</tbody>
</table>

All values are means ± standard deviations for quadruplicate experiments and those in the same row not sharing the same superscript letter are significantly different from each other (P < 0.05).

^A Expressed as the weight percentage of dried leaves over the wet weight of old tea leaves used to make that weight of dried tea leaves.

^B Expressed as mg of tea constituents per gram of dried tea leaves.

**Fig. 2.** Effect of blanching time on the levels of caffeine and catechins in old tea leaves. Samples of the old tea leaves were blanched at 100°C at a water-to-tea ratio of 20:1 mL/g for the indicated length of time and dried as in Fig. 1. The values for caffeine and total catechins (scale on the right y-axis) and the ratio of the total catechins to caffeine (scale on the left y-axis) are means ± standard deviations for quadruplicate experiments. The values sharing a superscript symbol or a letter are not significantly different at P < 0.05.

**Fig. 3.** Yield and recovery of catechins for green tea powders. The yield of powder (mg) was expressed as a percentage relative to the extractable solids (mg) per g of dried tea leaves. The recovery of catechins in the powders was expressed as a percentage relative to the content of catechins in the dried tea leaves. The values are means ± standard deviations for quadruplicate experiments and those not sharing a letter on top of the columns are significantly different at P < 0.05.
yields for freeze drying were 100% whereas the yields for spray drying were around 75%. Therefore, the drying process had a significant impact on the green tea powder yields, and this appeared to be mainly due to losses in the spray dryer’s chamber and piping. From the results in Table 1 for the extractable solids and the yields of the different powders (Fig. 3), scaling up to 100 kg of dried old tea leaves, approximately 25.5 kg of caffeinated freeze dried tea powder, 19.5 kg of decaffeinated freeze dried tea powder; 19 kg of caffeinated spray dried tea extract and 14.5 kg of decaffeinated spray dried tea powder could be expected.

Similarly, the results presented in Fig. 3 also showed that the decaffeination process did not significantly affect the recovery of the catechins. However, the drying method had a significant impact on their recovery; 100% of the catechins in the dried tea leaves were recovered in the green tea powders produced by freeze drying whereas only 75–79% of the total catechins in the dried tea leaves were recovered in the tea extracts produced by spray drying. Thus, freeze drying gave a higher yield of green tea powders and a higher recovery of catechins than spray drying.

3.2.2. Physical properties of the green tea catechin powders

The physical properties of the tea powders produced by the different processes are shown in Table 2. For each of the two drying conditions, the bulk density was not significantly different between the caffeinated and decaffeinated green tea powders. However, for both the caffeinated and decaffeinated powders, the bulk density of the powders produced by freeze drying (~0.17 g/cm³) was significantly lower than for the powders produced by spray drying (~0.25 g/cm³).

However, the results showed that all the green tea powders had very similar low moisture levels (<3%) and had little variation in water activity. In addition, all the tea powders had a similar solubility in water, with at least 96% of each powder able to be effectively dissolved in water at room temperature.

Table 2

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>Freeze dried caffeinated</th>
<th>Freeze dried decaffeinated</th>
<th>Spray dried caffeinated</th>
<th>Spray dried decaffeinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density (g/cm³)</td>
<td>0.16 ± 0.01a</td>
<td>0.17 ± 0.01a</td>
<td>0.25 ± 0.03b</td>
<td>0.25 ± 0.01b</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>2.28 ± 0.16</td>
<td>2.35 ± 0.30b</td>
<td>2.19 ± 0.13</td>
<td>2.22 ± 0.15</td>
</tr>
<tr>
<td>Water activity (a_w)</td>
<td>0.19 ± 0.02ab</td>
<td>0.21 ± 0.01b</td>
<td>0.17 ± 0.01b</td>
<td>0.18 ± 0.01b</td>
</tr>
<tr>
<td>Solubility (%)</td>
<td>96.92 ± 0.16a</td>
<td>96.70 ± 0.30a</td>
<td>96.97 ± 0.73b</td>
<td>95.98 ± 0.65a</td>
</tr>
<tr>
<td>Colour of green tea powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lightness</td>
<td>69.47 ± 1.13a</td>
<td>67.99 ± 1.87a</td>
<td>76.03 ± 0.61b</td>
<td>72.65 ± 0.63b</td>
</tr>
<tr>
<td>Chroma</td>
<td>25.99 ± 0.75a</td>
<td>27.31 ± 0.65b</td>
<td>33.24 ± 0.55b</td>
<td>33.43 ± 0.82b</td>
</tr>
<tr>
<td>Hue angle</td>
<td>89.11 ± 0.47a</td>
<td>86.69 ± 0.22a</td>
<td>90.38 ± 0.25b</td>
<td>88.98 ± 0.33b</td>
</tr>
<tr>
<td>Colour of reconstituted green tea powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lightness</td>
<td>46.50 ± 0.62a</td>
<td>45.78 ± 0.84b</td>
<td>45.37 ± 0.48b</td>
<td>44.58 ± 0.55b</td>
</tr>
<tr>
<td>Chroma</td>
<td>24.96 ± 0.38a</td>
<td>25.25 ± 1.98b</td>
<td>25.49 ± 0.47b</td>
<td>25.52 ± 0.56b</td>
</tr>
<tr>
<td>Hue angle</td>
<td>99.84 ± 0.14a</td>
<td>98.26 ± 0.30a</td>
<td>99.63 ± 0.85b</td>
<td>98.57 ± 0.38b</td>
</tr>
</tbody>
</table>

The values are means ± standard deviations for quadruplicate experiments and those in the same row not sharing the same superscript letter are significantly different from each other (P < 0.05).

A The green tea powder (0.4 g) was reconstituted in 100 mL of water.

![Fig. 4. Typical HPLC chromatograms for green tea caffeinated powder (A) and decaffeinated powder (B). The peaks detected at 280 nm for caffeine, the epistructured compounds epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), their non-epistructured counterparts gallatechin gallate (GCG), gallatechin (GC), catechin gallate (CG) and catechin (C) and L-tryptophan, the internal standard (IS), are indicated.](image-url)
The colour characteristics of the powders are presented in Table 2. The main differences were that the spray dried powders were lighter (Lightness) but had more intense colour (Chroma) than the freeze dried powders. For the colour tone values, the ‘Hue angle’ ranged between 86.5° and 90.5°, which reflected the yellowish brown colour of all the tea powders.

The colour characteristics of the reconstituted powder solutions are also presented in Table 2. The main differences were that the decaffeinated powders gave solutions which were lighter (Lightness) and had slightly higher colour tone values (Hue angle) than the solutions made with the caffeinated tea powders. However, even though the latter values were significantly different, all values were close to 100°, which reflected the solutions’ greenish yellow colour.

3.2.3. Chemical composition of the green tea catechin powders

Fig. 4 shows a comparison of HPLC chromatograms from a caffeinated and a decaffeinated spray dried powder. It can be seen that the peak for caffeine for the decaffeinated spray dried powder was much smaller than for the caffeinated spray dried powder whereas there was not much difference in the peaks for the catechins.

The composition of the green tea powders, obtained by HPLC analysis, is shown in Table 3. The level of caffeine in the decaffeinated tea powders was <6.5 mg/g and was <30% of the caffeine in the caffeinated powders. In contrast, the decaffeinated freeze dried and spray dried powders were found to have significantly higher levels of total catechins than the caffeinated powders, with the spray dried decaffeinated powder having the highest concentration. This was not due to differences in the epistructured catechins; these were significantly lower in the decaffeinated powders than in the caffeinated powders (Table 3). However, almost twice the levels of the non-epistructured catechins were measured in the decaffeinated tea powders in comparison with the caffeinated powders.

These results showed that the decaffeination process combined with spray drying, which includes a concentration step using a rotary evaporator run at 70 °C for 3–4 h, lead to a very significant increase in the conversion of the epistructured catechins to their non-epistructured counterparts. The ratio of epistructured to non-epistructured catechins decreased from 3.56/1 in the caffeinated dried tea leaves (Table 1) to 1.31/1 in the spray dried decaffeinated powder (Table 3).

Finally, although there was some variation and the values were low, detectable levels (6.8–7.2 mg/g) of theanine were measured in all the green tea powders (Table 3).

4. Discussion

The optimal blanching time for decaffeinating the old tea leaves was 10 min, which was longer than the 3 min needed to remove ~83% of the caffeine while retaining ~95% of the catechins, as reported by Liang et al. (2007) for new growth leaves (apical bud and up to 4 leaves down the stem). The longer blanching time needed to decaffeinate the old tea leaves is most likely to be due to their thicker, stronger and waxyer structure compared to young leaves, which is likely to reduce their permeability to water and thereby decrease the diffusion of the caffeine into the infusion water.

The level of caffeine in the dried tea leaves after blanching in water at 100 °C for 10 min was 1.72 mg/g (Table 1) and therefore, they can be classified as ‘decaffeinated’ because they have less than 4 mg caffeine per gram of tea leaves (Ye et al., 2007). However, the yield of leaves and the amount of extractable solids remaining in the leaves were decreased by the decaffeination process (Table 1). This meant that the amount of total solids, catechins and theanine extracted from the dried decaffeinated leaves was significantly lower than for the caffeinated leaves, which ultimately led to a lower yield of green tea powder from the decaffeinated leaves. Furthermore, the level of the non-epistructured catechins in the decaffeinated leaves was significantly higher than in the caffeinated leaves (Table 1).

Therefore, the disadvantages of the decaffeination process were: (1) some loss of water soluble substances other than caffeine including catechins and theanine; and (2) an increase in the conversion of the epistructured catechins to their non-epistructured forms. However, despite these drawbacks, this decaffeination method has marked advantages in comparison with the methods used previously such as organic solvent methods (Copeland et al., 1998; Jin et al., 2006), resin and activated carbon absorbent methods (Lu et al., 2010; Zhao et al., 2007; Ye et al., 2007), or supercritical fluid extraction with carbon dioxide (Park et al., 2007), which can leave undesirable residues behind in the decaffeinated products or are expensive to scale up to a commercially viable size.

In comparison, water is a safe, inexpensive and accessible solvent and the decaffeination process using hot water is very safe, simple, inexpensive and relatively easy to commercially scale up (Vuong et al., 2011a). In addition, the equipment required for the hot water decaffeination process is relatively inexpensive and the blanching process can easily be done because it only requires control over the temperature of the water and the length of time the blanching is done for.

Water at 80 °C, using methods previously optimised for dried commercial teas (Vuong et al., 2011a), was also effectively used to extract the catechins from the old tea leaves once they had been dried, whether the leaves had first been decaffeinated or not (Table 3). Although there were some slight differences, both freeze drying and spray drying of the water extracts resulted in similar concentrations of total catechins and theanine in the green tea powders. Furthermore, although old tea leaves generally have up to a 2.7-fold lower content of catechins compared to the younger leaves (Lin et al., 1996), the concentrations of total catechins in the green tea catechin powders (174–193 mg/g; Table 3) were similar to the content of polyphenolic compounds in a powder produced from young tea leaves (196 mg/g; Sinija et al., 2007).

The decaffeinated powders, especially the decaffeinated spray dried powder, had substantially lower concentrations of the natural epistructured catechins and higher levels of the non-epistructured catechins than the caffeinated powders (Table 3). These low levels of epistructured catechins were explained to a certain...
extent by their lower concentrations in the decaffeinated dried leaves compared to the cafffeinated tea leaves (Table 1).

However, the spray drying process also led to substantially lower concentrations of the natural epistructured catechins relative to their non-epistructured counterparts when compared to freeze drying (Table 3). The high temperatures the catechins were exposed to during the two processes, 100°C during decaffeination and the 170°C inlet and 110°C outlet air temperatures during spray drying are likely to have caused the increased conversion; it is well known that the conversion of the epistructured catechins into non-epistructured catechins increases as the temperature is increased (Vuong et al., 2011a; Wang and Hellwell, 2000). The concentration step by rotary evaporation at 70°C for 3–4 h, which is needed prior to spray drying, may also have contributed to the conversion of the catechins’ structure.

The yield of powder and the recovery of total catechins were also significantly higher (20–25% higher) for freeze drying compared to spray drying (Fig. 3), as observed by others who compared freeze drying with spray drying (Desobry et al., 1997; Moßhammer et al., 2006). Some of the powder was lost in the spray dryer’s chamber and piping; this material was not completely dry and physically adhered to the insides of the piping and the collection cylinder of the spray dryer. In addition, due to their sensitivity to high temperatures (Vuong et al., 2011a; Wang and Hellwell, 2000), the catechins may also have partially degraded due to the 170°C inlet and 110°C outlet air temperatures they experienced during spray drying. However, due to the high costs and the length of time needed for freeze drying (Mujumdar, 2000), spray drying cannot be fully discounted as a method for making green tea catechin powders, especially if conditions can be determined to preserve the catechins and their epistructures.

The moisture content of all the green tea catechin powders (<3%, Table 2) was also less than the <5% moisture content required for the stability of powders during packaging and storage (Nadeem et al., 2011; Sinija and Mishra, 2008). However, the powders were found to be hydrosopic and care needed to be taken to keep them dry. Finally, all the powders also had very high solubility in water (>96%, Table 2), which is similar to the solubility of a spray dried tea powder reported on previously (Nadeem et al., 2011).

Both the freeze-dried and spray-dried decaffeinated green tea powders had caffeine levels (6.42 and 6.11 mg/g, respectively) (Table 3). The moisture content of all the green tea catechin powders (<3%, Table 2) was also less than the <5% moisture content required for the stability of powders during packaging and storage (Nadeem et al., 2011; Sinija and Mishra, 2008). However, the powders were found to be hydrosopic and care needed to be taken to keep them dry. Finally, all the powders also had very high solubility in water (>96%, Table 2), which is similar to the solubility of a spray dried tea powder reported on previously (Nadeem et al., 2011).

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5. Conclusions

This study has shown that old green tea leaves, which are usually discarded, could be used as an underutilised source to make caffeinated and decaffeinated green tea catechin powders. It was also shown that water, a safe, economically sustainable and environmentally friendly solvent could be used along with freeze drying or spray drying to prepare caffeinated and decaffeinated green tea catechin powders from these otherwise wasted old tea leaves. These powders are likely to be acceptable to the food and health supplement industries and to health-conscious consumers and could thus add value to the tea industry.

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2.2.5. Preparation of decaffeinated and high caffeine powders from green tea

Quan V. Vuong, John B. Golding, Minh H. Nguyen and Paul D. Roach

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Preparation of decaffeinated and high caffeine powders from green tea

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ABSTRACT

The aims of this study were to develop optimal conditions for decaffeination and spray drying procedures to produce decaffeinated and high caffeine powders from green tea (Camellia sinensis). Blanching the tea leaves with water at 100 °C for 4 min at a water-to-tea ratio of 20:1 mL/g removed 83% of the caffeine while retaining 94% of the catechins. The optimal spray drying conditions, which gave the highest yield of green tea powder and the highest concentrations of the naturally occurring epistructured catechins were found to be 180 °C for the inlet temperature and 115 °C for the outlet temperature. Using these optimal conditions, a decaffeinated green tea powder (7 mg/g caffeine) and a high caffeine powder (95 mg/g) were produced. These two green tea powders had excellent physical properties and could be used as instant teas by consumers or utilized in the food, cosmetic and nutraceutical industries.

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1. Introduction

Green tea from the Camellia sinensis plant is a rich source of the strong antioxidant compounds called the catechins [1] and the unique amino acid theanine [2,3]. Numerous epidemiological studies have linked green tea and its constituents with many human health benefits such as prevention of cancers, cardiovascular diseases, microbial diseases, diabetes and obesity [4,5]. The catechins have also been found to prevent lipid peroxidation, a major problem in the food industry, which can cause undesirable rancidity and potentially toxic reaction products [6].

Dried green tea leaves also naturally contain high levels of the alkaloid caffeine, which can be as high as 5% (w/w). This is higher than the amount of caffeine normally found in coffee beans [7], which can be up to 2% (w/w). However, the concentration of caffeine in the green tea beverage is usually a third to a half of its concentration in the coffee beverage because less green tea than coffee is used to make the respective beverages. Caffeine has some positive physiological effects; it has been linked with enhancement of cognitive function, improvement of neuromuscular coordination, elevation of mood and relief of anxiety [8]. However, caffeine can also have negative effects such as irritation of the gastrointestinal tract and sleeplessness in some people [6,7].

As for coffee, several methods have been developed to produce instant green tea powders in order to offer convenience as well as the health benefits [9–11]. Furthermore, due to the health concerns some people have relative to caffeine [6,7], a range of techniques have been developed to remove the alkaloid from some green tea powders, which have included the use of organic solvents [12,13], activated charcoal [11], supercritical CO2 extraction [14,15], microwave-enhanced ice water extraction under vacuum [16], a lignocellulose column [17] and microbial or enzymatic systems [18]. However, all of these methods have limitations such as: risk of solvent residues, expensive equipment, long production times or being difficult to scale up for industrial production. In one study, a simple hot water blanching step was found to effectively remove caffeine from green tea leaves [19]. However, the impact of different lengths of blanching time on the chemical composition of the blanched tea leaves was not fully determined.

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Spray drying is the most widely used industrial method for the continuous production of dry solids in powder, granulate or agglomerate form [10]. However, spray drying is done using high inlet and outlet temperatures, which may be an issue with making green tea...
powders because the catechins are known to be sensitive to high temperatures; at high temperatures, the epistructured catechins tend to be converted to their non-epistructured counterparts or be degraded due to oxidation [1,6]. Consequently, if spray drying is to be used, the impact of the inlet and outlet temperatures during the spray drying process on the yield and stability of the green tea catechins needs to be evaluated. The feed rate is closely related to the inlet and outlet temperatures and therefore it is used to control the inlet and outlet temperatures [21].

Therefore, the aims of this study were to optimize the decaffeination and spray drying procedures for producing decaffeinated and high caffeine green tea powders. The optimal conditions for decaffeinating the tea leaves using hot water as solvent were investigated and the potential of using the caffeine thus extracted to make a high caffeine powder was evaluated. The impact of the inlet and outlet temperatures during spray drying on the yield and chemical composition of the tea powders was also examined.

2. Materials and methods

2.1. Materials

The apical bud, the top four leaves and the stem down to the fourth leaf of the Camellia sinensis var. sinensis Yabukita cultivar plant, referred to as tea leaves, were harvested at the end of the production season in March 2011 from an experimental green tea plantation on the NSW Department of Primary Industries research farm at Somersby, New South Wales (NSW), Australia (latitude 33° 22′ S; longitude 151° 21′ E). After plucking, the leaves were immediately stored at −18 °C before use to minimise the oxidation and degradation of the catechins.

The chemicals used for the analyses were: L-theanine, L-tryptophan (used as an internal standard), caffeine, the epistructured catechins, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (EGC), epicatechin (EC) and the non-epistructured catechins, galloatechin gallate (GC), galloatechin (GG), catechin gallate (CG) and catechin (C) from Sigma Chem. Co. (Castle Hill, NSW, Australia) and acetonitrile, orthophosphoric acid and tetrahydrofuran were purchased from Lambda Scientific (Taren Point, NSW, Australia). Ultrapure (type 1) de-ionised (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia Pty Ltd, North Ryde, Australia).

2.2. Methods

A schematic of the processes used to produce the decaffeinated and the high caffeine green tea powders is presented in Fig. 1.

2.2.1. Decaffeination of tea leaves

To optimize the decaffeination process, samples stored at −18 °C (apical bud, top four leaves and the stem down to the fourth leaf), were thawed at room temperature for 10 min. To remove as much caffeine as possible and to inactivate the oxidising enzymes, the thawed samples (500 g) were then blanched in water (10 L) for various lengths of time ranging from 0 to 15 min at the optimal temperature of 100 °C and at a water-to-tea ratio of 20:1 mL/g, as reported in a previous study [19].

The water was then decanted from the blanched tea leaves and the leaves were dried to constant weight at 70 °C in a vacuum oven (Thermoline Scientific Equipment, Smithfield, NSW, Australia) to give decaffeinated dried tea leaves. For comparison of the chemical composition of the decaffeinated dried tea leaves, a sample of caffeinized dried tea leaves was also prepared. For this, a tea sample was thawed at room temperature for 10 min, but instead of blanching at 100 °C to remove the caffeine, it was only quickly steamed for 30 s to stop oxidation and then dried to constant weight at 70 °C using a vacuum oven (Thermoline Scientific Equipment, Smithfield, NSW, Australia).

The decanted aqueous solution, containing the extracted caffeine from the blanched tea, was filtered using Whatman #1 filter paper (90 mm diameter) (Lomb Scientific, Taren Point, NSW, Australia) and then concentrated 16 times by boiling at 100 °C prior to spray drying to obtain a high caffeine green tea powder (Fig. 1).

2.2.2. Extraction of the decaffeinated dried tea leaves

To extract the decaffeinated dried tea leaves, the optimal extraction conditions reported in a previous study [22], for the efficient use of water and the high yield of catechins, were applied. The decaffeinated dried tea leaves were ground into particles <1 mm using a blender (John Morris Scientific Pty, Chatswood, NSW, Australia) and then sieved using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd, London, England). The ground tea leaves were brewed twice in water at 80 °C for 30 min, once with the ratio of water-to-tea of 12:1 mL/g and once with the ratio of water-to-tea of 8:1 mL/g. Hydrated leaves and fine suspended particles were separated from the tea extract by first filtering using cheese cloth, then centrifuging at 3000 × g for 10 min and finally filtering using Whatman #1 filter paper (90 mm diameter) (Lomb Scientific, NSW, Australia). The tea extract was then concentrated to 20% (w/v) at 60 °C under vacuum using a Buchi rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) (Fig. 1).

2.2.3. Spray drying of the green tea extracts

To prepare powders from the green tea extracts, a Buchi mini spray drier (Model B-480, Buchi Australia, Noble Park, VIC, Australia) was used.

To investigate the impact of the outlet temperature on the yield of green tea powder and on the stability of the catechins, the inlet temperature was fixed at 170 ± 1 °C, reported as optimal by Tang et al. [10], the aspiration rate was set at 100% and the compressed air flow was fixed at 301 L/h. The pump rate was then adjusted between 4 and 24% to obtain outlet temperatures ranging from 70 to 115 °C.

To determine the impact of the inlet temperature, the pump rate was adjusted between 4 and 32% to maintain the outlet temperature at 115 ± 1 °C, the aspiration rate was set at 100% and the compressed air flow was fixed at 301 L/h. The inlet temperatures tested ranged from 170 to 220 °C.

2.2.4. The composition of dried green tea leaves and powders

The composition of the decaffeinated and caffeinated dried green tea leaves was determined by high performance liquid chromatography (HPLC). For the analysis, 1 g of ground tea leaves (<1 mm) was brewed in 100 mL water at 80 °C for 30 min. The tea extract was then placed on ice to cool down to room temperature, diluted 1:1 with 500 μM L-tryptophan (internal standard) in deionized (DI) water and finally filtered and transferred into brown glass HPLC vials using a 5 μL syringe and a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty Ltd, NSW, Australia).

The composition of the green tea powders was determined by dissolving 0.4 g powder in 100 mL water at 80 °C and agitating on a vortex mixer for 5 min. The solution was then placed on ice to cool down to room temperature, diluted 1:1 with 500 μM L-tryptophan in DI water and finally filtered and transferred to the HPLC vials for analysis.

The composition of the dried green tea leaves and powders was determined using a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW, Australia) with a 250 × 4.6 mm Synergi 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd, Lane Cove, NSW, Australia). The solvents, HPLC conditions and quantification methods were as described previously [22].

2.2.5. Yield and physical properties of the green tea powders

The yield of spray dried green tea powder was calculated as described by Amiri-Rigi et al. [23] and was expressed as a percentage of the maximum yield possible. The physical properties of the green tea powders, moisture content, water activity, bulk density, colour
2.2.6. Statistical analyses

The Student t-test was used when two treatments were compared (Tables 1, 4 and 5) and the one-way ANOVA with the Fisher Least Significant Difference (LSD) post hoc test was used when comparing more than two treatments (Tables 2 and 3 and Fig. 2). The SPSS-PASW GradPack 18.0 for Windows was used to do the statistical tests and significance was set at \( p \leq 0.05 \).

3. Results and discussion

3.1. Decaffeination using hot water

As shown in Fig. 2, the respective extraction rates of caffeine and the catechins from the tea leaves into 100 °C water differed significantly. The percent caffeine remaining in the tea leaves decreased very quickly during the first 4 min to <20% and then stayed relatively constant at this level over the next 15 min of blanching. In contrast, the percent of catechins remaining in the tea leaves only gradually decreased to ~90% during the first 4 min and to ~60% after 15 min of blanching. Therefore, as shown in Fig. 2, the ratio of total catechins to caffeine considerably increased from ~4 to ~24 during the first 4 min and remained high (~20) at 15 min.

Based on the results in Fig. 2, the optimal blanching time was determined to be 4 min and it was therefore used for decaffeinating the green tea leaves. Table 1 describes the chemical composition of the decaffeinated dried tea leaves. Compared to unblanched (caffeinated) dried green tea leaves, blanching for 4 min was effective at selectively removing the caffeine from the dried tea leaves; 83% of the caffeine was removed whereas 94% of the catechins remained in the leaves.

The current findings are in agreement with the results reported by Liang et al. [19], who found that blanching leaves for 3 min removed

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**Table 1**

The composition of blanched and unblanched dried tea leaves.

<table>
<thead>
<tr>
<th>Dried Tea leaves</th>
<th>Theanine (mg/g)</th>
<th>Caffeine (mg/g)</th>
<th>EGC (mg/g)</th>
<th>ECG (mg/g)</th>
<th>EC (mg/g)</th>
<th>C (mg/g)</th>
<th>GCG (mg/g)</th>
<th>GC (mg/g)</th>
<th>CG (mg/g)</th>
<th>C (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unblanched</td>
<td>25.1 ± 0.1a</td>
<td>31.1 ± 0.1a</td>
<td>53.5 ± 2.7c</td>
<td>56.7 ± 0.3a</td>
<td>8.7 ± 0.6a</td>
<td>3.0 ± 0.2b</td>
<td>15.6 ± 0.5a</td>
<td>3.2 ± 0.2a</td>
<td>6.1 ± 0.4a</td>
<td></td>
</tr>
<tr>
<td>Blanched</td>
<td>17.2 ± 0.1b</td>
<td>5.3 ± 0.1b</td>
<td>44.9 ± 4.8b</td>
<td>51.7 ± 1.0a</td>
<td>6.2 ± 0.9b</td>
<td>2.3 ± 0.2b</td>
<td>23.7 ± 1.0b</td>
<td>2.4 ± 0.3b</td>
<td>6.4 ± 0.7a</td>
<td></td>
</tr>
</tbody>
</table>

The values are mean ± standard deviations for four preparations and those in the same column not sharing the same superscript letter are significantly different from each other at \( p < 0.05 \).
83% of the caffeine and retained 95% of the catechins. The minor difference in blanching time (1 min) could be due to differences in the cell-wall structure and chemical composition of the different tea varieties and different harvest locations and times of year between the two studies. However, because the values are so close, the difference in the optimal blanching time may simply be due to experimental variation.

Table 1 revealed that three of the epistructured catechins (EGCG, EGC and ECG) and two of the non-epistructured catechins (GCG and GEC) were significantly lower in the decaffeinated dried tea leaves in comparison with the unblanched caffeinated leaves. In contrast, the level of GC in the decaffeinated dried tea leaves was 50% higher than in the unblanched leaves (p < 0.05) while the level of EC and C

### Table 2

The impact of the spray dryer outlet temperature on the yield and composition of the decaffeinated green tea powder.

<table>
<thead>
<tr>
<th>Outlet temperature (°C)</th>
<th>Yield (%)</th>
<th>TC (mg/g)</th>
<th>ESC (mg/g)</th>
<th>NESC (mg/g)</th>
<th>Caffeine (mg/g)</th>
<th>Theanine (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>26.7 ± 0.8a</td>
<td>183.2 ± 3.1a</td>
<td>139.9 ± 3.3a</td>
<td>43.3 ± 0.4a</td>
<td>7.3 ± 0.2a</td>
<td>21.4 ± 0.5a</td>
</tr>
<tr>
<td>80</td>
<td>31.3 ± 1.9b</td>
<td>184.2 ± 3.9b</td>
<td>140.8 ± 3.8b</td>
<td>43.4 ± 0.5b</td>
<td>7.3 ± 0.2b</td>
<td>21.5 ± 0.3b</td>
</tr>
<tr>
<td>90</td>
<td>33.0 ± 1.3b</td>
<td>185.7 ± 3.9b</td>
<td>141.3 ± 4.3b</td>
<td>44.4 ± 0.6b</td>
<td>7.3 ± 0.1b</td>
<td>21.8 ± 0.5b</td>
</tr>
<tr>
<td>100</td>
<td>40.2 ± 1.5c</td>
<td>192.1 ± 5.7c</td>
<td>147.6 ± 4.4c</td>
<td>44.5 ± 1.2c</td>
<td>7.3 ± 0.2c</td>
<td>21.9 ± 0.6c</td>
</tr>
<tr>
<td>110</td>
<td>42.0 ± 0.2c</td>
<td>194.6 ± 2.6c</td>
<td>149.6 ± 2.4c</td>
<td>45.0 ± 0.6c</td>
<td>7.4 ± 0.2c</td>
<td>22.4 ± 0.7c</td>
</tr>
<tr>
<td>115</td>
<td>48.4 ± 1.1c</td>
<td>193.1 ± 3.5c</td>
<td>148.8 ± 2.9c</td>
<td>44.2 ± 0.9c</td>
<td>7.5 ± 0.2c</td>
<td>22.4 ± 0.1c</td>
</tr>
</tbody>
</table>

TC: total catechins; ESC: epistructured catechins; NESC: non-epistructured catechins. The values are mean ± standard deviations for four preparations and those in the same column not sharing the same superscript letter are significantly different from each other at p < 0.05.

3.2. Effect of the spray drying outlet temperature

The results for different outlet temperatures with a fixed inlet temperature of 170 ± 1 °C are shown in Table 2. These clearly showed that changing the outlet temperature of the spray dryer significantly affected the yield of green tea powder. Generally, as the outlet temperature was increased from 70 to 115 °C, the amount of powder obtained was increased; the yield was almost doubled at 115 °C (48.4%) in comparison with 70 °C (26.7%). The differences in yield were caused by an observed decrease in the stickiness of the tea powder as the temperature was increased. Most likely due to a decrease in the moisture content of the powder produced, it was observed that less of the powder stuck to the inside wall of the drying chamber and cyclone of the spray dryer. Such a decrease in wet sticky deposits on the spray dryer walls, which resulted in higher yields of powdered trehalose as the outlet temperature was increased, was also observed in a previous study [21].

Table 2 also shows that increasing the outlet temperature did not affect the stability of the catechins. The concentration of the total catechins and the epistructured catechins also generally increased as the temperature was increased; the total catechin concentration increased by 5.4% and the epistructured catechins by 6.4% when the outlet temperature was increased from 70 °C to 115 °C. In contrast, there was no significant effect of the outlet temperature on the concentration of the non-epistructured catechins, caffeine and theanine. However, despite apparent different effects of the outlet temperature on the epistructured and non-epistructured catechins, the ratio of the epistructured to non-epistructured catechins (~3:1), did not significantly change as the outlet temperature was increased.

Thus, these findings illustrated two points: 1) the yield of powder doubled and 2) the concentration of the total and epistructured catechins was slightly increased (~5%) as the outlet temperature was increased from 70 to 115 °C. Therefore, of the outlet temperatures tested, the optimal temperature was 115 °C and, for this reason,
115 °C was used for the outlet temperature in the subsequent experiments.

3.3. Effect of the spray drying inlet temperature

The results for different inlet temperatures with a fixed outlet temperature of 115 ± 1 °C are shown in Table 3. These clearly showed that changing the inlet temperature of the spray dryer significantly affected the yield of the decaffeinated green tea powder. There was a significant 6% increase in the yield of powder at 180 °C compared to 170 °C. However, as the inlet temperature was further increased from 180 to 220 °C, the amount of powder obtained was decreased; the yield was 30% lower at 220 °C (36.4%) in comparison with 180 °C (51.4%).

In contrast, the inlet temperature had no significant effects on the concentration of total catechins, epistructured and non-epistructured catechins, caffeine and theanine in the decaffeinated green tea powder (Table 3). Therefore, an inlet temperature of 180 °C was found to be optimal and was used, along with the outlet temperature of 115 °C, for subsequent production of tea powders.

The current observations for the spray dryer inlet temperature are similar to the results of a previous study, which found that higher inlet temperatures resulted in lower production yields for fruit powders [28]. Again, the yields were seen to be related to the stickiness of the powders. In this case, the yields were lower at the higher inlet temperatures, because these temperatures increased the adherence of the powder to the wall of the spray drying chamber [28,29].

The current findings show that the highest yield of decaffeinated green tea catechin powder, was obtained using our Buchi spray dryer system with inlet and outlet temperatures of 180 and 115 °C, respectively. Thus, the results indicated that the optimal spray drying conditions for production of green tea powder were: inlet temperature at 180 °C, outlet temperature at 115 °C, aspiration rate at 100% and compressed air flow at 301 L/h.

Using these settings for spray drying, decaffeinated and high caffeine green tea powders were prepared as outlined in Fig. 1. The decaffeinated green tea powder was produced from the extraction of blanched and dried tea leaves while the high caffeine powder was produced from the extract solution, which resulted from the blanching process. The composition (Table 4) and physical properties (Table 5) of the two powders were then compared.

### 3.4. Composition of the decaffeinated and high caffeine green tea powders

The caffeine concentration in the decaffeinated green tea powder (Table 4), at 0.73% (w/w), was well within the maximum permitted level of 1% (w/w) for classification as a decaffeinated powder [11]. As expected, the caffeine concentration was much higher (13 times) in the high caffeine powder than in the decaffeinated powder. This level of caffeine (95 mg/g) is also high compared to coffee; it is threefold higher than in instant coffee, which ranges from 29 to 38 mg/g [30]. Therefore, this high caffeine green tea powder could be used as an instant tea alternative for instant coffee. It could also be used as a starting material to isolate caffeine for use in the food and beverage industry.

The decaffeinated tea powder produced in this study had a total catechin concentration of 196 mg/g (Table 4), which was exactly the same as for a non-decaffeinated green tea powder produced by water extraction and freeze-drying in another study for potential use as an instant green tea [9]. The present decaffeinated tea powder also contained a relatively high concentration (151 mg/g) of the naturally occurring epistructured catechins, EGCg, EGC, EC and EGC and high levels of theanine (23 mg/g), which is important for taste [2,3]. Therefore, it has great potential for use as a decaffeinated instant tea. It could also be used as a low caffeine starting material for isolating the catechins and theanine for utilization in the food, cosmetics and pharmaceutical industries [1,3,6].

Surprisingly, the total catechin concentration was almost exactly the same for both the decaffeinated and the high caffeine powders. This was despite the high caffeine powder having 70% less EGCg, 63% less EGC, 58% less ECG, 45% less EC in (total, 63% less epistructured catechins) and 28% less GCG than the decaffeinated powder. This was balanced by the high caffeine powder having 3.5 times more GC, 3.6 times more Ca and 40% more CG (in total, 3.1 times more non-epistructured catechins) than the decaffeinated powder.

Notably, in the high caffeine powder, the concentrations of GC and C were 4.2 and 2.4 times the concentration of their respective naturally occurring epimers, EGC and EC. The latter two catechins, EGC and EC, are known to infuse quicker from green tea into water than the other two epicatechins [22,31]. Thus, they may have been preferentially extracted during the short 4 min blanching process and, because of the high temperature (100 °C) used for blanching and the lengthy concentration step (Fig. 1), they may have been epimerized to form GC and C [1,22]. Due to its high levels of GC and C (104.8 and 28.7 mg/g, respectively), this powder could be used as the starting material for isolating these two catechins.

The concentration of theanine was 15% lower in the high caffeine powder than in the decaffeinated green tea powder. However, this

### Table 4

<table>
<thead>
<tr>
<th>Tea powder</th>
<th>Caffeine (mg/g)</th>
<th>Total catechins (mg/g)</th>
<th>EGCg (mg/g)</th>
<th>EGC (mg/g)</th>
<th>ECG (mg/g)</th>
<th>EC (mg/g)</th>
<th>GCG (mg/g)</th>
<th>GC (mg/g)</th>
<th>CG (mg/g)</th>
<th>C (mg/g)</th>
<th>Theanine (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decaffeinated</td>
<td>7.3 ± 0.4a</td>
<td>195.8 ± 8.3a</td>
<td>54.3 ± 7.6a</td>
<td>67.7 ± 1.0a</td>
<td>7.6 ± 1.3a</td>
<td>21.5 ± 0.5a</td>
<td>3.6 ± 0.1a</td>
<td>30.0 ± 0.7a</td>
<td>3.2 ± 0.3a</td>
<td>8.0 ± 0.4a</td>
<td>22.7 ± 0.1a</td>
</tr>
<tr>
<td>High caffeine</td>
<td>94.8 ± 0.3b</td>
<td>196.9 ± 12.8a</td>
<td>16.5 ± 5.0b</td>
<td>24.8 ± 0.7b</td>
<td>3.2 ± 1.0b</td>
<td>11.8 ± 4.1b</td>
<td>2.6 ± 0.2b</td>
<td>104.8 ± 4.4b</td>
<td>4.5 ± 2.0b</td>
<td>28.7 ± 2.2b</td>
<td>19.2 ± 0.2b</td>
</tr>
</tbody>
</table>

The values are mean ± standard deviations for four preparations and those in the same column not sharing the same superscript letter are significantly different from each other at p<0.05.
meant that there was still a substantial amount of theanine in the
high caffeine powder to give this preparation ample umami taste [2,3].

3.5. Physical Properties of the decaffeinated and high caffeine green tea powders

The physical properties of the two tea powders are shown in
Table 5. Both powders had a very low moisture content and water activity ($a_w$). The moisture content, at $<$2%, was well within the moisture level of $<$5%, which is needed for good stability and effective packaging and storage of powders [9,25]. Both powders also had a high bulk density, which is also convenient for efficient packaging and transportation. Therefore, vacuum packing to maintain the low moisture content and to maximize the density of packing, would be a suitable method to prolong the shelf life of these two green tea powders.

In addition, the two tea powders had a similar and very high solubility in water (Table 5) at room temperature (~97%). Therefore, both powders could effectively be used as instant teas and would also be easy to use in the food, cosmetic and nutraceutical industries.

As shown in Table 5, the two powders also had very similar colour characteristics of Lightness and Chroma. With its Hue angle values of 87°, the high caffeine tea powder had a slightly more brownish yellow colour (90° = pure yellow), compared to the decaffeinated tea powder, which had a Hue angle of 95° and therefore a slightly greener yellow colour. When the powders were dissolved in water, the high caffeine powder was even browner in colour than the decaffeinated powder, as illustrated by its significantly lower Hue angle (Table 5).

In solution, the high caffeine powder also had a lower colour intensity than the decaffeinated preparation, as seen by its significantly lower Lightness and Chroma values (Table 5).

4. Conclusions

In summary, the Blanching conditions of 100 °C for 4 min at a water-to-tea ratio of 20:1 (mL/g) were optimal for decaffeinating the tea leaves, as 83% of the caffeine could be removed while 94% of the catechins were retained in the leaves. For spray drying, the optimal conditions were determined to be 180 °C for the inlet temperature and 115 °C for the outlet temperature. Using the above conditions, decaffeinated and high caffeine green tea powders were prepared which had very different bioactive compositions. However, both powders had excellent physical properties and therefore have potential for use as instant teas by consumers or use in the food, cosmetic and nutraceutical industries. They could also be used as starting materials for the further isolation of catechins, caffeine or theanine, the three major types of bioactive compounds in green tea.

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References


2.2.6. Improved extraction of green tea components from teabags using the microwave oven

Quan V. Vuong, Sing P. Tan, Costas E. Stathopoulos and Paul D. Roach

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Original research article

Improved extraction of green tea components from teabags using the microwave oven

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ABSTRACT

The green tea (Camellia sinensis) catechins are strong antioxidants linked with potential health benefits. Based on previous studies, it was hypothesised that the typical household conditions for brewing green tea in a teabag – 200 mL freshly boiled water for 2–3 min, as per the manufacturers' instructions – were not sufficient to extract all the catechins and that a household microwave oven could be used to improve the extraction. The catechins and the two other main green tea components, caffeine and theanine, were monitored by HPLC. The typical household conditions only extracted 62% (61 mg/g tea), 76% (24 mg/g) and 80% (10 mg/g) of the catechins, caffeine and theanine, respectively, from the five varieties of teabags analysed. However, using microwave assisted extraction (MAE) by first brewing a teabag in 200 mL freshly boiled water for 0.5 min before irradiation for 1 min in a microwave oven (hot MAE), improved the extraction of the catechins and caffeine to 80% (80 mg/g) and 92% (29 mg/g), respectively, although the extraction of theanine was not affected. Therefore, the hot MAE technique could help maximise the extraction of the catechins for those who consume green tea for the potential health benefits of the catechins.

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1. Introduction

Green tea (Camellia sinensis) is an abundant source of catechins, which are strong antioxidants that have been receiving considerable interest for their potential benefits in human health and food preservation (Khan and Mukhtar, 2007; Vuong et al., 2010, 2011d). For example, the catechins have been linked with protection against cardiovascular disease (CVD) (Kuriyama, 2008). Various catechin extracts from green tea have been shown to be active in humans; they lower low density lipoprotein (LDL) cholesterol, one of the major risk factors for CVD (Zheng et al., 2011). Other studies have shown that the mechanisms of actions for their cholesterol-lowering effect include an increase in the LDL receptor and a decrease in cholesterol synthesis (Bursill et al., 2001, 2007; Bursill and Roach, 2006, 2007).

Therefore, due to the potential benefits of the green tea catechins, several studies have been done on the various brewing conditions to maximise the extraction of these components from green tea. The results have defined optimal water brewing conditions including the temperature being maintained at 80 °C for 30 min and a ratio of tea to water of ≤1:20 g/mL (Komes et al., 2010; Lin et al., 2008; Peterson et al., 2005; Vuong et al., 2011b). However, these optimisation studies focused on loose-leaf green tea and were carried out under laboratory conditions. These conditions are very different from household brewing habits, where tea is simply brewed in boiled water and left at room temperature for a short time (≤3 min) before being consumed (Astill et al., 2001).

Although many people still traditionally prepare their green tea by brewing loose-leaf tea in boiled water, a more popular and convenient way of preparing green tea now is simply brewing a teabag in boiled water for 3 min, as suggested by the teabag manufacturers (Astill et al., 2001). However, compared to 30 min under optimal laboratory conditions, it was hypothesised that most of the catechins would not have time to infuse into the hot water during the short suggested brewing time of 3 min. This could be relevant because it may explain why results from epidemiological studies have shown that only high volumes (5–10 cups/day) of green tea are associated with health benefits (Kuriyama, 2008; Uji et al., 2009). In other words, consumers may not get the full health benefits of drinking green tea because the extraction of the catechins is not optimal under household brewing conditions.

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Therefore, this study investigated the impact of typical household teabag brewing conditions on the extraction of the catechins from green tea; the volume of boiled water used and the length of the brewing time were studied. Furthermore, microwave ovens are now ubiquitous in households, and in light of the finding that microwave assisted extraction (MAE) can be more effective at extracting bioactives from plant materials (Mandal et al., 2007), it was hypothesised that a household microwave oven could be used, in a time-efficient manner, to more effectively extract the catechins from green tea in teabags than simply following the current manufacturers’ instructions. The extraction of the two other main green tea components, caffeine and theanine, was also monitored.

2. Materials and methods

2.1. Materials

Five different commercial green teas (C. sinensis) in teabags were purchased from a local supermarket (Woolworths, Tuggerah, NSW, Australia) in 2011 May: (1) Twinings Pure Green Tea, (2) Twinings Green Tea Pear & Apple, (3) Woolworths Select Green Tea, (4) Lipton Pure Green Tea and (5) Lipton Jasmine Green Tea. Three different boxes, containing 50 teabags per box, were purchased for each type of green tea for the analyses. The chemicals used in this study, L-tryptophan, L-theanine, caffeine, catechin (C), catechin gallate (CG), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallic acid (GA), and galloyl catechin gallate (GCG) were purchased from Sigma–Aldrich, Castle Hill, NSW, Australia. The structures of the eight catechins listed above are illustrated in Fig. 1. Orthophosphoric acid, High Pressure Liquid Chromatography (HPLC) grade acetonitrile and HPLC grade tetrahydrofuran were obtained from Lom ard Scientific, Taren Point, NSW, Australia.

Ultra-pure (Type 1) de-ionised (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia Pty Ltd, North Ryde, Australia). The DI water was used as solvent for all the brewing experiments with teabags.

2.2. Optimal brewing conditions

To determine the maximum amount of the green tea components available in a teabag (extractable components), the teabags were brewed under optimal laboratory extraction conditions as reported in a previous study (Vuong et al., 2011b). One teabag was extracted in 200 mL water maintained at 80°C for 30 min while stirring constantly using a magnetic stirrer at 300 rpm. Triplicate extractions, each with a separate teabag, were done. The teabag was then moved up and down 10 times, removed from the brewing solution and squeezed. The resultant tea solution was placed on ice to cool it down to room temperature and then diluted 1:1 with 500 mM L-tryptophan in DI water, giving a final concentration for the internal standard of 250 mM. The solution was then filtered through a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty Ltd, Lane Cove, NSW, Australia) into brown glass vials for HPLC analysis.

2.3. Household brewing conditions

2.3.1. Impact of the volume of boiled water

To determine the impact of the volume of boiled water on the extraction of the tea components, the teabags were brewed in a range of water volumes from 100 mL, which is equivalent to the volume of a small cup, to 250 mL boiling water, which is equivalent to the volume of a mug as described by Astill et al. (2001). Water was brought to boiling in a household kettle and either 100, 150, 200 or 250 mL of the boiled water was added to a 250 mL glass beaker containing one teabag and left to brew at room temperature for 3 min without stirring. Triplicate extractions, each with a separate teabag, were carried out for each volume of boiled water. The teabag was then moved up and down 10 times, removed from the brewing solution and squeezed. The resultant tea solution was placed on ice to cool to room temperature and then diluted 1:1 with 500 mM L-tryptophan. The solution was finally filtered through a 0.45 μm cellulose syringe filter for HPLC analysis.

2.3.2. Impact of the length of brewing time

To determine the impact of the length of brewing time, a teabag was brewed in 200 mL of boiled water in a 250 mL glass beaker and allowed to stand at room temperature for various lengths of time ranging from 1 to 30 min. Triplicate extractions, each with a separate teabag, were done for each volume of boiled water. The teabag was then moved up and down 10 times, removed from the brewing solution and squeezed. The tea solution was placed on ice to cool to room temperature and then diluted 1:1 with 500 mM L-tryptophan. The solution was finally filtered through a 0.45 μm cellulose syringe filter for HPLC analysis.

The temperature of the tea solution for each of the triplicate teabag extractions at the end of each length of brewing time was also recorded using a DTM-3103 digital thermometer (Tecpel Co., Ltd, Taipei, Taiwan).

Fig. 1. Structure of the eight green tea catechins measured in this study.
2.4. Microwave assisted extraction (MAE)

2.4.1. Cold MAE

A teabag was put in 200 mL of room temperature water in a 250 mL glass beaker and placed in a microwave oven (Panasonic, Model NN-655, NSW, Australia) set at 500 W for various lengths of irradiation time ranging from 30 s to 120 s. Triplicate extractions, each with a separate teabag, were done for each irradiation time. The teabag was then moved up and down 10 times, removed from the brewing solution and squeezed. The tea solution was placed on ice to cool to room temperature and then diluted 1:1 with 500 mM L-tryptophan. The solution was finally filtered through a 0.45 μm cellulose syringe filter for HPLC analysis.

2.4.2. Hot MAE

Hot MAE is a combination of normal brewing and MAE. A teabag was put in 200 mL of boiled water in a 250 mL glass beaker and kept at room temperature for 0.5, 1, 2, 3 or 4 min. After each of these 5 lengths of brewing time, the beaker was then placed in a microwave oven (Panasonic, Model NN-655, NSW, Australia) set at 500 W and irradiated for 30, 45 or 60 s. Triplicate extractions, each with a separate teabag, were done for each of the 15 possible combinations of the normal and MAE lengths of brewing time. The teabag was then moved up and down 10 times, removed from the brewing solution and squeezed. The tea solution was placed on ice to cool to room temperature and then diluted 1:1 with 500 mM L-tryptophan. The solution was finally filtered through a 0.45 μm cellulose syringe filter for HPLC analysis.

2.5. HPLC analysis

The tea components, including eight individual catechins, caffeine and theanine were measured using a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW, Australia) using UV detection at 210 nm and 280 nm, and a 250 × 4.6 mm Synergy 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd, Lane Cove, NSW, Australia) which was maintained at 35 °C as previously described (Vuong et al., 2011b).

The mobile phases consisted of solvent systems A and B: solvent A was 0.2% (v/v) orthophosphoric acid:acetonitrile:tetrahydrofuran, 95.5:3:1.5 (v:v:v) and solvent B was 0.2% (v/v) orthophosphoric acid:acetonitrile:tetrahydrofuran, 73.5:25:1.5 (v:v:v). A gradient elution schedule was used: 100% A from 0 to 10 min; a linear gradient from 100% A to 100% B from 10 to 40 min; a linear gradient from 100% B to 100% A from 40 to 50 min, with a post-run re-equilibration time of 10 min with 100% A before the next injection. An auto injector was used to inject 20 μL of the tea solutions onto the HPLC and the flow rate was 1 mL/min.

The tea components were quantified by dividing the peak area of the tea components by the peak area of the internal standard, L-tryptophan, and determining the concentration of each tea component in the sample from a standard curve of the peak area ratios for increasing concentrations of the respective external standard and the same concentration for the internal standard, 250 mM L-tryptophan. The value for the concentration of the ‘total catechins’ was the sum of the concentration for each of the eight individual catechines measured (C, CG, EC, ECG, EGC, EGCG, GC and GCG).

The Shan green tea variety (C. sinensis var. pubilimba) from the Thai Nguyen region of Vietnam, obtained from the Vietnam Tea Corporation (Hanoi, Vietnam), is routinely analysed by HPLC in our laboratory (Vuong et al., 2011b) and served as a control green tea in this study, which allowed for the reproducibility of the HPLC measurements of the constituents to be monitored. Only runs with control tea values within ±2 standard deviations were deemed to be within the control parameters.

2.6. Data analysis

The data was analysed for statistical significance (p < 0.05) using the SPSS statistical software version 18.0 for Windows. The Student t-test was used when there were only two treatments to compare (manufacturers’ instruction vs. optimal hot MAE). For single factor experiments with more than two treatments, the one-way ANOVA and the Bonferroni post hoc tests were performed – e.g. comparing the various volumes of boiled water, the length of brewing time or the microwave irradiation times for cold MAE. For the experiment with two factors (length of brewing time in boiled water and MAE), the two-way ANOVA and the Bonferroni post hoc tests were used.

3. Results and discussion

3.1. Extraction from teabags under optimal brewing conditions

To determine the maximum amount of green tea components able to be extracted from the green tea in a teabag, the Twinings Pure Green Tea teabags were brewed under optimal laboratory conditions.
The impact of the volume of water used under household brewing conditions on the extraction of green tea components from teabags and comparison with optimal brewing conditions.

<table>
<thead>
<tr>
<th>Optimum conditions*</th>
<th>Household conditions*</th>
<th>Water (mL)</th>
<th>Extracted tea components (mg/g)</th>
<th>Catechins</th>
<th>Caffeine</th>
<th>Theanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>60.3 ± 0.6b</td>
<td>2.92 ± 0.3b</td>
<td>10.7 ± 0.7b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>60.5 ± 0.7b</td>
<td>24.0 ± 0.1h</td>
<td>10.9 ± 0.1b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>61.3 ± 0.8b</td>
<td>24.6 ± 0.5h</td>
<td>11.4 ± 1.0b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>62.7 ± 2.0b</td>
<td>25.5 ± 0.7h</td>
<td>11.7 ± 0.8b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One teabag of Twinings Pure Green Tea in 200 mL water maintained at 80 °C for 30 min.

3.2. Extraction from teabags under household brewing conditions

The manufacturers’ instructions for the five types of green tea used in this study are shown in Table 2. Usually, the instructions suggest the use of 200 mL of freshly boiled water for 2–3 min. However, not everybody follows the instructions accurately. For example, the volume of boiled water can vary depending on the size of the cup used and the length of brewing time can depend on how strong people like their tea.

Therefore, the impact of the volume of boiled water and the length of brewing time used under household brewing conditions on the extraction of the catechins, caffeine and theanine was investigated and compared to the maximum amounts of these green tea components, which were extracted under the optimal laboratory conditions, as described in Section 3.1.

3.3. Impact of the volume of boiled water

The effect of the typical range of water volumes likely to be used by consumers, from 100 mL (the volume of a small cup) to 250 mL (the volume of a mug), on the extraction of the catechins and the other two green tea components, caffeine and theanine is shown in Table 1.

The results show that there was no significant difference between the amounts of catechins and theanine extracted within this typical range of boiled water. However, the typical household brewing conditions only extracted 62–65% of the catechins and 71–78% of the theanine extracted using the optimal laboratory conditions (Table 1).

For caffeine, there was a general and statistically significant increase in the amounts extracted as the volume of boiled water was increased. However, as for the catechins and theanine, the amount extracted with the typical household brewing conditions only accounted for 64–72% of the caffeine extracted under optimal conditions (Table 1).

Therefore, because there was no significant impact of the volume of boiled water used under the typical household conditions on the extraction of the catechins, the volume most manufacturers recommend (200 mL) was used for all the subsequent experiments.

3.3.1. Impact of the length of brewing time

The manufacturers’ instructions for brewing the teabags used in this study suggest the use of 200 mL of freshly boiled water for 2–3 min, although one of the manufacturers does not specify a length of brewing time (Table 2). However, the length of brewing time can depend on how strong people like their tea. Therefore, the effect of the typical range of brewing durations likely to be used by consumers (0.5–5 min), as previous consumer studies conducted in Europe revealed (Astill et al., 2001), was investigated. The length of brewing times tested was also extended to 30 min to determine how long it would take to extract all of the extractable components from the Twinings Pure Green Tea teabags under these conditions.

The results in Fig. 3 also show that the length of brewing time significantly affected the extraction of the catechins, caffeine and theanine. The longer the brewing time, the higher were the amounts of catechins, caffeine and theanine extracted into the green tea solutions. Furthermore, it can be seen that >90% of the caffeine and theanine was extracted within 10 min while it took up to 20 min to extract >90% of the catechins.

These findings are in agreement with the results reported in previous studies (Astill et al., 2001; Komes et al., 2010; Sari and Velioglu, 2011; Vuong et al., 2011c; Yang et al., 2007). The quicker extraction of caffeine and theanine compared to the catechins most likely reflects their greater solubility in water and their faster partition into the tea solution because they have a lower molecular mass (194 and 174 kDa, respectively) compared to the catechins (290–458 kDa) (Vuong et al., 2010, 2011a).

The results in Fig. 3 also show that brewing for 3 min in 200 mL of freshly boiled water only extracted 64% of the extractable catechins, 69% of the caffeine and 76% of the theanine in the green tea in the teabags. This confirmed that the manufacturers’ instructions were not 100% effective in extracting the tea components from the teabags into the tea solutions. Furthermore, with ≤2 min of brewing, as used by the majority of European tea drinkers (Astill et al., 2001), the current study found that less than
Fig. 3. Impact of the duration of brewing on the extraction of the green tea components from teabags. The values for the green tea catechins, caffeine and theanine extracted from one teabag of Twinings Pure Green Tea brewed in 200 mL freshly boiled water over the indicated times are expressed as a percentage of the maximum amounts of these tea components extractable from the teabags determined under optimal conditions (Table 1). The temperature of the extraction solution was also monitored. The values are means ± standard deviations for triplicate extractions for each brewing condition.

Fig. 4. Impact of cold MAE on the extraction of the green tea components from teabags. The values for the extracted green tea catechins, caffeine and theanine extracted from one teabag of Twinings Pure Green Tea in 200 mL of room temperature water (cold MAE) over the indicated irradiation times are expressed as a percentage of the maximum amounts of these tea components extractable from the teabags determined under optimal conditions (Table 1). The values are means ± standard deviations for triplicate extractions for each brewing condition and those not sharing a letter (on top of the columns) are significantly different at p < 0.05.

42% of the extractable catechins and less than 57% of the extractable caffeine and theanine were extracted into solution (Fig. 3). Thus, with short brewing times (<2 min), more than half of the catechins remained behind in the teabags and their availability in the resultant green tea would therefore be low. This may explain why a high volume of green tea (5–10 cups) appears to be required daily in order to obtain health benefits, as shown in various epidemiological studies (Kuriyama, 2008; Sano et al., 2004; Ui et al., 2009).

Although >90% of the catechins were extracted when the duration of brewing was >20 min, this length of time is not practicable for tea drinkers mainly because, as seen in Fig. 3, the temperature of the green tea would drop below 60 °C under such prolonged brewing conditions. This would not normally be favourable for many tea drinkers because they generally like to drink their tea hot (Astill et al., 2001). Therefore, in the subsequent experiments, the use of the microwave oven was studied to determine whether it could be used to improve the extraction of the catechins within an acceptable length of brewing time.

3.4. Extraction from teabags using the microwave oven

Microwave ovens are now ubiquitous in households and recent studies have found that MAE is a time-saving and effective way of extracting bioactive components from plant materials (Mandal et al., 2007; Nkhili et al., 2009; Spigno and DeFaveri, 2009). Therefore, in this study, it was hypothesised that a household microwave oven could be used, in a time-efficient manner, to more effectively extract the catechins from green tea in teabags than simply following the current manufacturers’ instructions.

To address this hypothesis, the microwave oven was tested under two different conditions. In the initial experiment, the teabags were placed in room temperature water and irradiated in a microwave oven (cold MAE) and in the second study the teabag was first brewed in freshly boiled water from 0.5 to 4 min prior to microwaving the teabag in the same solution (hot MAE).

3.4.1. Impact of cold MAE

To investigate the impact of the cold MAE on the extraction of the catechins, caffeine and theanine, a teabag was placed in 200 mL water at room temperature and various MAE irradiation durations, ranging from 30 to 120 s, were applied. The longest MAE irradiation duration studied was 120 s because during preliminary trials (data not shown) it was found that longer irradiation times caused the teabags to break open and the green tea to spill out into the water, most likely due to overheating.

The results in Fig. 4 show that the amounts of the tea components extracted increased as the cold MAE irradiation duration was increased. However, the amounts extracted were low: even with cold MAE for 120 s, only 45% (43 mg/g) of the catechins, 50% (18 mg/g) of caffeine and 53% (8 mg/g) of theanine were extracted into the solution. These levels were also

Table 3

Impact of hot MAE on the extraction of the green tea components from teabags.

<table>
<thead>
<tr>
<th>Household brewing (min)</th>
<th>MAE (s)</th>
<th>Catechins (% extracted)</th>
<th>Caffeine (% extracted)</th>
<th>Theanine (% extracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>30</td>
<td>76.7 ± 0.9b</td>
<td>82.0 ± 1.0a</td>
<td>75.4 ± 1.0a</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>83.3 ± 2.7a</td>
<td>86.9 ± 1.2b</td>
<td>79.2 ± 0.8b</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>84.9 ± 4.1a</td>
<td>89.4 ± 0.9b</td>
<td>86.6 ± 1.6a</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>73.5 ± 8.9a</td>
<td>81.6 ± 1.6c</td>
<td>75.0 ± 1.0e</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>81.5 ± 8.8b</td>
<td>86.0 ± 2.5c</td>
<td>79.6 ± 3.3c</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>88.8 ± 1.2d</td>
<td>88.9 ± 1.0b</td>
<td>88.3 ± 3.1d</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>78.1 ± 2.5a</td>
<td>82.7 ± 0.6d</td>
<td>75.5 ± 0.8e</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>85.9 ± 6.6c</td>
<td>83.0 ± 0.8e</td>
<td>85.4 ± 4.3d</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>88.3 ± 2.1d</td>
<td>89.6 ± 0.7e</td>
<td>86.4 ± 1.5d</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>75.5 ± 4.3b</td>
<td>83.6 ± 1.0c</td>
<td>73.9 ± 1.1d</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>85.5 ± 1.9b</td>
<td>86.3 ± 2.0c</td>
<td>85.9 ± 1.1e</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>88.3 ± 3.0d</td>
<td>89.9 ± 0.7e</td>
<td>87.5 ± 0.3d</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>82.5 ± 4.7d</td>
<td>83.6 ± 1.0c</td>
<td>75.9 ± 0.7e</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>85.8 ± 1.7d</td>
<td>86.3 ± 0.2a</td>
<td>85.8 ± 1.2d</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>88.7 ± 1.1c</td>
<td>89.8 ± 0.9e</td>
<td>91.5 ± 1.6e</td>
</tr>
</tbody>
</table>

Manufacturer’s instructions:

- One teabag of Twinings Pure Green Tea brewed in 200 mL freshly boiled water over the indicated times (household brewing) followed by irradiation in a microwave oven for the indicated times (MAE).
- One teabag of Twinings Pure Green Tea brewed in 200 mL freshly boiled water and left at room temperature for 3 min as per the manufacturer’s instructions.

The values are expressed as a percentage of the maximum amounts of these tea components extractable from the teabags determined under optimal conditions (Table 1). The values are means ± standard deviations for triplicate extractions for each brewing condition and those not sharing a letter in a column are significantly different at p < 0.05.
Table 4
Impact of optimum hot MAE on the extraction of total catechins, caffeine and theanine from five varieties of commercial teabags.

<table>
<thead>
<tr>
<th>Brewing conditions</th>
<th>Extracted tea components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catechins (mg/g)</td>
</tr>
<tr>
<td></td>
<td>(% extracted)</td>
</tr>
<tr>
<td>Optimum conditions'</td>
<td>100.4 ± 12.9a</td>
</tr>
<tr>
<td>Hot MAE'</td>
<td>79.5 ± 7.7a</td>
</tr>
<tr>
<td>Manufacturers' instructions'</td>
<td>61.4 ± 4.2a</td>
</tr>
</tbody>
</table>

* One teabag brewed in 200 mL freshly water maintained at 80 °C for 30 min.
** One teabag brewed in 200 mL freshly boiled water for 0.5 min followed by irradiation in a microwave oven for 1 min.
*** One teabag brewed in 200 mL freshly boiled water and left at room temperature for 3 min.

The values are expressed as a percentage of the maximum amounts of these tea components extractable from the teabags determined under optimal conditions. The values are means ± standard deviations for five varieties of teabags (Table 2) and those not sharing a letter in a column are significantly different (p < 0.05).

Table 5
Impact of optimum hot MAE on the extraction of individual catechins from five varieties of commercial teabags.

<table>
<thead>
<tr>
<th>Brewing conditions</th>
<th>Extracted tea components (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGCG</td>
</tr>
<tr>
<td>Optimum conditions'</td>
<td>41.0 ± 5.3a</td>
</tr>
<tr>
<td>Hot MAE'</td>
<td>32.7 ± 2.1b</td>
</tr>
<tr>
<td>Manufacturers' instructions'</td>
<td>23.8 ± 2.5a</td>
</tr>
</tbody>
</table>

* One teabag brewed in 200 mL freshly water maintained at 80 °C for 30 min.
** One teabag brewed in 200 mL freshly boiled water for 0.5 min followed by irradiation in a microwave oven for 1 min.
*** One teabag brewed in 200 mL freshly boiled water and left at room temperature for 3 min.

The values are means ± standard deviations for five varieties of teabags (Table 2) and expressed as mg/g of green tea in the respective teabags. Values in the same column not sharing the same superscript letters are significantly different (p < 0.05).

... significantly lower than those extracted using the typical manufacturers' instructions – a teabag in 200 mL boiled water for 3 min (Fig. 4).

Therefore, the findings illustrated that irradiating the teabags in water at room temperature was not effective at extracting the green tea components and the procedure did not improve on the extraction of the catechins, caffeine and theanine compared to the typical household brewing conditions with freshly boiled water.

3.4.2. Impact of hot MAE

Due to the cold MAE not being effective at extracting the tea components, the process referred to in this study as hot MAE was investigated. In this process, instead of using room temperature water, a teabag was first brewed in 200 mL freshly boiled water and kept at room temperature for 0.5 to 4 min, as per normal household brewing conditions, before being irradiated in a microwave oven for 30, 45 or 60 s.

The analysis of the results shown in Table 3, using the two-way ANOVA, revealed that the length of time (0.5–4 min) a teabag was brewed in freshly boiled water had no significant impact on the extraction of the catechins after the MAE and that there was no significant interaction between the length of this brewing period and the duration of the MAE. However, the duration of the MAE (30–60 s) significantly affected the extraction of the catechins (Table 3); generally, the longer the microwave irradiation time was, the higher was the extraction of the catechins. In contrast, there were no significant effects of both the length of the brewing period in boiled water prior to MAE and the duration of the MAE and no interaction between the two for the extraction of caffeine and theanine (Table 3). However, in comparison with the typical manufacturers’ instructions (3 min in 200 mL boiled water without microwaving), all the MAE times (30, 45 and 60 s) considerably improved the extraction of the catechins and caffeine but not theanine (Table 3).

Therefore, from these results (Table 3), it was concluded that hot MAE was the best of the available household method for extracting the catechins from green tea in teabags. As there was no effect of the duration of brewing in boiled water, the shortest time tested for this (0.5 min) was deemed sufficient to be used before the MAE. Furthermore, based on the extraction of the catechins (Table 3), the longest of the microwave irradiation times tested (1 min) was determined to be optimal. Therefore, the optimal household brewing conditions for the extraction of the catechins were found to be first brewing a teabag in 200 mL of freshly boiled water for 0.5 min followed by 1 min in the microwave oven. Under these hot MAE conditions, the extraction of catechins, caffeine and theanine were improved by 34%, 29% and 14%, respectively, in comparison with the typical manufacturers’ instructions (3 min in 200 mL boiled water).

In Table 4, the ability of this hot MAE method to consistently extract higher amounts of the green tea components compared to the typical manufacturers’ instructions, was tested with the five different green teas listed in Table 2. The findings confirmed that the optimal MAE conditions consistently improved the extraction of the catechins by 29% and caffeine by 21% from the five commercial teabag preparations of green tea, while the extraction of theanine was not significantly different, in comparison with the typical manufacturers’ instructions.

Finally, Table 5 also shows that the optimal hot MAE method significantly improved the extraction of EGCG, GCG and GC compared to the manufacturers’ instructions, although not to the level of the optimum laboratory conditions, and that the same applied for both the epistructured and non-epistructured catechins. This last finding indicated that the hot MAE did not cause any significant conversion of the naturally occurring epistructured catechins to their non-epistructured counterparts, which can occur during exposure of the epistructured catechins to heat (Vuong et al., 2010).

4. Conclusions

As hypothesised, the short brewing time of 3 min suggested by the manufacturers was not long enough to extract all of the catechins, caffeine and theanine. Therefore, the low extraction of
the catechins under normal household brewing conditions could
be relevant to the many epidemiological studies, which have
shown that only high volumes (5–10 cups/day) of green tea are
related to beneficial health outcomes.

Also as hypothesised, it was found that a household microwave
oven could be used to more effectively extract the catechins,
caffeine and theanine from green tea in teabags in a time-efficient
manner. However, cold MAE with water at room temperature was
not even as effective as the manufacturers’ instructions. For MAE to
improve the extraction of the green tea components, the teabags
first had to be brewed for at least 0.5 min in freshly boiled water
before the microwave oven was used (hot MAE) and irradiation for
1 min was found to be optimal.

In general, the optimal hot MAE conditions are easy to apply
under household conditions and can extract the majority of the
extractable components from the green tea in teabags. Therefore,
for green tea drinkers, who consume the tea for the potential
health benefits ascribed to its catechins, this technique could help
maximise their catechin intake. However, it should be noted that
green tea prepared in this fashion will be strong because of the high
concentration of the main tea components, especially the
catechins. It may be more bitter and astringent than when
prepared as suggested by the manufacturers. Therefore, the taste
may not be favourable to many green tea drinkers and the addition
of other flavours may be required such as lemon, jasmine, pear and
apple, varieties that are already available on the market (Table 2).

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PART 3: GENERAL DISCUSSION
AND CONCLUSIONS
3.1. DISCUSSION

As a consequence of the concerns relative to the use of organic solvents in the food industry, water was the only solvent used in this study. Water, rather than other organic solvents, was selected as the solvent for this study because it is safe, environment-friendly, relatively inexpensive and accessible in comparison with the organic solvents. In addition, the efficient use of water was taken into account because of the possible consequent costs associated with heating the solvent to boiling and to dry the aqueous extracts into powders.

The main findings supported the hypothesis that the extraction of the three bioactive components, the catechins, theanine and caffeine, from loose leaf green tea or green tea in tea bags, could be improved and that aqueous extractions could be used to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders.

The aim to improve the aqueous extraction of the three main bioactive components, the catechins, theanine and caffeine, from loose leaf green tea was achieved in Research Result Papers VI-VIII. The current study was the first to comprehensively investigate the impact of six factors, which could affect the extraction of the catechins from green tea using water as the solvent: temperature, length of extraction, water-to-tea ratio, particle size of green tea, pH of the aqueous brewing solution and the number of times the same tea sample is extracted.

Although several studies had previously attempted to study the impact of various possible factors on the efficiency of extracting catechins from green tea using water (Yoshida et al., 1999; Perva-Uzunalić et al., 2006; Astill et al., 2001), these studies investigated the impact of only a few factors at a time with none comprehensively covering all six of the main possible factors.

In addition, most of the previous studies only studied the impact on the extraction of the epistructured catechins: EGCG, EGC, ECG, and EC, not all eight catechins (EGCG, EGC, ECG, EC, GCG, GC, CG, and C) as monitored in the present study. For example, Yoshida et al. (1999) only examined the effects of two factors: pH of the water and the water-to-tea ratio on the efficiency of the extraction of the epistructured catechins only. Perva-Uzunalić et al. (2006) studied the impact of three factors (temperature, length of extraction and the number of times the same sample is extracted) on the extraction of the epistructured catechins only. Similarly, Astill et al. (2001) investigated the impact of temperature, length of brewing time and water-to-tea ratio on the total catechins but they did not report on the individual catechins.
Moreover, none of previous studies took into account the efficient use of water as was done in the present study. The results in Paper VI showed that the yield and stability of the catechins extracted from green tea using water as the solvent were affected by all six of the factors investigated. In terms of the maximal extraction of catechins per gram of green tea, the best extraction efficiency was achieved with water at 80°C for 30 min, a tea particle size of 1 mm, a brewing solution pH < 6 and a water-to-tea ratio of 50:1 mL/g. However, in terms of efficient use of water in a single extraction, and the consequent cost-effectiveness of any drying process, a water-to-tea ratio of 20:1 mL/g gave the best results.

Similarly, at the water-to-tea ratio of 20:1 mL/g, the highest yield of catechins per gram of green tea was achieved by extracting the same sample of green tea twice. However, for the most efficient use of water, and for the lowest cost of any subsequent drying process, the best extraction was found to be once at a water-to-tea ratio of 12:1 mL/g and once at a water-to-tea ratio of 8:1 mL/g.

The current study was also the first study to comprehensively examine the impact of four factors (temperature, length of extraction, the ratio of water-to-tea and the tea particle size) on the yield of theanine extracted from green tea using water. In addition, the study also identified which of these factors had the most influence on the yield of extracted theanine and, using response surface methodology (RSM), attempted to further optimise the conditions for extracting theanine from green tea using water.

Several studies had previously reported methods for extracting theanine from fresh tea leaves and commercially available dried green, oolong and black teas (Alcázar et al., 2007; Chen et al., 2003; Peng et al., 2008; Syu et al, 2008). However, although these studies used specific extraction conditions for temperature, length of extraction time and the ratio of water-to-tea, the impact of these various extraction conditions were not extensively studied over a range of values in order to optimise the yield of theanine extracted from tea as it was in the present study.

The results in Paper VII showed that the temperature and the length of extraction had more impact on the yield of theanine extracted from green tea than the ratio of water-to-tea and the tea particle size. The yield of theanine was maximal with water at 80°C for 30 min, the same conditions found to give the maximum yield of the catechins in Paper VI. Furthermore, RSM supported the findings that these conditions were optimal and that they could not be substantially improved using this methodology. Also similar to the findings in Paper VI, the results showed that the optimum
water-to-tea ratio was 20:1 mL/g when both theanine yield and the efficient water use (cost-effectiveness) were taken into account. Similarly, the optimum tea particle size was 0.5–1 mm. However, this paper did not investigate the impact of pH and of the number of times the same sample is extracted, on the yield of theanine extracted from green tea.

The study reported in Paper VIII was also the first to comprehensively investigate the impact of the pH of the aqueous extraction solution on the yield of catechins, caffeine and theanine extracted from green tea. Importantly, the pH was tightly monitored using a temperature-calibrated pH meter and controlled at the intended values between 1 and 9 during the whole brewing period, using 0.1M HCl or 0.1M NaOH, to make sure it did not change because of the buffering capacity of the material infusing into the solution. Several studies had previously investigated the impact of the pH of the brewing solution but the range of pH studied was limited to between 4-7 (Kim et al., 1999) and 6-8 (Yoshida et al., 1999). Furthermore, in these studies, the pH was not controlled and in some was observed to have changed during the brewing period.

The results in Paper VIII indicated that the extraction and the stability of the catechins was significantly affected by the pH of the brewing solution; it was higher under acidic conditions (pH<6) than under more neutral and basic conditions. In addition, to maximise the epistructured catechins relative to their non-epistructured counterparts, the green tea needed to be brewed at pH between 3-5.3. In contrast, the extraction of caffeine and theanine was less influenced by changing the pH of the brewing solution.

Paper VIII also investigated the impact of the pH on the amount of solids extracted from green tea and on the amount of tea cream formed upon cooling the extract. In turn, these two elements can impact on the purity of the bioactive compounds obtained after extractions. It was found that more extractable solids were obtained when the pH of the brew solution was ≤ 2 or > 7. More tea cream was also obtained when the green tea was brewed at pH ≤ 2 but little cream was obtained at pH > 7.

The aim of preparing decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves using water as the only solvent for the extractions and freeze drying and spray drying to dry the aqueous extracts, was achieved in the Research Result Papers IX and X.

Firstly, the use of water for the decaffeination of both young leaves (the apical bud and the first four leaves down the stem of the growing shoot) and old mature leaves (from the fifth to the tenth leaf down the stem) was comprehensively investigated. A recent study by Liang et al. (2007) had
found that hot water could be used to remove caffeine from freshly picked young tea leaves. However, these young tea leaves are primarily used for making quality green teas and it would not be commercially viable to use them for preparing extracts and powders. On the other hand, the older, more mature leaves (from the fifth to the tenth leaf down the stem) are usually cut during pruning at the end of the growing season and left on the ground as mulch for the soil (Zandi and Gordon, 1999). Therefore, these could potentially be used to prepare green tea extracts and powders and thereby add value to the industry. However, studies on the use of these old green tea leaves have not been reported.

The results in Paper IX showed for the first time that the unutilised old tea leaves could be used to prepare green tea extracts and powders using water as the solvent. Furthermore, the results showed that decaffeinated green tea could be produced by simply blanching the old tea leaves in boiling water for 10 min at a water-to-tea ratio of 20:1 mL/g. After drying, the levels of caffeine, total catechins and theanine were 1.5, 49.3, and 1.7 mg/g, respectively. Compared to undecaffeinated green tea made from these old leaves, the values were 80%, 17% and 30% lower for caffeine, total catechins and theanine, respectively. Thus, the decaffeination method removed 80% of the caffeine while retaining 83% of the catechins. However, 30% of the theanine was lost.

The findings in Paper X, revealed that the optimum conditions for the decaffeination step differed slightly depending on whether young or old leaves were used. The blanching time required to decaffeinate the young tea leaves was shorter - 4 min compared to 10 min for the old leaves in Paper IX. For the decaffeinated green tea made from the young leaves, the levels of caffeine, total catechins and theanine were 5.3, 155.6 and 17.2 mg/g, respectively. Therefore, the decaffeination method remove 83% of the caffeine while retaining 94% of the catechins. However, there was a 31% loss of theanine.

The concentration of the three components was significantly higher than in the decaffeinated green tea produced from the old tea leaves because the young tea leaves contained higher amounts before decaffeination than the old tea leaves. By definition, decaffeinated green tea is required to have a caffeine content of ≤ 4 mg/g (Ye et al., 2007). Therefore, only the tea from old leaves (1.5 mg/g) could officially be referred to as decaffeinated green tea as the content of caffeine in the tea from the young leaves (5.3 mg/g) was higher than 4 mg/g.

This could be another reason for preparing decaffeinated green tea using the old tea leaves as starting material - leaves which are otherwise wasted. Alternatively, more caffeine could be
extracted from the young leaves by prolonging the blanching step; this would most likely lead to an increased loss of catechins but some of the catechins may have to be sacrificed to reach the target caffeine level in order to be make decaffeinated green tea from these young leaves. Therefore, this decaffeination method still has some limitations such as not extracting enough caffeine and the partial loss of some of the catechins and losing a substantial amount of the theanine. Therefore, further study is required if these limitations are to be overcome.

In Paper IX, normal caffeine and decaffeinated green tea powders were also produced. The dried green teas prepared from the old tea leaves, were extracted with water using the optimal conditions identified in Paper VI. The extracts were then filtered and concentrated to 20% (w/v) using a rotary evaporator at 60°C under vacuum. For the final step, two major drying techniques, freeze drying and spray drying were used to obtain the powders. The results showed that, although there were some slight differences, the powders produced by freeze drying and spray drying had similar physical properties and similar concentrations of the catechins and theanine.

However, as found in previous studies comparing freeze drying and spray drying (Desobry et al., 1997; Moßhammer et al., 2006), the yield of powder was higher for freeze drying compared to spray drying. There was a partial loss (20-25%) of powder and catechins during spray drying because some of the extract did not completely dry and adhered to the insides of the piping and the collection cylinder of the spray dryer. There was also an increased conversion of the epistructured catechins to their non-epistructured forms during spray drying. Ideally, as much as possible of the catechins in the powders should be in their epistructured form; therefore, the preservation of the epistructured catechins is an area which will require further studies.

Nonetheless, although the yield of powder and the recovery of catechins were lower than for freeze drying and there was an increased conversion of the epistructured catechins to their non-epistructured counterparts, spray drying can still be considered a suitable method for drying green tea powders because it has much lower costs and shorter times are needed for drying in comparison with freeze drying (Mujumdar, 2000). Therefore, to improve the yield of powder and the recovery of the catechins, the conditions for spray drying the green tea extracts were optimised in Paper X. The results revealed that, relative to powder yield, the optimal conditions for spray drying to be 180°C for the inlet temperature and 115°C for the outlet temperature.

The decaffeinated green tea powders produced in this study (Papers IX & X) had caffeine levels ranging from 6.4 to 7.3 mg/g, which were below the maximum permitted (10 mg/g) for
classification as a decaffeinated tea powder (Ye et al., 2007). Furthermore, these levels were similar to those in the powders produced using other decaffeination methods such as with activated charcoal (7.81 mg/g) (Ye et al., 2007) and significantly lower than the level of caffeine in the normal caffeine green tea powder produced in this study (21.3-21.8 mg/g) and in another study using organic solvent extraction (37.89 mg/g) (Dong et al., 2011). In contrast, the powder, produced from the water used to extract the caffeine from green tea in the blanching step (Paper X), had a much higher caffeine level of 95 mg/g.

In addition, the decaffeinated, normal caffeine and caffeine-enriched green tea powders had excellent physical properties such as high water solubility (≥96%) and low moisture content (<2.5%). Therefore, such green tea powders have potential for use as instant teas or as additives in the food and nutraceutical industries and may help people reach levels of bioactives equivalent to those in the large volume of tea (5-10 cups) shown in epidemiological studies to be required to obtain health benefits (Wu et al., 2003; Zaveri, 2006; Zhang et al., 2007).

The last aim of improving the extraction of the three green tea bioactive components from green tea in teabags using water and the microwave oven was achieved in Research Result Paper XI. Based on the results with loose leaf tea under optimum laboratory conditions (i.e. 30 min brewing time, Papers VI-X), it was obvious that the conditions suggested by the manufacturers for making the green tea beverage from teabags, brewing for 3 min at room temperature in 200 mL of boiled water, were not going to extract all the available catechins, theanine and caffeine from the tea.

Consequently, there was a low level of green tea bioactive compounds in the beverage produced in this fashion; the results in Paper XI indicated that the conditions suggested by the manufacturers only extracted 62% of the catechins, 76% of the caffeine and 80% of the theanine present in a teabag. Therefore, this low extraction of the bioactive compounds could be one of the reasons why a large volume of tea (5-10 cups) has been shown in epidemiological studies to be required to obtain health benefits (Wu et al., 2003; Zaveri, 2006; Zhang et al., 2007).

As the microwave oven had previously been found to increase the extraction of bioactive components from plants (Mandal et al., 2007) and because it is presently ubiquitous in households around the world, this study investigated the application of microwave assisted extraction (MAE) to determine whether this could improve the extraction of the green tea constituents from teabags.
The results in Paper XI indicated that using the MAE to brew teabags in water at room temperature (cold MAE) was less effective than using the manufacturers’ instructions. However, brewing the teabags in freshly boiled water for at least 0.5 min followed by irradiation for 1 min in a microwave oven (hot MAE) was effective at increasing the extraction of the tea bioactive components. With hot MAE, the extraction of the catechins, caffeine and theanine was improved by 34%, 29% and 14%, respectively, compared to the manufacturers’ instructions and 85% of the catechins, 90% of the caffeine and 87% of the theanine in the teabags could be extracted.

Therefore, most of green tea constituents were extracted using the hot MAE conditions, which could easily be applied in the home or office by tea drinkers who want to maximise their intake of the catechins for health reasons. However, it should be noted that the tea beverage prepared under these conditions can be strong because of the high concentration of catechins and caffeine, which bring astringent and bitter tastes to the green tea (Graham, 1992). Therefore, the taste such tea beverages may not be favourable to some tea drinkers. However, the addition to the green tea of flavours such as lemon, jasmine, lotus, pear and apple, some of which are already available on the market, may improve the taste of the resulting beverage.

Finally, to address the issue of people possibly not being able to drink the large volumes of tea (5-10 cups/day), which appears to be required for obtaining health benefits (Wu et al., 2003; Zaveri, 2006; Zhang et al., 2007), this study improved the methods for extracting the bioactive compounds from loose leaf green tea using water only under laboratory conditions and for producing decaffeinated, normal caffeine and caffeine-enriched green tea powders. This study also showed that the extraction of the bioactive components of green tea in teabags was poor using the manufacturers’ instructions and showed how the extraction could be improved using the common household appliance, the microwave oven.
3.2. CONCLUSIONS

As hypothesised, the aqueous extraction of the three bioactive components, the catechins, theanine and caffeine, from loose leaf green tea or green tea in tea bags, was improved and the aqueous extractions were used to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders with excellent physical properties.

The aim to improve the aqueous extraction of the three main bioactive components, the catechins, theanine and caffeine from loose leaf green tea was achieved in Papers VI to VIII. The optimal conditions for the extraction of the catechins were found to be once at 80°C, 30min, water-to-tea ratio of 12:1 mL/g and once at 80°C, 30min, water-to-tea ratio of 8:1 mL/g with tea particle size of 1 mm (Paper VI). The optimal conditions for the extraction of theanine were found to be 80°C for 30 min with a particle size of 0.5–1 mm and a water-to-tea ratio of 20:1 mL/g (Paper VII). In addition, to maximize the epistructured catechins relative to their non-epistructured counterparts, the green tea should be brewed at pH between 3-5.3 (Paper VIII). However, the extraction of caffeine and theanine was less influenced by changing the brewing solution pH.

The aim to prepare decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves using water as the only solvent for the extractions and freeze drying and spray drying to dry the aqueous extracts, was achieved in Papers IX and X. The optimal conditions for decaffeination of the old tea leaves were found to be 100°C, 10 min, and ratio of water-to-tea 20:1 mL/g (Paper IX) and the optimal conditions for decaffeination of the young tea leaves were found to be 100°C, 4 min, and ratio of water-to-tea 20:1 mL/g (Paper X). Using these conditions, decaffeinated dried green tea having 1.49 mg/g caffeine was produced from the old tea leaves but the caffeine content (5.3 mg/g) of the dried green tea of produced from young tea leaves was lightly above the threshold (4 mg/g) required for being called decaffeinated.

To produce green tea powders, the optimal spray drying conditions were investigated and were found to be 180°C for the inlet temperature and 115°C for the outlet temperature (Paper X). In terms of cost-effectiveness, spray drying is considered as a method of choice, although the recovery was found to be 20-25% lower than with freeze drying. Using spray drying, decaffeinated (6.42 – 7.3 mg/g) and normal caffeine (21.3-21.8 mg/g) green tea powders were produced and using the water used to extract the caffeine from green tea to make dried decaffeinated green tea, a caffeine-enriched (95 mg/g) green tea powder was also produced (Papers IX and X). All the powders had
excellent physical properties such as high water solubility (≥96%) and low moisture content (<2.5%) and would have potential for use as instant teas, supplements or as additives in the food and nutraceutical industries.

Finally, the aim to improve the extraction of the three green tea bioactive components from green tea in teabags using water was achieved using the common microwave oven in Paper XI. In this study, the manufacturers’ instructions were found to be inefficient for extracting the bioactive compounds. Use of the microwave oven improved the extraction when a teabag was first brewed at room temperature in boiled water for 0.5 min and then irradiated in a microwave oven for 1 min (hot MAE). Compared to the manufacturers’ instructions, hot MAE improved the extraction of the catechins, caffeine and theanine by 34%, 29% and 14%, respectively, and 85% of the catechins, 90% of the caffeine and 87% of the theanine in the teabags was thus extracted.

In summary, the hypothesis was supported and the aims and objectives were achieved in the current study. The aqueous extraction of the three main bioactive components, the catechins, theanine and caffeine from loose leaf green tea was improved. In addition, using water as the only solvent, this study developed methods, which were used to prepare decaffeinated dried green tea and decaffeinated, normal caffeine and caffeine-enriched green tea catechin powders from freshly harvested young and old green tea leaves. Finally, this thesis developed a method using the microwave oven, which improved the extraction of the three green tea bioactive components form green tea in teabags.
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