Determination of Marker Compounds to Assess the Quality of Green Tea

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by

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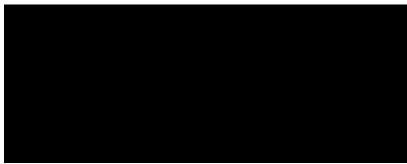
Statement of Authenticity

This thesis is submitted in fulfilment of the requirements for the Master of Research degree at the Western Sydney University; School of Health and Science. The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not previously submitted this material, either in full or in part, for a degree at this or any other institution. Unless otherwise stated, all of the data and observations presented here are the results of my own work.



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Abstract

Green tea, as a commonly consumed beverage worldwide, has a long history of use as a dietary supplement for various conditions. Green tea products have been shown to possess various biological activities, ranging from cardiovascular protection to anticancer effects. The bioactivity of green tea is largely attributed to catechin polyphenols, which make up around on third of the dry leaf weight of green tea. However, the quality of green tea is variable, which may be attributed to the variation in contents of the catechins and other polyphenols, thus the content of these polyphenols may be a suitable determinant of the quality of green tea. The aims of this project are to develop analytical methods to determine the concentration of antioxidant compounds in various green tea products, as well as their antioxidant activities. The findings may be useful for the assessment of green tea product quality and used for quality control of green tea products.

Eleven (11) commercial green tea products were sourced from the local market, including dried tea leaves, tea powders and TGA listed tablets and sachet. The green tea products were extracted and subjected to HPLC analysis of selected chemical markers, using a Shimadzu HPLC with a photo-diode array detector, and LC-MS analysis, using a Waters Xevo TQ with electrospray ionisation source and Waters Aquity UPLC. An *in vitro* DPPH radical scavenging assay was used to determine the antioxidant activity of green tea samples. The total phenolic content (TPC) was determined using the Folin Ciocalteu (FC) method. In addition, an online antioxidant screening assay was developed using HPLC and post column derivatization.

The chemical markers , gallic acid, gallocatechin, theobromine, epigallocatechin, epigallocatechin gallate, caffeine, epcatechin and epicatechin gallate in the green tea

samples were quantified by HPLC. The content of each marker compound varied in different green tea products. The highest contents of markers were observed for a TGA listed product (S11), while the lowest content was also observed for another sachet product (S10). Among the different products, the traditional tea leaf products (S3, S4, S5, S6 and S7) showed a higher level of marker contents than the tea powder products (Matcha, S1 and S2). In addition, the content of L-theanine, determined by LC-MS, varied significantly among different products, with the highest level observed in sample 7 and 5, followed by Sample 1. L-theanine was not detectable in TGA listed green tea tablets (S8 and S9), indicating that the manufacture procedure may affect the level of L-theanine in the final product. These results indicate that the level of marker compounds varies significantly among different green tea products. It is surprising to observe that one green tea sachet product (S10), which claimed (on packaging) to be potent and equal up to 20 cups of green tea per sachet is actually the less potent among the products tested- including the raw teas. Thus the consumer should be wary when choosing the green tea products based on the product labels. More random testing should be conducted by the regulatory authorities to ensure the listed products have quality matching their labels.

Similar results were obtained for the antioxidant activity of green tea products, with the highest antioxidant activity observed for S11, followed by S9 and S8, and the lowest antioxidant activity was observed for the green tea sachet product (S10), indicating the antioxidant activity of the green tea products is related to the content of marker compounds studied. This is further supported by series of correlation analysis which showed a significant positive correlation between the total content of group catechin markers (GA, EGC, EC, ECG) with the antioxidant activity, indicating these compounds may directly link to the antioxidant activity of green tea products. In

contrast, other compounds present in tea, methylxanthines, caffeine and theobromine, showed no a correlation with the antioxidant activity. Similar results were obtained for L-theanine, no correlation was observed for its content with the antioxidant activity. These results indicate that there is a strong correlation between the concentrations of selected catechin marker compounds in the tea samples and the antioxidant activity *in vitro*.

Finally, a specific online assay has been developed with efficient separations and clear antioxidant responses. It is also consistent with the results of the *in vitro* antioxidant activity assay, with the markers (eg GA, EC, EGC and EGCG) strongly correlated with the in vitro anti-oxidant activity also found to be active in the online FRAP assay. Thus, the online method is efficient to identify known or unknown component/compounds with antioxidant activities. This approach is potentially a powerful tool to study the antioxidant activity of complex natural mixtures, and makes it possible to identify specific ingredients/compounds with antioxidant activity which otherwise might not be able detect using crude extracts and conventional antioxidant assays.

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Abbreviations and Acronyms

Abbreviation	Full term
(+) ESI	ESI operating in positive ion mode
$[M + H]^+$	Molecular plus H ion
$[M – H]^-$	Molecular minus H ion
$[M]^+$	Molecular ion
AR	Analytical reagent
CVD	Cardiovascular Disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
Caf	Caffiene
Thea	Theanine
Theo	Theobromine
ELSD	Evaporative Light Scattering Detection
ESI	Electrospray ionisation
EtOH	Ethanol
FRAP	Ferric reducing ability of plasma
F-C	Folin-Ciocalteu
GA	Gallic Acid
GC	Gallocatechin

g	Gravitational acceleration
h	Hour
HPLC	High performance liquid chromatography
I.D.	Internal Diameter
LPS	Lipopolysaccharide
m/z	Mass-to-charge ratio
Max.	Maximum
MeOH	Methanol
min	Minute
Min.	Minimum
MS	Mass spectrometer
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
TGA	Therapeutic Goods Administration
TPC	Total phenolic content

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Chapter 1: Introduction

Herbal medicines have been used to treat a variety of chronic diseases with reduced side effects. Many individuals suffer adverse effects of Western pharmaceuticals or mistrust and therefore herbal medicines are a viable option for many. However, many factors affect the chemical composition of the herbs and inconsistencies in the final herbal products have been widely observed. Consistency concerns with herbal medicines must be resolved before they can be accepted as safe and reproducibly effective. Herbal products are complex, typically containing hundreds of compounds. It is difficult to monitor all these compounds in routine quality control. It is also often unclear which of the components are biologically active and responsible for the herbs activity. To simplify the quality assessment of herbs major compounds are selected as markers of quality.

1.1 Marker compounds and quality control of Complementary Medicines

Complementary medicines often have a complex chemical composition, and in many cases the nature of so called “active” compounds in those products are not known. Thus, in order to compare their chemical similarity and efficacy, the assessment of certain chemical markers is used as a measure for quality control. Marker compounds often include those molecules that are present in reasonable quantities in the product, and link to the action or efficacy of the products. The usual criteria that a suitable marker compound for complementary medicines needs to fulfil are that is present in the mixture in high abundance and that there is a significant proven benefit to the disease of interest(1). In some cases, however, the link of the marker compounds to the product efficacy has not been established. Thus, it is important to establish if the

markers compounds are suitable and capture the activity of the products to be compared as proper marker compounds can provide a basis to rank the quality of natural products(2).

The Therapeutic Goods Administration (TGA) is the governing regulation body that oversees medicines and supplements in Australia and provides guidelines for manufacturers to adhere to in order to be able to sell their product. Currently, manufacturers only need to quantify marker compounds that are on the label as an active. This leaves the door open for less specific claims. For example, claims such as “14x extract” can be made based on the production extraction without the extract being containing 14 times the concentration of the chemical components in the raw material. Product claims of this type are not subject to regulated quantitative testing and are therefore open to exploitation. Cost saving practice of re-extraction or extraction of diluted materials are purported to occur within the industry. This can result in misleading and low quality finished products. The chemical composition of the herbal marker can be used to access this; however, natural variations will exist. To determine if these variations are significant testing of biological activity is required.

1.1.1 Phytoequivalence

Given natural variation, it is extremely unlikely that any 2 herbs will be identified as chemically exactly the same- there will always be minor variation. Based on chemical abundance alone it is difficult to predict how minor variations in the chemical composition affect the biological activity. It is therefore crucial that for any 2 herbs to be considered equivalent they must have equivalent chemical composition and biological activity; this is termed phytoequivalence. Bioequivalence is a term used in

pharmacokinetics that describes two pharmaceutical products that are biologically equivalent, meaning, that the same dose of each of the two products will exhibit the same pharmacological effect(3). Similarly, this is described in natural products as phytoequivalence. This comes from a recognition that there is no way to comprehensively determine what the components of the herbs do when consumed. As a result, it is difficult to determine which of the components are responsible for the biological activity (or modulating the biological activity), and therefore all compounds need to be monitored to maintain pharmacological consistency. To ensure that there is a level of consistency, there needs to be a matching phytochemical composition and biological activity.

Marker compounds can be quantified to account for some of the activity of a product to allow for product comparison. The question that must be answered is, do these marker compounds capture the activity of the herb, if the answer is yes, then it is a suitable marker compound to determine the quality of similar herbal products. Marker compounds can provide a basis to rank the quality of natural products. The usual criteria that a suitable marker compound needs to fulfil are that is present in the mixture in high abundance and that there is a significant proven benefit to the disease of interest(1).

1.2 **Green tea**

1.2.1 Traditional use

Green tea has been used for centuries as a medicine to treat a variety of diseases. One of the earliest mentions of green tea is taught in Chinese mythology, where in 2737 B.C.E, the emperor Shen Nung first discovered the medicinal properties of tea. Until

the 'Tang dynasty in 618-906 C.E(4), tea was only considered as a medicine, but soon after became popular as a beverage. Throughout this era it is recalled that tea was consumed in vegetable soups with various other ingredients such as ginger and orange peel. A method of brewing used by ancient Himalayans is brick tea, this is still a popular brewing method in modern Mongolia. During the "Song" dynasty (10th-13th centuries), this method became less widespread and was replaced by a powered form of tea that was whipped into froth. This form of tea is a bright emerald green colour with lower astringency than normal tea. This preparation method of tea survives today in Japan as Mattcha(5). The medicinal properties of green tea were also recorded in the pharmacopeia, "Ben Cao Gang Mo", written by Shi Zheng Li in the Ming dynasty (16th century)(6). The use of tea has continued into the modern era.

1.2.2 Modern use

Tea is one of the most widely consumed beverages in the world, second only to water(7). In the USA alone 320g of tea (equivalent to 35-40 L) are consumed per capita. In India, this number is almost doubled, and Ireland consumes this amount ten-fold(4). It is made from the leaves of *Camellia Sinensis*, an evergreen plant, native to China and many other Asian countries (Fig. 1). There are two major varieties of the plant, the *Sinensis* variety, popular in China, Japan and other parts of Eastern Asia, and the *Assam* variety is more popular in tropical regions such as India. These plants are characterized by their larger leaf size, up to 20cm compared to 5 to 12 cm of the *Camellia Sinensis* leaves(4,8). In most cases, the *Sinensis* variety is used for the preparation of green teas and the *Assam* variety is used in the production of black teas(9). There are various types of green tea products, such as oolong, green and black teas which are related to the different extraction /manufacture process involving

withering, bruising, fermentation and drying (see Fig 2). It is estimated that about 78% of the tea production is black tea, followed by green tea (20%) and other types of teas.s There is an extensive history of green tea intake by humans, its medicinal use dates back thousands of years and continues in modern times.

1.3 **Health benefits of green tea**

The health benefits of green tea have had a growing interest in the last few decades. The annual sales of dietary supplements globally in 2013 was $67 billion, according to a publication in the JAMA Internal medicine(10). The article also concluded that this usage was not limited to a particular age group or gender, therefore its popularity is widespread.

Green teas use as a supplement may be based on traditional uses however there are recent studies that support the use of green tea as a supplement. There have been various studies on the potential health benefits of green tea, such as; anticancer(11), cardioprotective(12), neuroprotective, , anti-diabetic, antimicrobial, anti-viral and anti-inflammatory effects(13). Most effects are thought to be related to the antioxidant properties of chemical ingredients in the green tea mainly polyphenols, especially catechins such as epigallocatechin gallate (EGCG)(14). These polyphenols are present in all forms of tea, however they are present in much higher concentrations in green and white teas then they are in black teas, thus green tea is the preferred source of antioxidants.

The major health benefit of green tea is its antioxidant activity(14). An antioxidant is a compound that is able to prevent or slow down the oxidation of another compound(15). Antioxidant activity is complex; there are many antioxidants that act

by different mechanisms on different oxidative agents. Oxidation can be caused by free radicals. Antioxidant activity may be assessed by the DPPH (2,2-di-phenyl-picrylhydrazyl) assay(16,17), and the Folin-Ciocalteu assay(18). The DPPH reagent is a radical compound with a strong absorbance at 515nm. Hydrogen-donating antioxidants reduce the DPPH free radical, therefore lowering the absorbance reading at 515nm and visualised as a colour changed from deep purple to yellow. FC assay is a colorimetric method that uses a mixture of phosphomolybdate and phosphotungstate, that shows a colour change from blue to yellow when it is reduced.

Some of the most exciting research that has been done on tea is benefits toward cancer research. There have been clinical trials that has been undertaken on the contribution of green tea in fighting different types of cancer as reviewed by Raymond Cooper (14).

1.3.1 Cancer

Cancer is a common disease affecting a larger proportion of the global population(11). According to statistics by the Australian Cancer Council, 1 in 2 Australians will be diagnosed with cancer by the age of 85, and this year it is estimated that there will be 138,000 new cases of cancer diagnosed, with this number set to rise to 150,000 by 2020. In 2014 cancer was the second leading cause of death in Australia, accounting for about three in every 10 deaths. This has risen since then and this year is expected to end with 48,000 cancer related deaths(19). Green tea has been used to prevent and treat a variety of cancers including breast, liver and prostate cancers. In a Canadian report by Boon et. al. (2007) green tea was the most commonly used for "self-treatment" of breast cancer(20). According to Yang (1993) the main reason for this is the that polyphenols inhibit cancer cell proliferation(21). However, there has been limited evidence to suggest that there is a significant causal link between the

consumption of green tea and a lower incidence of cancer. A review by the International Agency for Research on Cancer Working Group(22), determined that there was not enough clinical evidence to suggest the tea drinking could lower the carcinogenicity in humans and animals. There have however been significant advances for *in vitro* studies(21).

Despite widespread use the efficacy and mechanism for green teas against cancer is yet to be established. Green tea is used and has shown some promise in the treatment of other diseases.

1.3.2 Cardiovascular diseases

Cardiovascular diseases (CVD) are a major burden on the healthcare sector worldwide(7). According to the World Health Organisation (WHO 2011), almost one third of the total global deaths can be attributed to CVD. A variety of studies have led to a conclusion that the consumption of green tea can have a positive effect on cardiovascular function. One of the major contributors of cardiovascular disease is atherosclerosis, CVD can be caused when the arteries become blocked. When the arteries become blocked by a blood clot or when the blood flow is restricted, this limits how much blood and oxygen can be delivered to the organs or tissue. Based on a literature review (14,28,29), compounds in green tea such as theobromine have been found to have cardiotonic properties. This could indicate why green tea has been traditionally effective in the prevention of such diseases.

1.3.3 Cognitive Function

In addition to these theobromine and polyphenols, green tea also contains the amino acid, L-theanine that is unique to tea(8). L-theanine also has the potential to cross the blood-brain barrier (BBB)(8,30). It has been shown to have a calming effect on the brain and negating the effect of caffeine. This effect has been suggested through very limited pharmacological studies on the effect of the caffeine from tea as opposed to a regular dose of caffeine and observing the different effects.

One significant study on the pharmacological effect of L-theanine monitored the anti-stress effect on mice after consuming Gyokoru, a variety of tea that is rich in the amino acid L-theanine. The effect was compared with standard sencha tea and sencha tea with lowered caffeine(30). The study showed adrenal hypertrophy was significant lowered in the mice that ingested theanine. This dosage (0.32mg/kg) was much lower than the theanine in standard sencha tea (5mg/kg), which suggested that there was a component of the tea that had a suppressive effect on the anti stress effect of theanine. There have been previous studies that have found that caffeine antagonizes theanine(31). Although there were also findings by Unno et al that suggested that ECGC also suppressed the action of theanine(30).

Further studies of L-*theanine* have found that this amino acid present in green tea can improve cognitive impairment in mice that have been injected with Aβ peptides(32). In this study, the mice were treated these peptides which induced cell neuron death. The study found that the treatment of L-*theanine* was able to repair the cognitive damaged caused by the Aβ peptide treatment and therefore improve the cognitive health of the mice.

Green tea and it components have been shown to have a variety of beneficial effect on health, it is therefore not surprising that green tea is widely used to improve health.

1.3.4 Weight loss

Obesity is a growing health concern in the global population and a priority for a lot of prevention and early intervention healthcare strategies. Obesity has been defined by the World Health Organisation (WHO) as "abnormal or excessive fat accumulation that may impair health"(23). The clearest way to determine whether or not someone is overweight or obese is by their body mass index (BMI). An adult with a BMI of 25 kg/m^2 is classified as overweight. An adult with a BMI of $30kg/m^2$ or higher is classified to be obese. The average BMI differs among different countries. For example the average BMI in some Asian countries is often lower(24).

Tea, high in bioactive flavonoids, has been found to assist with weight loss,(25). Green tea has been found to have a history of uses in the management of weight and has been observed and claimed in some cases since the early 1990s, to increase the energy output of some individuals, thus stimulating weight loss. Research by Dullo, A.G et al. suggests that the combined activity of the polyphenol EGCG and caffeine has a more profound effect on weight loss than the caffeine alone(26). In this study, the subjects were treated with one of three dosages; a mixture of 50mg of caffeine and 90mg of EGCG, 50mg of caffeine, as well as a placebo. The subjects were tested on three occasions over a 24 h period for their respiratory quotient and urinary excretion of nitrogen and catecholamines in 10 healthy men. On three randomly selected occasions, the test subjects were assigned to 3 different treatments as described above.

The study found significant metabolic differences between the groups treated with caffeine and caffeine/ECGC combo, compared to the placebo.

Green tea weight loss supplements reportedly contain a higher concentration of catechins, thus increasing the potency of the antioxidants in comparison to the tea leaf products(26). However there have been various toxicological studies on green tea weight loss supplements that have been linked to various cases of liver damage as well as heart problems, such as acute left ventricle(27). It is unclear whether these effects are due to high catechin content or other components, as they are typically poorly characterised.

In contradiction to these safety concerns green tea has been suggested to be beneficial in the treatment of cardiovascular problems.

1.4 **Safety of green tea supplements**

Most green tea dietary supplements are "listed medicines" by the Therapeutic Goods Administration (TGA). Listed medications are slight different from registered medications in that the products that are of lower risk to the consumer than registered medicines and are usually for a consumer's self-medication. In Australia, TGA-listed medications are labelled with an "Aust-L" number and TGA-registered products are labelled "Aust-R". Listed medicines will only contain ingredients that have been well established and have a long history of use or the condition.

Various report suggests that high doses of EGCG can lead to hepatotoxicity(33). These reports may be linked to other contaminates in the products as in highly regulated markets this toxicity has not been observed. The TGA monitor the adverse events reported for green tea products sold in Australia. There are over 267 products the vast majority have been available and sold for years. While international concern of liver

harm are noted by the TGA no concerns have been recorded for product sold and monitored in Australia(34). The safety and benefits of the green tea products are linked to their chemical composition.

1.5 **Chemical profile of green tea**

The major components of green tea are its flavonoids and poly phenols, of which make up 50 to 60% of the dry weight of the tea leaves(8,35). The five major classes of polyphenols include phenolic acids, stilbenes, flavonoids, lignans, tannins and flavan-3-ols(8). Catechins are the major flavanols in green tea, namely, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate(2).

Figure 1 illustrates the main polyphenols in green tea and their chemical structure. The major catechins in green tea: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG).

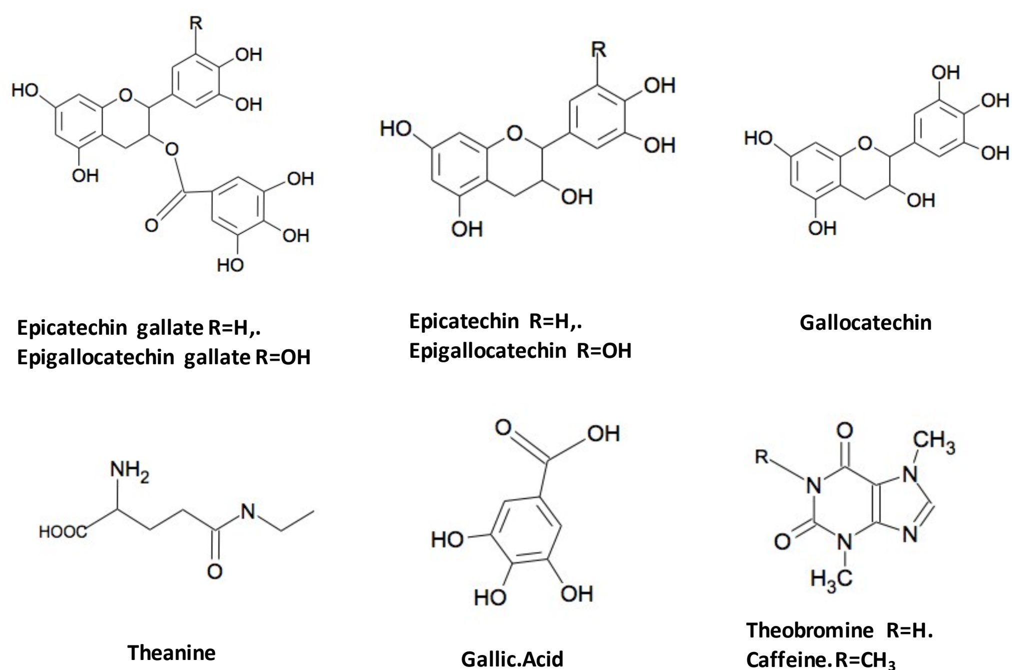


Figure 1:The major compounds in green tea and their chemical structures

Catechin and epicatechin both are monomeric flavanol, epicatechin has an ortho-dihydroxyl group in the B-ring at carbons 3' and 4' and a hydroxyl group at carbon 3' on the C-ring. EGC has trihydroxyl group at carbons 3', 4', and 5' on the B-ring. ECG has gallate moiety esterified at carbon 3 of the C-ring. However, EGCG has both trihydroxyl groups at carbons 3', 4', and 5' on the B-ring and a gallate moiety esterified at carbon 3' on the C-ring(36) on the chemical backbone of flavonoids(37). These catechins account 30-42% of the dry weight of green tea and EGCG the main catechins accounts 50-80% of the total catechins (35). It was reported that radical-scavenging ability of EGCG was higher than that of other catechins due to the presence of a gallate moiety in the C ring(38). Catechins are ordered based on their antioxidant activity as indicated by a number of studies: EGC ~ EGCG >> ECG = EC > catechins and the number of hydroxyl groups is the major factor contributing to antioxidant potential of catechins of green tea(39,40).

There are five major types of flavan-3-ols in green tea, including catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (EGCG). Table 1 shows an outline of the composition of the main polyphenols in green tea.

Table 1: Composition of major polyphenols in green tea(6).

Catechin	2%
Epicatechin	6%
Epicatechin gallate	6-12%
Epigallocatechin	20-30%

Epigallocatechin gallate	33-50%

1.6 **Factors affecting the chemical composition of green tea**

Tea comes from different variations of the evergreen shrub known as *Camellia Sinesis*. There is a large amount of variation in the chemical constituents, particularly between green and black tea. These arise from variations in the climate, the harvesting and the variety of the tea.

The variety of the tea that contains the highest concentration of catechins is white tea. This tea is prepared by harvesting the young leaves or buds from the tip of the plant and the leaves are blanched and dried as very quickly in order to stop the oxidation process. Green tea leaves are still for the most part un-oxidised although the more mature leaves are picked from plant, therefore the polyphenol content is not as high as that of white tea.

Black tea is the fully oxidised form of tea, the leaves are withered and rolled to allow the fermentation process to take place(8,41). This process produces a by-product, theaflavins and thearubigens. Because of this, the antioxidant effect of black tea is greatly reduced compared to that of catechin-rich green tea.

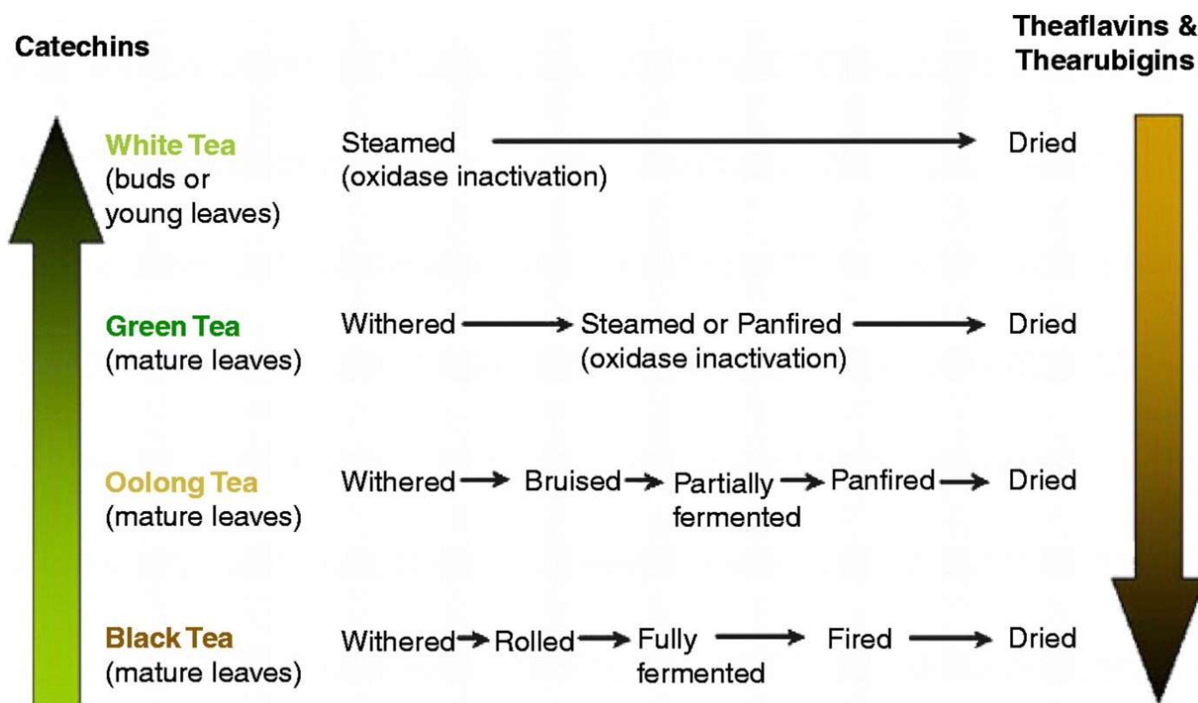


Figure 2: Schematic of the tea extraction processes. Adopted from Cooper R (2005) Medicinal Benefits of Green Tea: Part 1. Review of Noncancer Health Benefits(7).

The oxidation (or fermentation) process is catalysed by the enzyme phenol oxidase(8). After this process of fermentation, the content of polyphenols, the main antioxidants in tea, are reduced and the molecules known as theaflavins and thearubigens. According to a review by Harbowy and Balentine(41), green tea contains on average 3 times the catechin content of black tea. Differences in the fermentation process can influence the chemical composition of the tea.

It has been demonstrated that different processes in the green tea production affect the chemical compositions of green tea products. For example, unfermented green tea contains the highest concentration of flavan-3-ols. During tea fermentation, flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerisation, resulting in the formation of theaflavins and thearubigins(42). Oolong tea is a partially fermented product and therefore contains a mixture of flavan-3-ols, theaflavins, and thearubigins.

Black tea is the most fermented tea, and as a result, contains abundant theaflavins and thearubigins, and limited or no flavan-3-ols(43).

A study on the effect of the levels of these bioactives in green tea by Lee, L et al, has discovered that over a two (2) month plucking period, the contents in tea of epicatechin, epigallocatechin gallate, and epigallocatechin significantly increased, whereas the contents of catechin, gallocatechin gallate, theanine, theobromine and caffeine were decreased significantly(44). The antioxidant activity, determined by ABTS, FRAP and DPPH assays showed an increase in the total antioxidant content with the increased age of the harvested leaves. To address the factors effecting the consistency of green tea marker compounds are used.

1.7 Selection of green tea marker compounds

Marker compounds are selected to reflect the quality of a herbal extract without quantifying all chemicals present. There are a variety of way marker compounds are selected, it can be based on convenient factors availability of commercial reference standards or abundance. It is ideal to have markers that are not necessarily based on convenience but reflect the activity of the herb. Selection of marker compounds should be based on pharmacological activity(1). These selection criteria are primarily designed to determine the quality of complex herbal formulas, although can be applied to many complex herbal materials such as green tea. The purpose of these criteria is to consider the components of a herbal mixture and to determine how likely each component is to be a major contributor to the activity of the herb. A number of factors should be considered;

- The Traditional use of the herb

- Relationship between the action and disease of interest
- The bioavailability of the compounds
- The quality of the research conducted
- The amount of research to support the action of the compound
- Toxicity of the compound and safety limits applied to its use
- Physical and chemical properties of the compound
- The part of the plant used in the product and the relative concentration of each analyte present

The constituent needs to demonstrated bioactivity (*in vitro/in vivo*), this is difficult and sometime unachievable for more complex formulas, making it difficult to determine the active compound.

The marker compounds can be ranked on a scale from 1, being the lower end, and 5 being upper end. For an analyte to have a rank of 5, the first thing that needs to be investigated is the concentration of the analyte in the sample. The compound should be abundant in the herb to have a larger effect. For example, a compound that is present at 2000ppm is more likely to be more active than a compound that is present at concentrations <1ppm, although other factors also play into its activity. This carries onto the next criterion, that the compound should have an activity that is related to the disease or condition of interest. The activity that has been proven in the literature should be related to the disease of interest, as well as the traditional or current use of the herb being used for this same or similar purpose. Green tea, as described in section 1.2, has in the past been linked to various health benefits that help a variety of health conditions and more recently there has been work done on the mechanism of action of the chemical constituents in tea to confirm why these health benefits took place.

1.8 **Aims and objectives of the study**

There are three main aims of the study. The first of which is to determine the contents of marker compounds of green tea products/batches to assess the quality of these products.

The second aim is to determine the phytoequivalence of various green tea extracts/ products, the chemical equivalence in terms of the marker compounds and the pharmacological equivalence in terms of the antioxidant activity.

The third aim is to develop an online assay to identify the compounds responsible for the antioxidant activity of various ingredients in green tea extracts/products.

In order to achieve these objectives, ten different samples of tea will be selected from the available market.

The objectives of this study are to

1. Develop a suitable method to assess the phytochemical variation between the different batches of Green tea products;
2. Utilise chemo metric data analysis to identify any correlations in chemistry between the products;
3. Develop activity-based assays to assess the pharmacological variations between the products; and, identify chemical sources of the pharmacological variation, if observed.

Chapter 2: Methodology

2.1 Materials

2.1.1 Reagents and Primary Standards

The primary standards; gallic acid, caffeine, epicatechin gallate, epigallocatechin gallate and theobromine were of analytical standard grade and purchased with a certificate of analysis from Sigma Aldrich (Castle Hill, New South Wales, Australia). The primary standards gallocatechin, epigallocatechin and epicatechin were of analytical standard grade and purchased with a certificate of analysis purchased from Biopurify Phytochemcials Ltd (Chengdu, Sichuan, China).

The Folin Ciocalteu (FC) reagent used in the total phenolics bulk assay was sourced from Sigma Aldrich (USA). The DPPH reagent used in the bulk antioxidants assay was sourced from Sigma Aldrich (Germany).

2.1.2 Samples

The test samples are shown in Table 2. Tea leaf samples were purchased off the shelf from a local shopping centre. The powdered matcha tea and TGA listed (Aust-L) tablet extracts, were also selected off the shelf from the local pharmacy.

Table 2: Sample information

Code	Type	Label claim ingredient	Expiry*
S1	Tea Powder	ECGC, Catechins	09/03/19
S2	Tea Power	No specific claims	31/1/18
S3	Tea leaves	No specific claims	10/7/2020
S4	Tea leaves	No specific claims	01/02/2020
S5	Tea leaves	No specific claims	23/06/2019
S6	Tea leaves	No specific claims	29/7/18
S7	Tea leaves	Caffeine	20/8/2020
S8	Tablet	ECGC, catechins	01/01/2018
S9	Tablet	ECGC	01/10/2019
S10	Sachet tea powder	1 sachet tea equivalent	01/08/2018
S11	Tablet	Catechin, incl EGCG	01/12/2018
S12	Tablet	No details.	No details.

***all testing was complete before expiry date**

2.2 Equipment

2.2.1 HPLC Instrumentation:

HPLC analysis was performed on a Shimadzu LC 30AD (Rydalmere, NSW, Australia), with a photo-diode array detector (Shimadzu SPD-M20A) and operated using Shimadzu Lab Solutions software. The chromatographic column used was a Phenomenex Luna C-18 (150 x 4.6 mm with 3 µm particle size).

Figure 3: The HPLC system in the NICM Herbal Analysis Laboratory located at Western Sydney University, Campbelltown

2.2.2 HPLC-ESI-MS-MS Analysis

LC-MS analysis was performed using a Waters Xevo TQ with electrospray ionisation (ESI) source and Waters Acquity UPLC. A Waters Acquity PDA detector was connected in parallel to the MS detector. Separation was achieved on an Acquity UPLC BEH C18 (2.1 x 50mm with 1.7µm particle size) column. Data was analysed using MassLynx/Targetlynx version 4.1, SCN876.

2.2.3 HPLC and Post Column Antioxidant Screening Analysis

The online antioxidant screening was performed on a Shimadzu HPLC system, equipped with a Shimadzu SCL-10A controller, SIL-10AD auto injector, LC-20AD pump, FCV-10AL switching valve, a SPD-M10A PDA detector and a Phenomenex (Lane Cove, NSW, Australia) DG-4400 degasser. One Shimadzu LC10AD pump, fitted with an inline degassing unit (Phenomenex DG-4400 (Lane Cove, NSW, Australia)) was used to deliver the post-column derivatisation reagent. An additional Shimadzu LC-10AT pump was used for the addition of the FRAP reagent post column. A splitter system was used to simultaneously direct eluent to the PDA and fluorescence detectors (FLD) in one HPLC run. The detectors acquired data in parallel, with the split set to divert 0.5 mL/min to the PDA and 0.5 mL/min to the FLD from the 1.0 mL/min column flow rate. A Hypersil GOLD column (150 × 4.6 mm, particle diameter 5 µm) from Thermo Fisher Scientific (Runcorn, Cheshire, United Kingdom).was used for the reaction flow analysis(45). A schematic diagram of the instrument configuration is shown in Figure 4.

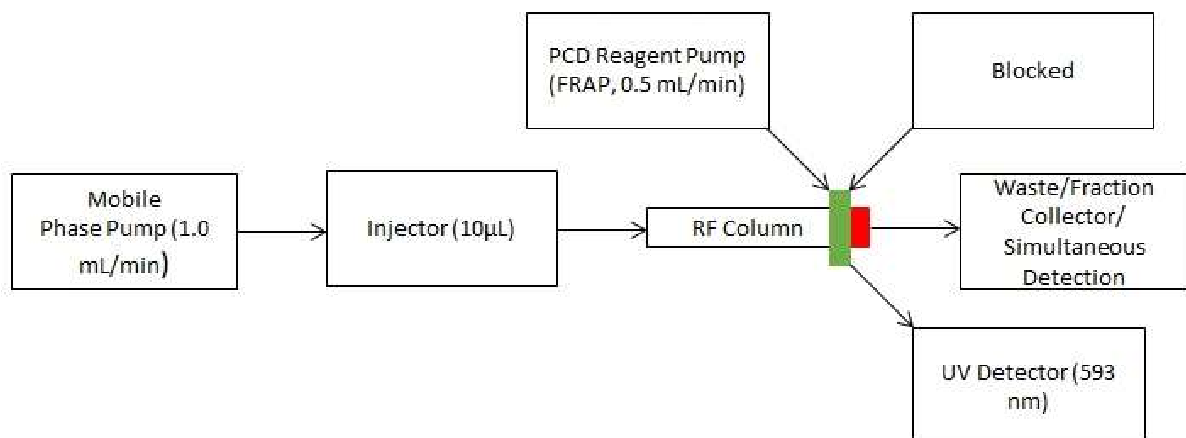


Figure 4: This is an illustration of the instrumental setup of the online system.

2.2.4 Miscellaneous Equipment

The extracts and chemical reagents were weighed using an Adam (Australia) AFA-210 HPLC analytical balance when the mass being weighed was >100 mg. The standards, extracts and some reagents were weighed using a Sartorius (Germany) SE2 ultra-micro-balance when the mass being weighted was <100 mg.

The ultrasonic water bath that was involved in the extraction of the samples was a Power Sonic 420 from Thermoline Scientific (Australia). The purified water used in analysis was prepared using a Milli-Q Direct 8/16 Water Purification System. Filtered using a 0.22µ Millipore filter. The automatic pipettors P100 (10 – 100 µL) and P1000 (100 – 1000 µL) series 2100 were from Eppendorf (Germany). The multi-channel pipettor Finnpipette (1 – 10 µL) was from Thermo Scientific (USA). Volumetric pipettes and volumetric flasks used were class A specification purchased from Duran (Germany). The plate reader used for the antioxidant assays was a FLUROstar OPTIMA reader from BMG Labtech (Germany).

2.3 Experimental

2.3.1 Selection of marker compounds

The compounds selected as potential chemical markers for green tea quality were selected based on selection criteria(1).

2.3.2 HPLC-PDA

The concentration of the marker compounds in the samples was quantified by HPLC. A standard stock solution was prepared by accurately weighing 1g of each standard and dissolving in 10 mL of 50% aqueous methanol. The samples were extracted in two different extraction solvents and were tested based on the determined optimum extraction solvent (50% aqueous methanol) as well as water extracts to mimic cosmopolitan tea drinking.

The water extracts were prepared by grinding the tea leaves (unless already powdered) using a herb grinder and accurately weighing out 1 gram of the powder. The powder was then dissolved in boiling water and cooled before then filtering the resulting concoction.

The aqueous methanoic extracts were prepared by dissolving 1 gram of the powdered product in 50% aqueous methanol and then ultrasonicating for 30 minutes at room temperature.

The flow rate was set at 1.00 mL/min with a gradient mobile phase consisting of aqueous formic acid (0.4 % v/v): Methanol. The injection volume of each sample and standard solution was 5µL. The gradient elution program used is given in Table 3.

Table 3: Mobile phase program used for the HPLC quantification of Green teas.

Time (min)	Segment	Flow (mL/min)	Solvent A (%) [a]	Solvent B (%) [b]
Initial	Analysis	1.00	95	5
30	Analysis	1.00	0	100
34	Wash	1.00	0	100
34.1	Equilibration	1.00	95	5
37.1	Equilibration	1.00	95	5

[a] 0.4% v/v aqueous formic acid; [b] Methanol.

The $\lambda_{\mathbf{det}}$ of the marker compounds used was 270 nm. This wavelength appeared to give the best compromise between sensitivity of detection and the baseline noise. The column temperature was maintained at 30 ºC under the specified chromatographic conditions to yield back pressures between 2300 - 2700 psi.

2.3.3 HPLC-ESI-MS/MS Analysis

The concentration of L-theanine in the samples was quantified by LC-MS. The samples and standards prepared for the HPLC analysis (2.3.1) were analysed by LC-MS. The LC conditions are shown in Table 4.

Table 4: HPLC and MS conditions for the quantitation of green tea

Time (min)	Segment	Flow (mL/min)	Solvent A (%) [a]	Solvent B (%) [b]
Initial	Analysis	0.6	95	5
0.5	Analysis	0.6	95	5
2	Analysis	0.6	50	50
2.1	Wash	0.6	0	100
4.0	Equilibration	0.6	95	5
5.0	Equilibration	0.6	95	5

[a] 0.4% v/v aqueous formic acid; [b] Methanol.

The for the MS analysis, a targeted analytical run was performed. The direct infusions of the pure standards were performed and optimum precursor and product ions were determined for each analyte and these are summarised in Table 5.

Table 5: Parameters for targeted analysis from direct infusions of the pure standards

Compound	Formula Mass	Parent *m/z*	Cone Voltage	Daughters	Collision energy	Ion Mode
Theobromine	180.164	181.13	36	137.85	18	ES+
		181.13	36	163.04	18	ES+
		181.13	36	67.01	28	ES+
Gallic Acid	170.12	169.09	30	124.9	14	ES-
		169.09	30	78.34	20	ES-
		169.09	30	78.92	24	ES-
EGCG	458.37	459.15	74	139.00	18	ES+
		457.15	96	168.94	20	ES-
		459.15	74	289.03	8	ES+
EC	290.27	291.17	28	139.00	16	ES+
		291.17	28	122.99	14	ES+
		291.17	28	165.0	12	ES+
Caffeine	194.194	195.16	40	138.03	20	ES+
		195.16	40	110.04	22	ES+
		195.16	40	42.06	30	ES+
Theanine	174.2	175.23	20	84.01	18	ES+
		175.23	20	158.04	12	ES+
		175.23	20	46.04	12	ES+
ECG	442.37	441.15	38	168.94	18	ES-
		443.15	22	122.99	14	ES+
		441.15	387	289.04	16	ES-
EGC	306.27	307.17	20	139.01	14	ES+
		305.17	38	124.95	24	ES-
		305.17	38	178.97	16	ES-
GC	306.27	307.17	26	139.01	14	ES+
		305.11	36	124.95	26	ES-

		307.17	26	150.98	10	ES+

2.3.3 Antioxidant assays

2.3.3.1 Total Phenolics Assay

The total phenolic content of the tea samples was determined using the Folin Ciocalteu (FC) method. The samples were prepared the same way as in section 2.3.1. These concoctions were then diluted 1 in 10 in a 2mL Eppendorf tube. 2.573 mg of the gallic acid standard was dissolved in 10mL of methanol to make a 1.5mM stock solution. The sodium carbonate buffer (0.7M) was prepared by dissolving 752mg of sodium carbonate in 10ml of Milli-Q water. The stock solution was used to make gallic acid standards 1.5, 1.2, 0.9, 0.6, 0.3mM by serial dilutions. 20µL of the standards, samples and blank were pipetted onto a 96-well micro plate along with 140µL of water and 20µL of F-C reagent. The contents were vortexed before the reaction was ceased by adding 30µL of sodium carbonate solution to give the well a final volume of 200µL. The plate was shaken at 37ºC for 30 mins in the dark before the absorbance was measured at 765nm using POLARstar OPTIMA, BMG. The values were expressed as milligram gallic acid equivalents per gram of dried extract (mg GAE/g DE).

2.3.3.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging capacities of the tea samples were determined by a method adapted from Blois *et al.* and Molyneux *et al*(16,46). All samples and reagents were prepared in methanol. The gallic acid calibration curve was obtained by preparing 0.3, 0.6, 0.9, 1.2 and 1.5 mM working standards, by diluting the 1.5mM stock solution.

Samples were prepared the same way as for the HPLC analysis, 2g of sample in 200 mL. Dilutions 1:10 of the stock tea sample solutions and 1:100 of the stock G.T.E tablets were also prepared. Water was used as the reagent blank. DPPH reagent (250 μM; 180 μL) and 20 μL of sample, standard or blank were applied to each well of a 96 well microtitre plate to give a final volume of 200 μL. The plate was shaken for 30 min in the dark prior to measuring the absorbance at 515 nm (POLARstar OPTIMA, BMG). All values were expressed as milligram gallic acid equivalents per gram of dried extract (mg GAE/ g DE). All data was expressed as the mean ± standard error of the mean of three independent experiments performed in triplicates.

2.3.4: Online FRAP:

The FRAP reagent was adapted from the work performed by Benzie and Strain(47). The acetate buffer (300mM, pH 3.6) was prepared by dissolving 40.8g of sodium acetate trihydrate in 500mL of Milli-Q water with the aid of ultrasonic agitation. The pH of the solution was then adjusted to 3.6 (±0.1) with glacial acetic acid and diluted to 1L with Milli-Q water. HCl (40mM) was prepared by diluting 3.3 mL of the concentrated hydrochloric acid to 1L milli-Q water. TPTZ (10mM) was prepared by by dispersing 62.5 mg TPTZ in 20 mL of 40 mM HCl with the aid of ultrasonic agitation. Ferric chloride (20 mM) was prepared by dissolving 108.1 mg ferric chloride hexahydrate in 20 mL of Milli-Q water with the aid of ultrasonic agitation. The final FRAP reagent was prepared by combining 500 mL of 300 mM acetic acid buffer pH 3.6, 20 mL of 10 mM TPTZ and 20 mL of 20 mM ferric chloride. The derivatisation reagent FRAP, was prepared daily and filtered through a 0.22 μm filter before use(48). The LC conditions for the Online FRAP assay are shown in Table 6.

Table 6: HPLC conditions for the online antioxidant assay

Time (min)	Segment	Flow (mL/min)	Solvent A (%) [a]	Solvent B (%) [b]
Initial	Analysis	1.0	95	5
30	Analysis	1.0	0	100
34	Wash	1.0	0	100
34.1	Equilibration	1.0	95	5
38.6	Equilibration	1.0	95	5

[a] 0.4% v/v aqueous formic acid; [b] Methanol.

Chapter 3: Results and Discussion:

3.1 Ranking of marker compounds

The marker compounds were selected based on the on pharmacological activity, commercial availability and abundance (Bensoussan et al., 2015). Table 7 lists the potential marker compounds that, based on the literature, describe the quality of green tea and have therefore been selected for this investigation.

Table 7: Potential marker compounds to describe the quality of green tea

Compound	Reported Concentrations (ppm)	Action	Ranking	Commercially Available
Catechin	85.2-132000	Antioxidant, anticoagulant, antiinflamatory, antimutagent, antiperoxidant(49),	5	Yes
Epicatechin	149 – 21250	Antioxidant, Antidiabetic, antimutagenic, antiperoxidant(49), antiinflamatory, antibacterial	5	Yes
Epicatechin gallate	5417 – 44000	Antioxidant, free radical scavenger, Antiperoxidant(49)	5	Yes
Epigallocatechin	203- 10866.7	Antioxidant, free radical scavenging activity.	5	Yes
Epigallocatechin gallate	16600-54300	Antioxidant, free radical scavenging activity.	5	Yes
Gallo catechin	12876.00 (highest reported concentration)	Antioxidant, free radical scavenging activity.	5	Yes
Gallic acid	3500 (highest reported concentration)	Antioxidant, free radical scavenging activity.	5	Yes
Theobromine	500 – 1100	Cardiotonic, simulant, vasodilator, diuretic, Bronchodilator	5	Yes
Caffiene	3810.0 – 93000	Antiobesity, CNS stimulant, antioxidant, diuretic, Vasodilator,	5	Yes
Theanine	5000 – 13500	Antithromboxane, Hypocholesterolemic Antioxidant, free radical scavenging activity, stress reduction(30).	5	Yes

3.2 HPLC Profile of Green Tea products

To access the quality of the tea samples a HPLC method was developed that adequately separate marker compounds. The marker compounds, including gallic acid, gallocatechin, theobromine, epigallocatechin, epigallocatechin gallate, caffeine, Epcatechin and epicatechin gallate, were studied. A representative chromatogram of a mixture of these marker compounds is shown in Figure 3-1

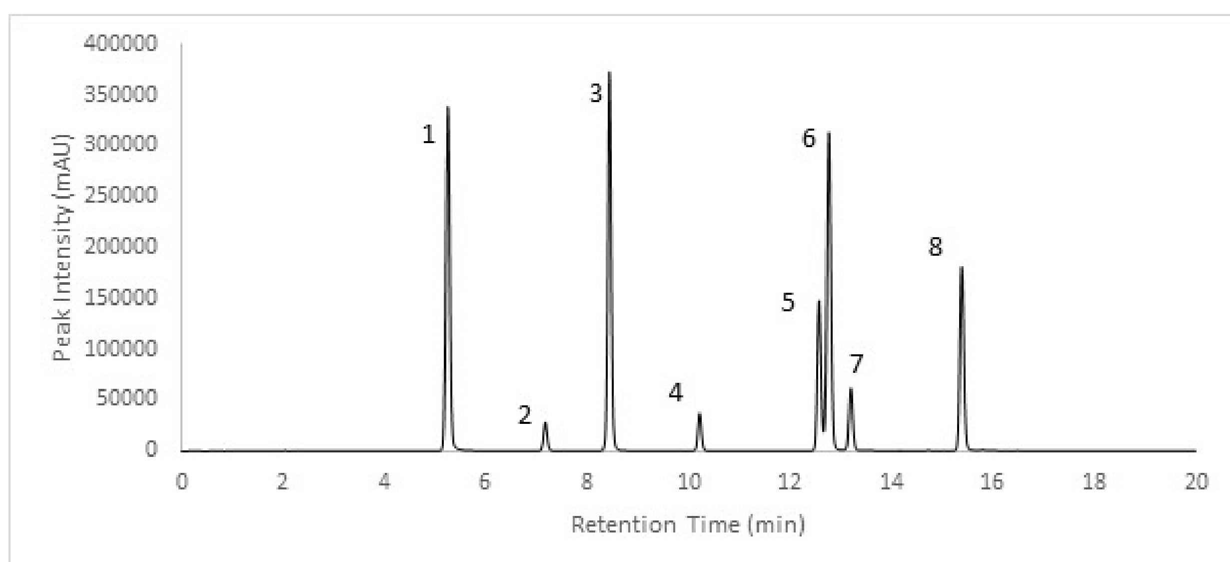


Figure 5: HPLC chromatogram of chemical markers: gallic acid (1), gallocatechin (2), theobromine (3), epigallocatechin (4), epigallocatechin gallate (5), caffeine (6), epicatechin (7) and epicatechin gallate (8). The HPLC condition for determination of these compounds is described in section 2.3.2.

As shown in the Figure, all marker compounds were efficiently separated under the experimental condition, indicating that the HPLC method used in this study is adequate for the proposed research aims. The determination of catechins by HPLC in green tea

products was previously validated by Naldi et al and shown to be sensitive and accurate(50).

To determine the appropriate wavelength to use for analysis of marker content in green tea products, the heat map of a green tea exact (S4) was obtained (Figure 6). It illustrates that the absorbance of various compounds was wavelength-dependent. The wavelength selected for this analysis was 270nm. This wavelength is also commonly used in the literature for the examination of green tea. The reason for this is that the lambda (λ) maxima for all of the selected compounds is within this range. This is shown in figure 7, where the pure standard of the marker compound (pink) is overlayed with the corresponding peak in the tea sample (black). The graphs below also show a peak at around 230nm as well as some methods in the literature. While this still would be an optimum wavelength for the compounds with a lower peak intensity at 270nm, it can be shown on the heat map in figure 6 that there is significant baseline interference at the lower wavelength and therefore 270nm is the more appropriate wavelength for the analysis.

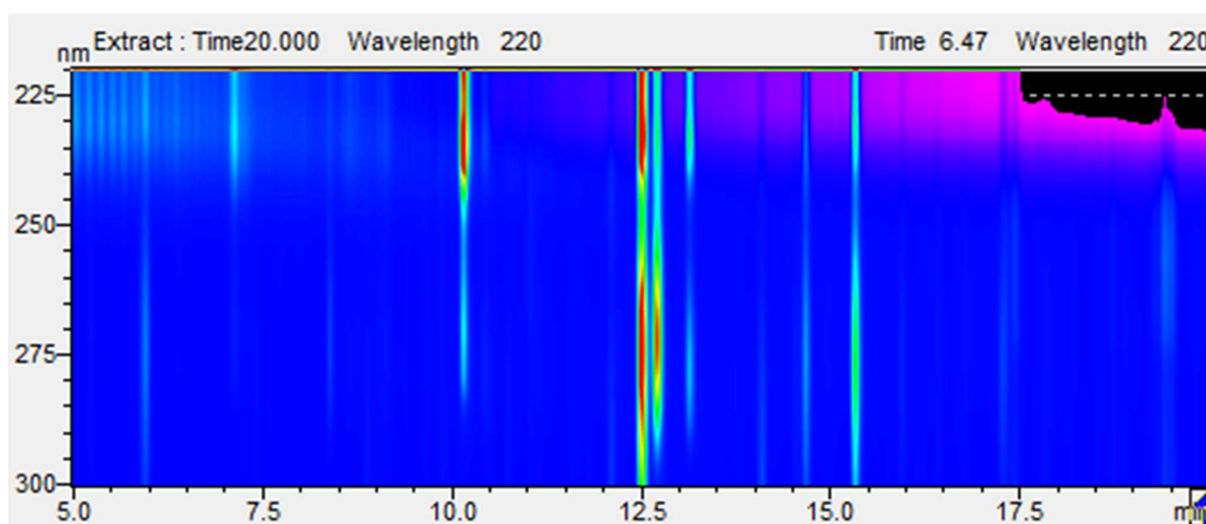


Figure 6: Heat map of green tea sample.

The heat map in Figure 6 confirm the ideal wavelength of 270nm that is commonly reported is appropriate. There are some compounds that did not absorb as efficiently, however, the chosen wavelength gave the best representation with minimum interference from the baseline noise.

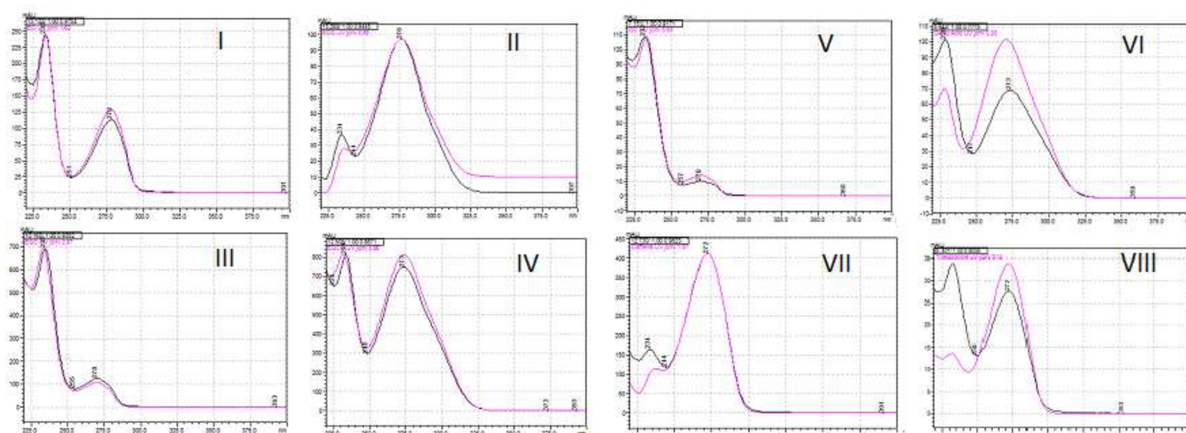
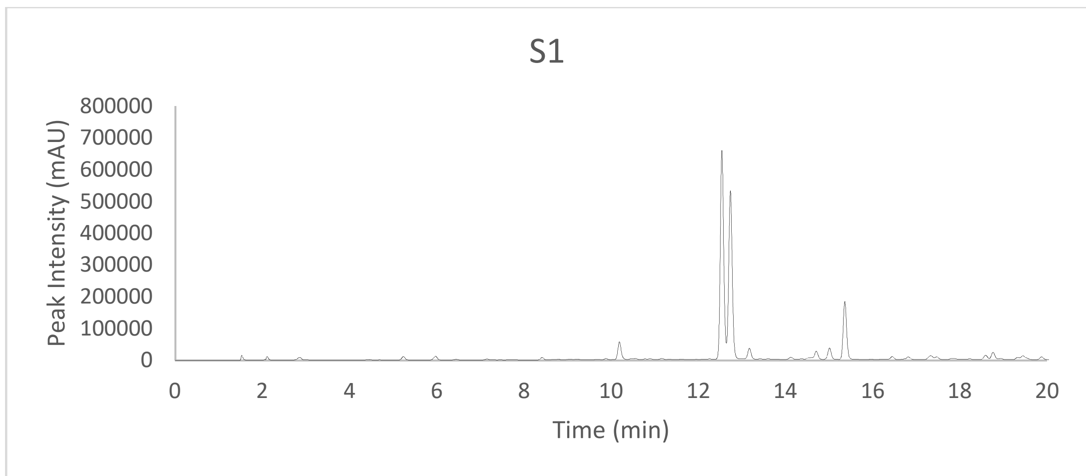
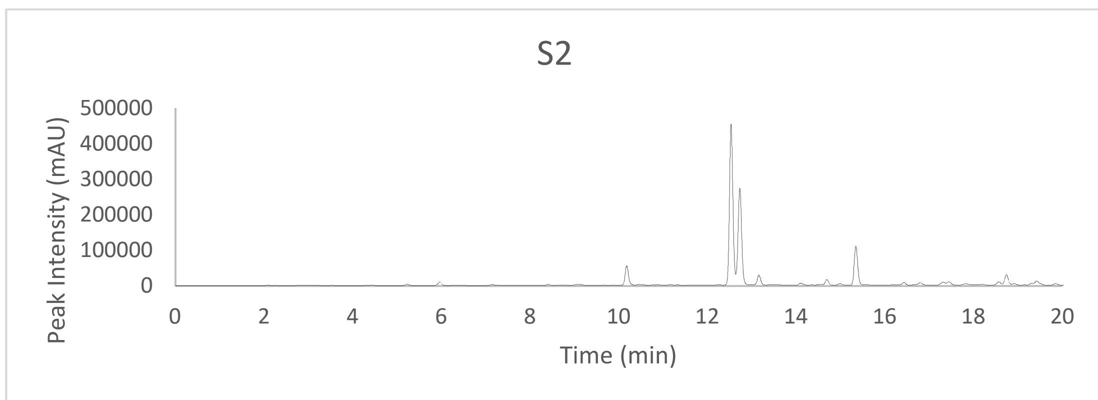


Figure 7: comparison of HPLC chromatogram of green tea extract (sample 4, A) and marker compounds (B). The number 1-8 indicate the relevant peak for marker compounds. I-VIII show UV UV spectrums for Epicatechin (I), Epicatechin gallate (II), Epigallocatechin (I)

3.2 HPLC profile of tea samples

Using the established HPLC method, the HPLC profiles of various green tea product samples were studied, including two matcha products (S1, S2), five traditional tea leaf products/tea bag (S4,S5,S6,S7), tea sachet supplement (S10) and three TGA listed tablets (S8,S9,S11). These products were sourced locally. The identity of manufacturer of each product was recorded but not revealed in this thesis. Due to this study's time limit, these samples are only a representative of a large number of green tea products on the market. It is not our intention to include all commercial green tea products in this study. Nevertheless, the list covers several main types of green tea products, including traditional tea leaves/bags, tea power, tea extracts-derived products (tablets and sachet) as TGA listed complementary medicines. The representative HPLC chromatograms of these green tea samples are shown in Figures 8-10





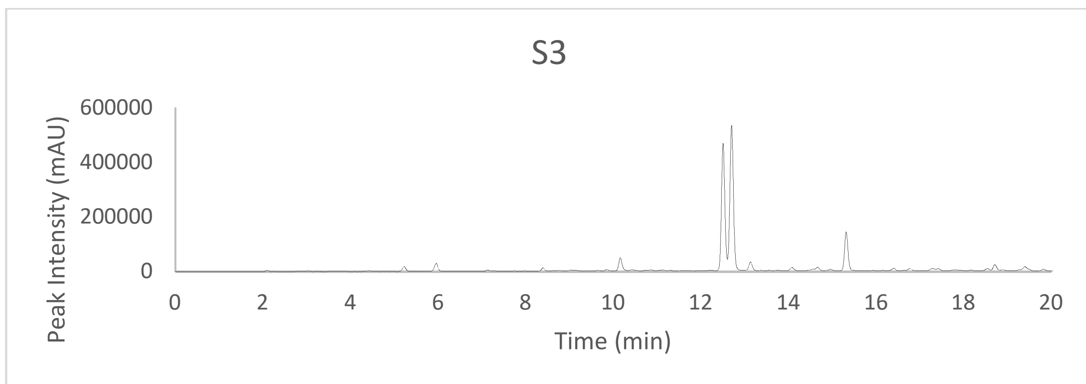
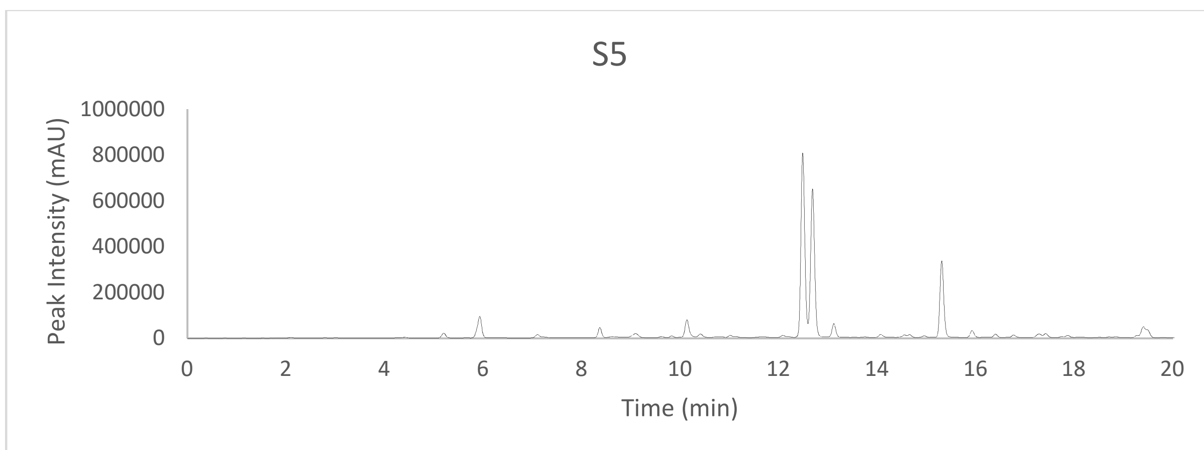
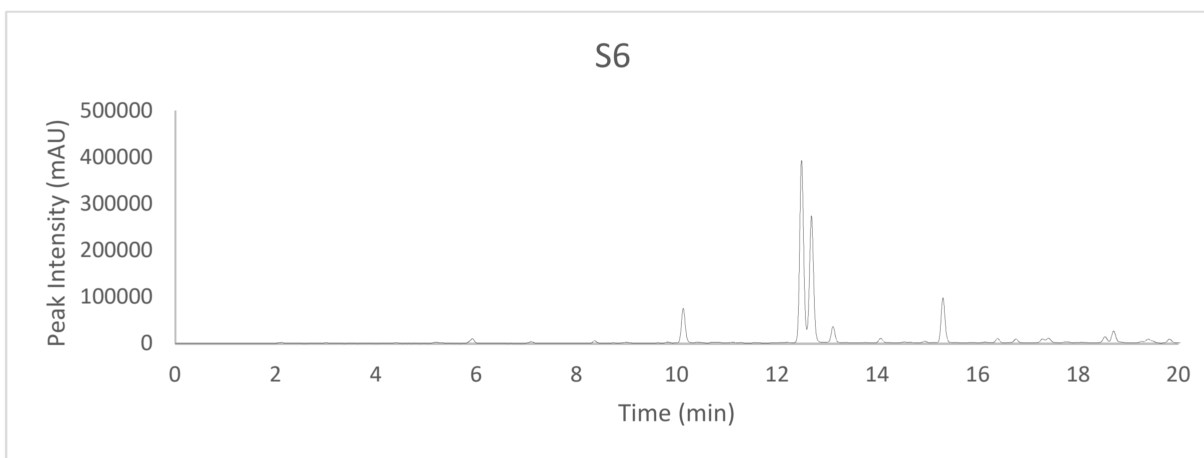


Figure 8: Representative HPLC chromatogram of green tea products. Samples include matcha sample 1 (S1), matcha sample 2 (S2), and green tea bag (S3)





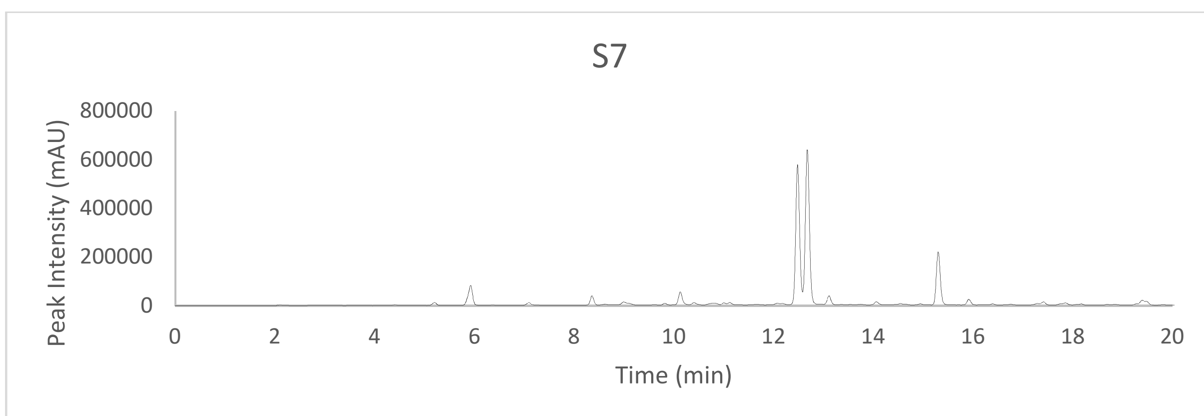


Figure 9: Representative HPLC chromatogram of green tea products. Samples include traditional tea leaves (tea bag) (S5, S6, S7).

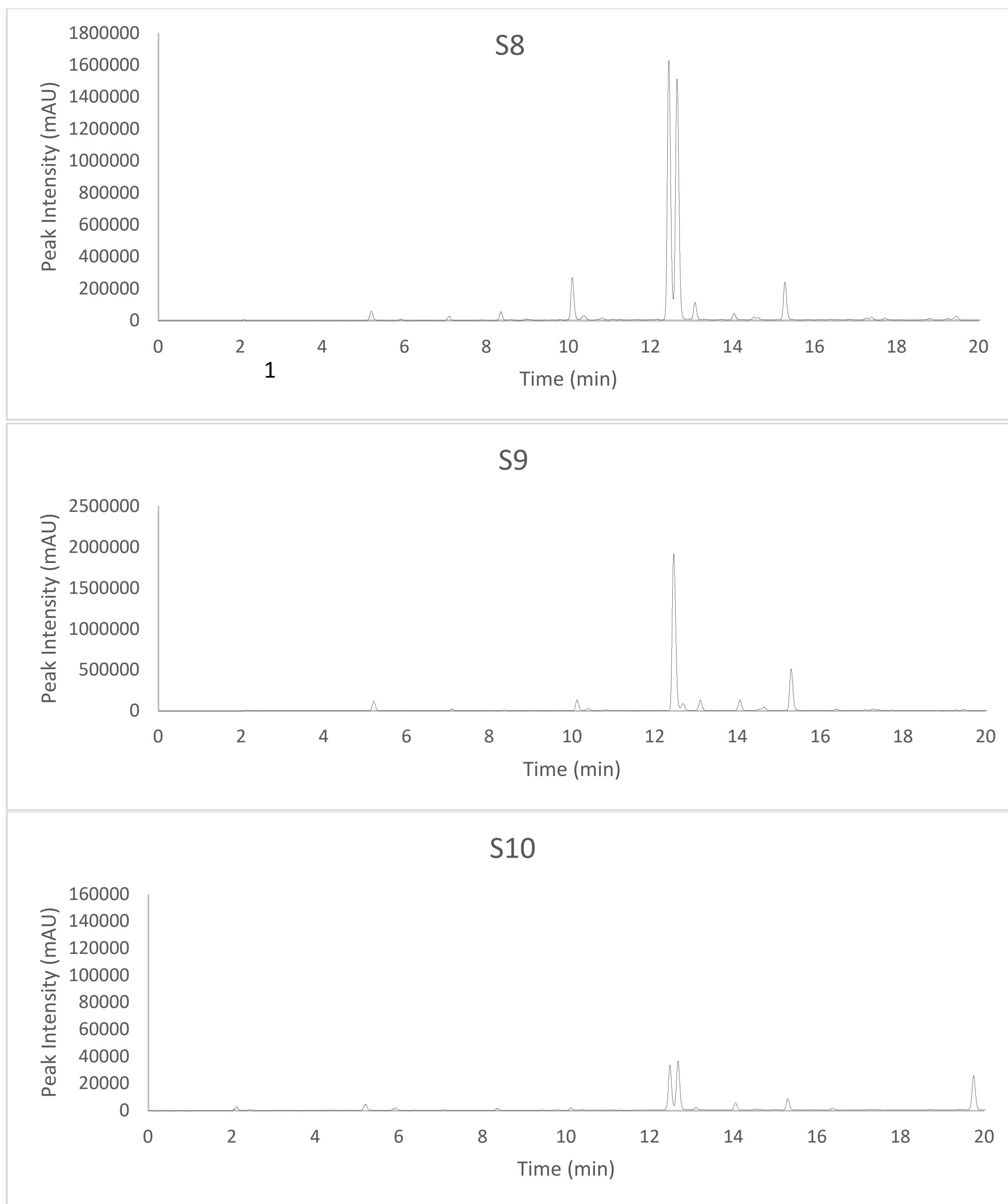


Figure 10: Representative HPLC chromatogram of green tea products. Sample 8-10, including TGA listed tablets (S8,S9,) and sachet (S10). The HPLC condition for determination of these compounds was described in Chapter 2.

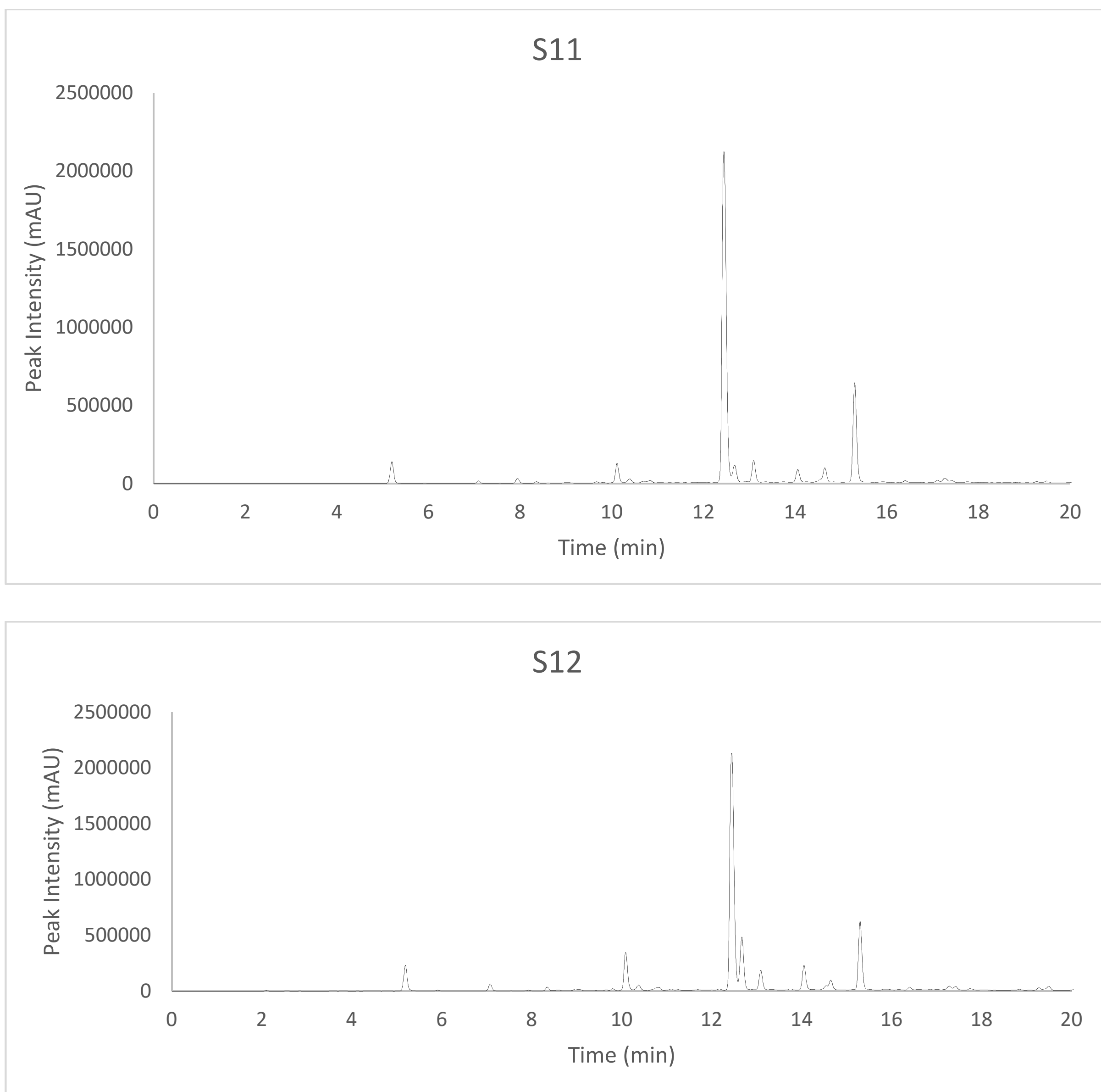


Figure 11: Representative HPLC chromatogram of green tea products. Sample 8-12, including TGA listed tablets (S11, S12)The HPLC condition for determination of these compounds was described in Chapter 2.

These chromatograms tentatively identify the peaks of the marker compounds based on retention time and comparison of UV spectrum as shown in Figure 3-6 with S4 as an example.

The peaks with the matching retention times to the mixed standards have similar UV spectrum. The identification of the small peaks is considered only tentative as some are close to detection limit as the lower concentration of the compounds in the sample makes it more difficult to compare the UV spectrum as at low absorbance. In general, the results indicate that the catechin markers exist in green tea samples tested. However, the profile these markers varied significantly, with certain maker's absence in several products. Thus, further quantification analysis of these markers was conducted to assess the changes of their contents and relationship to the quality of green tea products.

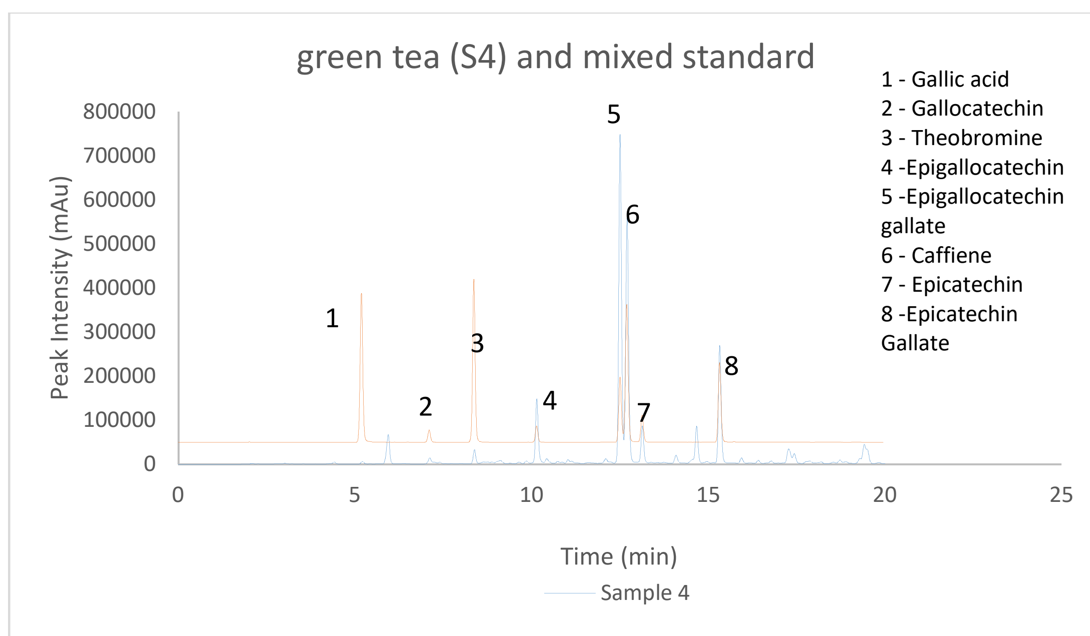


Figure 12: HPLC overlay of S4 and the mixed standard

The peaks having matching UV spectrum and RT compared to the reference standards were quantified using unique standard curves.

3.3 Quantification of marker compounds by HPLC-PDA

In order to quantify the content of maker compounds in the green tea products a series of dilution of the marker compounds are used to establish a calibration curve. For quantification purposes the calibration curve should be linear over the range used for quantification. Figure 11 shows the calibration curves of each marker compound determined using the method described in Chapter 2.

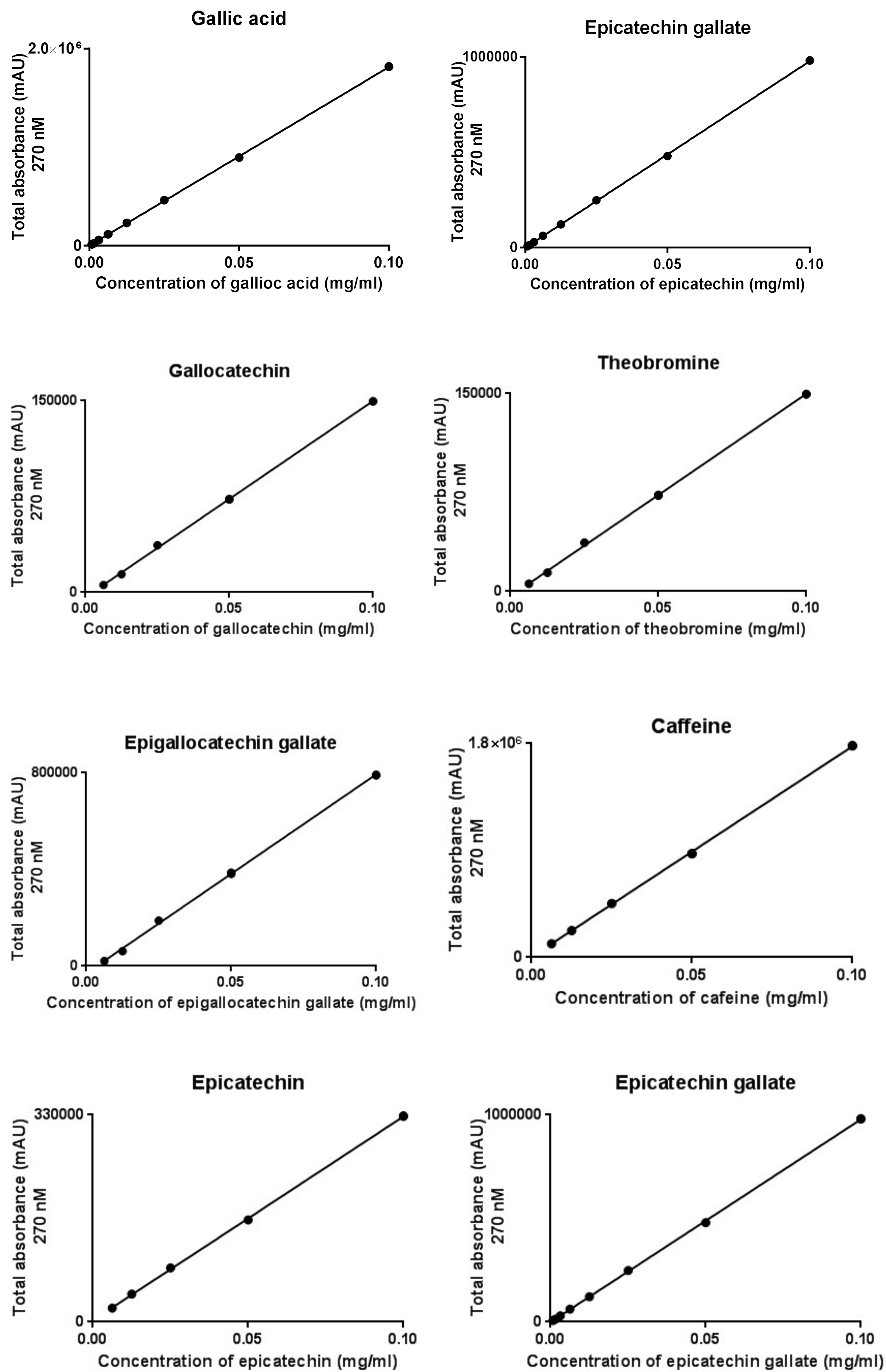


Figure 13: Calibration curves of purified chemical markers for quantification of marker compounds. n=3, r2=0.9993-0.9999.

Excellent concentration- peak area linear relationship were observed for all marker compounds, with r^2 between 0.992 to 0.999. The relevant equations were obtained (Table 8) based on the calibration curve of each compound, and used to calculate the concentration of each analyte in the green tea samples.

Table 8: Calibration equations for calculation of the content of marker compounds in green tea samples, based the data shown in Figure 3.6-8.

Marker compound	Equation	r^2 value
Gallic Acid	y= 16790155.3959x - 8.9194	0.9999
Gallocatechin	Y=1535455.5914x – 3671.6042	0.9995
Epicatechin	Y= 32473152688x +2654.3333	0.9998
Epicatechin gallate	y = 9,791,007.8731x - 859.2312	0.9998
Epigallocatechin	y = 2,018,035.6989x - 10,574.0833	0.9986
Epigallocatechin gallate	y = 8,235,102.1505x - 29,739.7083	0.9992
Caffeine	y = 17,729,821.9355x + 3,190.0000	0.9998
Theobromine	y = 18,522,407.7583x + 4,228.5724	0.9999

The results of the HPLC analysis for each sample of green tea are shown in Figure 3-9.

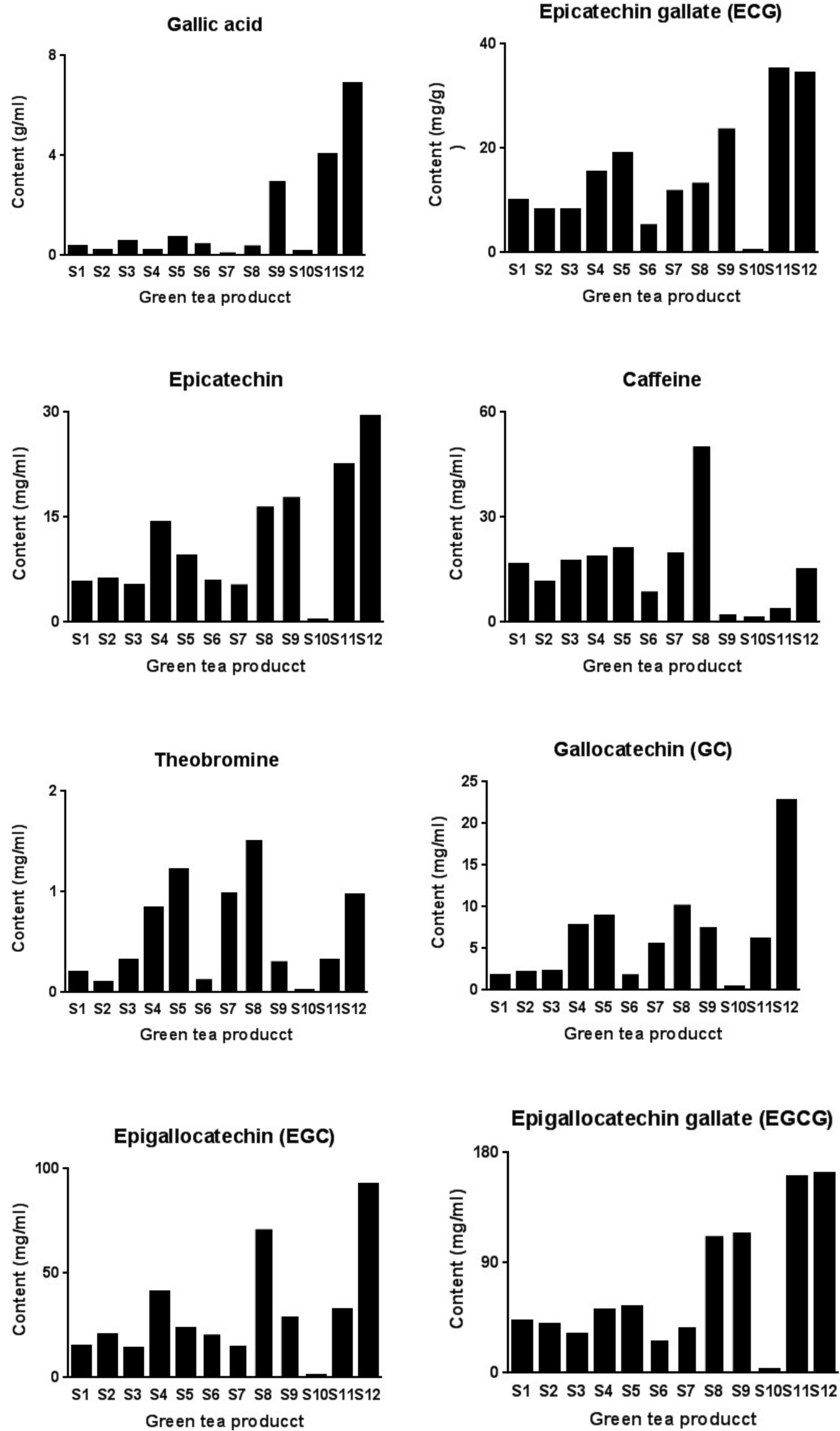


Figure 14: Content of marker compounds in green tea products, determined using the method as descried in Chapter 2. S1-S12 indicate samples of green tea products (see Chapter 2)

In general, the content of each marker compound varied with different green tea products. EGCG account for ~50% of taotal catchin, in agreement with (35). The highest contents of markers were observed for two manufactured TGA listed products (S11 and S12), while the lowest content was also observed for sachet product (S10). This product is claimed as 20-50 times more potent than the traditional green tea products, while in fact it is the lowest quality tested, with all marker compounds below the traditional tea leave products/tea bags. Among the different products, the traditional tea leaf products (S3, S4, S5, S6 and S7) showed a higher level of marker contents than the tea powder products (Matcha, S1 and S2). Within the same product group, eg traditional tea leave products, S4 and S5 showed a higher level of most marker compounds than other products. The overall ranking of the products in each group are as follows: tea leave products: S4>S5>S7>S6=S3; tea powder product: S2≥S1; The TGA listed products: S9>S8>S11. For each marker compound, the rank orders are as follows. Gallic acid: S12>S11>S9>S5>S3>S6; ECG: S11=S12>S9>S5>S4>S1; Epicatechin: S12>S11>S9>S8>S4; Caffeine: S8>S5>S4=S7=S1=S12; Theobromine: S8>S5>S7>S12=S4; GC: S12>S8>S5>S4>S9>S11; EGC: S12>S8>S4>S11; EGCG: S12=S11>S9>S8>S5=S4;

The results are shown in Table 9.

Table 9: Chemical content among different products

Sample number	Gallic Acid (mg/g) ± SD	GC (mg/g) ± SD	Theobromine (mg/g) ± SD	EGCG (mg/g) ± SD	EGC (mg/g) ± SD	Caffeine (mg/g) ± SD	ECG (mg/g) ± SD	EC (mg/g) ± SD
S1	0.37±0.00	1.73±0.14	0.22±0.00	44.09±0.07	18.68±0.01	19.30±0.09	9.94±0.01	6.17±0.00
S2	0.21±0.00	2.12±0.00	0.11±0.00	41.41±0.08	25.03±0.00	13.53±0.03	8.20±0.01	6.59±0.16
S3	0.62±0.00	2.27±0.03	0.36±0.00	32.84±0.30	17.44±0.08	20.40±0.05	8.17±0.05	5.64±0.23
S4	0.22±0.00	7.87±0.01	0.93±0.00	53.06±0.10	50.69±0.14	21.77±0.07	15.42±0.04	15.30±0.04
S5	0.77±0.00	8.99±0.01	1.35±0.00	56.29±0.26	29.00±0.1	24.82±0.04	18.91±0.06	10.11±0.02
S6	0.16±0.03	1.93±0.00	0.15±0.00	27.57±0.11	25.41±0.07	10.31±0.0	5.46±0.01	6.58±0.02
S7	0.43±0.00	5.76±0.00	1.10±0.00	38.79±0.14	18.80±0.02	23.54±0.01	12.00±0.01	5.80±0.00
S8	0.13±0.00	0.64±0.00	0.10±0.00	7.15±0.00	5.39±0.00	3.65±0.00	0.83±0.00	1.09±0.00
S9	0.26±0.00	0.61±0.00	0.03±0.00	9.52±0.02	2.89±0.01	0.22±0.00	1.90±0.00	1.53±0.00
S10	0.10±0.00	0.26±0.02	0.01±0.00	1.56±0.01	0.72±0.01	0.79±0.00	0.27±0.00	0.20±0.01
S11	0.35±0.00	0.49±0.00	0.03±0.00	13.26±0.03	3.21±0.01	0.34±0.00	2.81±0.01	1.93±0.00

Some of the products had claims made about the concentration of the extract. The results have been calculated as a percentage of the claimed value and shown in table 10

Table 10: Percentage of label claims

Sample number	Label claim Ingredients	Total Catechins (%)	ECGC (%)	Caffeine (%)	Gallic Acid (%)	GC (%)	Theobromine (%)	EGC (%)	ECG (%)	EC (%)
S1	Matcha, ECGC, Catechins	80.98%	111.62%	95.70%	84.09%	32.25%	28.28%	66.08%	82.89%	71.03%
S2	Matcha, no claim	87.02%	41.41	0.670864736	47.73%	36.81%	14.14%	88.55%	68.38%	75.87%
S3 –S7	Average tea	96.02	41.71	20.168	0.44	5.364	0.778	28.268	11.992	8.686

Sample number	Label claim Ingredients	Total Catechins (%)	ECGC (%)	Caffeine (%)	Gallic Acid (%)	GC (%)	Theobromine (%)	EGCG (%)	EGC (%)	ECG (%)	EC (%)
S8	ECGC, total catechins.	2.61%	2.45%	74.48%	121.59%	49.10%	52.89%	78.47%	0.28%	0.52%	2.61%
S9	ECGC	17.40%	5.17%	0.08%	4.28%	0.82%	0.28%	0.74%	114.81%	127.64%	17.40%
S10	5g of dry leaf powder	1.77%	2.24%	2.35%	13.61%	2.90%	0.77%	1.53%	1.35%	1.38%	1.77%
S11	EGCG, caffeine, catechin	6.29%	7.21%	0.12%	5.76%	0.66%	0.28%	0.82%	1.70%	1.61%	6.29%

It can be seen in Figure 14 and Table 9 that the levels of most the marker compound are well below the expected value based on the lable claims.

These results indicate that the level of marker compounds changes significantly among different green tea products. Given that these marker compounds are related to the quality of green teas (see Chapter 1), the results further indicate that the quality of green tea products on the market varies significantly. It is surprising to observe that one green tea sachet product, which claimed to be potent and equal up to 20 cups of green tea per sachet is actually the lowest quality among the product tested, even lower than the traditional tea bags. Thus the consumer should be alerted when choosing the green tea products, not totally believe the product labels. There is not a necessary link between high price and high quality. Based on these findings further tests are warranted to analyse a wider range of green tea products for their quality. More random testing should be conducted by TGA and authorities to ensure the listed product has a quality match their labels.

Many of the products make claims around the concentration in terms of equivalence to raw green tea. Making statements like “20X extract”. The Phytochemical results were normalised to account for the concentration of the extract based on the label claims. For example, a 20X extract claim was divided by 20 to allow direct comparison to the raw green tea. Sample 1-7 are raw teas and 8-12 were adjusted based on their claim. The results are shown in figure 15.

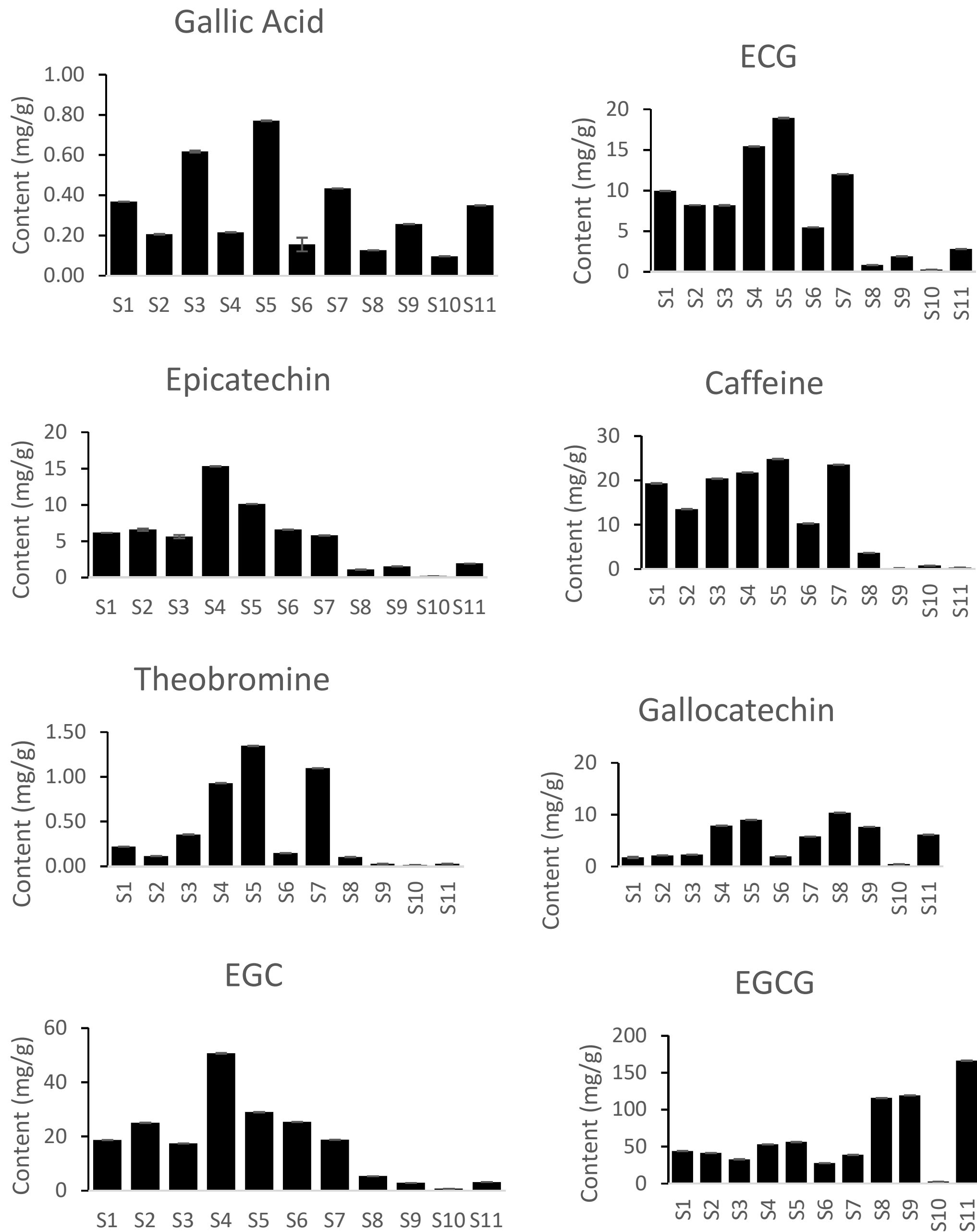


Figure 15: The content of selected marker compounds with green tea extracts (S8-S11) normalised to account for the concentration of the extract based on the label claims

It can be seen in Figure 15 that when compenstated for their label claims most products and markers are well below the expected abundance-in comparison to raw tea samples.

HPLC is a common and accurate technique for the qantificaition however it cannot detect all compounds. One of the major reported active in green tea is an ammino acid L-theaninie that cannot be detected at the levels present in green tea by HPLC-PDA. Therefore a LC-MS method was develop to quantify it.

3.4 Quantification of L-theanine by LCMS

L-theanine is an amino acid and commonly found in green tea leaves(8). Theanine is an important marker compound because there is a lot of research interest in the calming effect of the amino acid L-Theanine and is an important response for the activity of green tea(8,14,30,51).

Although HPLC-PDA is a relatively straight forward method and has been used for the other analytes, there are some limitations. Photo-Diode Array (PDA) detection requires the molecule of interest to have a chromophore so it absorbs the UV light. This part of the molecule is usually defined by areas of conjugation in the chemical structure. The chemical structure of theanine lacks a chromophore, thus it does show a peak response on the PDA.

The analysis of *L-theanine* cannot be conducted by conventional HPLC-PDA method as the L-theanine lack chromophores it must be present in large quantities to be quantified (52). To overcome these problems, in this study we used a LC-MS method

which is more sensitive and accurate, and has been sued in previous studies in analysis of tea products.

Figure 3-11 shows the calibration curves of *L-theanine*. Based on this, the equation was calculated and used to determine the content of *L-theanine* in the green tea samples, and shown in Fig 3.8B.

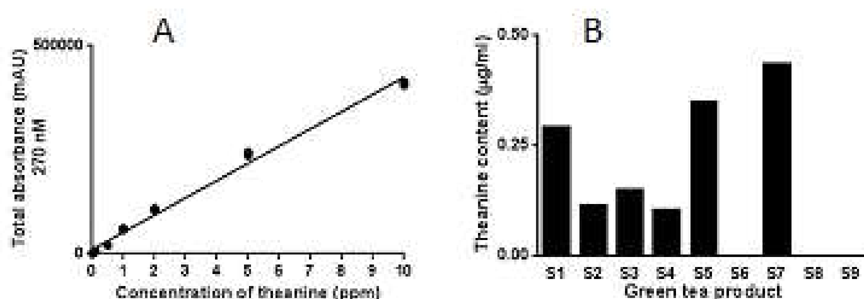


Figure 16: Determination of L-theanine in green tea products. Calibration curves (A) of purified compound and content determination in green tea products (B). Data were presented as means of duplicate

As Figure 14 shows, the content of *L-theanine* changed significantly among different products, with the highest level observed in sample 7 and 5, followed by Sample 1. Three samples (S6, S8 and S9) showed undetectable level of *L-theanine.*This is to be expected because the extract was not standardized to contain L-*theanine*. The L-*theanine* seems to be present in high concentrations in the green tea leaves, rather than the powdered Matcha and the green tea extract tablets. Matcha is expected to be different in composition from tea leaves, as it is prepared by being compressed and powdered as opposed to dried leaves.

The green tea leaves also contain caffeine at relatively high concentrations in comparison to the other sample types based on the HPLC data, these may be related.

In a study discussed in section 1.4.5, theanine has been discovered to have the opposite effect of caffeine(30).

Interestingly, *L-theanine* was not detectable in TGA listed green tea tablets (S8 and S9) which contained higher level of other marker compounds (see section 3.3*)*. This indicates the manufacture procedure may affect the level of *L-theanine* in the final product. Further study is needed to test more manufactured product to confirm this hypothesis.

3.4 Antioxidant Activity

3.4.1 DPPH Radical Scavenging Assay

The total antioxidant activity of the green tea products were studies using the method described in Chapter 2. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a radical compound with strong absorbance at 515 nm. Hydrogen donating antioxidants reduce DPPH free radicals, diminishing their absorbance at 515 nm. The antioxidant activity of green tea samples was expressed as equivalent of a standard antioxidant gallic acid. Figure 15 shows the standard concentration response curve of gallic acid, with a liner repression equation calculated which was used to calculate the antioxidant activity of green tea samples. The eight-point calibration curve was linear (R^2 = 0.9998) from 4.1 to 52.4 μg/mg gallic acid.

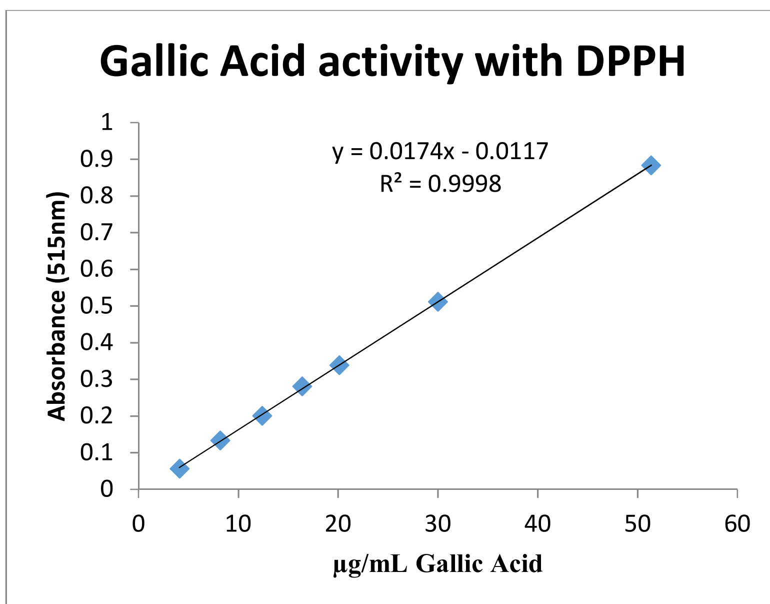


Figure 17: Antioxidant activity of gallic acid - concentration – response curve

The antioxidant activity of green tea products were calculated as gallic acid equivalent (µg) / green tea material (mg), calculated for each sample and shown in Figure 16.

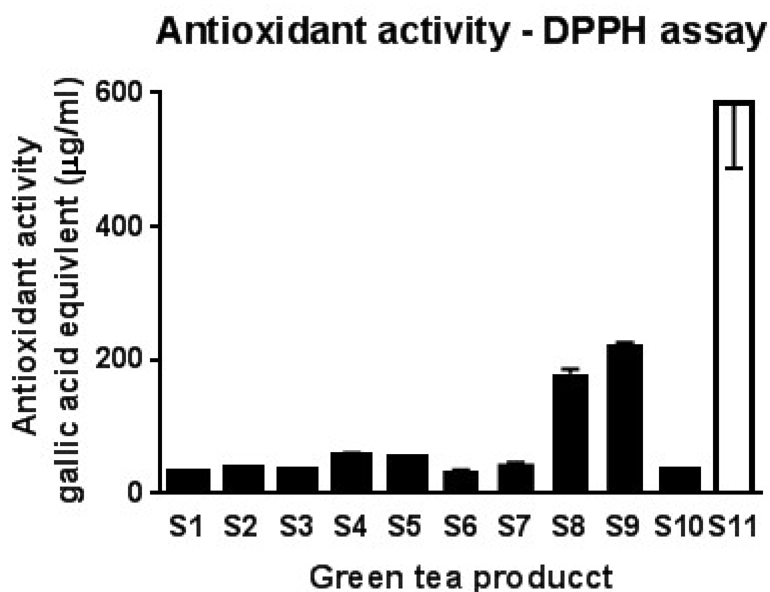


Figure 18: Antioxidant activity of green tea products determined by DPPH assay. See Chapter 2 for method. Data were expressed as means and s.e.m. n=?=3

The µg (gallic acid) / mg (herbal material) was calculated for each batch using the calibration curve. The results are shown in Table 10.

Table 11: Antioxidant activity of green tea products (DPPH)

Sample Number	Gallic acid equivalence (µg/mg ± SD) n=9
S1	34.2 ± 0.2
S2	38.8 ± 0.3
S3	37.2 ± 0.5
S4	58.3 ± 1.3
S5	52.9 ± 1.3
S6	35.1 ± 0.5
S7	45.4 ± 0.9
S8	180 ± 19
S9	223.6 ±5.6
S10	35.8 ± 0.6
S11	584 ± 290

As shown, the antioxidant activity varies with different green tea products. The highest antioxidant activity was observed for S11, followed by S9 and S8. This was not surprising as the S8,S9 and S11 are all manufactured products using concentrated green tea extract as "active" components. However, the lowest antioxidant activity was doing for another manufactured product S10. These results are consistent with the findings on marker content as described above. This it is highly possible that the antioxidant activity of the green tea products is related to the content of marker compounds studied.

DPPH is one measure of chemical measure of antioxidants. Antioxidants can occur via different mechanisms; therefore, the FC assay was used as a second measure of antioxidants.

3.4.2 Folin Ciocalteu assay of phenolic content

Folin Ciocalteu assay measures the total phenolic content (TFC) or anti-oxidant capacity of testing samples. Using the method as described in Chapter 2, The total phenolic content of the green tea samples were measure and shown in Figure 17 and Table 11. In these experiments S10-S12 were not tested due to the availability of the sample and to remaining consistent for comparison with the online phenolics assay.

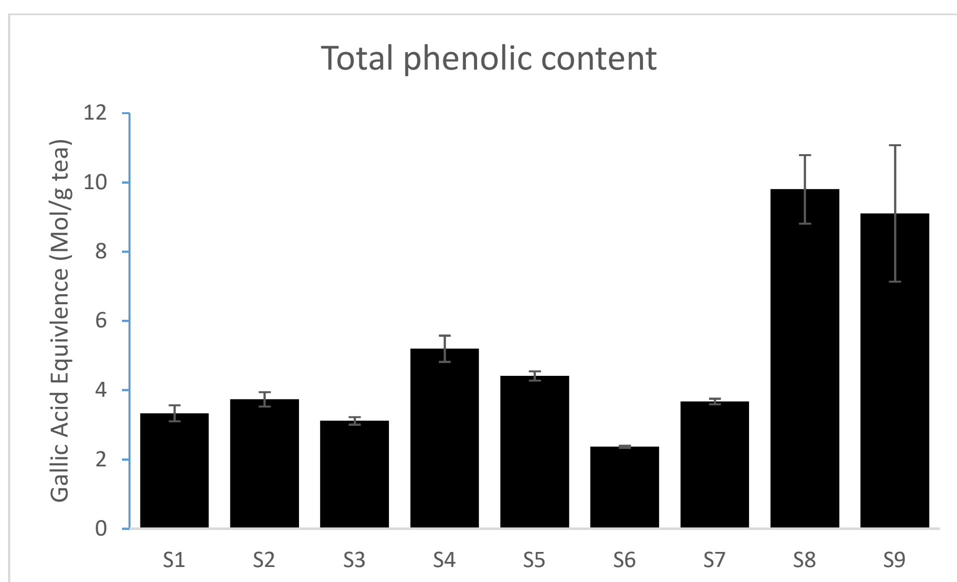


Figure 19: Total phenolic content of green tea products determined by Folin Ciocalteu assay. See Chapter 2 for method. Data were expressed as means and s.e.m. n=?=3

Table 12: Antioxidant activity of green tea products (TP)

Sample Number	Gallic acid equivalence (µg/mg) n=9
S1	3.3 ± 0.2
S2	3.7 ± 0.2
S3	3.1 ± 0.1
S4	5.2 ± 0.4
S5	4.4 ± 0.1
S6	2.4 ± 0.1
S7	3.7 ± 0.1
S8	9.8 ± 1.0
S9	9 ± 2

The highest TPC was observed for the manufactured products (8-9). The traditional green tea leaf and powder products (S8 and S9) showed a lower TPC. This is expected as seen with the results of in vitro anti-oxidant activity. For example, S8 and S9 contained relative high level of catechins, which correlated to a relatively high TPC. This further suggests that phenolic content plays a large role in the antioxidant activity of natural products, as learned from previous works on the chemical composition of natural products such as teas and fruit extracts, and it is not clear what type of green tea extract was used in these products. Further characterisation of phenolic compounds in these products may shed more light on this.

3.5 Correlation of the antioxidants and Marker compounds.

In order to establish the relationship of maker compounds to the antioxidant activity of the green tea products, series of correlation analysis were conducted.

Figure 3-15 shows the correlation analysis between the content of catechin markers to the antioxidant activity (DPPH assay).

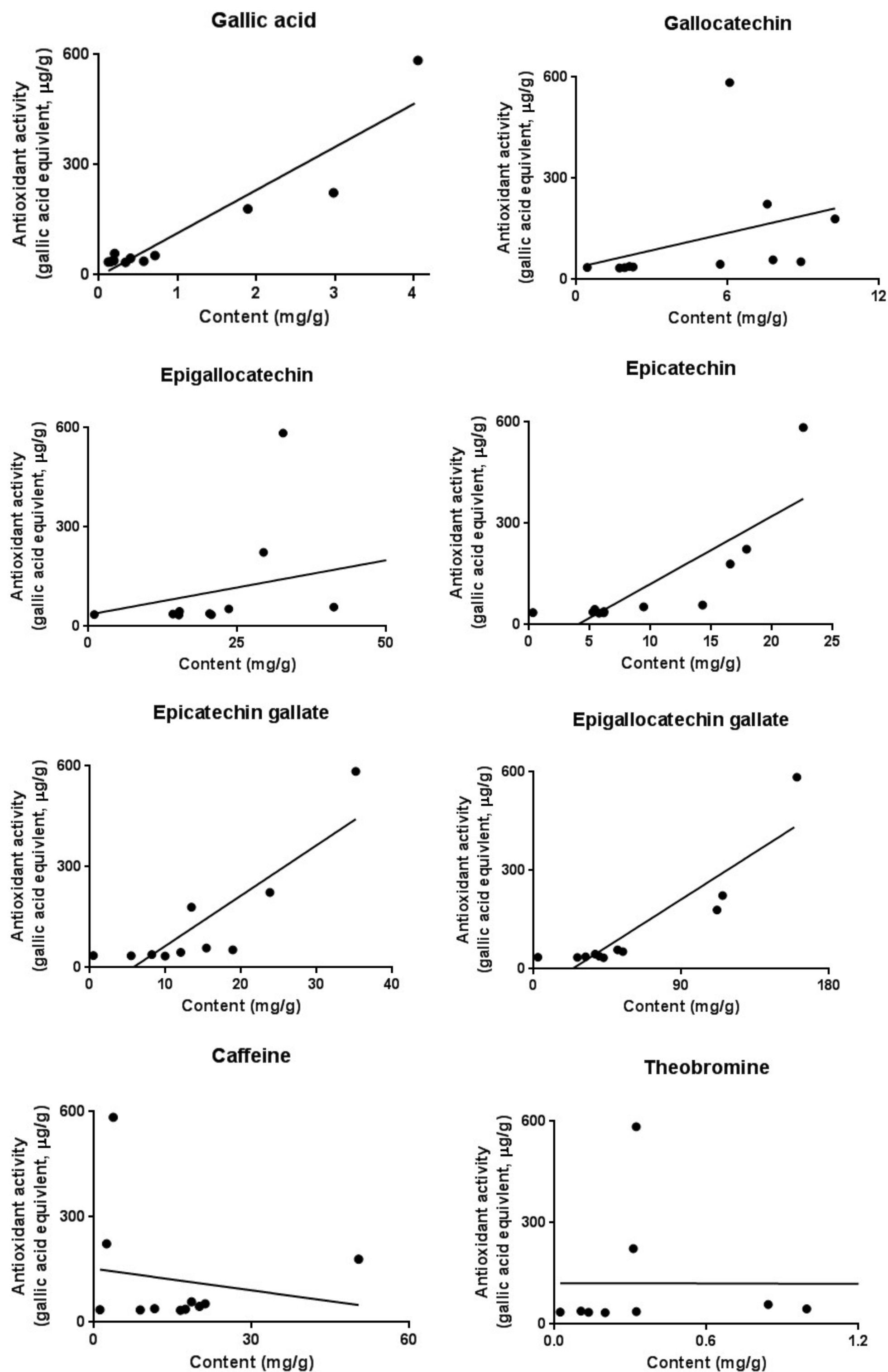


Figure 20: Correlation between the content of individual marker compounds and the antioxidant activity, based on the data in Figure 3.7 and Figure 3.10. Significant correlations were observed for GA (r=0.938, p<0.0001); EC (r=0.821, p<0.01); ECG (r=0.859, p<0.00

There is a significant positive correlation between the total content of group catechin markers (GA, EGC, EC, ECG) with the antioxidant activity, indicating these compounds may directly link to the antioxidant activity of green tea products. Individual analysis revealed that a strong correlation between the content of GC, GA and EGC with the antioxidant activity, less with that of EGCG, ECG and EC. The present findings support the use of gallic acid as a marker for the antioxidant content of many extracts such as fruits, berries and tea(15). In addition, the findings indicate some catechin compound such as GC may be a better maker than GA. Based on these results it can be observed that there is a strong correlation between the concentrations of these marker compounds in the tea samples and the antioxidant activity *in vitro*. These marker compounds were selected and compared because they all are part of the same class of compounds, catechin polyphenols.

In contrast, other compounds present in tea, methylxanthines caffeine and theobromine, did not show a significant correlation with the antioxidant activity as shown in Figure 3.14. Similar results were obtained for L-theanine, no correlation was observed for its content with the antioxidant activity (Figure 18).

When the selected marker compounds were combined (GA+EC+ECG+EGCG), a significant correlation was observed (Figure 19).

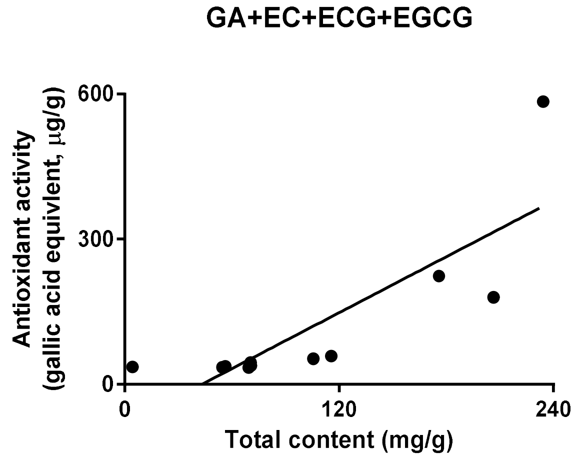


Figure 21: Correlation between the content of selected markers (GA+EC+ECG+EGCG) and the in vitro antioxidant activity. r=0.8229, p<0.01

However, the correlation coefficient value (r^2) was not further improved, indicating that using well selected individual markers, for example gallic acid or EGCG, can achieve the same or even better predication of the in vitro antioxidant activity then using a group of marker compounds. These results further indicate that these markers can be used as a quality control indicator for the antioxidant activity of the green tea products. It should be pointed that the correlation of the marker compounds with the anti-oxidant activity also depends on the type of green tea products. For example, the green tea leaf and powdered tea products showed a stronger correlation between the content of the caffeine and theobromine with the antioxidant activity of the samples, whereas TGA listed tablet products did not show a correlation with caffeine content and antioxidant activity since the caffeine appears to have been intentionally reduced in some of these products as indicated in the product label, or part of the manufacturing process of these products may have affected caffeine content.

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There was a strong positive correlation (r^2=0.97) between the content of EGCG and the total phenolic content. This is similar to the DPPH antioxidant result, which shows that phenolic compounds have a significant effect on the antioxidant activity of green tea. It was expected that ECGC had the strongest correlation as it is commonly found in a number of studies as a major active component of green tea and quote as the active ingredient in a number of TGA listed green tea dietary supplements, such as sample 9-11 that were tested in this study.

The methylxanthine compounds, caffeine and theobromine, did not have as a strong of a correlation due to the nature of this class of compounds. In contrast to the catechins and gallic acid, these compounds do not contain hydroxyl groups, which are characteristic of the type of phenolic compounds that can be detected by this method and there for are not suited to this method.

The results of the total phenol assay match the correlations observed for DPPH. Figure 20 shows the correlations.

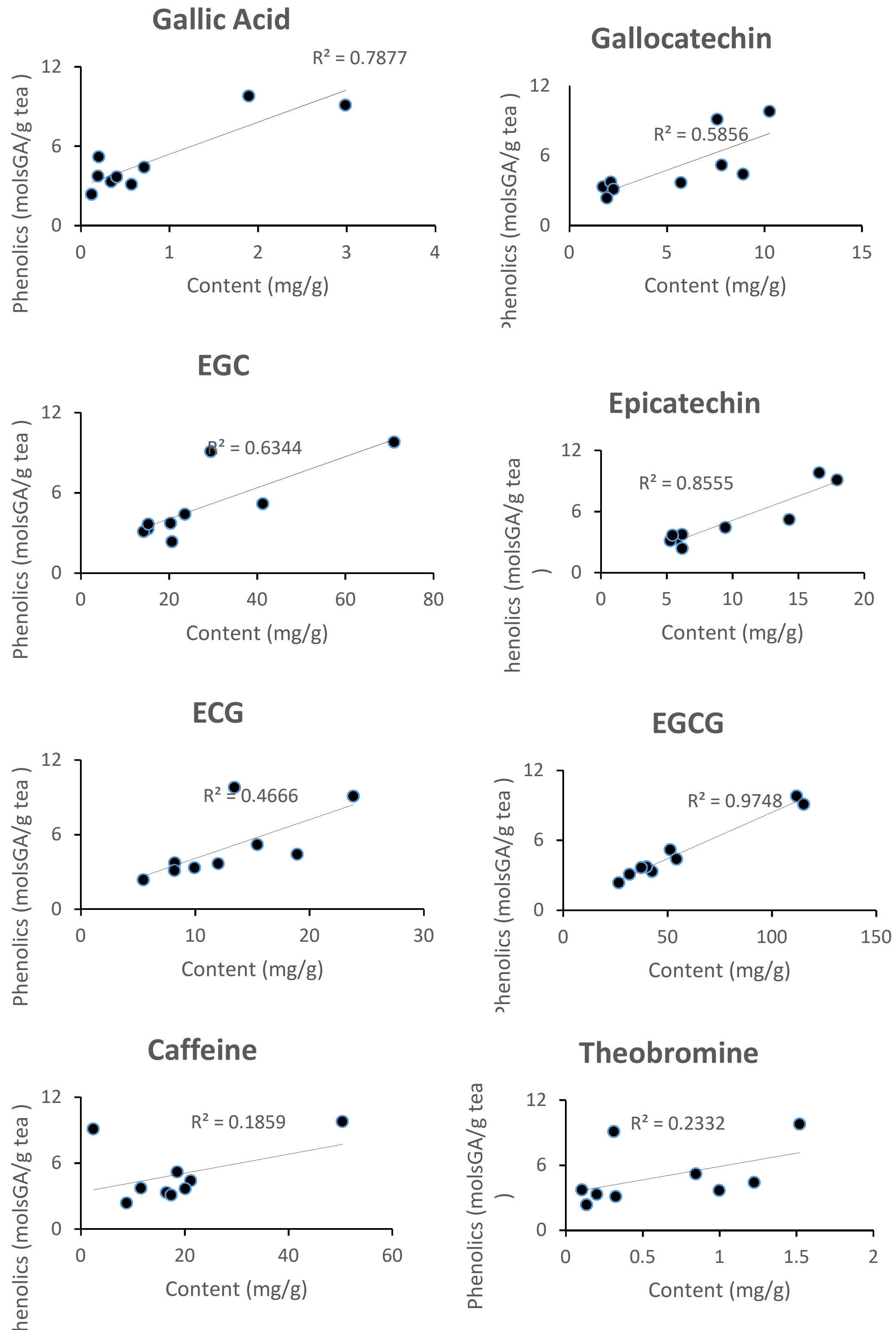

Figure 22: Correlation between the content of marker compounds and the total antioxidant capacity (total phenolic content), based on the data in Figure 3.7 and Figure 3.11. Significant correlations were observed for EC p<0.05), ECG (p<0.01) and EGCG (p<0.01).

The amino acid L-theanine showed no significant correlation with the DPPH or TC antioxidant results as can be seen in Figure 21.

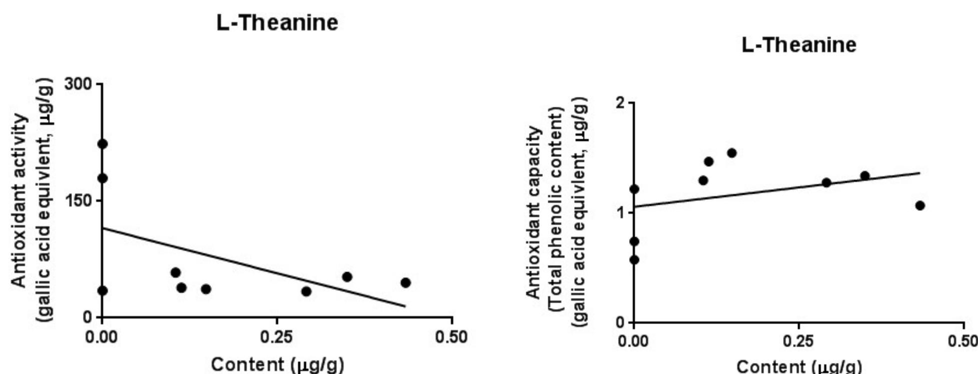

Figure 23: Correlation between the content of L-theanine and the in vitro antioxidant activity (r=-0.53) / total antioxidant capacity (r=0.35). P>0.05.

3.7 Online Antioxidant Assay

3.7.1 Online FRAP

Using the method described in the Chapter 2.3.4, a specific online assay was developed and shown to be effective to identify the potential antioxidant component/compound in complex green tea extracts. Figure 3.17 shows the HPLC chromatogram of the UV (280nm) and FRAP (593nm) responses for the green tea product (sample 4). As it shows, efficient separations and clear antioxidant responses were observed for the main peaks of the green tea sample. This result is consistent with those from the in vitro antioxidant activity assay as discussed above, with the markers (eg GA, EC, EGC and EGCG) strongly correlated with the in vitro anti-oxidant activity was also found to be active in the online FRAP assay. The results indicate that the online method is efficient to identify known or unknown component/compounds with antioxidant activities. One peak of significant interest in this investigation is peak 2, gallocatechin.

This peak showed a low absorbance in the UV range, in contrast with a relatively large response with the FRAP reagent.

Most previous studies on the antioxidant potential has been determined by conventional colorimetric assays such as DPPH, FRAP and ORAC assays(44,53–55). These "benchtop" assays measure the total antioxidant potential of a herbal mixture such as green tea, but lack the specificity in determining the marker compound that is contributing to the response.

The online antioxidant assay is a relatively novel approach and powerful tool to study the antioxidant activity of complex natural mixtures, and make it possible to identify particular ingredients/compounds with antioxidant activity which otherwise not be able detect using crude extracts and conventional antioxidant assays. It provides a much better understanding of the efficacy of each marker compound and its suitability in being an ideal marker for the quality of green teas in terms of antioxidant potential. Due to limitations in the cost of this method in terms of availability of additional instrumentation and resources, as well as addition training in more complex method, this method of antioxidant screening less widespread in the commercial assessment of green tea quality.

In the experiment, ferric reducing/antioxidant power (FRAP) assay was used due to complications with the DPPH reagent and the online system in our initial experiment. Further study will be needed to sue the DPPH online assay to compare with the results from conventional in vitro antioxidant assay. Nevertheless, no obvious discrepancy was observed these initial experimental results. Previous studies also demonstrated

that DPPH and FRAP assays did not show vast differences in their determination of the antioxidant content of guava fruit extracts (56).

Similarly, described in the Chapter, an online assay was also explored for the Folin Ciocalteu assay. As show in Figure 19 and Figure 20, efficient separations and clear phenolic content responses were observed for the main peaks of the green tea sample. The result is consistent with those from the in vitro antioxidant activity assay as discussed above, with the markers (eg EC, ECG and EGCG) correlated with the TPC was also found to be active in the online FRAP assay. The results indicate that the online method is efficient to identify components/compounds potentially affecting the antioxidant capacity.

In figure 22, where there is a FRAP peak with the same retention time as peak number 4, for example, in the UV chromatogram, this means that peak 4 is responsible for the then the substance indicated by that peak is responsible for the FRAP activity. This is the same for the other peaks of interest labelled in the figure and the corresponding response for FRAP.

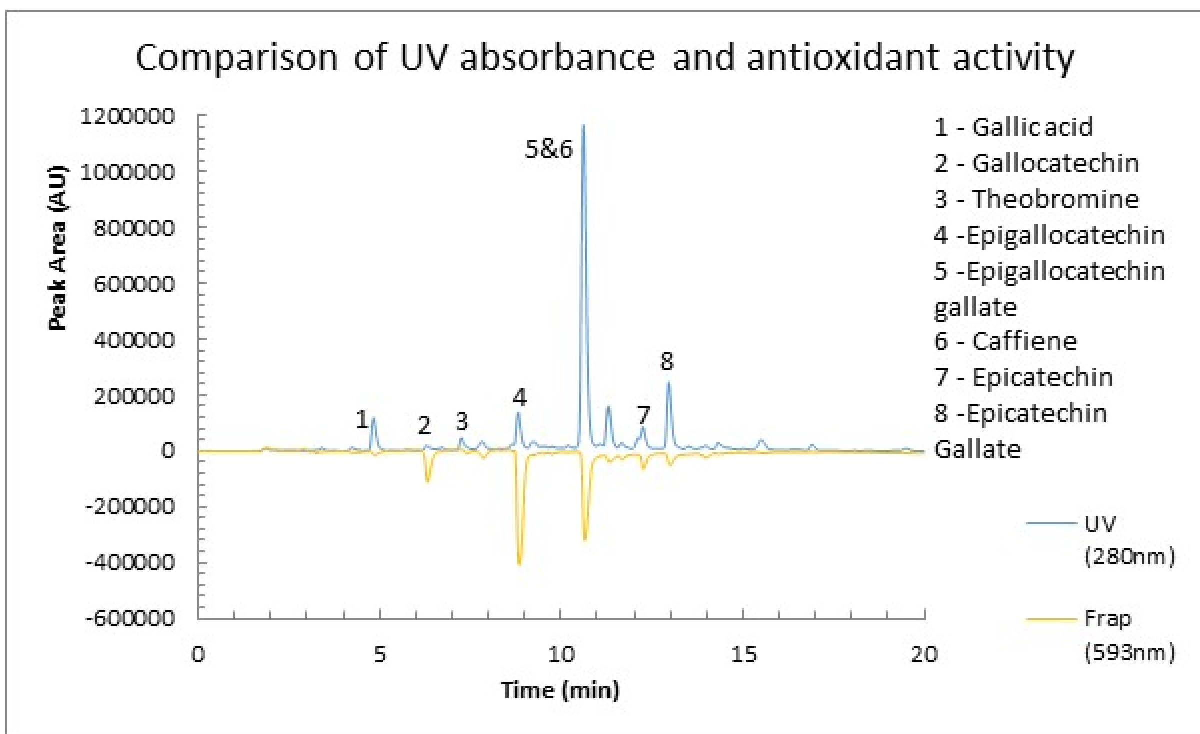

Figure 24: HPLC chromatogram of the UV (280nm) and FRAP (593nm) responses for green tea product (sample 4).

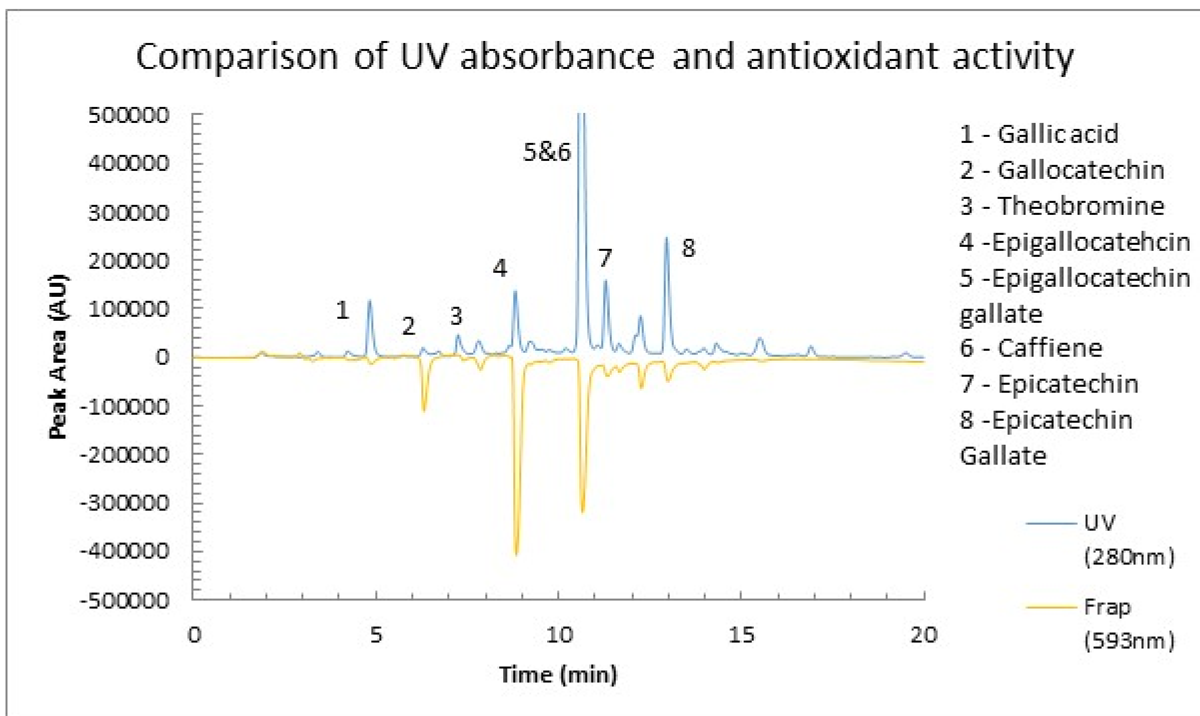

Figure 25: HPLC chromatogram of the UV (280nm) and FRAP (593nm) antioxidant responses for the green tea product (sample 4), zoomed into 500mAU.

The chromatogram in figure 23 is a chemical profile of a typical green tea sample and its antioxidant activity, zoomed to 500mAU.

Catechins are ordered based on their antioxidant activity as indicated by a number of studies: EGC ~ EGCG >> ECG = EC > catechins and the number of hydroxyl groups is the major factor contributing to antioxidant potential of catechins of green tea(39,40). This is in agreement with our results.

It would be ideal to have used DPPH in the online assay to compare to the benchtop assays, however due to complications with the DPPH reagent and the online system, this was not possible. Based on the work of Thaipong et al. the DPPH and FRAP assays did not show differences in their determination of the antioxidant content of guava fruit extracts (56). It is possible that similar conclusions can be drawn for tea. DPPH and FRAP both detect antioxidants. Direct comparison to the same antioxidant reaction is preferred and was achieved for the TPC.

3.5.2 Online assay on phenolic content

To directly compare to the TPC result and online TP HPLC assay was developed for green tea. The chromatogram is shown in Figure 26. Sample 4 was selected as an example as it had the highest phenolic response in the F-C assay as well as having relatively high concentrations of the marker compounds. This figure shows that there is a high UV absorbance of EGCG and Caffeine which corresponds to a higher phenolic response in comparison to other peaks in the sample. This is expected as in

previous studies, ECGC has been found to be an abundant polyphenol in green tea and as a result will give a larger response(2,57).

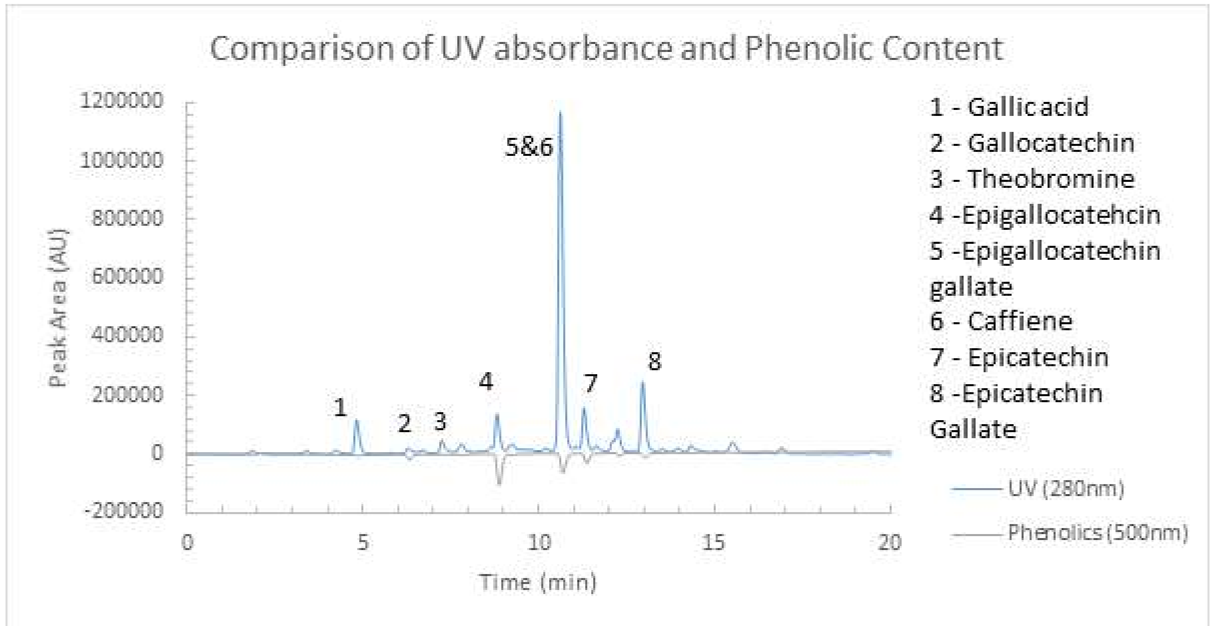

Figure 26: HPLC Chromatogram of the UV (280nm) and phenolic content (500nm) of the green tea sample 4.

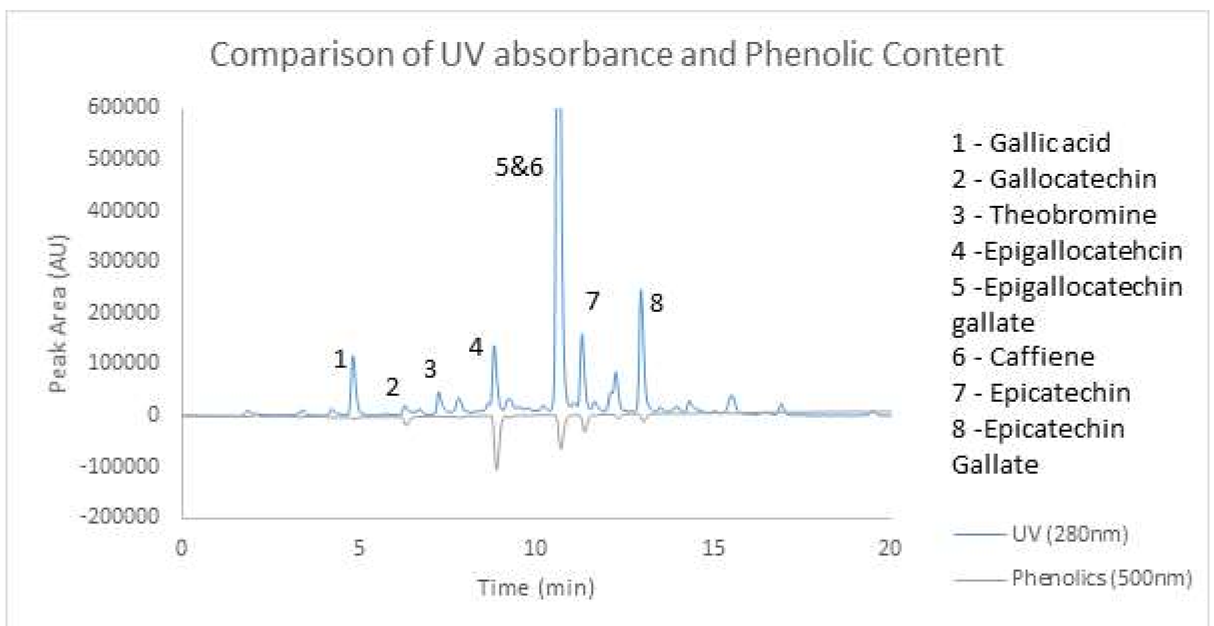

Figure 27: HPLC Chromatogram of the UV (280nm) and phenolic content (500nm) of the green tea sample 4, zoomed to 600mAU

The online antioxidant assays allowed the identification of the chemicals responsible for the antioxidant activity in the green tea.

Future Work

There are some limitations of the present study. Firstly, due to the time limit, we have only sourced limited number of green tea samples. It is not clear if the correlations as demonstrated in this study can be extended to other green tea products. Thus the findings from this study need to be further confirmed by analysis of more green tea products. Secondly, the antioxidant activity assay used (DPPH assay) is useful but it is *in vitro* in nature. It has been known that some findings *in vitro* can be transformed directly into an *in vivo* situation. Thus, further research is needed to study the relationship of the marker compounds with *in vivo* antioxidant activity to confirm the correlation established has a general implication. Thirdly the online antioxidant assay is promising, but it is only a pilot study. Further research is needed to optimize the assay condition and then apply to various green tea samples for testing. It is highly possible the online method may reveal important aspects of quality markers and important links between chemical compositions to bioactivities. In addition, we did not fully characterise the chemical compositions of each green tea product, thus it is not clear if any other known or unknown compounds are involved in the observed antioxidant activity. It is possible that further research may discover more active compounds in the green teas which may have a better correlation to the antioxidant activity *in vitro* and *in vivo*. A further limitation is that the methods have not had inter-

laboratory validation and therefore will need to be confirmed by other laboratories for confirmation of the content of products.

Conclusion

In conclusion, the findings from this study have clearly demonstrated that there is a close relationship between the content of certain chemical markers to antioxidant activity of green tea products. Those markers, in particular certain catechins such as gallic acid, EGCG, EC, EGC, can be used as quality markers for determining the quality of various green tea products. In addition, two online assays have been successfully developed, which can be used to future research to determine the link between known and unknown components/compounds to the antioxidant activity of various complex natural extracts/products. Further research in this area may lead to new approaches to study and identify the bioactive compounds from natural products including green tea and various herbal products.

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