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Identification of digallated and methylated catechins using UPLC/MS/MS and development of a rapid analysis method for theanine in tea [*Camellia sinensis* (L.) O. Kuntze] utilizing evaporative light scattering detection

by

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DECLARATION

I declare that the dissertation that I hereby submit for the degree in Biochemistry at the University of Pretoria has not previously been submitted by me for degree purposes at any other university and I take note that, if the dissertation is approved, I have to submit the additional copies, as stipulated by the relevant regulations, at least six weeks before the following graduation ceremony takes place and that if I do not comply with the stipulations, the degree will not be conferred upon me.

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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AMPA	Amino-methyl propionic acid
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
BHA	Butylated hydroxyanisole
C	(+)-Catechin
CC	Column Chromatography
CDER	Centre for Drug Evaluation and Research
CID	Collision-Induced Dissociation
CITP	Capillary Isotachophoresis
CCC	Counter-Current Chromatography
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
dddwater	Double Distilled De-ionized Water
DNA	Deoxyribonucleic Acid
DNFB	2,4-Dinitrofluorobenzene
DPPH	1,1 Diphenyl-2-picrylhydrazyl
EAAT	Excitoray Amino Acid Transporter
EC	(-)-Epicatechin
E.C.	Enzyme Commission
ECg	(-)-Epicatechin gallate
EGC	(-)-Epigallocatechin
EGCg	(-)-Epigallocatechin gallate
ECgg	(-)-Epicatechin-3,5-digallate
EGCgg	(-)-Epigallocatechin-3,5-digallate
EGCmetG	(-)-Epigallocatechin-3-(3"-O-methyl) gallate
ECmetG	(-)-Epicatechin-3-(3"-O-methyl) gallate
ELS	Evaporative Light Scattering
ELSD	Evaporative Light Scattering Detector
ESI	Electrospray Ionization
ESR	Electron Spin Resonance

EU	European Union
FAO	Food and Agriculture Organization
FDA	US Food and Drug Administration
FMOC-Gly-Cl	9-Fluorenylmethoxycarbonyl glycine chloride
GABA	γ-Aminobutyric acid
GC	(+)-Gallocatechin
GC	Gas Chromatography
Gln	Glutamine
GlnT	Glutamine Transporter
Glu	Glutamate
HILIC	Hydrophilic Interaction Chromatography
HPCE	High Performance Capillary Electrophoresis
HPLC	High Performance Liquid Chromatography
HSCCC	High Speed Counter-Current Chromatography
ISO	International Standards Organization
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LS	Light Scattering
MAE	Microwave-Assisted Extraction
MEKC	Micelllar Electrokinetic Chromatography
mGluR	Metabotropic Glutamate Receptor
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NBD-F	4-Fluoro-7-nitro-2,1,3-benzoxadiazole
NDA	Naphthalene-2,3-dicarboxaldehyde
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
OPA	O-Phthalaldehyde
PC	Polyphenol Content
PITC	Phenyl Isothiocyanate
PLE	Pressurized Liquid Extraction
PPO	Polyphenol Oxidase
PVPP	Polyvinylpolypyrrolidone
RDA	Retro-Diels-Alder Reaction

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAS	Statistical Analysis Software
SDS	Sodium Dodecyl Sulfate
SFE	Supercritical Fluid Extraction
SIM	Selected Ion Monotoring
TEAC	Trolox Equivalent Antioxidant Capacity
LOD	Limit of Detection
LOQ	Limit of Quantification
TGA	Therapeutic Goods Administration
TLC	Thin Layer Chromatography
TQD	Tandem Quadruple Mass Spectrometry Detector
UN	United Nations
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
UV-vis	Ultraviolet-visible

Chapter 1

Introduction

1.1 Tea: A Popular Beverage

Next to water, tea is the most popular non-alcoholic beverage consumed in the world. According to the UN Food and Agriculture Organization (FAO), global tea production amounted to 3.6 million tons in 2006. Tea is cultivated in approximately 52 countries worldwide and is generally consumed in the form of black (78%), green (20%) or oolong (2%) tea. Green tea is preferred in oriental countries, including a number of countries in North Africa and the Middle East, while black tea is the drink of choice in Western countries (Cooper *et al.*, 2005). Due to a growing awareness of the beneficial health properties of green tea, it is now gaining popularity in the western world as well.

Hundreds of types of commercial teas are produced but they are generally classified into 3 categories: non-fermented green tea, semi-fermented oolong tea and fully fermented black tea. Tea is manufactured from the tender top leaves of an evergreen shrub, *Camellia sinensis* (L.) O. Kuntze. Fresh, green tea leaves contain 30 to 40% (w/w) polyphenolic compounds, among which catechins are the most abundant as illustrated by Figure 1.1 (Del Rio *et al.*, 2004). During the processing of tea leaves an enzyme, polyphenol oxidase (PPO E.C. [Enzyme Commission] 1.10.3.1), is released that oxidises the catechins present in the leaves leading to the formation of theaflavins and thearubigins. This enzymatic oxidation process is referred to as fermentation.
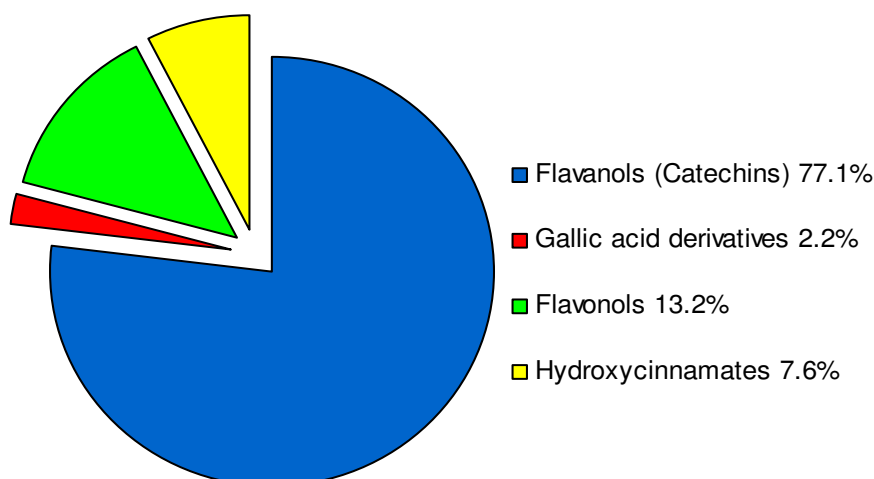
Figure 1.1. Percentile composition of the different classes of phenolics in green tea (Adapted from Del Rio *et al.*, 2004).

During the manufacturing of green tea, PPO is inhibited to prevent the oxidation of the catechins. These catechins are responsible for the characteristic flavour and astringency of green tea. Inhibiting PPO results in the leaves retaining their green colour and almost all of their original polyphenol contents. In order to inactivate PPO, freshly collected leaves are steamed, roasted, parched, oven heated, etc. (Gulati *et al.*, 2003). Similar to green tea, white tea is also unfermented. White tea is prepared from very young tea leaves and buds covered with tiny, silvery hairs that are harvested only once a year in early spring. After picking, the young buds and leaves are steamed and dried immediately to prevent oxidation, giving the tea a light, delicate taste (Rusak *et al.*, 2008). Oolong tea is allowed to undergo limited fermentation and as a result contains a mixture of catechins, theaflavins and thearubigens. Black tea is fully fermented and most of the catechins are converted to theaflavins and thearubigens, which are responsible for the distinct aroma and colour of this tea. Table 1.1 illustrates the typical levels of major phenolic constituents found

in green and black tea; while Figure 1.2 demonstrates the effect different processing

methods (for production of different types of tea) have on the different phenolic

constituents.

Table 1.1. Typical levels of constituents found in black and green tea (Borzelleca *et al.*, 2006; Cooper *et al.*, 2005). LOD = limit of detection. a) Calculated form the difference between catechin in green tea and catechin and theaflavin content in black tea.

Compound	Green Tea (cup)†	Black Tea (cup)†
Catechins	60 -125 mg	30 - 60 mg
Theaflavins	<LOD	3 - 6 mg
Thearubigens[a]	<LOD	27 – 59 mg
Caffeine	20 - 50 mg	30 - 60 mg
L-Theanine	20 - 40 mg	20 - 40 mg

†A typical cup of tea uses 2 - 3 g of dried tea leaves
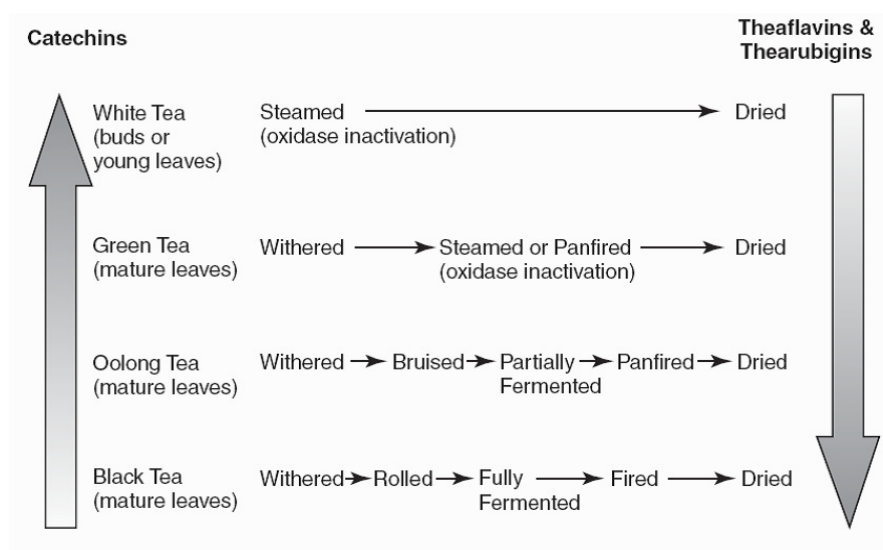
Figure 1.2. Schematic representations of the effect of processing on tea composition (Cooper *et al.*, 2005).

The quality of tea is mainly assessed through its appearance (colour, colour intensity

and clarity), its taste (astringency, bitterness and sweetness) and its aroma (floral,

sweet, grassy, etc.). The most important chemical constituents that influence the flavour and taste of tea infusions are catechins, polyphenols, caffeine, sugars, organic acids, amino acids and volatile aroma compounds. However, the components that influence the quality of green and black teas differ. Black tea quality depends on the contents of theaflavin, thearubigen, catechin and caffeine, while the quality of green tea is more dependant on amino acid (especially theanine), catechin and caffeine contents (Le Gall *et al*., 2004; Liang *et al*., 2003; Nakagawa, 1975).

Recently an enormous amount of research has demonstrated the therapeutic function of tea extract in a wide range of diseases. The wide range of physiological functions of tea is largely due to the presence of catechins, caffeine and theanine. It has been shown that catechins have anti-mutagenic, anti-tumorgenic, anti-hypertension, anti-vascular disorders, anti-inflammatory properties etc., while theanine induces relaxation and enhances cognitive abilities (Cooper *et al*., 2005; Coyle *et al*., 2008; Haskell *et al*., 2008; Jain *et al*., 2006; Kumara *et al*., 2007).

The following sections will focus on theanine and catechins.

1.2 Theanine

Theanine is a unique free amino acid that was first discovered and isolated from tea leaves (*Camellia sinensis*) by Kito *et al*. in 1949. It was named after the former scientific name of green tea, *Thea sinensis*. This amino acid is found only in two other species, namely *C. japonica* and *C. sasanqua,* of the *Camellia* genus (Juneja *et*

al., 1999) and a mushroom called *Xerocomus badius* (Casimir *et al.*, 1960). Theanine represents 50% of the total free amino acids found in tea (Syu *et al.*, 2008). It is responsible for the sweet, brothy unami taste of green tea and therefore plays an important role in the quality of green tea.

1.2.1 The Chemical Properties of Theanine

Theanine is a water soluble compound with the molecular formula $C_7H_{14}N_2O_3$ and a molecular weight of 174.2 daltons. It has a melting point of 117 °C and absorption maximum (λ_{max}) of 194 nm. The chemical structure is γ-ethylamino-L-glutamic acid (Figure 1.3) and theanine is also known as γ-glutamylethylamide or N-ethyl-L-glutamine. Theanine standard is stable in solution over a pH range of 3.0 to 6.6 and it has been shown to be stable in both neutral (pH = 6.5) and acidic (pH = 3.0) conditions over a 12 month storage period below 25 °C (Juneja *et al.*, 1999).
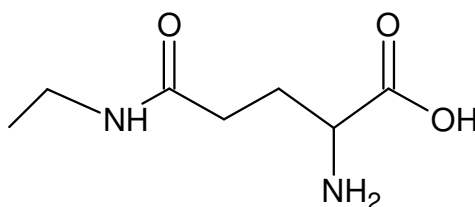
Figure 1.3. The structure of theanine.

1.2.2 The Biochemical and Health Properties of Theanine

Since ancient times it has been said that drinking tea brings relaxation. This property of tea is attributed to the presence of theanine. Theanine readily crosses the blood-brain barrier in a dose-dependent manner within 30 minutes after consumption,

reaching maximal levels after 5 hours post-administration in animals (Terashima *et al.*, 1999; Yokogoshi *et al.*, 1998a,b). It increases the generation of alpha waves in the brain which is associated with a relaxed yet alert mental state (Gomez-Rameriz *et al.*, 2007; Juneja *et al.*, 1999; Kelly *et al.*, 2008). However, alpha brainwave activity is only an indirect measure of relaxation. Studies that investigated the effects of theanine on self-reported anxiety and stress, as well as physiological stress responses such as heart rate and salivary imunoglobulin A levels, also support the relaxing effects of theanine (Lu *et al.*, 2004; Kimura *et al.*, 2007).

The pharmacology of theanine is relatively unknown, but animal studies have shown numerous effects on different neuro-chemical systems. These include increasing the levels of dopamine and γ-aminobutyric acid (GABA – a neurotransmitter associate with the regulation of responses) and decreasing levels of norepinephrine (probably as a result of increased GABA levels). A increase in serotonin levels in the stratum, hippocampus and hypothalamus has been observed although general serotonin release is suppressed. These reported effects on serotonin and GABA supports the hypothesis that theanine plays a role in the regulation of anxiety. The observed increase in dopamine and serotonin levels also supports studies that have shown the potential cognitive enhancing abilities of theanine since these monoamine neurotransmitters are closely associated with learning and memory (Graziano *et al.*, 2002; Juneja *et al.*, 1999; Yamada *et al.*, 2009; Yokogoshi and Terashima, 2000).

Furthermore, a few animal studies demonstrated that theanine reduced post-ischemic neural death in the hippocampus, reduced the size of cerebral infracts and inhibited neural death caused by brief exposure to glutamate (Cho *et al.*, 2008; Egashira *et al.*, 2004; Kakuda *et al.*, 2002; Nagasawa *et al.*, 2004). The results form

these studies imply that theanine may have neuroprotective effects against brain damage. In addition, theanine appears to antagonize the stimulatory effects of caffeine (Kakuda *et al.*, 2000a; Rogers *et al.*, 2008), displays anti-hypertensive properties (Yokogoshi and Kobayashi, 1998), seems to be effective against alcoholic liver injury (Sadzuka *et al.*, 2005) and plays a role in the anti-obesity effects of green tea by influencing feeding hormones (Yamada *et al.*, 2008). Theanine has even been found to increase the anti-tumor activity of some chemotherapeutic agents such as doxorubicin and idarubicin and to ameliorate their toxicity to normal cells (Sadzuka *et al.*, 2006; Sugiyama and Sadzuka, 2004).

1.2.2.1 Possible Mechanisms of Action of Theanine

Glutamatergic Neurons.

The glutamine/glutamate cycle, neuronal death and theanine.

Glutamate is the most common excitatory neurotransmitter in the brain. Glutamate receptors are ionotropic (ion channel coupled) and metabotropic (G-protein coupled) receptors. The ionotropic receptors are the amino-methyl propionic acid (AMPA), the N-methyl-D-aspartate (NMDA) and the kainate receptor. The NMDA receptor has at least 5 binding sites which includes binding sites for glutamate and glycine. To terminate the action of glutamate released in the synapses, it is reabsorbed directly into neurons or taken up by astrocytes (glial cells) via excitatory amino acid transporters. It is widely accepted that this active transport is vital to prevent the accumulation of extracelluar glutamate to a neurotoxic level (Kakuda *et al.*, 2008). In astrocytes glutamate is converted to glutamine by glutamine syntethase, a molecule that cannot cause excitotoxicity (damaging effects due to excessive excitatory neurotransmitter release). Glutamine is exported to extracelluar spaces and imported

into neurons through glutamine transporters. In the neurons glutamine is hydrolyzed to glutamate by the phosphate-dependant enzyme glutaminase. The glutamate is then incorporated into synaptic vesicles for release. It is known that excessive glutamate concentrations in the central nervous system causes neuronal death in cases such as ischemic neuropathology and Alzheimer's disease (Meldrum, 2000). Ischemia induces a large scale glutamate surge (excitotoxicity) causing a large influx of Ca^{2+} into ischemic neurons through NMDA and non-NMDA glutamate receptors. Accumulation of Ca^{2+} implies that the system is not maintaining the balance, as Ca^{2+} levels are usually regulated and recycled depending on the excitation level of the cell. Excessive Ca^{2+} accumulation activates various intracellular enzymes and increases superoxide radicals which cause oxidative damage resulting in neuronal death (Figure 1.4).
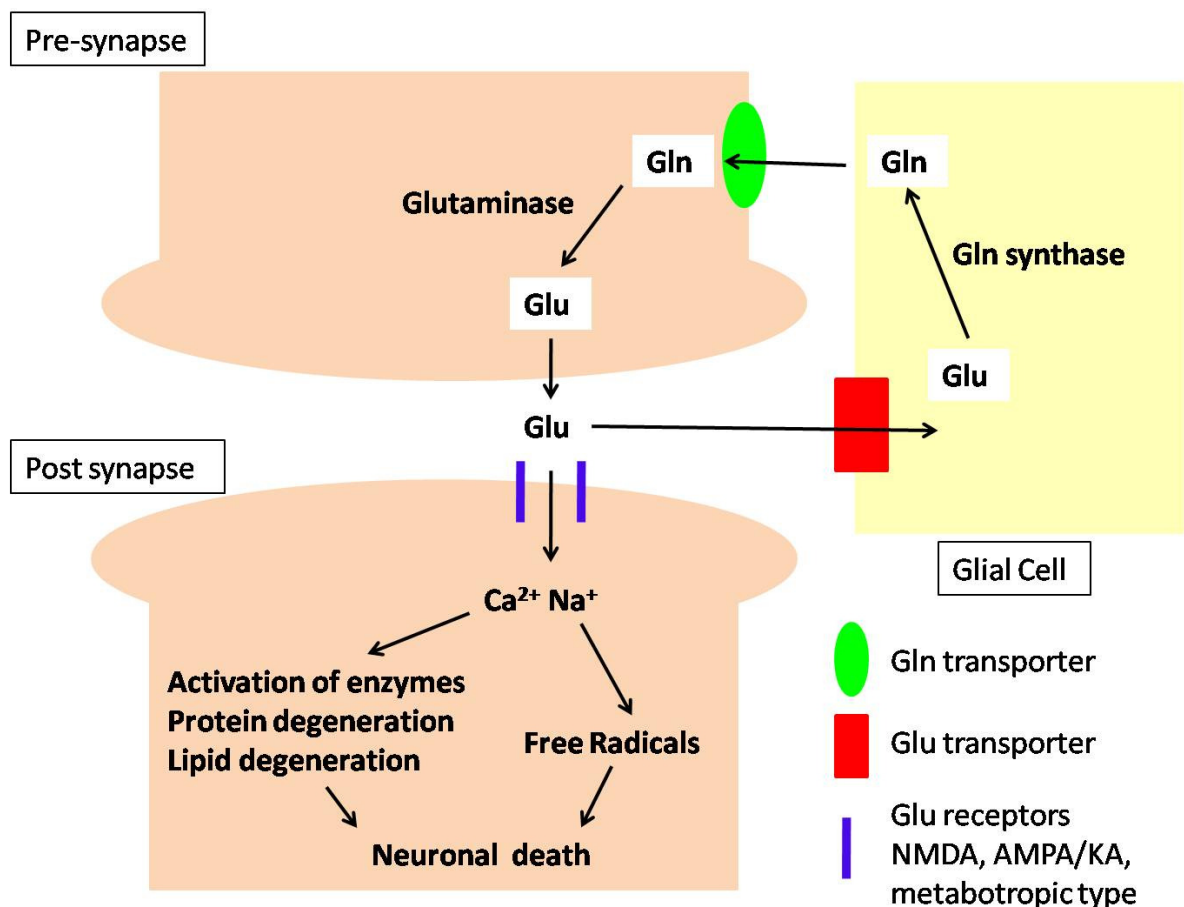
Figure 1.4. A schematic representation of the Glutamate/glutamine cycle, the glutamate receptors and neuronal cell death (Adapted from Kakuda, 2002).

In strokes and other forms of brain ischemia, excitotoxicity due to glutamate is one of the major destructive processes seen. Recently, the protective effect of theanine on ischemic delayed neuronal death in gerbils and glutamate-induced cell-death in cortical neurons has been reported (Egashira *et al.*, 2004; Kakuda *et al.*, 2000b). The structural similarity of theanine to glutamate has led researchers to propose that theanine interacts with glutamate receptors as antagonist, eliciting neuroprotection. However, it has been shown that the binding capacity of theanine to glutamate receptor subtypes AMPA, NMDA and kainate is considerably lower than that of glutamate, implying that the antagonist effect of theanine is very mild (Kakuda *et al.*, 2002). Nagasawa *et al.* (2004) demonstrated that group 1 metabotropic glutamate receptor (mGluR) antagonists inhibit the suppression of delayed death of neurons (caused by brief exposure to glutamate) by theanine. This suggests that binding of theanine to group 1 mGluRs might contribute to the neuroprotective effects of theanine. Furthermore, Kakuda *et al.* (2008) hypothesised that theanine could elicit neuroprotection through glutamine-mediated mechanisms since theanine is a structural analogue of glutamine. The results of their subsequent study supported this hypothesis and suggested that theanine inhibits glutamine transporters in neuron and astrocyte plasma membranes, suppressing the glutamate/glutamine cycle that maintains the neurotransmitter pool of glutamate in glutamatergic neurons (Figure 1.5). This could explain the beneficial effects theanine has on a variety of neurodegenerative and neuropsychiatric disorders relevant to over activation of glutamatergic neurotransmission and neurotoxicity in the brain.
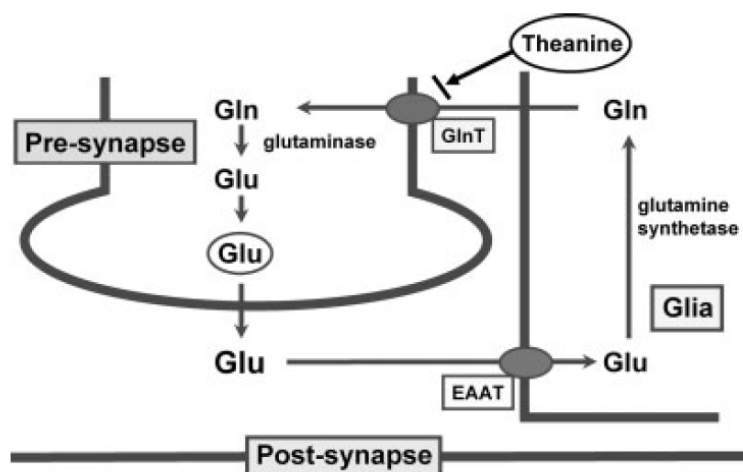
Figure 1.5. A schematic representation of the neuroprotection mechanism of theanine in the brain as proposed by Kakuda *et al*. (2008). This mechanism suggests that theanine suppresses the glutamate/glutamine cycle by inhibiting glutamine transporters (GlnT) which mediate the import of extracelluar glutamine into neurons. (The glutamate/glutamine cycle maintains the neurotransmitter pool of glutamate in glutamatergic neurons, also see Figure 1.4). EAAT = Excitatory amino acid transporter.

Dopaminergic Neurons.

Yokogoshi *et al*. (1998a) reported that theanine administration caused a significant increase of dopamine release in a dose-dependant manner in rats. Dopamine affects some physiological functions, behaviour and mood. It is known that dopamine release is enhanced by increased levels of glutamate (Moghaddam *et al*., 1990). However, Yamada *et al*. (2005) found that the effect of theanine on dopamine release is independent of a glutamate excitatory neurotransmission pathway, but did discover that theanine enhanced the levels of glycine, an inhibitory neurotransmitter. On further investigation they found that theanine induced pronounced increases in glycine levels accompanied by increased dopamine levels (Yamada *et al*., 2009). Blocking NMDA receptors at the glycine binding site did not influence the increased dopamine levels induced by theanine, whereas blocking glycine receptors not associated with the NMDA receptors did suppress the dopamine levels, although not completely. AMPA receptor antagonists suppressed the theanine-induced increase in

dopamine and glycine concentrations. Based on these results they proposed that theanine-induced changes in perfusate glycine levels involve AMPA/kainate receptors being directly affected by theanine (see Figure 1.6). In other words, it seems that theanine influences inhibitory neurotransmission, but further research is necessary to clarify the detailed mechanisms involved.
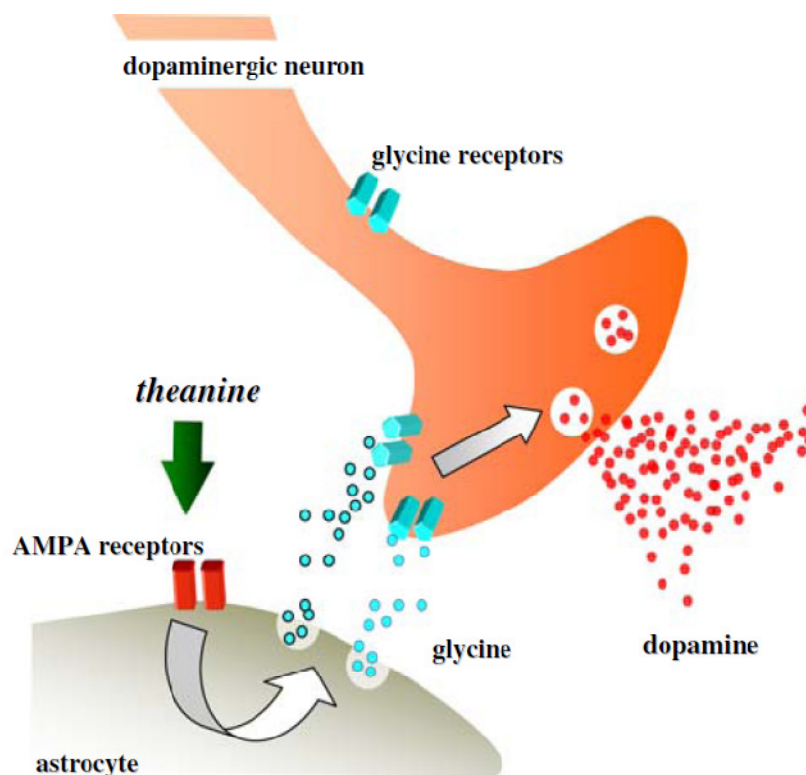
Figure 1.6. Hypothesis of the action mechanism of theanine on neurotransmitter concentration in the striatum as proposed by Yamada *et al.* (2009). Theanine binds to AMPA receptors, facilitating the release of glycine, which in turn mediates the release of dopamine.

Parkinson's disease is a dopaminergic neural disorder. The precise mechanisms of dopaminergic neural degeneration are unknown. However, post mortem studies showed that dying cells displayed signs of apoptosis, particularly chromatin

condensation, DNA fragmentation, oxidative damage, mitochondrial dysfunction and caspase activation (Nagatsu and Sawada, 2007). An increasing number of studies recognize a variety of environmental factors, in particular pesticides such as rotenone and dieldrin that increases the risk of non-familial Parkinson's (Sherer *et al.*, 2007; Singh *et al.*, 2007). The precise mechanisms involved in rotenone and dieldrin induced dopaminergic neuronal degeneration remain unclear however, oxidative stress mediated apoptosis has been shown to play a key role. Cho *et al.* (2008) demonstrated for the first time that theanine protects human dopaminergic neuronal cells against rotenone and dieldrin induced cytotoxicity. Theanine mediated neuroprotection in dopaminergic neurons entailed (a) modulation of specific neurotrophic factors associated with protective and reparative effects on the dopamine system, (b) blocking the decrease in extracelluar signal-regulated kinase1/2 (the anti-apoptotic molecule) and (c) significantly reducing the morphological changes of nuclei. In addition theanine prevented rotenone and dieldrin induced up-regulation of heme oxygenase-1 (a cellular protein expressed in brain and other tissues in response to reactive oxygen species [ROS]), implying that theanine can suppress ROS overproduction. This study opens a novel clinical approach for treatment of neurodegenerative diseases such as Parkinson's disease, though further studies are warranted to elucidate the mechanisms involved.

1.2.3 Extraction, Purification and Separation of Theanine

Compared to the other major components of tea, such as catechins and caffeine, there are significantly fewer reports in literature describing the extraction, purification

and separation of theanine. No comprehensive review could be found by the author and the following summary of the topic is unique to date.

1.2.3.1 Extraction Methods

Theanine is very soluble in water. Most researchers seems to exploit this property by using water at 80 °C, 90 °C or boiling point to extract theanine form fresh or dried tea leaves, thus avoiding the use of hazardous organic solvents. The factors affecting the extraction efficiency are the tea:water (w/v) ratio, temperature and extraction time (Lin *et al.*, 2004).

1.2.3.2 Purification Methods

A sample clean-up or purification step is often required before theanine analysis due to co-extractives from the tea leaves interfering with the analysis by co-eluting with theanine. Catechins, often the main interfering compounds, are removed with water/ethyl acetate partitioning (Ekborg-Ott *et al.*, 1997; Syu *et al.*, 2008; Zhang *et al.*, 2004). The catechins extract into the ethyl acetate phase while theanine and the other amino acids remain in the water phase. Solid phase extraction with strong cation resins are used to pre-concentrate theanine from crude tea extracts (Ekborg-Ott *et al.*, 1997; Zhang *et al.*, 2004). However, these resins are usually expensive. The polymeric adsorbent polyvinylpolypyrrolidone (PVPP), is an inexpensive alternative used to remove interfering polyphenolic compounds before analysis of theanine and other amino acids present in tea (Kato *et al.*, 2003). Recently, the use of a molecularly imprinted polymer as solid phase extraction sorbent for the clean-up

of water extracts of green tea was reported (Lachova´ *et al*., 2007). By imprinting L-theanine on the polymer it becomes selective for theanine.

1.2.3.3 Separation Methods

High Performance Liquid Chromatography.

High performance liquid chromatography (HPLC) along with high performance capillary electrophoresis (HPCE) are the dominate techniques used for separation of theanine in tea samples. Since theanine and most other amino acids lack a suitable chromophore, chromatography with post- or pre-column derivatization is usually carried out.   Reverse phase HPLC using O-phthalaldehyde (OPA) pre-column derivatization with fluorescence detection is the preferred method for quantification of amino acids in tea (Alcázar *et al*., 2007; Thippeswamy *et al*., 2006; Ying *et al*., 2005; Zhu *et al*., 2004).   Other reagents proposed for reverse phase HPLC with fluorescence detection of theanine include phenyl isothiocyanate (PITC), dabsyl chloride and 9-fluorenylmethoxycarbonyl glycine chloride (FMOC-Gly-Cl) (Ekborg-Ott *et al*., 1997; Syu *et al*., 2008; Thippeswamy *et al*. 2006). Low stability of amino acid derivatives, relative complexity of the reaction system or reagent interferences in pre-column derivatization are among the shortcomings of these methods. Alternative detection methods used (with reverse phase HPLC) include atmospheric pressure chemical ionization or electrospray ionization mass spectrometry and evaporative light scattering detection (Desai and Armstrong, 2004; Li Y *et al*., 2005). Simultaneous determination of catechins and other polyphenolics, purine alkaloids and underivatized theanine using reverse phase HPLC with UV detection at 210 and 280 nm and/or ESI mass spectrometry has also been reported (Peng *et al*., 2008; Zhu *et al*., 2004).   Alternatively, anion-exchange HPLC coupled to integrated pulsed

amperometric detection was used by Ding *et al.* (2002) for the analysis of theanine in tea samples.

High Performance Capillary Electrophoresis.

HPCE is widely used in many fields due to the fact that it is a highly efficient, economic and environmentally friendly technique. Several capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) methods using UV detection have been developed for the analysis of theanine, separately and together with other compounds found in tea (Aucamp *et al.*, 2000; Chen *et al.*, 2003; Horie and Kohata, 1998; Horie *et al.*, 1997). A disadvantage of these methods is that UV detection has to be carried out at short wavelengths (190 to 200 nm) as theanine lacks a suitable chromophore. Consequently, interference and poor sensitivity is an issue. To overcome this problem, MECK with fluorescence detection of the 2,4-dinitrofluorobenzene (DNFB) derivative (Li P *et al.*, 2005) or CZE with naphthalene-2,3-dicarboxaldehyde (NDA) as the derivatization reagent (Hsieh and Chen, 2007) is used. Furthermore, microchip-based electrophoresis with fluorescent detection for short time analysis of amino acids was developed to analyze theanine in Japanese green tea using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as derivatization reagent (Kato *et al.*, 2003).

Alternative Methods.

High resolution nuclear magnetic resonance (NMR) has been effectively used for simultaneous analysis of theanine and other amino acids, phenolics, fatty acids and sugars (Le Gall *et al.*, 2004; Fuijwara *et al.*, 2006). This technique is effective but expensive and not available in all laboratories. Capillary isotachophoresis (CITP) has also been successfully used to analyze theanine in tea extracts (Kvasnička and

Krátká, 2006). The main advantage of this technique is the markedly lower running costs compared to HPLC.

1.2.4 Green Tea Quality Assessment and Theanine

Many characteristics are taken into account to evaluate tea quality (Paul *et al.*, 2008). Teas of different origin have their own distinctive characteristics but in general high quality green teas are described as delicate or sweet (Nakagawa, 1975). The sweetness is attributed to amino acids, especially theanine which has a sweet, brothy or unami taste. Since theanine accounts for more than 50% of the free amino acid contents in tea, it is considered an important flavour component that influences the quality of green tea (Nakagawa, 1975). Other important components are caffeine and catechins that contribute to the characteristic astringency and bitterness of green tea.

The quality and price of tea is established through the judgement of professional tea tasters, but there is growing need to assess the quality of tea by some form of analytical measurement. Standard international analysis methods for the quantification of caffeine and catechins already exist but there is no standard analysis method for the important flavour component theanine (Liang *et al.*, 2008).

1.3 Catechins

Catechins are flavanols, a subclass of flavanoids that constitute up to 20% of the dry leaf weight of tea (Labbé *et al.*, 2006, Wang and Helliwell, 2000). The 6 main catechins present in tea are catechin (C), epicatechin (EC), gallocatechin (GC),

epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg), Figure 1.7. EGCg is the most abundant catechin found in green tea. In some cultivars of tea less abundant catechins are also present, these include epigallocatechin-3,5-digallate, epicatechin-3,5-digallate, epicatechin-3-(3"-O-methyl) gallate and epigallocatechin-3-(3"-O-methyl) gallate (Amarowicz *et al.*, 2005; Coxon *et al.*, 1972).

1.3.1   The Chemical Properties of Catechins

Catechins are colourless compounds which impart bitterness and astringency to tea infusions. They reside in the vacuole of the tea leaves and are both water and lipid soluble (Labbè *et al.*, 2006), an important factor to consider in understanding the antioxidant activities of catechins.

The stability of green tea catechins is temperature and pH dependant. Thermal degradation and oxidation of catechins increases with temperature. In alkaline solutions catechins are very unstable and decompose in a few minutes, whereas in acidic solutions below a pH of 4 they are stable (Lun Su *et al.*, 2003; Wang *et al.*, 2006).

Epimerization of catechins, the conversion into their corresponding isomers, occurs at temperatures above 80˚C in aqueous solutions. At higher pH conditions (≥ 6) the major catechins, EC, EGC, ECg and EGCg, tend to epimerize into the minor catechins C, GC, Cg and GCg. This process is not only influenced by temperature and pH, but also by heating time (Wang and Helliwell, 2000; Yoshida *et al.*, 1999).
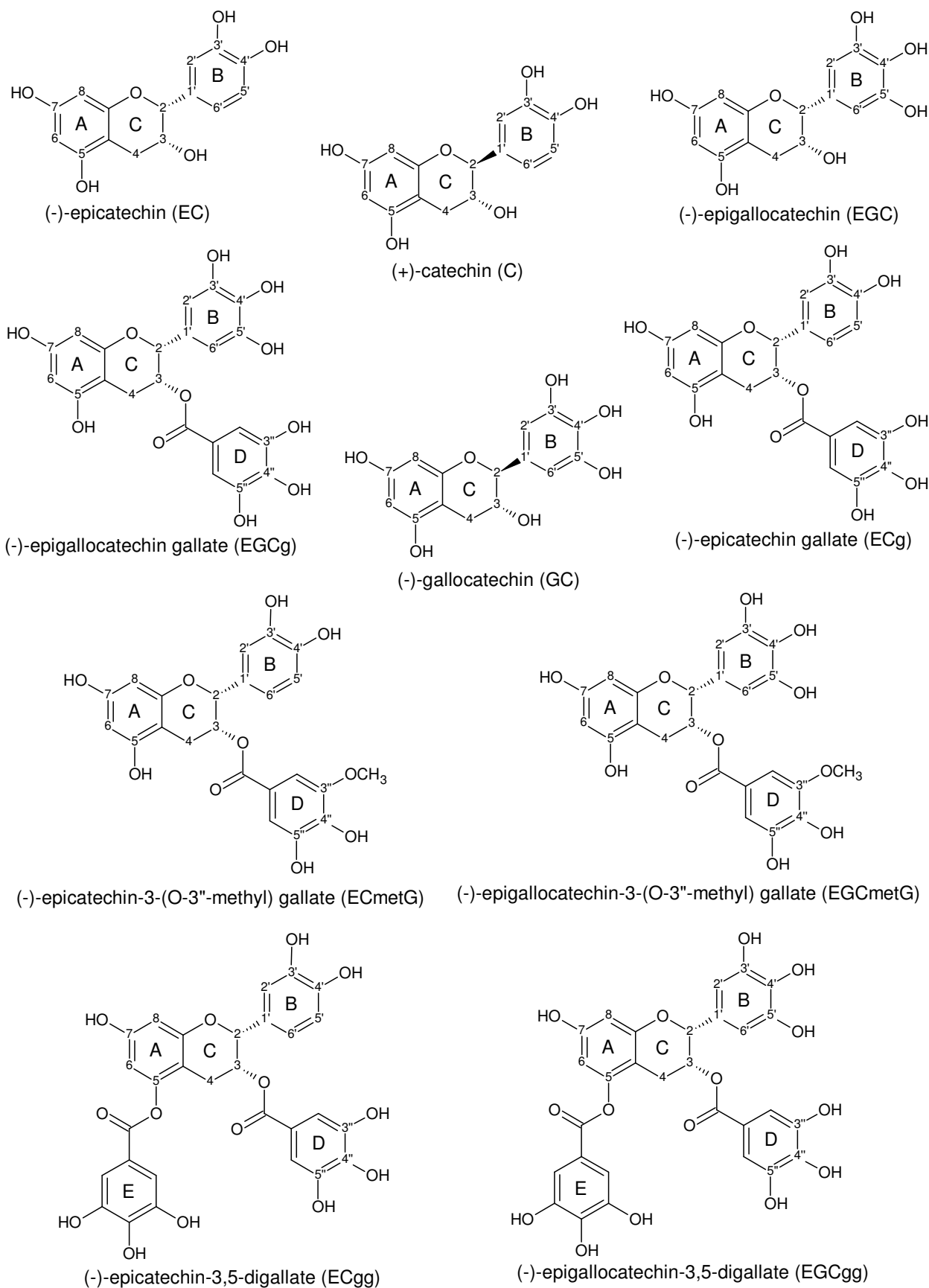
Figure 1.7. The structures of green tea catechins.

1.3.2 The Antioxidant and Health Properties of Catechins

Oxidative stress is the term used to describe the damage caused by free radical oxygen and nitrogen species to cell structures such as lipids, proteins and nucleic acids. These reactive oxygen species (ROS) and reactive nitrogen species (RNS) are:

- products of metal catalyzed reactions,

- by-products of mitochondria catalyzed electron transport reactions,

- produced by neutrophils and macrophages during inflammation,

- generated by irradiation with UV light, X-rays and gamma-rays,

- and are present in the atmosphere as pollutants.

Oxidative damage to DNA, proteins, lipids and other macromolecules has been hypothesized to play a key role in the development of age-related diseases such as arthritis, cancer, cardiovascular disease and neurodegenerative disorders. The harmful effects caused by these free radical oxidants are countered by the activities of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase. Examples of non-enzymatic antioxidants are vitamin C or ascorbic acid, vitamin E or α-tocopherol, flavonoids, carotenoids, thiol antioxidants such as glutathione, etc. (Valko *et al.*, 2006).

Green tea catechins have been identified as potent antioxidants and active scavengers of free radicals. They have been found to be effective in the prevention of various free-radical-induced and –mediated diseases such as cardiovascular diseases and arthritis (Coyle *et al.*, 2008; Jain *et al.*, 2006).

1.3.2.1 Possible Mechanisms for the Antioxidant Effects of Catechins

Radical and Oxidant Scavenging.

Over the past two decades there has been considerable interest in the mechanisms of antioxidant activities of flavonoids. Several studies have shown that antioxidative activities of catechins can be attributed to their ability to scavenge for reactive oxygen and nitrogen species (Burda and Oleszek, 2001; Dugas *et al.*, 2000; Haenen *et al.*, 1997; Jain *et al.*, 2006; Salah *et al.*, 1995; Sang *et al.*, 2002; Schroeter *et al.*, 2002). An important characteristic that influences this ability of a compound to act as a free radical scavenger is the standard one-electron reduction potential ($E^{\circ\prime}$). The lower the value of $E^{\circ\prime}$ the less energy is necessary for hydrogen and electron donation and therefore the better the scavenging ability. The reduction potentials of tea catechins (Table 1.2) are similar to that of vitamin E, but vitamin C has a lower reduction potential, implying a superior hydrogen donating ability.

Table 1.2. Standard one-electron reduction potentials for tea catechins (Adapted from Frei and Higdon, 2003). Standard reduction potential at pH 7.0 and 20 ˚C.

Antioxidant	Reduction Potential (mV)
Ascorbic Acid (Vitamin C)	280
Tocopherol (Vitamin E)	480
Glutathion	920
(-)-Epigallocatechin gallate	430
(-)-Epigallocatechin	430
(-)-Epicatechin	570
(-)-Epicatechin gallate	550
Theaflavin	510
Theaflavin digallate	540

Structural features defining the reduction potential of catechins include a 3'4'-dihydroxy catechol structure or a 3',4',5'-trihydroxyl group in the B ring, a gallate group esterified at position 3 in the C ring and hydroxyl groups at positions 5 and 7 in the A ring, see Figure 1.8 (Frei and Higdon, 2003). Due to the heterocyclic ring being saturated, there is no electron delocalization between the A and B rings.
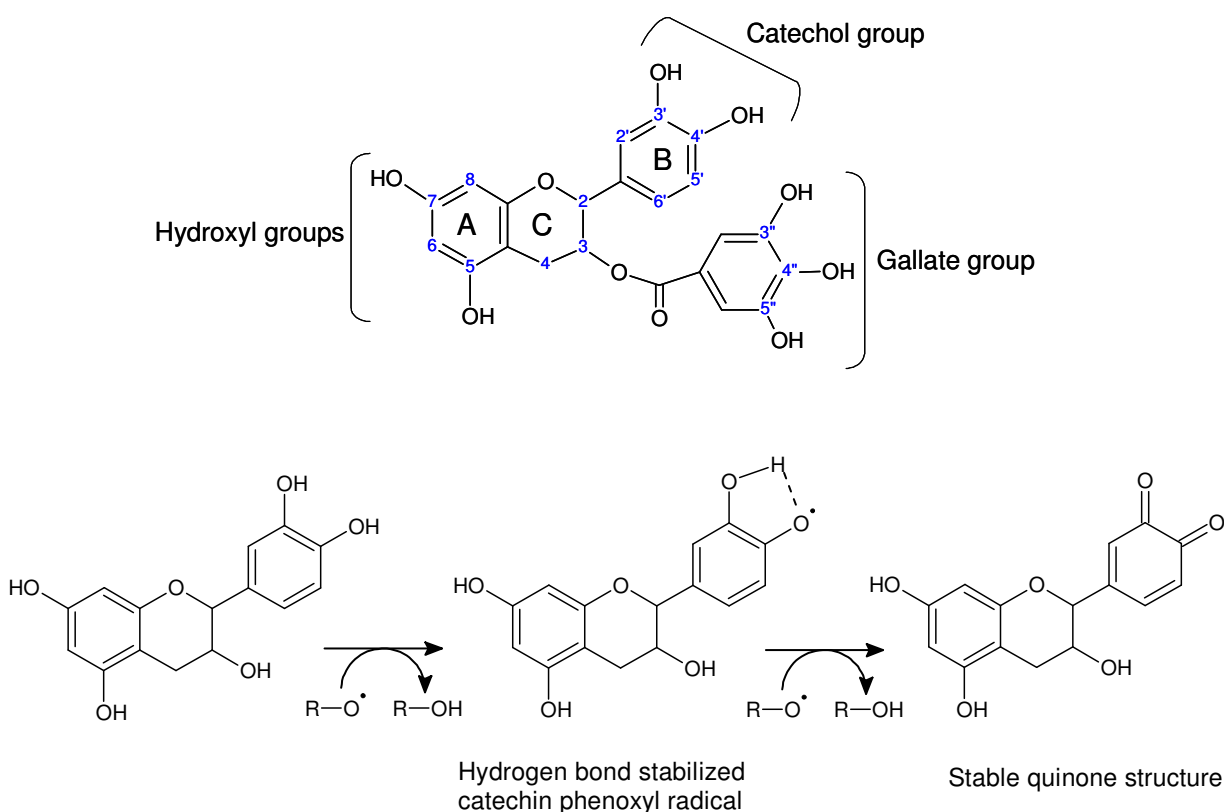
Figure 1.8. Functional groups important to the antioxidant activity of catechins, example EGCg, and the mechanism of antioxidant action, example catechin (Adapted from Frei and Higdon, 2003; Amić *et al.*, 2007).

The structures of the catechins and catechin gallate esters are shown in Figure 1.7. Gallocatechins have 3 hydroxyl groups in the B ring and the gallated catechins have a gallic acid esterified at position 3 in the C ring. The relative antioxidant potentials against radicals in the aqueous phase, expressed as the Trolox (a water-soluble

vitamin E analogue) equivalent antioxidant capacity (TEAC), are shown in Table 1.3. From these it is evident that the antioxidant activity of catechins are governed by the number and location of hydroxyl groups on the flavonoid ring system (Cao *et al.*, 1997; Salah *et al.*, 1995; Lien *et al.*, 1999). In contrast to the predictions made based on the standard redox potentials, catechin and epicatechin have TEAC values of 2.4 and 2.5 mM, respectively, antioxidant capacities more than twice as effective as vitamins C and E (TEAC, 1 mM). This superior radical-scavenging potential was confirmed by the results of a 1,1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant capacity assay (Figure 1.9), (Jain *et al.*, 2006). The gallated catechins have higher TEAC values than the non-gallated catechins, reflecting the contribution of the gallic acid in position 3. This correlates with the results of Guo *et al.* (1999), they investigated the scavenging effects of tea catechins and their epimers with electron spin resonance (ESR) and found that the scavenging abilities of gallated catechins (EGCg and ECg) are stronger than the non-gallated catechins (EGC, GC, EC, C). This suggests that the gallate group at position 3 plays an important role in the free radical scavenging abilities of the catechins. In addition, it was found that the scavenging effects of GC and GC were stronger than those of EC and C. This implies that the additional hydroxyl group at the 5' position in the B ring also contributes to the scavenging activities of catechins.

Table 1.3. Relative antioxidant potentials (Trolox Equivalent Antioxidant Activities) of tea catechins (Salah *et al.,* 1995).

Antioxidant	TEAC (mM)
Epicatechin gallate	4.93
Epigallocatechin gallate	4.75
Epigallocatechin	3.82
Epicatechin	2.50
Catechin	2.40
Gallic Acid	3.01
Quercetin	4.72
Rutin	2.42
Vitamin E	1
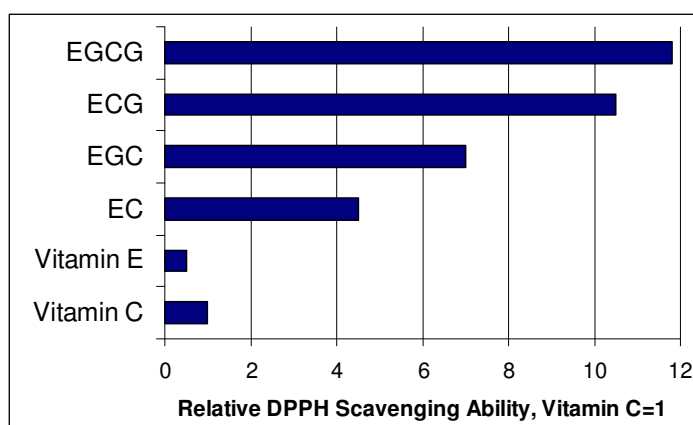
Figure 1.9. Relative radical scavenging potency of catechins of the DPPH radical (Jain *et al.*, 2006).

Catechins are also effective antioxidants in lipophilic environments. It was shown that EGCg inhibited lipid peroxidation, induced by boiling lard at 97.8 ˚C and bubbling air through, 20 times more effective than α-tocopherol and 5 times more effective than butylated hydroxyanisole (BHA), a phenolic antioxidant added to foods to preserve fats (Jain *et al.*, 2006).

Metal Chelation.

The chelating properties of catechins contribute to their antioxidant activity. Catechins chelate divalent metals, such as iron and copper, preventing these redox-active transition metals from catalyzing free radical formation (Ryan *et al.*, 2007). A chelating complex with divalent metal cations is formed between the 3'- and 4'- OH on the B-ring as shown in Figure 1.10.
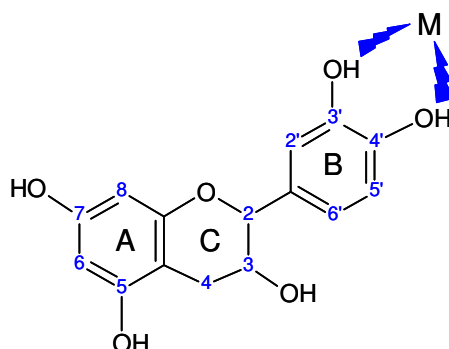
Figure 1.10. The structure of catechin and a possible position for a coordinated metal ion (M).

Other Mechanisms.

Other mechanisms related to the antioxidant activities of catechins include inhibition of redox-sensitive transcription factors, such as nuclear factor-κB and activator protein-1 (Frei and Higon, 2003). Catechins also inhibit "pro-oxidant" enzymes, such as lipoxygenases and cyclo-oxygenases, that are capable of co-oxidizing molecules other than their regular substrates, potentially increasing oxidative damage in some tissues (Jain *et al.*, 2006). Furthermore, catechins can induce phase II metabolic enzymes, e.g. glutathione S-transferases, that are involved in detoxification of potentially toxic, carcinogenic or free radical inducing chemicals (Zhen *et al.*, 2007).

1.3.2.2 Other Health Effect of Catechins

A wide array of health promoting properties is displayed by catechins. These include anti-cancer, antiviral, antibacterial, anti-allergic and anti-inflammatory effects. In animal models, cancer preventive effects reported include a decrease in tumor incidence, multiplicity and size. It was observed that catechins can suppress cancer growth by blocking angiogenesis (Cooper *et al.*, 1995; Khan and Mukhtar, 2007). Catechins also have bactericidal activity and show synergism with antibiotics. (Hamer, 2007). For viral infections such as influenza, it has been reported that infectivity and proliferation is inhibited by catechins by blocking absorption, haemmagglutiantion and virus assembly (Jain *et al.*, 2006). It has even been demonstrated that catechins suppress glucose uptake by inhibiting the sodium-dependant transporter SGLT1 mechanism, preventing diabetes (Khan and Mukhtar, 2007).

1.3.3 Extraction, Purification and Separation of Catechins

1.3.3.1 Extraction Methods

Water and organic solvents such as methanol, ethanol, chloroform, acetone, ethyl acetate, acetonitrile and combinations of these solvents and water are used to extract green tea catechins. Extraction conditions, such as solvents used, temperature, pH, time, ratio of solvent-to-sample and the number of extractions influences the extraction efficiency and the quality of extract obtained (Bazinet *et al.*, 2007; Lin *et al.*, 2008; Perva-Usunalić *et al.*, 2006; Ruzak *et al.*, 2008; Yoshida *et al.*, 1999).

Water extraction seems to be the choice of most researchers. The extraction efficiency of major catechins with water as extractant, is a function of the extraction time, temperature, pH and the tea:water (w/v) ratio. During water extraction at higher pH, epimerization and degradation of catechins occur. As a result, extraction efficiency decreases from more than 90% at a pH of 6 to 40% with an increase in pH to 7.2. An increase in tea concentration causes an increase in pH, consequently extraction efficiency declines when the tea:water (w/v) ratio exceeds 1% (Yosihida *et al.*, 1999). With prolonged extraction time, the content of major catechins in the water extract decreases due to thermal degradation, epimerization and oxidation (Perva-Usunalić *et al.*, 2006). Multiple extraction steps, between 2 to 5, significantly enrich the extract in major catechin content (Perva-Usunalić *et al.*, 2006; Sharma *et al.*, 2005).

Organic solvents generally extract catechins more efficiently than water, without epimerization of major catechins (Lun Su *et al.*, 2003; Yoshida *et al.*, 1999). In literature, most publications report that methanol, ranging from 70 to 100%, extracts qualitatively and quantitatively higher amounts of catechins than acetonitrile, ethanol, chloroform and ethyl acetate (Perva-Usunalić *et al.*, 2006).

However, the use of large amounts of organic solvents poses health and safety risks and is environmentally unfriendly. Alternative methods that reduce or eliminate the use of organic solvents includes microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). Table 1.4 summarizes these alternative methods.

Table 1.4. Alternative extraction methods for catechins.

Methods	Advantages, Disadvantages, Comments
Microwave-Assisted Extraction (MAE)	MAE combines microwave energy and the use of solvent. The advantages of MAE are shorter extraction times, less solvent use and high extraction efficiencies.
Supercritical Fluid Extraction (SFE)	Supercritical carbon dioxide is used in SFE. It has gas-like characteristics that help the liquid diffuse to the matrix and access the catechins, while the liquid-like characteristics offer good solvation. Supercritical carbon dioxide extraction has been successfully used to obtain caffeine-free green tea catechins (Chang *et al.*, 2000; Kim *et al.*, 2008; Park *et al.*, 2007). However, this is a very expensive technique.
Pressurized Liquid Extraction (PLE)	In PLE high pressure and elevated temperatures are applied to achieve fast and efficient extractions. Piñeiro *et al.* (2004) demonstrated that pressurized liquids can extract catechins effectively and found extraction for 10 minutes with methanol at 130 ˚C and 100 atm, produced the highest yields.

1.3.3.2 Purification Methods

Often after extraction a sample clean-up, purification or pre-concentration step is incorporated before analysis. Preliminary purification is achieved by using water/chloroform (1:1, v/v) partitioning followed by water/ethyl acetate (1:1, v/v) partitioning (Lun Su *et al.*, 2003). Caffeine and pigments readily extract into the chloroform layer and the catechins move into the ethyl acetate layer, leaving behind all the water soluble impurities. Precipitation of catechins from a tea extract using calcium carbonate or calcium hydroxide is another method employed to accomplish preliminary purification (Zhang *et al.*, 2006). Catechins and other polyphenolics complex with the divalent calcium ion and form a precipitate. Solid phase extraction, usually with Sephadex LH-20 as resin, is used to obtain a purified catechin fraction from the crude/semi-purified tea extract (Amarowicz *et al.*, 2005; Amarowicz and Shahidi, 1996; Row and Jin, 2006).

Recently, lignocellulose prepared from woody tea stalks was successfully used as adsorbent to isolate decaffeinated catechins from tea extracts (Ye *et al.*, 2009). Tea stalk lignocellulose preferentially absorbed catechins, showing good selectivity.

1.3.3.3 Separation Methods

High Performance Liquid Chromatography.

High performance liquid chromatography (HPLC) has generally been the method of choice for the analysis of tea catechins and a vast amount of literature exists about this method (Neilson *et al.*, 2006; Peng *et al.*, 2008; Sharma *et al.*, 2005; Yang *et al.*, 2007; Yao *et al.*, 2004; Zhu *et al.*, 2004; Zuo *et al.*, 2002). A typical HPLC method includes the use of a reverse phase C_{18} column, a UV-vis diode array detector (DAD) and a binary solvent system. The solvent system usually consists of acidified water (solvent A) and a polar organic solvent (solvent B). Solvent A typically includes aqueous acids or additives such as phosphate, whereas solvent B is usually pure or acidified methanol or acetonitrile. The acid in the mobile phases is essential for complete resolution of catechins and elimination of peak tailing. The analysis time is anything from 10 to 150 minutes per sample and flow rates vary from 1 to 1.5 ml/min. Hyphenated techniques are used to identify and quantify catechins present in trace amounts, such as in biological samples. For example, HPLC-DAD-mass spectrometry (MS) provides high sensitivity for quantification of catechins at low concentrations (Del Rio *et al.*, 2004; Pelillo *et al.*, 2004; Poon *et al.*, 1998; Wang *et al.*, 2008). However, MS is expensive and not commonly available in most laboratories.

High Performance Capillary Electrophoresis.

Even though HPLC is the most dominating separation technique for catechins, high performance capillary electrophoresis (HPCE) is gaining popularity. HPCE has several advantages: high efficiency due to non-parabolic fronting, short analyzing time, low cost (especially with capillary zone electrophoresis) and small amounts of organic solvent is used. In capillary zone electrophoresis (CZE) borate based buffers are employed to separate the major catechins. The separation of catechins is based on their complexation with the borate ions present in the running buffer, via the ortho-hydroxy groups in the B ring of the catechin structure (see Figure 1.11). Micellar electrokinetic capillary chromatography (MEKC) utilizes sodium dodecyl sulfate (SDS) in borate based running buffers. The surfactant SDS is present at critical micellar concentrations and readily forms micelles with the hydrophilic head groups orientated to the surface and the lipophilic tails projecting into the interior of the micelle. Compounds with different partition coefficients (K) distribute differently between the micelles and the mobile phase, adding an additional separating mechanism. Several studies reported the separation of major green tea catechins using CZE or MEKC (Bonoli *et al*., 2003; Larger *et al*., 1998; Lee and Ong, 2000; Vaher and Koel, 2003; Wörth *et al*., 2000).
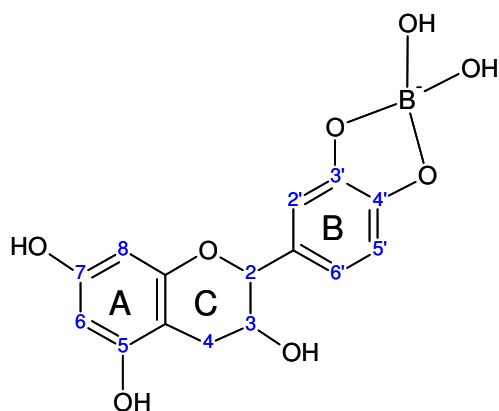
Figure 1.11. Complexation between a borate ion and the ortho-hydroxyl groups in the B ring of catechin results in the formation of a negatively charged borate complex (acidic pH) (Adapted from Spiro and Price, 1986). In capillary zone electrophoresis (CZE) borate based buffers are employed to separate the major catechins present in tea.

Other methods successfully used for the separations and/or isolation of catechins in tea extracts are summarized in Table 1.5.

Table 1.5. Separation methods of catechins, excluding the popular techniques HPLC and HPCE.

Method	Advantages	Disadvantages	Comments
Conventional chromatography: Paper, Packed column (CC) and Thin-layer (TLC) (Amarowicz and Shahidi, 1996; Amarowicz *et al.*, 2005)	No expensive instrumentation required.	Poor separation efficiency and difficulty in detection and quantification.	TLC and CC are used in the preliminary purification, separation and fractionation of catechins, before separation, identification and quantification by HPLC or other high-performance separation techniques. During CC fractionation TLC is often used to monitor the eluent.
Gas Chromatography (GC) (Shadkami *et al.*, 2008; Dalluge *et al.*, 2000)	High sensitivity and good resolution.	A derivatization step is required to convert the catechins to volatile compounds.	The derivatization step is labour intensive and time consuming. This, along with the fact that large scale separation and purification with GC is problematic, results in GC being a less popular separation method than HPLC.
High Speed Counter-Current Chromatography (HSCCC) (Baumann *et al.*, 2001; Cao *et al.*, 2001; Kumar *et al.*, 2005; Yanagida *et al.*, 2006)	Elimination of sample loss due to adsorption to the solid stationary phase, easy scale-up and low cost as no expensive absorbent or column is used.	Large volumes of organic solvents are used.	Counter-current chromatography (CCC) separates compounds based their on partition coefficients (*K*). HSCCC is a rather new technology and is the most advanced form of CCC in terms of efficiency and separation time. It is aided by pressure and centrifugal force and does not use solid support as stationary phase.

1.3.4 Digallated and Methylated Catechins

Recently, there is an accumulating body of evidence showing that methylated forms of catechins have potent inhibitory activities to allergies (Chiu and Lin, 2005; Fujimara *et al.*, 2007; Maeda-Yamamoto *et al.*, 2004; Maeda-Yamamoto *et al.*, 2007). However, knowledge of the pharmacology of these catechins is still limited. These methylated catechins have been detected as minor components in Chinese, Vietnamese and Indian green teas (Saijo and Takeda, 1999).

In addition to the methylated catechins, another relative novel group of catechins exists, namely digallated catechins. Digallated catechins were first discovered in green tea in 1959 by Vuataz *et al.* though, at that stage the identity of the compound was still unknown. Later in 1972, Coxon *et al.* repeated the work of Vuataz *et al.* and identified the unknown compound as epigallocatechin-3,5-digallate using UV spectra and NMR analysis. They also identified a second digallated catechin, epicatechin-3,5-digallate. Since then very little research has been done on these specific flavanols, possibly as they are usually present in very small amounts, if at all. The medicinal value of these compounds is not yet known, but it is reasonable to speculate that the antioxidant capacity of these compounds will be superior to that of epigallocatechin gallate (EGCg) due to the 3 additional hydroxyl groups provided by the second gallic acid moiety (see section 1.3.2.1). The antioxidant capacity of flavonoids increases with the number of hydroxyl groups on the ring structure (Rice-Evans *et al.*, 1996; Rice-Evans, 2001), see Figure 1.12. Catechins also broadly follow this tenet as illustrated by Figure 1.13.
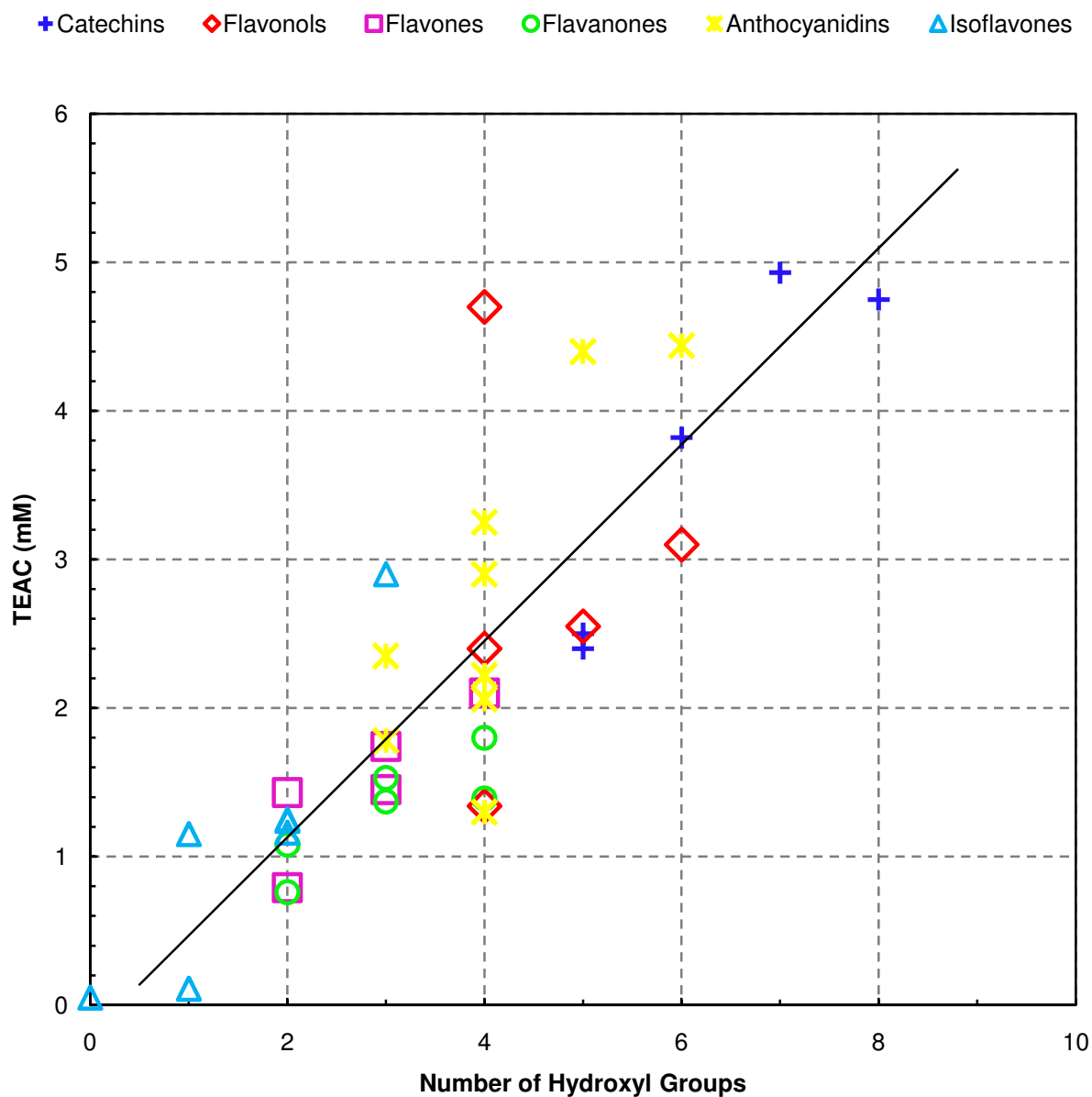
Figure 1.12. The antioxidant capacity of flavonoids generally increases with the number of hydroxyl groups on the ring structure (Adapted from Heim *et al.,* 2002; Lien *et al.*, 1999). The 6 subclasses of flavonoids are indicated by different symbols and colours.
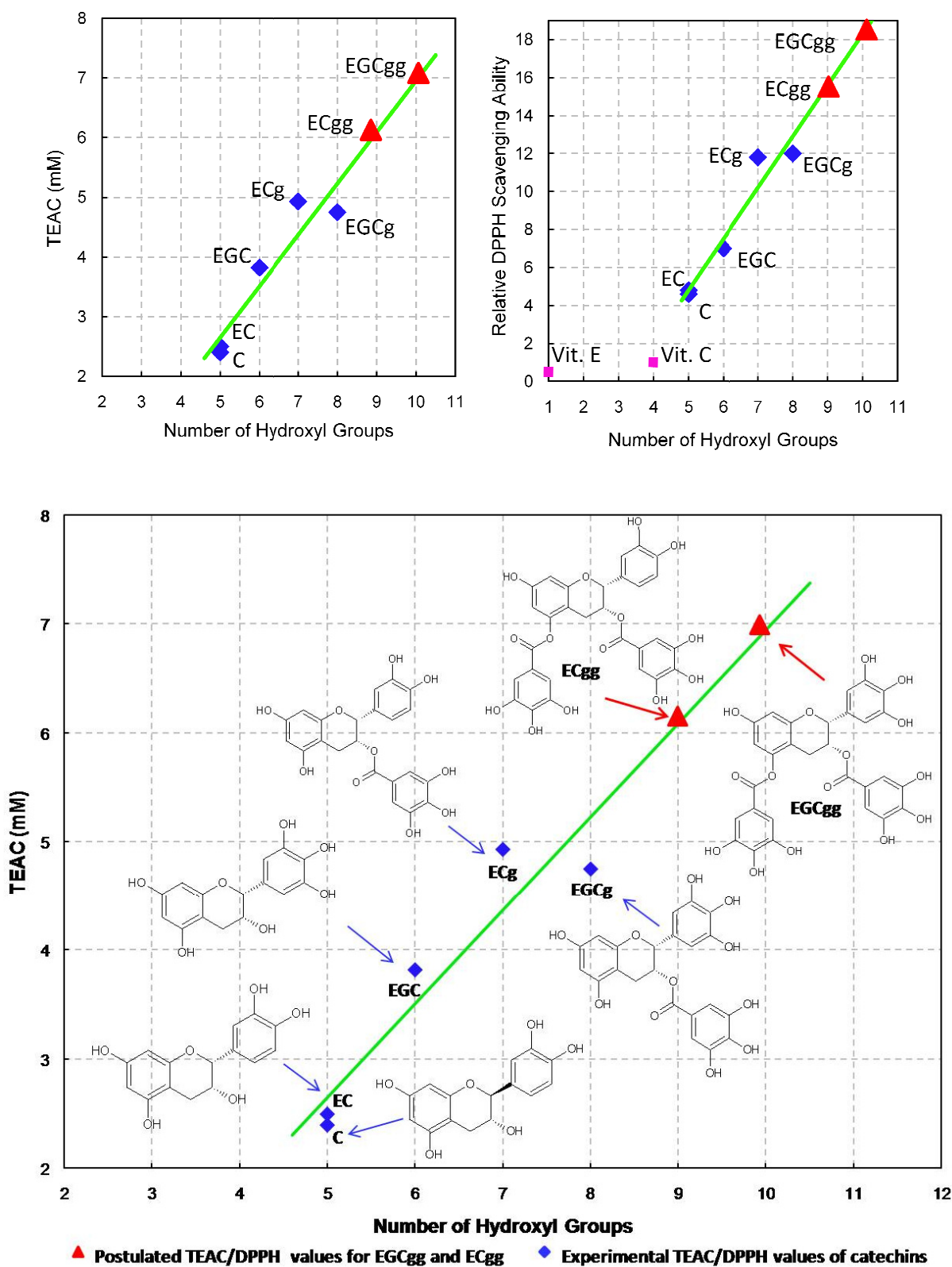
Figure 1.13. Influence of the number of hydroxyl groups on the TEAC and DPPH values of catechins. By extrapolating the trend, the TEAC and DPPH values of the digallated catechins is postulated to be even higher than the TEAC and DPPH value of the powerful antioxidant, EGCg. (TEAC values of catechins obtained from Salah *et al.*, 1995; DPPH values obtained from Jain *et al.*, 2006). **NOTE:** The relative DPPH value of vitamin C is 1 and vitamin E is 0.5.

In order to detect digallated and methylated catechins present in green tea extracts for which standards are not available, separation and detection techniques are required that provide sufficient structural information to confirm the identity of these compounds.

1.4 Aim

The main goal of this study is to develop and validate a simple but reliable and sensitive method for the analysis of theanine that can be adapted in quality control laboratories worldwide (Chapter 2).

The second goal of this research project is the detection and identification of digallated and methylated catechins in crude green tea extract using UPLC/MS/MS to assist in future large scale extraction from fresh green tea leaves (Chapter 3).

1.5 Hypothesis

Hypothesis I: Reverse phase HPLC with evaporative light scattering detection will be a simple, fast and reliable method for analysis of theanine in tea samples:

- The analysis time will be shorter than the analysis time of the current HPLC-ELSD method
- The intra- and inter-day coefficient of variation will be less than 5%
- The recovery will be more than 95%

Hypothesis II: Application of UPLC/MS/MS will aid in the identification of digallated and methylated catechins in a crude tea extract.

Chapter 2

Rapid Analysis of Theanine Utilizing Evaporative Light Scattering Detection in Tea (*Camellia sinensis*)

2.1 Introduction

Theanine, also known as γ-glutamylethylamide (Figure 2.1), is a free, non-protein amino acid found in *Camellia sinensis* (tea) and certain other species in the *Camellia* genus (*C. japonica* and *C. sasanqua*). The only other known natural source of theanine is *Xerocomus badius*, an edible mushroom common in Europe and North America (Casimir *et al.*, 1960).
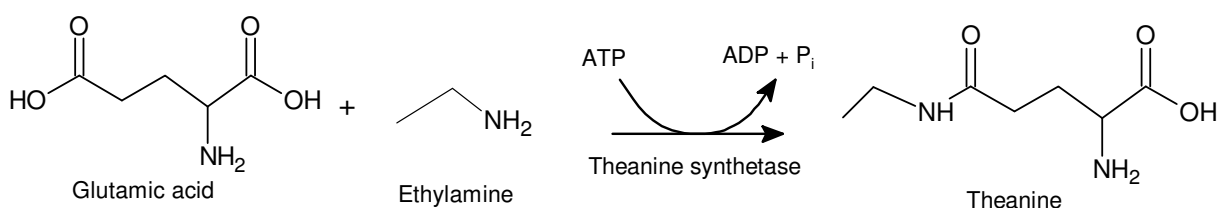
Figure 2.1. Theanine biosynthesis from glutamic acid and ethylamine.

Theanine synthetase (E.C. 6.3.1.6) synthesizes theanine mainly in the roots of tea plants from glutamic acid and ethylamine. From there it is transported to young leaves where it serves as a major source of soluble nitrogen for alkaloids (caffeine)

and other carbon skeletal compounds (Ying *et al.*, 2005). It is also an essential precursor for the biosynthesis of catechins (Deng *et al.*, 2008; Kito *et al.*, 1968).

Theanine is responsible for the unique, sweet, brothy umami taste of green tea and while other free amino acids are only found at trace level, theanine ranges from 1 to 2% of the dry weight. This constitutes up to 50% of the total free amino acid content of green tea, an important quality control index of green tea. Higher grade green teas have been found to contain higher amounts of theanine (Ekborg-Ott *et al.*, 1997; Horie *et al.*, 1997; Kaneko *et al.*, 2006; Kato *et al.,* 2003; Nakagawa, 1975; Nakagawa, 1970). In black tea however, theanine is a less important flavour component (Ekborg-Ott *et al.*, 1997; Ying *et al.*, 2005).

Lately, there has been an increased interest in theanine, not only because it is an important quality parameter, but because of its various pharmacological and physiological effects. Several studies reported the co-operative effects of theanine and anti-tumor agents, such as doxorubicin and idarubicin, *in vitro* and in animal studies (Sadzuka *et al.,* 2006; Sadzuka *et al.*, 2000a,b; Sugiyama and Sadzuka, 2004; Sugiyama and Sadzuka, 2003). Yokogoshi and Kobayashi (1998) demonstrated that administrating theanine to spontaneously hypertensive rats decreased their blood pressure. Theanine was also reported to enhance learning abilities in animals (Juneja *et al.*, 1999; Yokogoshi *et al.*, 2000), inhibit caffeine stimulation (Kakuda *et al.*, 2000) and induce relaxation effects in humans, probably through its ability to affect serotonin, dopamine and other neurotransmitters (Juneja *et al.*, 1999; Kimura *et al.,* 2007; Rogers *et al.*, 2008; Yamada *et al.*, 2005). The findings of a study by Kakuda *et al.* (2000) indicated that theanine may be clinically useful for preventing ischemic neuronal damage. Furthermore, a study by Cho *et al.*

(2008) suggested that theanine provides neuroprotection against Parkinson's disease-related neurotoxins and may be clinically useful for preventing Parkinson's disease symptoms.

Numerous methods for the analysis of theanine in tea have been reported. These include reverse phase high performance liquid chromatography (HPLC) of the phenylthiocarbamoyl, o-phthalaldehyde, 9-fluoromethoxycarbonylglycine chloride, 2,4-dinitrofluorobenzene and isothiocyanate derivatives (Ekborg-Ott *et al.*, 1997; Friedman *et al.*, 2007; Thippeswamey *et al.*, 2006; Ying *et al.*, 2005) and capillary electrophoresis of the 2,4-dinitrofluorobenzene and 4-fluoro-7-nitro-2,1,3-benzoxadiazole derivatives (Kato *et al.,* 2003; Li P *et al.*, 2005). Analysis without derivatization includes nuclear magnetic resonance spectrometry (Fujiwara *et al.*, 2006; Le Gall *et al.*, 2004), anion exchange chromatography coupled to mass spectrometry or integrated pulsed amperometric detection (Ding *et al.*, 2002), capillary electrophoresis and micellar electrokinetic capillary electrophoresis chromatography with UV detection (Aucamp *et al.,* 2000; Chen *et al.*, 2003; Horie and Kohata, 1998; Horie *et al.*, 1997) and reverse phase HPLC coupled to mass spectrometry (Desai and Armstrong, 2004; Zhu *et al.*, 2004).

Some of these methods reported require specialized columns and/or sophisticated instrumentation not present in general quality control laboratories and most of these methods involve complex and time consuming sample preparation, which includes clean-up and/or derivatization procedures. Eliminating derivatization (pre- of post-column) procedures simplifies these methods, but like most amino acids with the exception of a few that contain an aromatic group, theanine does not fluoresce. Direct detection by UV absorbance is only possible at a very low wavelength where

background absorption interference and poor sensitivity becomes a problem. All this limits the use of the above mentioned methods for the routine analysis of theanine. A simple alternative is evaporative light scattering (ELS) detection. It allows direct detection of all non-volatile compounds regardless of their chemical structure, making it ideal to detect compounds lacking a chromophore/suitable chromophore.

2.1.1 Functioning, Advantages and Limitations of the Evaporative Light Scattering Detector

An evaporative light scattering detector (ELSD) functions as follows (Figure 2.2). The eluent from the chromatographic column enters the nebulizer where it is transformed to an aerosol with the aid of a carrier gas. From there the fine droplets are carried into the heated drift tube by the gas where the solvent is evaporated leaving behind small particles of pure solutes. At the end of the drift tube a monochromatic or polychromatic light beam passes through the sample cell and is scattered by the particles present in the gas flow. Three light scattering mechanisms can occur depending on the ratio of the particle diameter (D) to the wavelength (λ) of the light source. Rayleigh scattering occurs when $D/\lambda < 0.1$, Mie scattering when $0.1 < D/\lambda < 10$ and reflection-refraction occurs when $D/\lambda > 10$ (Charlesworth, 1978; Van der Meeren *et al.*, 1992). The scattered light is detected by a photomultiplier tube, positioned at a 120° angle to the direction of the incident beam. The measured light is proportional to the amount of sample in the cell and is not dependant on a chromophore or a specific functional group (Christen and Veuthey, 2001). Therefore, similar to refractive index detectors, ELSDs are considered to be universal. But the ELSD has an added advantage in that it is compatible with a wider range of solvents and modifiers. It also produces stable baselines during gradient elution

chromatography because the response is independent of the spectral properties of the analyte and the solvents used (Stolywho *et al.*, 1984; Stolywho *et al.*, 1983).
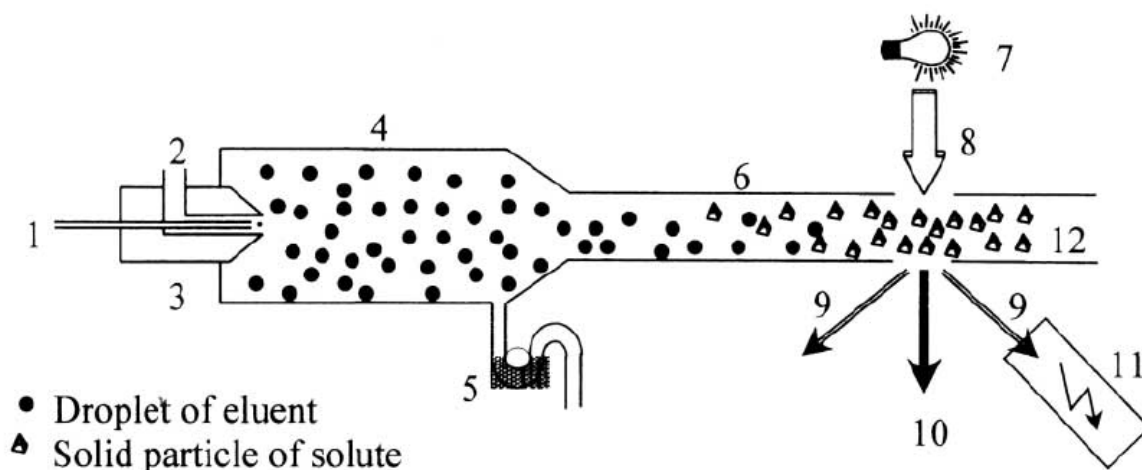
Figure 2.2. Schematic representation of an evaporative light scattering detector (Christen and Veuthey, 2001). 1: Effluent. 2: Nebulizing gas. 3: Concentric nebulizer. 4: Nebulizing chamber. 5: Waste liquid (settled droplets). 6: Heated drift tube. 7: Light source. 8: Light beam. 9: Diffracted light. 10: Transmitted light. 11: Photo-multiplier. 12: Gas exhaust.

The main limitations of the ELSD are the relatively low sensitivity, the non-linear response and the difficulties in determining very volatile compounds such as ethanol in wine (Stolywho *et al.*, 1983; Stolywho *et al.*, 1984). Small-molecule sensitivity with the ELSD is limited. The general observed limit of detection is 50 to 100 ng. By comparison some UV and fluorescence detectors yield limits of detection in the fentogram range (Young and Dolan, 2003). The non-linear response of the ELSD is a result of the various light scattering mechanisms and the particle size distribution. The ELSD response does not obey Beer's law, but instead is described by the empirical model (Stolywho *et al.*, 1983):

$$y = am^b$$

where y represents the observed peak area, m is the amount analyte injected, b is the slope of the response line and a is the response factor. Logarithmic values for y and m will produce a linear trend (Young and Dolan, 2003):

$$\log y = a + b \log m.$$

2.1.2 A Unique HPLC-ELSD Method for the Analysis of Theanine in Tea Samples

Many studies have demonstrated the relationship between the quality and the amino acid content of green tea (Kaneko *et al.*, 2006; Kato *et al.,* 2003; Nakagawa, 1975; Nakagawa, 1970). Theanine is the predominant free amino acid present in green tea, rendering it an important quality control parameter but to date there is no international standard method for the analysis of theanine (Liang *et al.*, 2008 and to the knowledge of the author). As mentioned before, several methods with different advantages and disadvantages have been reported for the analysis of theanine but most of them are unsuitable for routine quality control analysis. Therefore, it is important to develop a simple but reliable and sensitive method that can be adopted in quality control laboratories worldwide.

The objective of this study was to develop a simple reverse phase HPLC method for the routine analysis of theanine in tea leaves/commercial tea samples with ELS detection, eliminating the need for any time consuming derivatization processes or expensive detection techniques such as mass spectrometry.

Using ELS detection for theanine analysis is a relative novel approach. A method reported by Li Y *et al.* (2005) was the only other published method found in literature

that uses ELS detection for determination of theanine in tea samples. The Li Y *et al.* method uses reverse phase HPLC with gradient elution and requires no sample clean-up. The method reported in this study also uses reverse phase HPLC but is simpler because it uses isocratic elution. It is also better suited to high sample-through-put due to the analysis time that is only a third of the analysis time of the Li Y *et al.* method. The shorter analysis time was achieved by including a simple, fast but effective sample clean-up procedure utilizing polyvinylpolypyrrolidone (PVPP).

Specifically, the present study describes the development and validation of a HPLC-ELSD method for the analysis of theanine in tea samples unique to date.

2.2 Materials and Methods

2.2.1 Materials

All reagents were of analytical grade unless otherwise indicated. Theanine, leucine, glutamic acid, gallic acid monohydrate and polyvinylpolypyrrolidone (PVPP), Acetonitrile E CHROMASOLV® HPLC grade and methanol CHROMASOLV® for HPLC were purchased from Sigma Chemical Company (Steinheim, Germany). Acetic acid (Ultra $\geq$ 99.5%) was purchased from Fluka (Steinheim, Germany). Pharmaceutical grade L-glutamine was bought from Evox Advanced Nutrition (Johannesburg, Republic of South Africa). Folin-Ciocalteu reagent and anhydrous sodium carbonate were purchased from Merck (Darmstadt, Germany). Whatman® filter paper circles 595, $\varnothing$ 125 mm, were obtained from Schleicher & Schuell Microscience (Dassel, Germany) and Minisart hydrophilic 0.2 µm syringe filters were bought from Sartorius (Hannover, Germany). All solutions and dilutions were prepared with double distilled de-ionized water (dddwater), produced by an ELGA PURELAB Ultra water purification system from Veolia Water Systems Ltd (High Wycombe, United Kingdom) unless otherwise stated.

2.2.2 Tea Samples

In this study, commercial tea and specialty tea samples were purchased from the local supermarket. Japanese Green tea samples were kindly donated by Chajin, the famous tea salon in Paris, France.

2.2.3 Instrumentation

A Waters Alliance 2690 HPLC (Milford, MA, USA) equipped with a Waters 2966 Photodiode Array detector and a Waters 2420 Evaporative Light Scattering detector (ELSD) was used. Waters Empower 2 software (Build nr 2154) was used for data acquisition and processing. A sonication bath (7.2 L Ultrasonic Cleaner, 40 KHz operating frequency, Optima Scientific, Republic of South Africa) was used to degas all mobile phases under vacuum prior to use. For sample preparation, a Heraues Labofuge 300 centrifuge from ThermoScientific (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.2.4 Method Development

2.2.4.1 Sample Extraction

Black and green tea liquors were prepared from the commercial brand used as standard as 1% (w/v) tea solutions. The water extracts were made by adding 240 ml boiling water to a tea bag (2.4 g) in a thermal flask that was allowed to stand for 10 minutes with frequent shaking (Aucamp *et al*., 2000). The extracts were filtered through filter paper to remove particulate matter. For hydrophilic interaction chromatography (HILIC) samples were extracted at 70 ℃ with 100% methanol and the standards were made up in 100% methanol (HILIC requires samples in 100% organic solvent).

2.2.4.2 Chromatographic Conditions

Separations were carried out on three columns:

a) A reverse phase Luna 5 μm phenyl-hexyl column (250 x 4.6 mm) purchased from Phenomenex® (Torrance, CA, USA) and fitted with a Phenomenex® SecurityGuard™ guard column containing phenyl-hexyl cartridges (4 x 3 mm).

b) A reverse phase Luna 5 μm C_{18} column (250 x 4.6 mm) also from Phenomenex® (Torrance, CA, USA), protected by a Phenomenex® SecurityGuard™ guard column containing C_{18} cartridges (4 x 3 mm).

c) An Atlantis 5 μm HILIC column (150 x 4.6 mm) obtained from Waters (Milford, MA, USA).

Reverse phase chromatography.

Three mobile phases consisting of acetonitrile/acetic acid/water (1:2:97 v/v/v), acetic acid/water (2:98 v/v) and 100% water were used for the reverse phase chromatography. The flow rate of the mobile phase was 1 ml/min during this phase of method development. The column temperature was set at 30 ℃ and for each analysis 50 μl of sample or standard was injected.

Hydrophilic interaction chromatography (HILIC).

Acetonitrile (solvent A) and water (solvent B) was used for HILIC analysis. Different linear gradients were experimented with by varying the time over which the gradient was applied and by increasing the percentage of solvent B at the starting point of each gradient (time 0 minutes) but always ending with 95% solvent B. A flow rate of 1 ml/min was used. The injection volume was 50 μl and the column temperature was 30 ℃.

Detection.

To detect theanine with the ELSD during this phase of method development, the gain was set at 10 and the gas pressure at 45 psi. The drift tube and nebulizer temperatures were set at 70 °C and 46%, respectively.

2.2.4.3 Optimization of ELSD Conditions for Theanine Detection

Thirty micro litres of theanine standard at 50% of the test concentration (0.1 mg/ml) was injected and eluted with 100% dddwater in short 10 minute runs. For theanine detection a set gain of 20 with various combinations of nebulizer gas pressure, drift tube and nebulizer temperatures (Table 2.1) were used. Optimum detection condition was determined by evaluating the response obtained for the different conditions used and applied from this point onwards.

Table 2.1. The combinations of nebulizer gas pressure, drift tube and nebulizer temperatures used for optimization. This was repeated for nebulizer temperature percentages of 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40 and 35, respectively.

Drift Tube Temperature (°C)	Nebulizer Temperature (%)	Gas Pressure (psi)
100, 90, 80, 70 or 60	100	60
100, 90, 80, 70 or 60	100	55
100, 90, 80, 70 or 60	100	50
100, 90, 80, 70 or 60	100	45
100, 90, 80, 70 or 60	100	40
100, 90, 80, 70 or 60	100	35
100, 90, 80, 70 or 60	100	30
100, 90, 80, 70 or 60	100	25

2.2.4.4 Finding a Suitable Internal Standard

In order to find a suitable internal standard 50 µl of 1 mg/ml alanine, arginine, aspartic acid, asparagine, cysteine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, methionine, proline, serine, tryptophan, threonine and valine was injected and analyzed. These injections also served as a preliminary selectivity test for possible amino acid interference during analysis.

2.2.4.5 Sample Pre-treatment Utilizing PVPP

Preparation of a PVPP Pre-column

PVPP previously suspended in dddwater was packed into a short column (10 x 4.6 mm) and washed for 10 minutes with mobile phase (Horie *et al.*, 2002; Nakakuki *et al.*, 1999).

PVPP Batch Treatment Optimization.

Determining the Polyphenol Content of the Tea Samples.

Method adapted from ISO/DIS 14502-1 (Tea: Methods for determination of substances characteristic of green and black tea, 2005). The Folin-Ciocalteu method was employed to determine the total polyphenol content of the tea samples after PVPP treatment. A gallic acid dilution range (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) was used as calibration standard. Samples were diluted one hundred times. Two hundred micro litres of standard/diluted sample were pipetted into a 2 ml eppendorf tube and 500 µl Folin-Ciocalteu reagent (diluted v/v 1:10) and 400 µl of 7.5% (w/v) sodium carbonate (anhydrous) were added. For the blank 200 µl of dddwater was used instead of sample. The reaction mixture was vortexed and was incubated at room

temperature for 60 minutes before the optical density was measured at 765 nm using a Unico 1100 spectrophotometer (United Products and Instruments Inc., New Jersey, USA). Determinations were performed in duplicate. A standard curve was drawn and the polyphenol content of the samples was calculated.

Determining the Concentration of PVPP Required.

PVPP powder (25, 50, 70, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg) was suspended in dddwater (24 ± 1 ml) 24 hours before use to ensure complete hydration. The water was decanted after centrifugation for 10 minutes at 1500 x *g*. Tea sample (10 ml) was added to each quantity of PVPP. The suspension was incubated for 60 minutes with frequent mixing followed by centrifuged at 1500 x *g* for 10 minutes to remove the PVPP. The colorimetric method using Folin-Ciocalteu reagent was used to determine the amount of polyphenols remaining in the supernatant after PVPP treatment.

Determining the Optimum Contact Time.

PVPP powder (500 mg per 50 ml tube) was suspended in dddwater (24 ± 1 ml) 24 hours before use to ensure complete hydration. After centrifugation for 10 minutes at 1500 x *g* the water was decanted. Tea sample (10 ml) was added to each tube and incubated at room temperature for 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes, respectively. To ensure that the tea sample was not exposed longer to the PVPP than the specific contact time, a small amount of sample was immediately filtered through a syringe filter to remove the PVPP after the time period elapsed. The amount of polyphenolic compounds remaining in the filtrate after PVPP treatment was determined using the Folin-Ciocalteu method. It was expressed as a percentage of the polyphenol content (PC) of the untreated tea sample (the control):

$$\% \text{ Remaining polyphenols in filtrate} = \left(X_{sample} \middle/ X_0 \right) \times 100$$

X_{sample} is the amount of polyphenols (µg) in the tea sample after treatment for a specific time period

X_0 is the amount of polyphenols (µg) in the untreated tea sample (control)

2.2.5 Method Validation

The two methods using the phenyl-hexyl and the C_{18} columns were validated. Tea samples used for method validation were prepared according to the method described in section 2.2.6.2

2.2.5.1 Selectivity

Potential interferences were evaluated by analyzing several tea samples in triplicate. To further evaluate the selectivity of the methods 50 µl of a standard mixture containing 0.1 mg/ml of L-glutamic acid, L-glutamine and L-theanine was injected. Three injections were made.

2.2.5.2 Precision

Intra-day (n=8) and inter-day (10 days; n=8 per day) precision was evaluated by determining the coefficient of variation for the peak area of 0.1 mg/ml theanine standard and the peak area of theanine in PVPP treated 1% tea extracts. The injection volume for each analysis was 30 µl.

2.2.5.3 Linearity, Limit of Detection and Limit of Quantification

Linearity of the methods was evaluated by injecting 30 µl of standard theanine at concentrations ranging from 0.02 to 0.6 mg/ml. A calibration curve was constructed over 10 different concentrations (0.02, 0.03, 0.05, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/ml), 3 injections were made at each level and peak area was plotted against concentration. Linear regression analysis was used to generate the standard curve.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the peak area response and slope of the linear calibration curve.

$$LOD = \frac{3.3\sigma}{S} \qquad\qquad LOQ = \frac{10\sigma}{S}$$

S is the slope of the calibration curve
σ is the standard deviation of the response (the standard deviation of the y-intercept of the calibration curve was used)

2.2.5.4 Accuracy

Accuracy was determined by spiking 1% tea extracts before or after PVPP pre-treatment with known amounts of theanine standard at 25, 50, 80, 100 and 120% of the pre-determined sample level (three replicates at each level). The amount recovered theanine was compared to the amount added and expressed as percent recovery:

$$\% \: Recovery \: = \left(X_{determined} \middle/ X_{theoretical} \right) \times 100$$

$X_{determined}$ amount theanine (µg) in tea sample determined after spiking
$X_{theoretical}$ pre-determined amount theanine (µg) in tea sample plus the added
              amount of theanine standard (µg)

2.2.5.5 Sample Stability

The stability of theanine in PVPP pre-treated and untreated 1% tea extracts was evaluated after 1 to 7 freeze-thaw cycles at -20 °C and after 1 to 7 days storage at 4 °C (n=3). Before analysis, samples were allowed to reach room temperature. Samples were also vortexed and filtered through a 0.2 µm syringe filter.

2.2.6 Sample Analysis

2.2.6.1 Moisture Content

Two grams of powdered tea sample were weighed in triplicate and placed in a 103 °C oven to dry overnight (~16 hours) or to constant weight. Samples were removed from the oven and placed in desiccators and left to cool to room temperature before weighing again. The moisture content was determined by comparing the original weight with the oven dried weight and was expressed as the percentage mass lost.

2.2.6.2 Theanine Content

Sample Preparation.

Extraction.

The method was adapted from the method described by the ISO/CD 14502-2 (2003). Tea samples were powdered using a homogeniser (Fritsch Pulverisette®, Germany) and sifted through a 355 µm Test Sieve (Labotec, Johannesburg, Republic of South Africa) to ensure uniform powdered samples. Tea liquors were prepared from the powdered tea samples as 1% (w/v) tea solutions. The tea sample (100 mg) was extracted with 5 ml of dddwater for 10 minutes in a 90 °C water bath, vortexing every 5 minutes. The extract was centrifuged at 1500 x *g* for 10 minutes after which the supernatant was decanted. The pellet was extracted again with 5.5 ml of dddwater for 10 minutes in a 90 °C water bath, vortexing every 5 minutes, followed by centrifugation for 10 minutes at 1500 x *g*. The supernatant was decanted and added to the supernatant of the first extraction and made up to a final volume of 10 ml.

Pre-treatment (PVPP Clean-up Procedure).

PVPP was swollen overnight in dddwater in 50 ml tubes (250 mg/tube), centrifuged at 1500 x *g* for 10 minutes and the water decanted and discarded. Tea extract (10 ml) was added to the swollen PVPP (250 mg dry weight), vortexed and incubated at room temperature for 10 minutes, after which the slurry was centrifuged at 1500 x *g* for 10 minutes. The supernatant was decanted and filtered through a 0.2 µm syringe filter to remove particulate matter. The filtrate was used as the tea sample for HPLC analysis.

Analytical Conditions.

Chromatographic separations were performed on the reverse phase phenyl-hexyl column. Isocratic elution with dddwater as mobile phase was used. The flow rate of the mobile phase was 0.8 ml/min and the column temperature was set at 30 ℃. The injection volume for each analysis was 30 µl and all samples were spiked with internal standard to give a final concentration of 0.2 mg/ml leucine. Dddwater was used as blank. For detection with the ELSD the gain was set at 20 and the gas pressure at 40 psi. The drift tube and nebulizer temperatures were set at 100 ℃ and 100%, respectively.

Calculating the Percentage (w/w) Theanine per Tea Sample.

The theanine content, expressed as a percentage of the dry weight of the sample, was calculated with the following formula (ISO/CD 14502-2, 2003):

$$\% \ Theanine \ = \ \frac{(A_{sample} - A_{intercept}) \times V \times d \times 100}{slope \times m \times MC \times 10 \ 000}$$

A_{sample} is the peak area of theanine
$A_{intercept}$ is the peak area at the point the standard calibration line intercepts the y-axis
$slope$ is the standard calibration line slope
V is the sample extraction volume in millilitres
d is the dilution factor of the sample
m is the mass, in grams, of the sample used
MC is the moisture content, expressed as a mass fraction in percent

2.2.7 Total Polyphenol Content

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Sample Preparation.

The same method was followed for sample extraction as described in section 2.2.6.2, with the exceptions of the extraction solvent being 70% methanol and the extraction temperature 70 °C.

Determining Total Polyphenol Content.

The Folin-Ciocalteu method described in section 2.2.4.5 was employed to determine the total polyphenol content of the tea samples analysed.

2.2.8 Statistical Analysis

For all the measurements a minimum of 3 to 5 replicates were taken for data analysis. The ANOVA, MEANS and REG procedures from SAS (SAS Institute Inc., Cary, NC, USA) statistical software (version 9.2, 2008) were used to analyse the data. Mean values and the coefficients of variation for the replicates were reported.

2.3 Results and Discussion

2.3.1 Method Development

2.3.1.1 Chromatographic Conditions

Three analytical columns were tested during method development: 1) A phenyl-hexyl column, chosen because it is used for routine analysis of tea catechins and caffeine in our research laboratory in accordance with ISO/CD 14502-2 and using the same column for theanine analysis seemed an attractive possibility; 2) A regular C_{18} reverse phase column chosen since it is present in most laboratories, rendering a method using it generally more widely applicable; 3) A HILIC column to determine the suitability of hydrophilic interaction chromatography (HILIC) for theanine analysis in addition to the reverse phase approach, since this column is more suitable for hydrophilic compounds such as amino acids.

For the reverse phase mode various combinations of acetonitrile, acetic acid and water were evaluated as mobile phase. Finally, good chromatographic separation of theanine in tea samples was achieved in less than 10 minutes, using the Phenomenex$^{®}$ Luna 5 μm phenyl-hexyl and C_{18} columns with convenient isocratic elution with 100% water as mobile phase (Figure 2.3).

Even though a 100% aqueous mobile phase was used with a C_{18} and a phenyl-hexyl column, no retention time reproducibly problems were experienced over the course of this study (one year). Conventional hydrophobic stationary phases can exhibit a sudden loss of retention time, attributed to the proposed phenomenon of hydrophobic

phase collapse, when using highly aqueous mobile phases (usually < 5 to 10%

organic solvent depending upon the bonded-phase characteristics). Preventative

measures were taken to avoid promoting phase collapse, these include: purging the

column regularly with 10 column volumes of 65% acetonitrile in between runs and

never allowing the column to stand in the water mobile phase - always rinsing it with

20 column volumes of 65% acetonitrile before storage (65% acetonitrile was

recommended by the manufacturer as storage solvent).

For HILIC, the mobile phase consisted of acetonitrile and water. HILIC, a variation of

normal phase chromatography, uses a polar stationary phase (negatively charged

silanols) with a mobile phase consisting of a high concentration non-polar organic

solvent and a low concentration polar aqueous solvent. The aqueous polar solvent is

the strong solvent component of the mobile phase and compounds elute in order of

increasing hydrophilicity (Alpert, 1990). After several attempts the separation of

theanine was achieved by increasing the water content (solvent B) of the mobile

phase from 20% to 95% with a linear gradient over 10 minutes. Theanine eluted

after 7.9 minutes (Figure 2.4). Re-equilibrating the column for the next injection plus

the analysis time of 10 minutes, resulted in a total run time of 35 minutes per injection

compared to the reverse phase columns' total run time of 30 minutes per tea sample

injected (10 minute analysis, 20 minutes wash and re-equilibration) and only 10

minutes per standard injected. In an effort to reduce the run time the linear gradient

was applied over shorter time periods (down to 5 minutes) increasing the strength of

the mobile phase earlier in the run, but the retention time of theanine remained

around 8 minutes (Figure 2.4). The longer run time, along with the observed peak

tailing, led to the decision to continue the study only with the phenyl-hexyl and C_{18}

columns using isocratic 100% water elution.
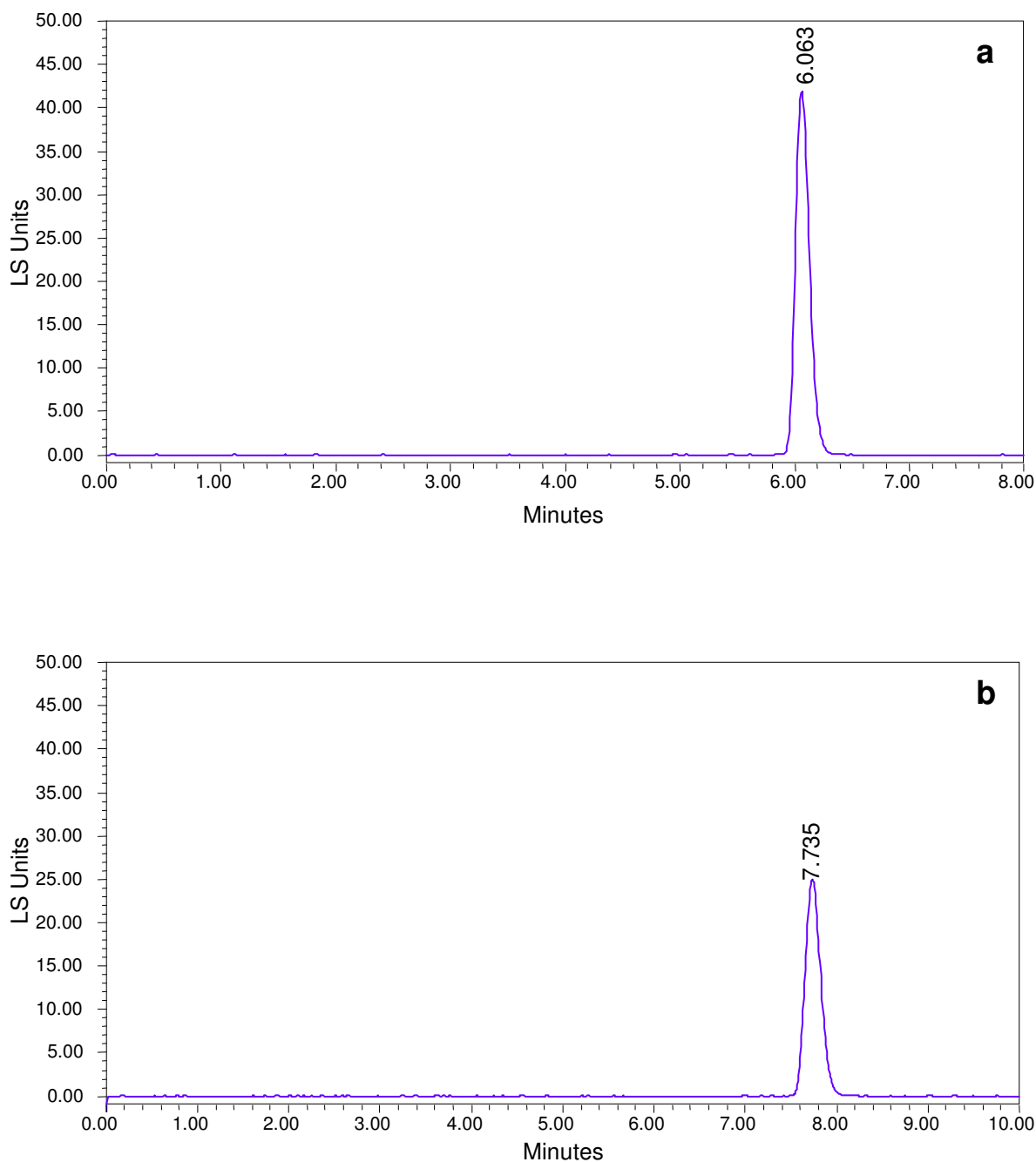
Figure 2.3. Typical reverse phase HPLC chromatogram of theanine standard (0.1 mg/ml) using isocratic water elution with the (a) phenyl-hexyl column and (b) C_{18} column. Flow rate: 0.8 ml/min. Injection volume: 30 µl. Detector drift tube temperature: 100 °C. Nebulizer temperature: 100%. Gas pressure: 40 psi. Signal gain: 20. Note: these are the optimized chromatographic and detection conditions. (LS units: light scattering units).
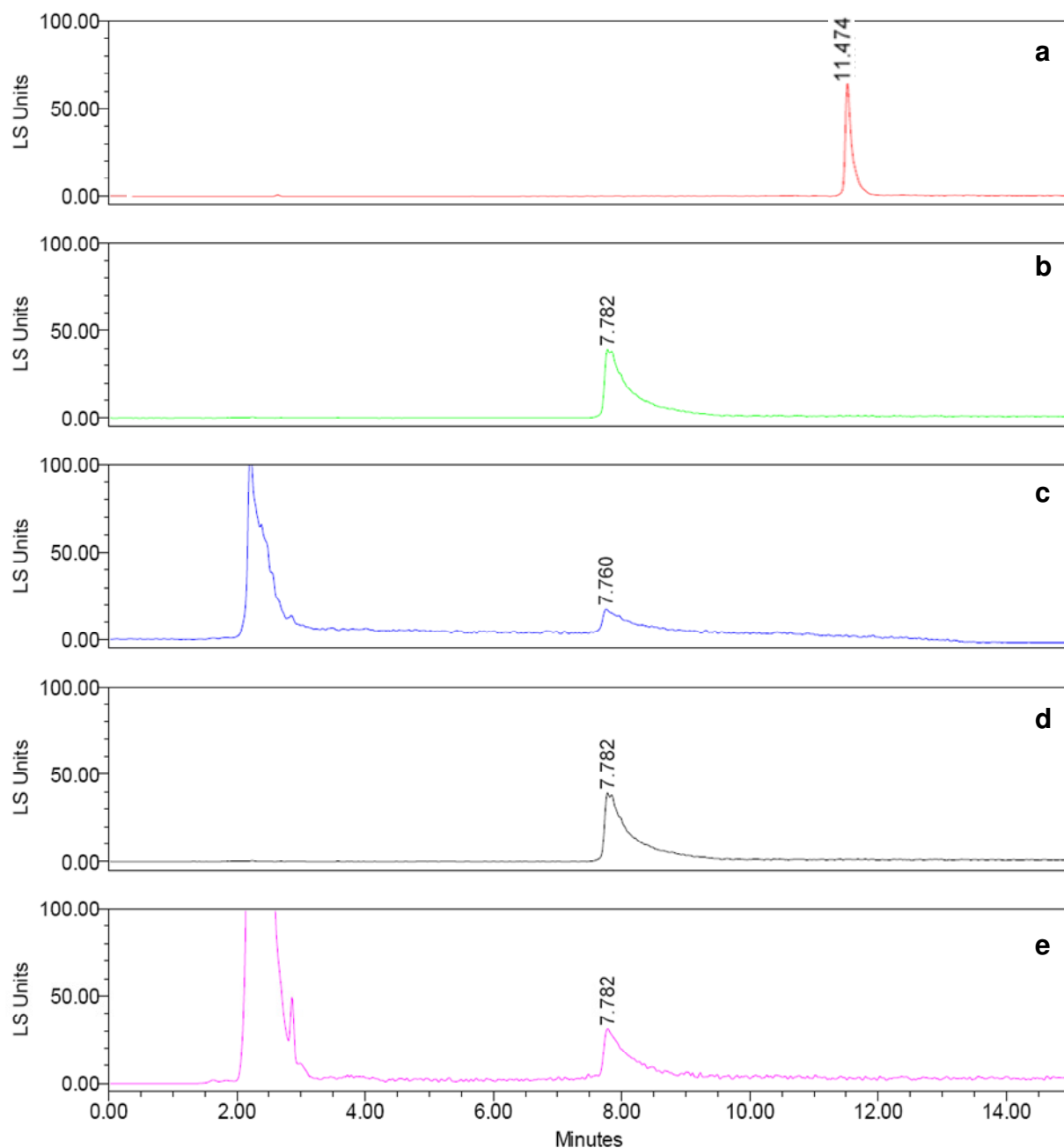
Figure 2.4. HILIC chromatograms of theanine standard (a, b and d) and theanine in a 2% green tea sample (c and e) using different gradients showing the influence on the retention times. The gradient conditions were as follows with solvent A 100% Acetonitrile and solvent B 100% water:

(a) 0 – 10 min 5 – 95% B; 10 – 15 min 95% B,

(b) & (c) 0 – 10 min 10 – 95% B; 10 – 15 min 95% B,

(d) & (e) 0 – 5 min 10 – 95% B; 5 – 15 min 95% B.

Flow rate: 1 ml/min. Injection volume: 50 µl. Detector drift tube temperature: 70 °C. Nebulizer temperature: 40%. Gas pressure: 45 psi. Signal gain: 10. (LS units: light scattering units).

2.3.1.2 Optimization of ELSD Conditions for Theanine Detection

ELS detection is almost universal, similar to a refractive index detector, just more sensitive (Stolywho *et al.*, 1984). Several studies have shown ELS detection to be a good choice for the analysis of underivatized amino acids (Camel *et al.*, 1992; Chaves-das-Neves *et al.*, 1997; Petritis *et al.*, 1999).

ELSD drift tube temperatures are chosen as close as possible to the boiling point of the mobile phase used, without it leading to significant destruction of the analyte, to eliminate interference by unevaporated mobile phase during detection. Using 100% water, the drift tube temperature was set at 100 °C. No significant reduction in the response signal was observed compared to the 70 °C used during initial method development. Although theanine is still successfully detected using lower drift tube temperatures (down to 70 °C), condensation and incomplete evaporation leads to water build up in the ELSD which interferes with detection. With the gain set at 20 and the drift tube at 100 °C, the response signal for numerous combinations of the nebulizer temperature (%) and nebulizer gas pressure (psi) were evaluated (Figure 2.5). The optimum nebulizer temperature and gas pressure was found to be 100% and 40 psi, respectively.
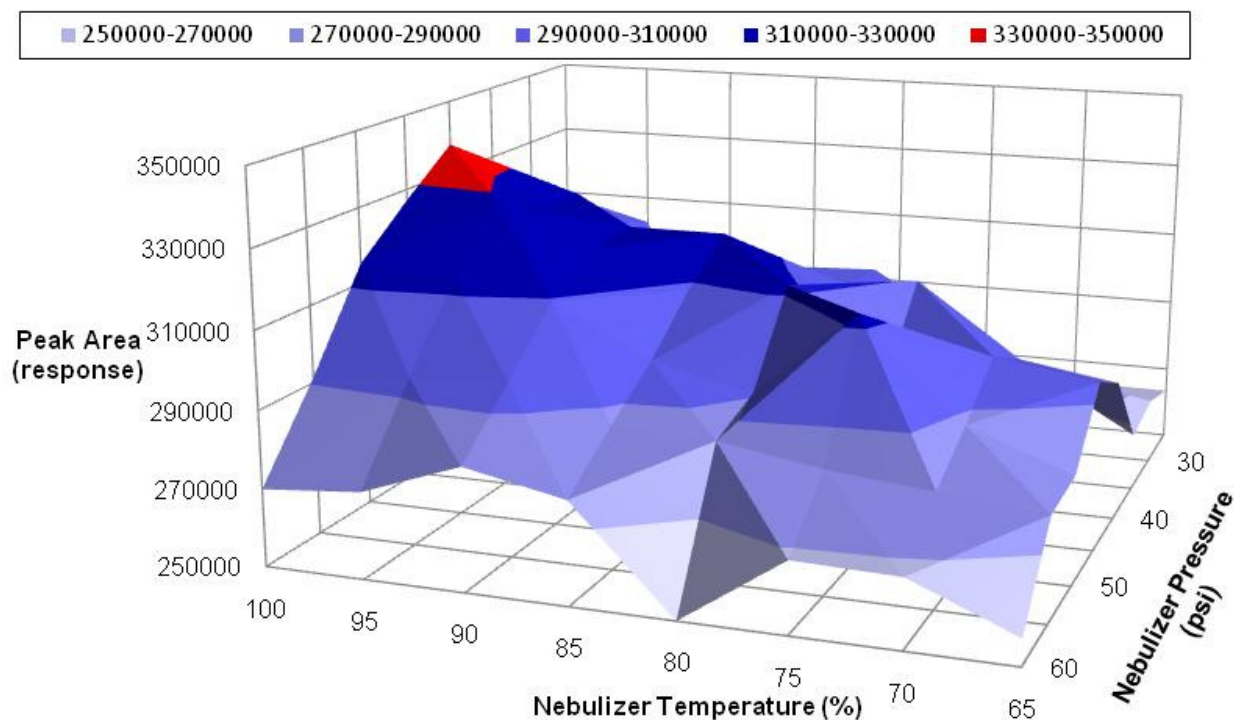
Figure 2.5. The optimum conditions for theanine detection. The drift tube temperature was set at 100 °C and the gain at 20. The optimal nebulizer temperature percentage and pressure was found to be 100 and 40, respectively.

It was noticed that the drift tube temperature started dropping after only a few hours of running mobile phase through the detector. The flow rate was reduced from 1 ml/min to 0.8 ml/ml to decrease the volume of cold mobile phase flowing through the drift tube per unit time. The reduction in flow solved the problem and the detector was able to maintain the drift tube temperature for extended periods of time. The retention time of theanine increased slightly due to the change in flow rate.

2.3.1.3 Finding a Suitable Internal Standard

Because theanine is an amino acid, another amino acid was chosen to serve as internal standard. Elution times of several amino acids were compared (Table 2.2). Most amino acids co-eluted with the 3 big unknown peaks present in tea samples at

2 to 4 minutes, with the exception of isoleucine, leucine, phenylalanine and tryptophan. Tryptophan and phenylalanine were deemed unsuitable because these 2 amino acids eluted only after 11 minutes on both the phenyl-hexyl and C_{18} column. Leucine was chosen as internal standard and not isoleucine due to the fact that isoleucine elutes closely together with theanine on the phenyl-hexyl column. Leucine elutes at 7.2 and 10.2 minutes compared to theanine at 6.1 and 7.6 minutes on the phenyl-hexyl and C_{18} column, respectively.

Table 2.2. The retention times of amino acids on two reversed phase columns in ascending order on the phenyl-hexyl column.

Amino Acid	Retention Time (minutes)	
	Pheyl-hexyl	C_{18}
Aspartic Acid*	3.31	3.20
Glycine	3.46	2.89
Asparagine	3.49	2.93
Serine	3.49	2.91
Arginine	3.52	2.26
Alanine	3.53	2.93
Threonine	3.60	2.97
Glutamine	3.61	3.73
Lysine	3.64	3.00
Cysteine	3.84	3.05
Histidine	4.16	3.18
Proline	4.41	3.19
Valine	4.43	3.50
Glutamic Acid*	4.61	4.20
Methione	5.52	4.21
Theanine	6.08	7.94
Isoleucine	6.64	5.50
Leucine	7.21	10.22
Phenylalanine	19.07	12.84
Tryptophan	44.19	27.44
Aspartic Acid*	3.31	3.20
Glycine	3.46	2.89
Asparagine	3.49	2.93
Serine	3.49	2.91
Arginine	3.52	2.26
Alanine	3.53	2.93
Threonine	3.60	2.97

*Elutes as broad peak with poor detection (< 4 LS Units)
under optimized conditions for theanine detection.

2.3.1.4 Sample Pre-treatment Utilizing PVPP

The isocratic solvent system developed for theanine analysis is simple and convenient but the catechins, theaflavines and other polyphenols that are present in a tea sample are not removed by the 10 minute run with water as mobile phase. Although these compounds do not interfere with the chromatographic separation itself, they remain bound to the column after a run. This means including a column wash step in between every couple of samples to remove these compounds to prevent peak broadening and damage to the column. To eliminate the time consuming column wash step a sample clean-up procedure was necessary. The conventional methods in the tea industry to remove these interfering compounds during analysis of methylated xanthines and amino acids include liquid-liquid and solid phase extraction. Liquid-liquid extraction can be harmful to the environment and human health due to the solvents used, while solid phase extraction is effective in removing polyphenols but expensive and time consuming. The popular, cost effective alternative is polyvinylpolypyrrolidone (PVPP) sample pre-treatment.

PVPP (Figure 2.6) is a water-insoluble, hydrophilic polymer that mimics a protein backbone to which polyphenols adsorb. Polyphenols interact with carbonyl sites either on protein or adsorbents mainly through hydrogen bonding between the proton donor from the polyphenol and the carbonyl group. Polar interactions, π-bond overlap (delocalized electrons) and hydrophobic interactions also contribute to the adsorption of polyphenols by PVPP (Sarioğlu, 2007; Thompson *et al.*, 2003).
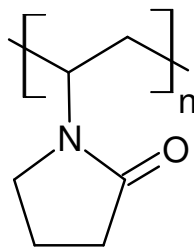
Figure 2.6. Chemical structure of polyvinylpolypyrrolidone.

Generally, PVPP pre-treatment entails mixing the powder with tea extract for more than 30 minutes and then removing it by centrifugation. Most of the polyphenols are removed by this batch treatment method. However, Nakakuki *et al.* (1999) reported a method using a PVPP pre-column for the online removal of polyphenols during HPLC analysis of methylated xanthines (caffeine and theobromine) that is more effective to remove catechins and theaflavines than the batch treatment. Horie *et al.* (2000) also reported that the online treatment of tea extracts during caffeine analysis was more effective than batch treatment and it was decided to investigate this method.

Performance of the PVPP Pre-column.

Before connecting the PVPP pre-column upstream of the analytical column, it was washed with mobile phase to equilibrate and to ensure that the pressure did not rise significantly. The pressure did rise significantly when the pre-column was connected upstream of the analytical column due to the PVPP compacting progressively as mobile phase washed through. PVPP is a soft polymer and compresses under high pressures (Horie *et al.*, 2002). Neither repacking the column several times nor lowering the flow rate solved the problem. Lowering the flow rate further in order to obtain lower back pressure to prevent compression of the PVPP resulted in an increased analysis time that was not acceptable as high sample through-put was one of the main aims of this study. Horie *et al.* (2002) also experienced compacting of the

PVPP pre-column. They solved the problem by choosing a shorter column of 75 mm x 4.6 mm (5 μm) as opposed to a 250 mm x 4.6 mm (5 μm) column used in this study. The resulting lower pressure prevented too tight packing of the PVPP. The online treatment of tea samples with a PVPP pre-column was abandoned and it was decided to use the conventional PVPP batch treatment method.

PVPP Batch Treatment.

Determining the Concentration of PVPP Required.

A colorimetric assay using Folin-Ciocalteu phenol reagent was used to determine the amount of polyphenols remaining in the tea extracts after treatment with various concentrations of PVPP. The reagent contains phospho-tungstic acids that are reduced by phenolic hydroxy groups yielding a blue colour with a broad maximum absorption at 765 nm (ISO/DIS 14502-1, 2005). The adsorption efficiency of polyphenols contained in 1% black and green tea extracts on PVPP was evaluated by determining the percentage decrease in the absorbance at 765 nm using the following equation described by Sarioğlu (2007):

$$\% \text{ Adsorption} = \left(\frac{A_0 - A}{A_0} \right) \times 100$$

A_0 is the absorbance of the tea sample before treatment at 765 nm
A is the final absorbance of the same tea sample after PVPP treatment

Figure 2.7 shows that the adsorption increases initially with the PVPP concentration and reaches a plateau as expected. The adsorption of polyphenols increases as the available adsorption surface increases up to the level were the adsorption process for the specific concentration of polyphenols in a 1% tea extract has reached equilibrium

(Doner *et al.*, 1993; McMurrough *et al.*, 1995; Sarioğlu, 2007). At this point, 100 mg PVPP per 10 ml 1% tea sample, 95 to 98% of the polyphenols was removed from the tea extract and increasing the concentration of PVPP did not increase this percentage significantly. While the efficiency of removing green and black tea polyphenols was effectively unchanged using 500 to 1000 mg PVPP per 10 ml 1% tea sample, 500 mg PVPP per 10 ml 1% tea extract (or 5 mg PVPP per 1 mg tea, dry weight) was employed for the pre-treatment from a practical point of view. Incorporating this 5 fold safety margin also made the method more robust.
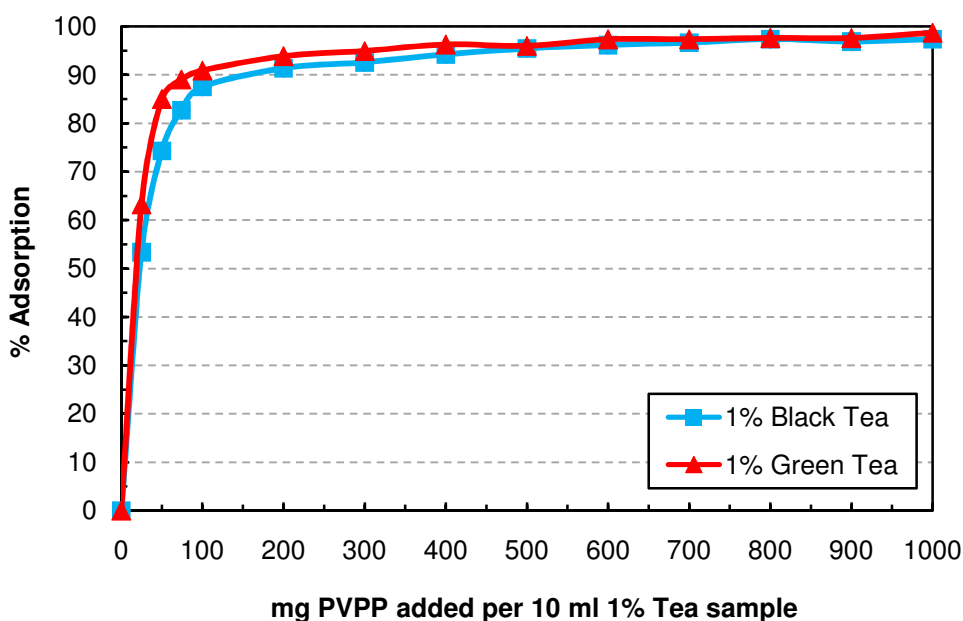
Figure 2.7. The adsorption of polyphenols from 1% black and green tea extracts at room temperature (22 °C).

Determining the Optimum Contact Time.

Figure 2.8 shows that PVPP, at 500 mg/10 ml 1% tea extract, was effective in removing polyphenols at all contact times between 1 and 60 minutes. With 3.3 to 6.2% polyphenols remaining in the tea extracts even after an hour contact time (due

to the adsorption process reaching equilibrium), 10 minutes was chosen as contact time purely for practical reasons.
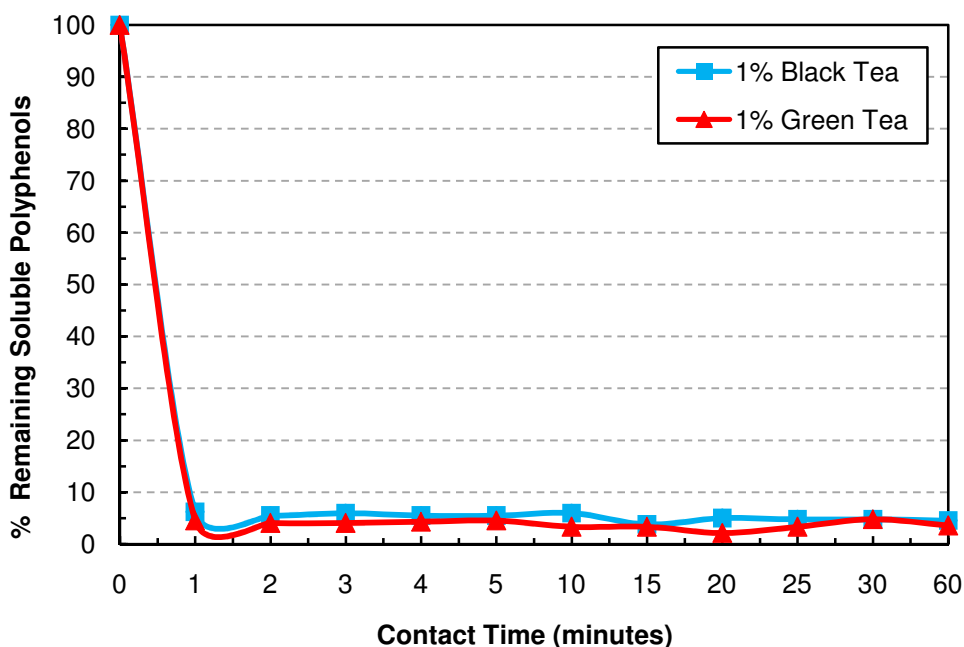
Figure 2.8. The effect of contact time on polyphenol uptake from 1% black and green tea extracts at room temperature (22 °C).

Effect of PVPP Batch Treatment on HPLC analysis.

The chromatograms of a green tea and black tea sample before and after PVPP treatment are shown in Figure 2.9. The peak areas obtained for theanine after PVPP treatment are almost the same as those obtained before PVPP treatment, meaning that theanine is not absorbed on PVPP. The peak areas of the polyphenolic compounds however, are significantly reduced. After 20 consecutive injections of tea sample with no wash step in between, no significant peak broadening or carry-over interferences were observed. Treatment of samples with PVPP prior to HPLC analysis proved to be successful in removing most of the polyphenolic compounds and effectively reduced the run time with 20 minutes by eliminating the need for a

column wash step after each sample. To protect the analytical column and extent its

life time, a 30 minute wash and re-equilibration step was included after every 10 tea

samples injected. Caffeine, which is not removed by PVPP treatment, elutes in the

wash step after every 10 samples.
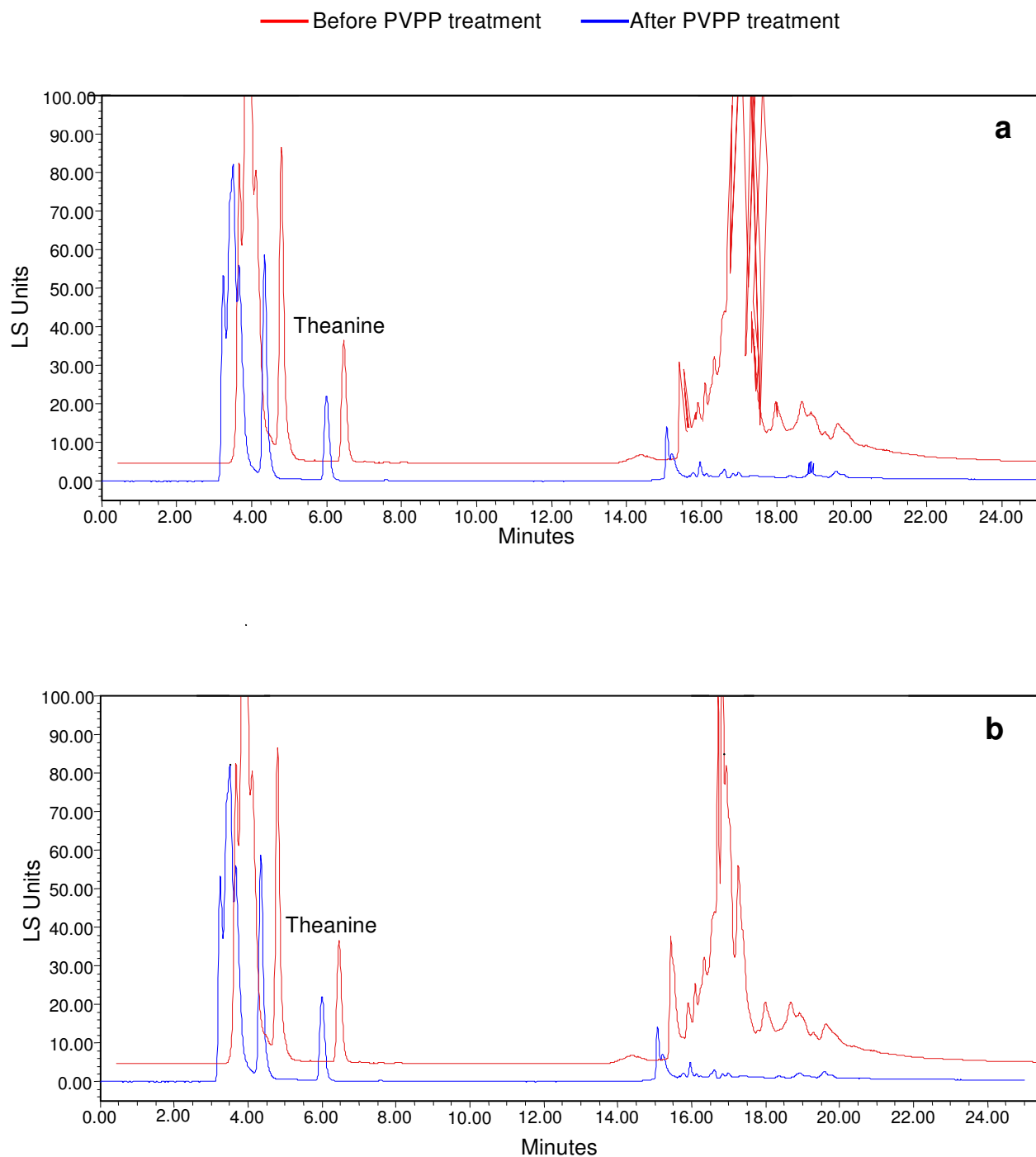
Figure 2.9. Overlaid chromatograms of (a) 1% green tea and (b) 1% black tea samples PVPP pre-treated and untreated. The polyphenolic compounds eluting between 14 – 22 minutes are significantly reduced in the PVPP pre-treated samples. Mobile phase: water. Flow rate: 0.8 ml/min. Injection volume: 30 µl. Detector drift tube temperature: 100 °C. Nebulizer temperature: 100%. Gas pressure: 40 psi. Signal gain: 20. (LS units: light scattering units).

2.3.2 Method Validation

To yield reliable results that could be properly interpreted, the developed methods using the phenyl-hexyl and C_{18} column were validated according to Reviewer Guidance Validation of Chromatographic Methods promulgated by the Centre for Drug Evaluation and Research (CDER) at the Food and Drug Administration (FDA), as well as the EU guideline for the Validation of Analytical Procedures Methodology as adopted by the Therapeutic Goods Administration (a division of the commonwealth Department of Health and Ageing) in 2002. The following parameters were examined: selectivity, precision, linearity, limit of detection and quantification, accuracy and sample stability.

2.3.2.1 Selectivity

An analytical method is selective if it can accurately measure the analyte in the presence of interference such as precursors, excipients, enantiomers and known or likely degradation products that may be expected to be present in the sample matrix. Besides theanine, there exist structurally similar amino acids in tea infusions e.g. glutamine, that generally interfere with the chromatographic separation. Kato *et al.*, (2003) reported inadequate separation of glutamine and theanine using microchip electrophoresis and fluorescent detection. Anion exchange chromatography with a ternary gradient elution system and integrated pulsed amperometric detection, resulted in the co-elution of glutamine and theanine (Ding *et al.*, 2002). Though in both reports, under optimized conditions separation was achieved but not with baseline resolution.

To investigate the selectivity of the method, using the phenyl-hexyl and C_{18} column, the elution profiles of a mixture of L-glutamine and L-theanine was evaluated. Under the given chromatographic conditions standard theanine was well resolved from glutamine (Figure 2.10).
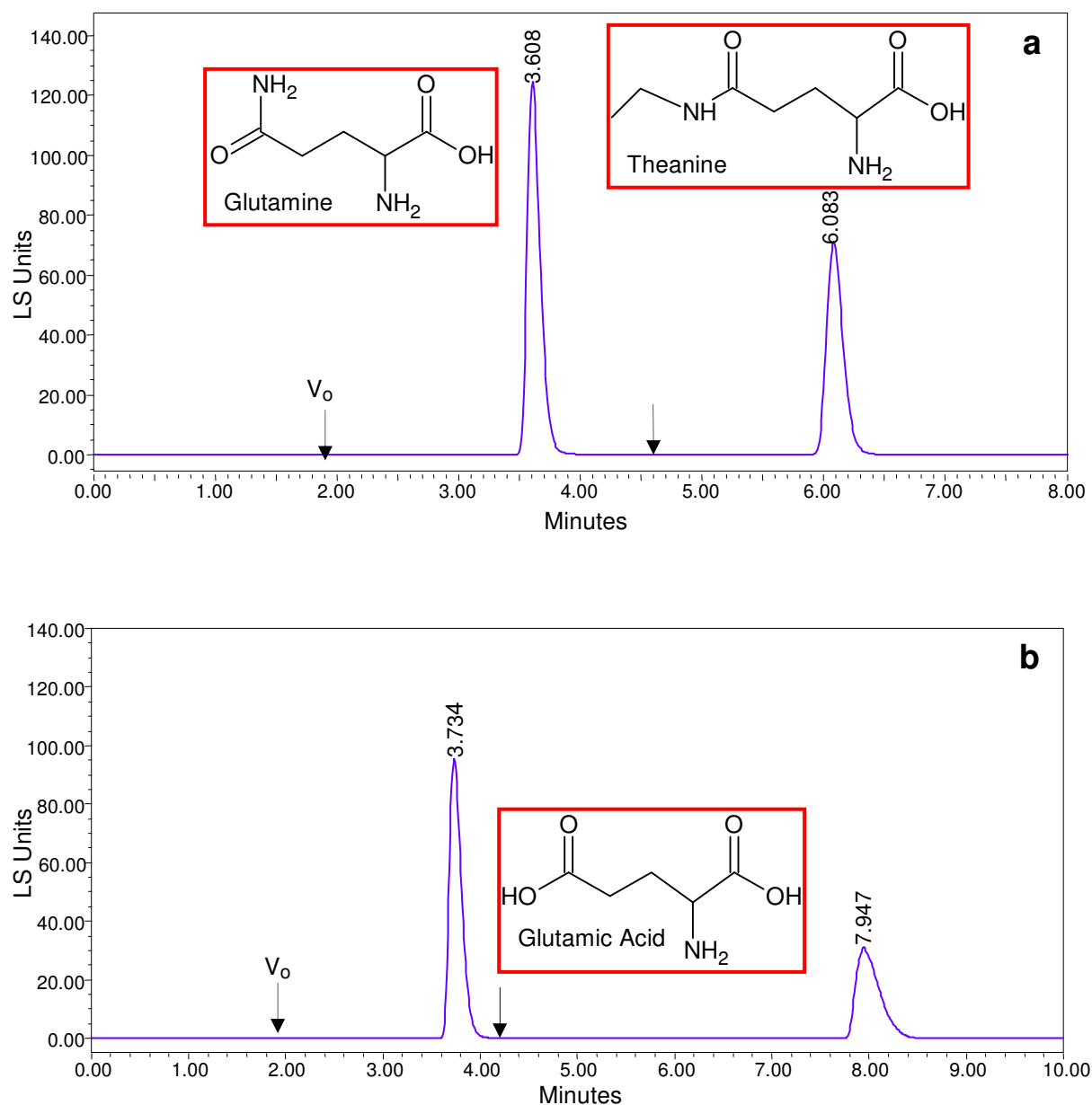
Figure 2.10. The structures of theanine and glutamine and their separation with the developed method using the (a) phenyl-hexyl and (b) C_{18} column, demonstrating the selectivity of the method. Glutamine elutes at 3.608 and 3.734 minutes compared to theanine that elutes at 6.083 and 7.947 minutes. At the optimized detection conditions for theanine, glutamic acid is not effectively detected; the arrows indicate where glutamic acid elutes. Mobile phase: water. Flow rate: 0.8 ml/min. Injection volume: 30 µl. Detector drift tube temperature: 100 °C. Nebulizer temperature: 100%. Gas pressure: 40 psi. Signal gain: 20. (LS units: light scattering units).

The retention times of glutamine were 3.6 and 3.7 minutes compared to 6.1 and 7.9 minutes for theanine using the respective columns. Glutamic acid, a precursor of theanine and possible interfering amino acid, eluted at the 4.2 and 4.6 minutes on the respective columns as a broad peak with poor detection (< 4 LS units) by the ELSD under the optimized conditions for theanine detection. No other interfering peaks were noted around the retention time of theanine in the control samples used to assess the selectivity of the method (representative chromatograms are shown in Figure 2.11).
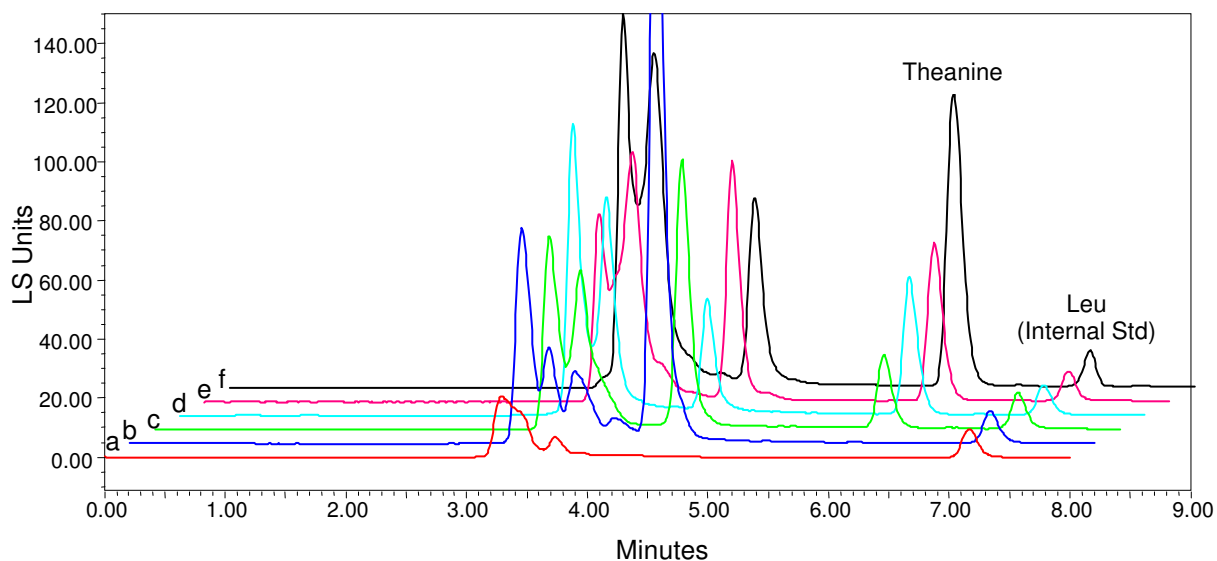
Figure 2.11. Chromatographic overlay of representative tea samples. (a) Peacock Rooibos tea (b) Peacock Honey Bush tea, (c) Twinning White tea, (d) Peacock Yunnan Green FOP, (e) Gyokuro Zuiun™®, (f) Matcha-Koicha Ichigo Ichie™®. No interfering peaks are observed around the retention time of theanine, confirming the selectivity of the method. Column: phenyl-hexyl. Mobile phase: water. Flow rate: 0.8 ml/min. Injection volume: 30 µl. Detector drift tube temperature: 100 °C. Nebulizer temperature: 100%. Gas pressure: 40 psi. Signal gain: 20. (LS units: light scattering units).

2.3.2.2 Precision

To evaluate the precision of an analytical method repeatability, intermediate precision and reproducibility must be determined.

The repeatability (intra-day variation) was determined by 8 injections of theanine standard at 100% of the test concentration and 8 injections of PVPP treated 1% green tea extract. The coefficients of variation ranged from 1.01 to 3.29% and 0.38 to 2.74% for the phenyl-hexyl and C_{18} column, respectively. The percentage coefficient of variation for theanine falls well within the accepted range of ≤ 5%. The results are presented in Table 2.3.

The intermediate precision (inter-day variation) was assessed for theanine standard at 100% of the test concentration and PVPP treated 1% tea extract (prepared fresh daily) with 8 replicates each. The assays were carried out over 10 days. The values obtained demonstrate that the intermediate precision of the method using either the phenyl-hexyl or C_{18} column is sufficient for the determination of theanine in tea samples (Table 2.3).

Reproducibility, defined as the precision obtained between different laboratories, is beyond the scope of this study and it is not normally expected if intermediate precision is accomplished (Center for Drug Evaluation and Research, Food and Drug Administration Reviewer Guidance, Validation of Chromatographic Methods, 1994).

Table 2.3. Intra- and inter-day precision (coefficient of variation) of the method using the phenyl-hexyl and C_{18} column for the determination of theanine standard (100 µg/ml) and PVPP treated 1% green tea extract.

Phenyl-hexyl			
	Day	**Coefficient of Variation (%)**	
		Theanine Standard	**PVPP Treated 1% Green Tea**
Intra-day (n=8)	1	1.20	2.29
	2	2.34	2.76
	3	3.29	2.18
	4	1.39	2.70
	5	1.59	1.62
	6	1.79	1.29
	7	2.26	1.01
	8	1.64	1.98
	9	1.74	1.58
	10	1.37	1.63
Inter-day (n=8/day)	10 days	1.85	3.86
C_{18} Column			
	Day	**Coefficient of Variation (%)**	
		Theanine Standard	**PVPP Treated 1% Green Tea**
Intra-day (n=8)	1	1.73	1.14
	2	1.66	1.10
	3	1.55	1.43
	4	1.95	2.74
	5	2.21	1.14
	6	1.54	1.37
	7	1.81	0.91
	8	0.38	1.61
	9	1.72	2.13
	10	1.37	1.63
Inter-day (n=8/day)	10 days	2.88	3.55

2.3.2.3 Linearity, LOD and LOQ

Linearity was studied in the concentration range of 0.02 to 0.6 mg/ml, corresponding to approximately 10 to 300% of the test concentration (the expected amount of theanine in a green tea sample is 1 to 2% (w/w)). Linear regression analysis was performed to determine the slope, intercept and correlation coefficient of the standard curve. The standard curve for theanine was linear over the tested concentration range with correlation coefficients (R^2) of 0.9954 and 0.9966 using the phenyl-hexyl and C_{18} column, respectively (Figure 2.12).

The LOD and LOQ were calculated statistically based on the standard deviation of the response and the slope obtained from the linearity plots. For the estimation of the standard deviation of the response, the standard deviation of the y-intercept was used. The LOD and LOQ of the method using the phenyl-hexyl column were 11.83 and 39.44 µg/ml, respectively. Utilizing the C_{18} column, the LOD was 10.53 µg/ml and LOQ 35.10 µg/ml. The linearity data are presented in Table 2.4.

Table 2.4. Parameters of linearity for theanine.

Column	Calibration Range (µg/ml)	y-intercept	Slope	R²	LOD (µg/ml)	LOQ (µg/ml)
Phenyl-hexyl	20 - 600	-133147	5321	0.9954	11.83	39.44
C_{18}	20 - 600	-123992	4861	0.9966	10.53	35.10
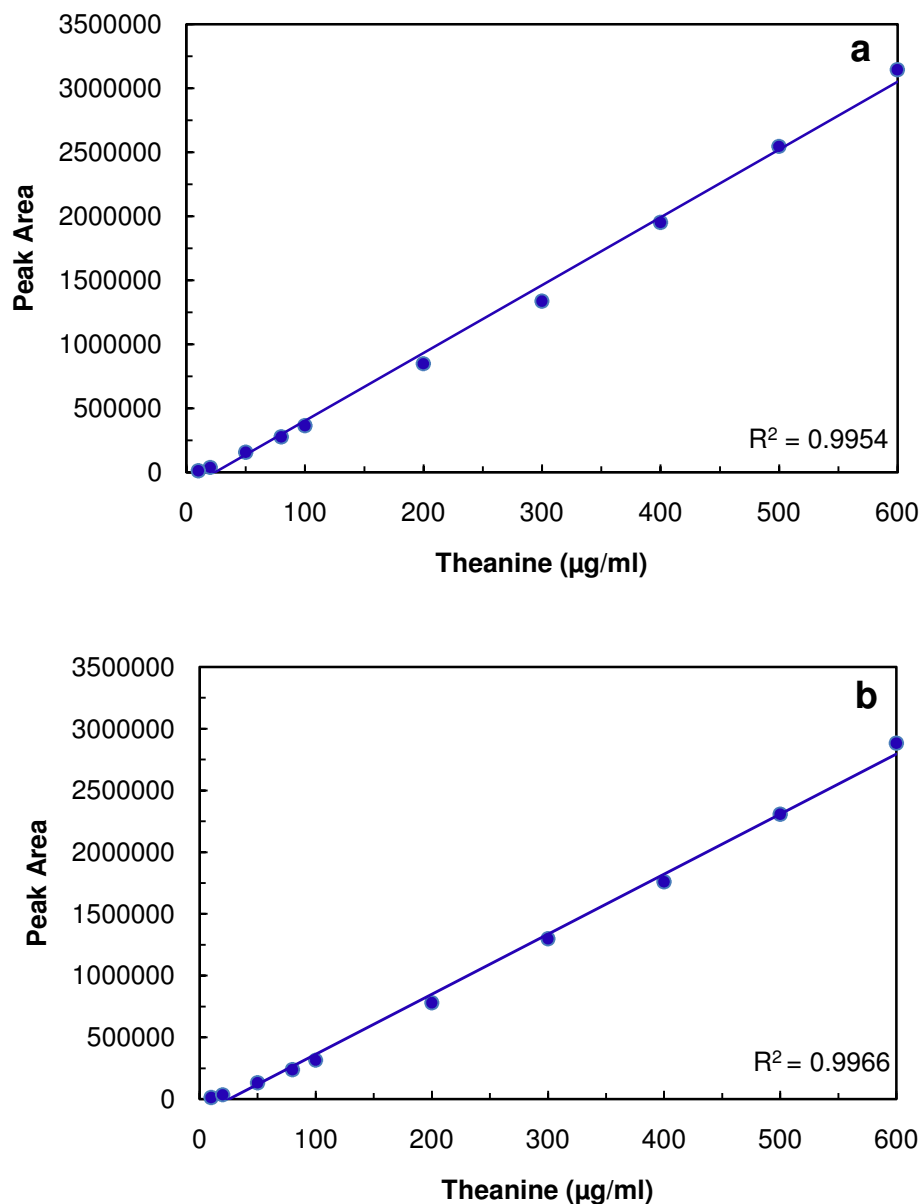
Figure 2.12. Calibration curve for measuring theanine for (a) the phenyl-hexyl and (b) C_{18} column.

2.3.2.4 Accuracy

The accuracy, the measure of how close the experimental value is to the true value, was determined by the use of commercial tea samples spiked with known amounts of theanine standard at 25, 50, 80, 100 and 120% of the pre-determined level. The amount of the recovered analyte was compared to the amount added and expressed

as percent recovery. The mean recoveries ranged from 92 to 106% and coefficients

of variation were less than 5% for samples spiked with standard before or after PVPP

treatment.   The recovery of theanine after PVPP treatment was found to be

satisfactory, proving it a reliable and effective method to remove polyphenols from a

tea sample before HPLC analysis of theanine. The recovery results are summarized

in Table 2.5.

Table 2.5. Recovery of theanine in 1% tea samples.

Theanine Concentration Level (%)**	Recovery (%)*			
	Phenyl-hexyl Column		C_{18} Column	
	Before Treatment	After Treatment	Before Treatment	After Treatment
25	92.58	101.48	97.56	104.89
50	105.07	97.27	98.88	103.53
80	97.69	95.15	98.03	102.11
100	108.42	96.59	101.35	103.21
120	105.06	96.73	106.62	106.65

*n=3
**Percentage of the expected theanine concentration level of 1 to 2% (w/w) in a green tea sample

2.3.2.5 Sample Stability

The stability of theanine in 1% tea samples was tested.   Several samples, PVPP

treated and untreated, were prepared and stored at 4 °C or -20 °C. Samples stored

at 4 °C were stable for 4 days. Except for after 3 days of storage, the mean theanine

content of the samples was not significantly different from each other after 0 to 5

days of storage (Table 2.6). Based on the results, it is concluded that the difference

observed after 3 days of storage may be attributed to coincidence rather than lack of

stability. Samples stored at -20 ℃ for 7 days with 1 freeze-thaw cycle showed significant degradation of theanine (Table 2.7). No significant degradation of theanine was observed for untreated samples after 4 days of storage at -20 ℃ and treated sample were stable for 3 days at -20 ℃ (Table 2.7). No changes in the chromatography of the stored samples were found and no additional peaks appeared when compared to freshly prepared tea samples.

Table 2.6. Evaluation of theanine stability in samples stored at 4 °C. ANOVA Duncan's multiple range test was used at 95% confidence levels. Values followed by the same letter indicate no statistical significant difference. (CV = Coefficient of Variation).

Storage at 4 ℃								
1% Green Tea					**PVPP Treated 1% Green Tea**			
Days	**Theanine* % w/w**	**Recovery %**	**CV %**	**Duncan Grouping**	**Days**	**Theanine* % w/w**	**Recovery %**	**CV %**	**Duncan Grouping**
0	0.82	100	1.25	A	0	0.66	100	1.33	A
1	0.82	100	4.04	A	1	0.65	98	0.09	A
2	0.82	100	0.88	A	2	0.65	98	1.30	A
3	0.75	92	1.43	B	3	0.53	82	2.21	C
4	0.80	98	0.11	A, B	4	0.64	98	0.23	A
5	0.79	96	1.34	A, B	5	0.64	98	2.79	A
6	0.75	92	0.91	B	6	0.58	89	4.74	B
7	0.74	90	1.17	B	7	0.59	91	2.33	B

*n=3

Table 2.7. Evaluation of theanine stability in samples stored at -20 °C. ANOVA Duncan's multiple range test was used at 95% confidence level. Values followed by the same letter indicate no statistical significant difference. (CV = Coefficient of Variation).

Storage at -20 °C								
1% Green Tea					PVPP Treated 1% Green Tea			
Days	Theanine % w/w	Recovery %	CV %*	Duncan Grouping	Days	Theanine % w/w	Recovery %	CV %*	Duncan Grouping
0 (0)	0.82	100	1.25	A	0 (0)	0.70	100	1.33	A
1 (1)	0.82	100	2.69	A	1 (1)	0.68	97	1.78	A
2 (2)	0.82	100	3.20	A	2 (2)	0.67	96	1.27	A
3 (3)	0.78	95	4.62	A, B	3 (3)	0.66	94	1.74	A, B,C
4 (4)	0.78	95	1.55	A, B	4 (4)	0.65	93	2.04	B, C, D
5 (5)	0.77	94	4.81	B	5 (5)	0.64	91	3.07	C, D
6 (6)	0.74	90	3.03	B	6 (6)	0.63	90	1.41	D
7 (7)	0.74	90	2.23	B	7 (7)	0.63	90	2.34	D
7 (1)**	0.74	90	2.23	B	7 (1)**	0.63	90	2.34	D

*n=3
[†]The number of freeze-thaw cycles is in dictated in brackets
**The sample was stored for 7 days at -20 °C followed by analysis, the sample was subjected to only 1 freeze thaw cycle

2.3.2.6 Comparison of the Newly Developed Method with a Published Method

As mentioned before, a method reported by Li Y *et al*., (2005) is the only other published method found for the determination of theanine in tea samples utilizing an ELSD. The method developed in this study compares well with this method as can be seen in Table 2.8. Both methods use reverse phase HPLC and were validated as simple, accurate and selective. The LODs and LOQs are also similar, but the new method, using a latter model ELSD, has a wider linear range than the Li Y *et al*. method. The new method has the advantage of a shorter analysis time due to a simple isocratic one solvent system and a PVPP sample pre-treatment procedure compared to the gradient two solvent system with no sample clean-up procedure as

reported by Li Y *et al*. The shorter analysis time facilitates a higher sample throughput. Approximately 40 samples can be prepared in an 8 hour day with overnight analysis by HPLC-ELSD compared to approximately 16 samples that can be analysed overnight (8 hours) using the Li Y *et al*. method.

The ELSDs are from different manufactures and different solvent systems are used for the chromatographic separation of theanine, making it difficult to compare theanine detection conditions, though both methods use a drift tube temperature of 100 °C.

Table 2.8. Comparison of the new method and the method reported by Li Y *et al*. (2005).

	Li Y *et al*. (2005) Method	**New Method**
HPLC mode	Reverse phase	Reverse phase
Columns	Polaris C_{18}	Luna C_{18} Luna Phenyl-hexyl
Mobile phases	0.1 % Trifluoroacetic acid water 0.1% Trifluoroacetic acid acetonitrile	Water
Flow rate	0.5 ml/min	0.8 ml/min
Elution	Gradient elution	Isocratic elution
Retention time of theanine	15 minutes	7.73 minutes* 6.06 minutes[†]
Sample clean-up	None	PVPP pre-treatment
Total analysis time	Total run time of 30 minutes for each sample and standard injected. Polyphenolics are removed during the run. Extra re-equilibration time required in between runs to obtain reproducible results.	Total run time of 10 minutes if samples are PVPP pre-treated; after every 10 samples a 20 minute column wash step is incorporated to protect the column. With no sample pre-treatment the total run time is 30 minutes: 10 minutes theanine analysis plus a 20 minute wash to remove polyphenolics and re-equilibrate the column.

(Table 2.8 continued)

	Li Y *et al.* (2005) Method	New Method
Detection	Alltech ELSD 2000 Drift tube temp: 100 °C Gas flow: 2.5 ml/min	Waters ELSD 2420 Drift tube temp: 100 °C Gas flow: 40 psi
Linear range	62 - 450 µg/ml (7.2 fold)	20 - 600 µg/ml (30 fold)
LOD and LOQ	12 µg/ml and 48 µg/ml	10.53 µg/ml and 35.10 µg/ml* 11.85 µg/ml and 39.44 µg/ml[†]
Validation	Simple, accurate and selective	Simple, accurate and selective

*Phenyl-hexyl column
[†]C_{18} column

2.3.3 Sample Analysis

The reliability and practicability of the method was verified by analyzing 39 commercial and Japanese speciality green tea samples. The theanine contents of the various tea samples are listed in Table 2.9. It is observed that the theanine content varies from 0.35 to 2.77% of the dried leaf weight in *Camellia sinensis* teas. These differences reflect the natural abundance of theanine in different cultivars but climate, soil, growth altitude and horticulture practices, plucking season, grading of the tea leaves, processing and storage of the tea also influences the theanine content. The results from two brands of rooibos tea (*Aspalathus linearis*) and two brands of honey bush tea (*Cyclopia spp.*) confirm that these plant species do not contain theanine.

Table 2.9. Theanine content of several tea infusions.

Commercial Teas bought at a local supermarket in South Africa		
Sample Name	**%Theanine*** **(mg/100 mg dry weight)**	**Coefficient of Variation (%)**
China Organic White Tea	0.85	0.97
Freshpak Honey Bush	<LOD	-
Lipton Black Tea	0.83	1.83
Lipton Green Tea	0.77	1.94
Lipton Rooibos	<LOD	-
Peacock Assam	0.69	2.79
Peacock Ceylon BOP	0.66	3.96
Peacock China Keemun	0.58	1.88
Peacock Darjeerling	0.44	1.22
Peacock Earl Grey	0.40	0.87
Peacock English Breakfast	0.72	0.81
Peacock Formosa Oolong	0.41	1.56
Peacock Gun powder Green Tea	0.50	3.04
Peacock Honey Bush	<LOD	-
Peacock Japan-Sencha Fukujyu	0.91	2.56
Peacock Kenyan GFBOP Marinyn	1.03	4.18
Peacock Lapsang Souchong	0.35	1.08
Peacock Mate (Herbal Tea)	0.32	0.14
Peacock Organic Tea China Chun Mee	0.99	4.15
Peacock Rooibos	<LOD	-
Peacock Yunnan Green FOP	1.28	1.55
Twinings White Tea	0.81	4.59
Speciality Green Teas from Japan		
Barcha Hojicha	0.57	2.20
Chajin no Sencha™®	0.76	2.40
Gyokuro Oubaku™®	1.89	2.75
Gyokuro Tokujo™®	2.21	2.99
Gyokuro Tokusen™®	2.19	1.40

(Table 2.9 continued)

Speciality Green Teas from Japan		
Sample Name	**%Theanine* (mg/100 mg dry weight)**	**Coefficient of Variation (%)**
Gyokuro Zuiun™®	2.32	3.64
Hojicha Tokujo™®	0.33	0.61
Matcha "C" Powder	2.41	2.86
Matcha Pun	0.85	3.42
Matcha-Koicha Ichigo Ichie™®	2.34	4.34
Setsuki™®	1.14	3.79
Shincha Haru Kazé™®	1.42	4.92
Sincha Hachi Ju Hachi Ya™®	1.59	5.05
Tencha "A" (Ichigo Ichie)	2.77	2.19
Tencha "C" leaves Kotobuki	1.19	2.97
Ujikabusecha™®	1.44	0.19

*n=3

From the samples analysed, the high quality green tea from Japan called Gyokuro and the Ceremonial Tea Matcha (a powdered form of Gyokuro) have higher theanine contents per dry weight than the other tea samples (Table 2.9), in agreement with published data (Horie *et al.*, 1997; Nakagawa, 1975) with the exception of Tencha "A" (Ichigo Ichie). Kito *et al.* (1968) discovered that theanine is a significant precursor of catechin biosynthesis and that light is one of the limiting parameters of this conversion reaction. Therefore, shading tea plants can lead to the accumulation of theanine. Considering that Gyokuro is partly grown in the shade, it is expected that the resultant tea has high theanine and low catechin content (Horie *et al.*, 1997; Nakagawa, 1975). The biosynthesis of catechins with theanine as a precursor also explains the inverse relationship observed in Figure 2.13 between theanine and polyphenol content, since most of the polyphenolic compounds found in tea (70 to

80%) are catechins as illustrated in Figure 1.1 (Del Rio *et al.*, 2004; Astill *et al.*, 2001).
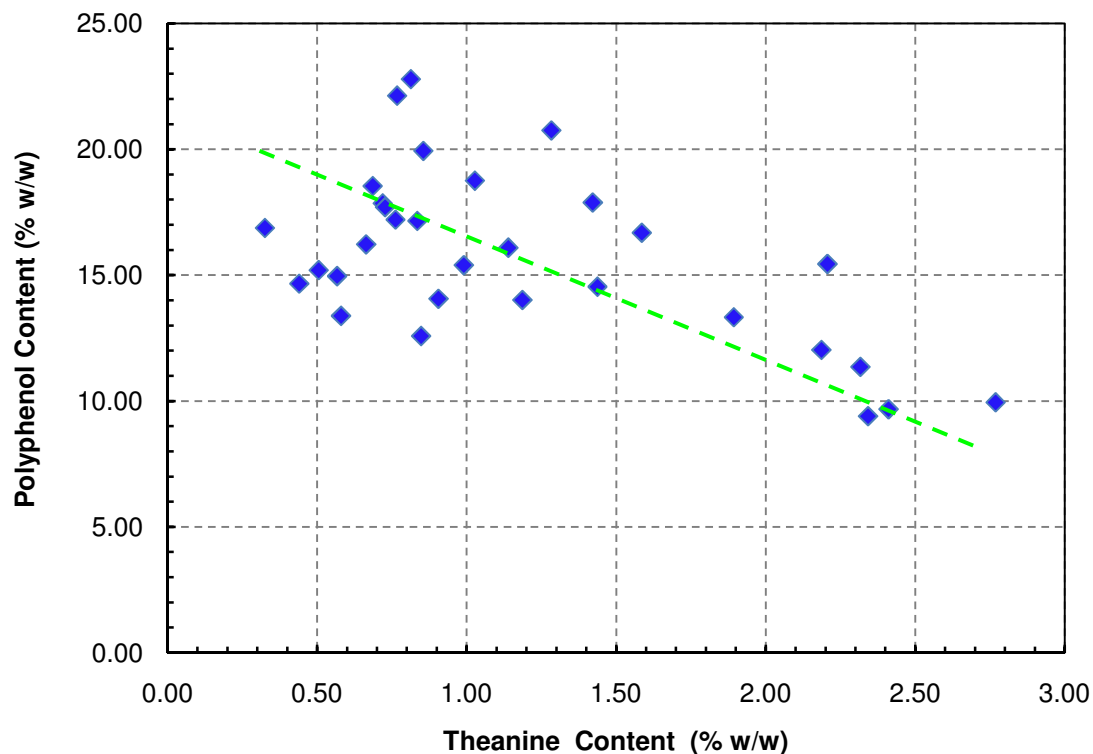
Figure 2.13. Comparison of the polyphenol and theanine content of various teas. An inverse relationship is observed, in agreement with the role of theanine as a precursor of catechin biosynthesis.

Many characteristic are considered to evaluate the quality of tea, but it is mainly assessed through its appearance (colour, colour intensity and clarity), aroma (sweet, grassy, floral etc.) and taste (sweetness, astringency and bitterness). Several studies have investigated the relationship between the quality and chemical components of tea. Free amino acids, catechins, caffeine and ascorbic acid were found to be qualitative important components of green tea, while black tea quality depended more on the content of theaflavins, thearubigins, catechins and caffeine (Ying *et al.*,

2005; Le Gall *et al.*, 2004; Ekborg-Ott *et al.*, 1997; Nakagawa, 1975; Nakagawa, 1970).

The highest quality teas fetch the highest prices and since the above mentioned components influence the quality of tea, price is also inevitably influenced by these components. Theanine is the predominant free amino acid in green tea and its relationship to quality is well established in literature (Kaneko *et al.*, 2006; Horie *et al.*, 1997; Nakagawa, 1975; Nakagawa, 1970). Consequently, it is comprehensible that there should be a notable relationship between theanine content and price. Though results from this investigation were inconclusive due to too small sample size, it seems from Figure 2.14 that the higher priced speciality green teas from Japan generally have higher theanine content. It is predicted that if this study is repeated with a larger number of samples a noteworthy trend will be observed. There was no correlation between theanine content and price of teas bought in South Africa. Such a correlation was not expected since theanine is a less important quality component in these teas, as illustrated by Figure 2.15 (Nakagawa, 1975; Nakagawa, 1970).
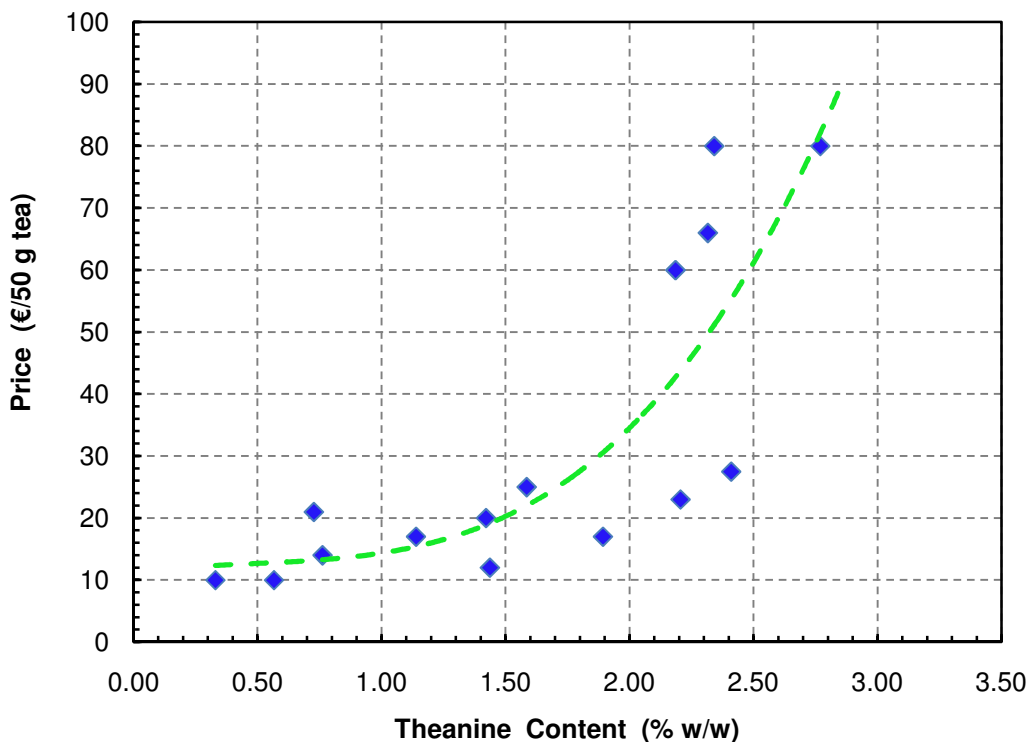
Figure 2.14. The correlation between theanine content and the price of speciality green teas from Japan: Price (€) = 17.427(theanine content %w/w)^2 - 28.328(theanine content %w/w) + 23.06, R^2 = 0.6734. It seems that the high priced green teas have higher theanine content, but due to a too small sample size the results are inconclusive. (Price (€ per 50 g tea) obtained from Chajin Tea Saloon).
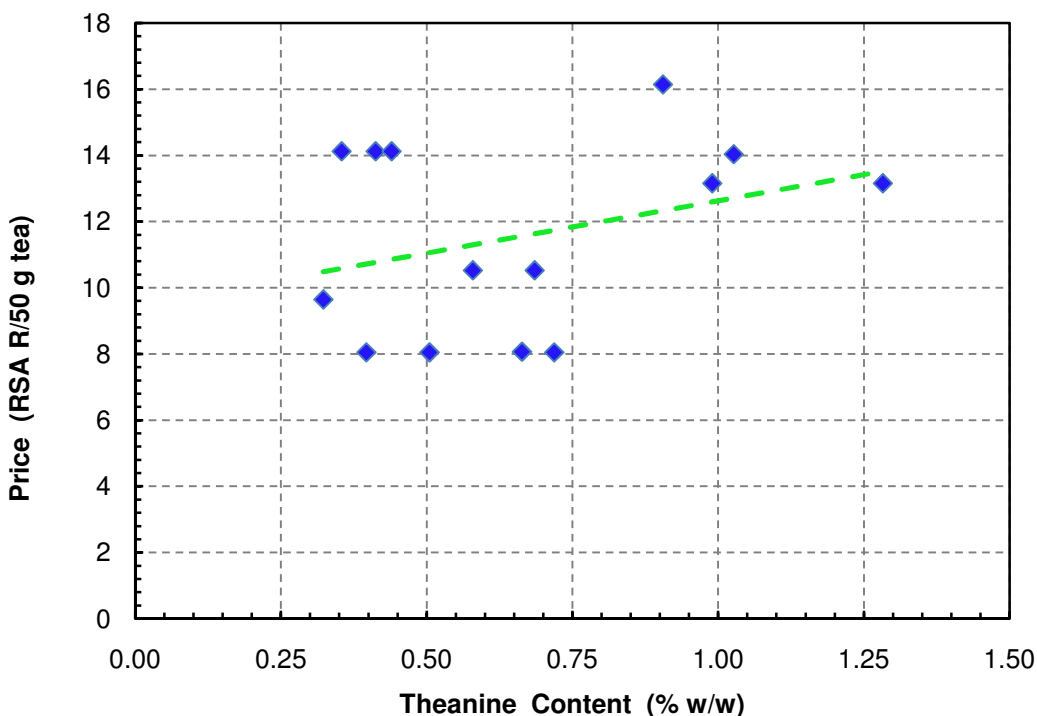
Figure 2.15. The correlation between theanine content and the price of the commercial teas bought in South Africa: Price (RSA Rand) = 3.1641(theanine content % w/w) + 9.4588; R^2 = 0.1043.   No significant trend is observed between theanine content and the price of teas bought at the local supermarket. This is expected due to most of these teas being black and oolong teas and theanine is a less important quality parameter in these teas.

2.4 Conclusion

Chromatographic separation of theanine was achieved by reverse phase HPLC using a phenyl-hexyl column, isocratic water elution and evaporative light scattering detection in commercial tea samples in less than 8 minutes. The method requires no sample derivatization and includes simple sample clean-up. Applying the same chromatographic conditions to a widely available C_{18} column, separation of theanine was achieved within 10 minutes. The successful application of the C_{18} column facilitates the wide application of this method in quality control laboratories worldwide. The method is linear over several orders of magnitude and the LOD are 11.53 and 10.83 µg/ml and the LOQ are 39.44 and 35.10 µg/ml for the phenyl-hexyl and C_{18} column, respectively. Simple but effective sample preparation and PVPP pre-treatment, along with the short analysis time facilitates high sample throughput (~40 samples can be prepared in an 8 hour day with overnight analysis by HPLC-ELSD). The method is selective, precise, accurate and practical for the quantification of theanine in tea extracts and was successfully used to determine theanine content in a variety of tea samples. The sensitivity and simplicity of this method renders it suitable for use in routine theanine analysis in quality control laboratories worldwide.

Chapter 3

Identification of Digallated and Methylated Catechins in Tea (*Camellia sinensis*) using UPLC/MS/MS

3.1 Introduction

Catechins are naturally occurring flavanols that are found in a variety of foods of plant origin including fruits, wine, beer and chocolate, but it is the most abundant in tea (*Camellia sinensis*). The 6 major catechins present in tea leaves are catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg), see Figure 1.6. In some cultivars of tea less abundant catechins are also present, specifically epigallocatechin-3,5-digallate (EGCgg), epicatechin-3,5-digallate (ECgg), epicatechin-3-(3"-O-methyl) gallate (ECmetG) and epigallocatechin-3-(3"-O-methyl) gallate (EGCmetG) (Amarowicz *et al.*, 2005; Chiu *et al.*, 2005; Coxon *et al.*, 1972; Dou *et al.*, 2007).

Tea catechins are powerful antioxidants and several *in vitro* and *in vivo* studies have demonstrated their numerous biological and pharmacological properties, which include anti-mutagenic, anti-vascular disorders, anti-diabetic, anti-inflammatory, antiviral and neuroprotective effects (Adhami *et al.*, 2007; Anderson and Polansky, 2002; Hamer, 2007; Zavari, 2006). The general mechanism of action is thought to be through scavenging for endogenously formed free radicals (Pirker *et al.*, 2007). Structurally important features for the antioxidant activities of catechins include the 3'4'-dihydroxy catechol structure or a 3',4',5'-trihydroxyl group in the B ring, the

gallate group esterified at position 3 in the C ring and hydroxyl groups at positions 5 and 7 in the A ring (Frei and Higdon, 2003).

It has been demonstrated that the scavenging effects of trihydroxyl catechins (EGC and GC) are stronger than those of the dihydroxyl (catechol) catechins (EC and C) (Guo *et al.,* 1999). Guo *et al.* (1999) also found that the scavenging abilities of the gallated catechins (EGCg and ECg) are stronger than the non-gallated catechins (EGC, GC, EC, C). This suggests that the gallate group esterified at position 3 in the C ring plays a key role in enhancing the free radical scavenging abilities of catechins. This supports other studies that found that the scavenging abilities of catechins increase with the number of hydroxyl groups of the structure (Rice-Evans et al., 1996; Rice-Evans, 2001). Subsequently, it is reasonable to expect that digallated catechins will have enhanced antioxidant abilities compared to EGCg, ECg and the other catechins since the second gallic acid moiety adds 3 more hydroxyl groups to the catechin structure. (Figure 1.7 illustrates the structures of the individual catechins and Figure 1.13 demonstrates the relationship between the number of hydroxyl group and the scavenging abilities of catechins, as well as the expected increased scavenging abilities of digallated catechins).

Methylated derivatives of catechins (minor components of some tea infusions) have been reported to inhibit type I (hypersensitivity to pollens, mold spores, dust mites etc.) and type IV (delayed onset hypersensitivity caused by metal, jewellery etc.) allergic reactions (Maeda-Yamamoto *et al.*, 2007; Sano *et al.*, 1999; Suzuki *et al.*, 2000). It is speculated that type I allergic reactions are inhibited through the inhibition of multiple protein kinases in mast cells (Fujimura *et al.*, 2007; Maeda-Yamamoto *et*

al., 2004). However, knowledge of the biological activities of methylated catechins is still very limited.

There is very little literature describing the separation and determination of digallated and methylated catechins in tea extracts. This is probably due in part to most of the published methods lacking the sensitivity and selectivity to detect these minor catechin components in tea. However, a few studies reported the successful identification of digallated and methylated catechins in tea using high performance liquid chromatography (HPLC) with atmospheric pressure chemical or electrospray ionization tandem mass spectrometry (MS/MS) (Del Rio *et al.*, 2004; Wang *et al.*, 2008; Zeeb *et al.*, 2000). Other studies identified these minor catechins components in tea extracts based on their individual UV spectra (Nishitani and Sagesaka, 2004; Sajio and Takeda, 1999; Yao *et al.*, 2004). However, several other phenolic compounds with similar UV spectra are also present in crude tea extracts. Digallated and methylated catechins are not commercially available to use as standards during the analysis, rendering this method unreliable since identification by comparison with a standard is not possible.

Recently a relative new liquid chromatography technique, ultra performance liquid chromatography (UPLC) coupled to MS/MS was effectively applied in the analysis of plant and food products. It was used for the analysis of phenolic acids in beverages such as white wine, grapefruit juice and green tea (Gruz *et al.*, 2008) and orientin-derived flavonoids found in *Trollius ledibouri* flowers (Li *et al.*, 2006). UPLC is an advanced form of liquid chromatography. It uses columns packed with 1.7 µm particles and mobile phase delivery systems operating at high back-pressures of up to 15,000 psi (103,421 kPa or 1034 bar; ACQUITY UPLC System Operator's Guide).

The major advantages of UPLC over conventional HPLC are enhanced resolution and selectivity which results in decreased analysis time and solvent consumption (Yu *et al.*, 2006). Coupling UPLC with electrospray ionization tandem mass spectrometry thus offers a superior alternative to conventional HPLC/MS/MS.

In this study a tandem quadrupole, atmospheric pressure ionization (API) mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source was used (TQD). The quadrupole is the most widely used mass analyzer due to its ease of use, mass range cover, good linearity for quantitative work, good resolution and quality of the mass spectra obtained. The TQD consists of two quadrupoles separated by a collision cell (see Figure 3.1). The sample from the liquid chromatography system is introduced into the ionization source at atmospheric pressure. The ions generated travel to the first quadrupole. The first quadrupole is used to select a molecular or precursor ion, which is fragmented in the collision cell. This is typically achieved in the collision cell by accelerating the ions in the presence of a collision gas (argon, helium etc.). The energy of the collision with the gas can be varied to allow different degrees of fragmentation. The resulting fragments are analyzed by the second quadrupole, used either in selected ion monitoring (SIM) or in scan mode. The ions (which are in the gas phase) are separated according to their mass to charge ratio (*m/z* value).
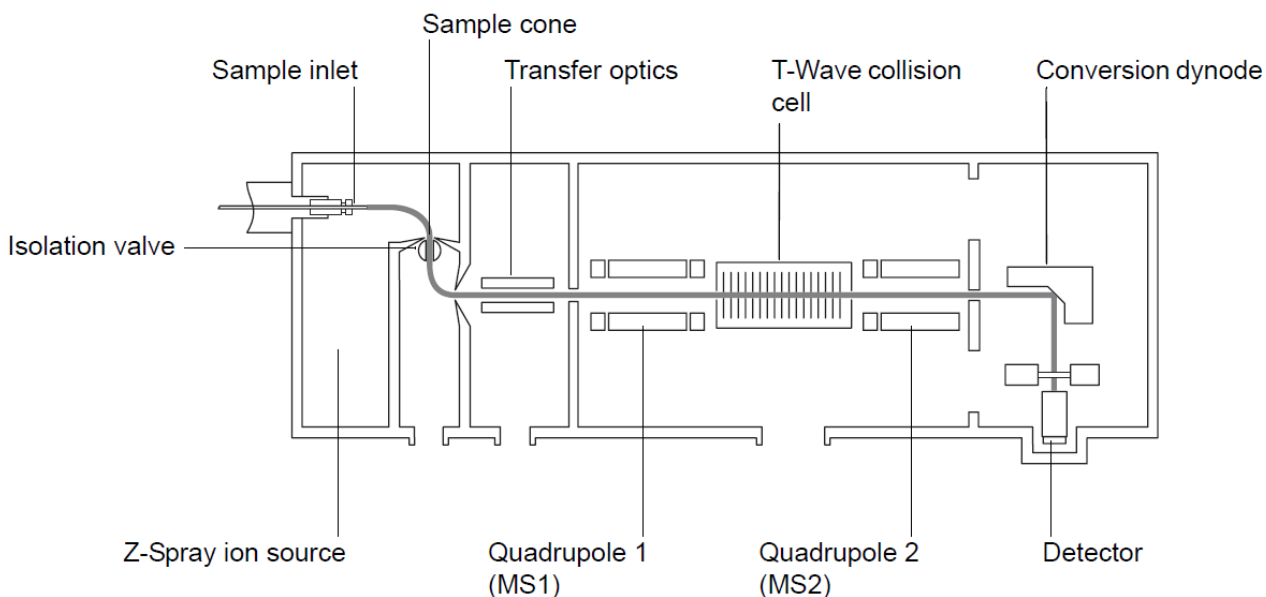
Figure 3.1. Schematic representation of the tandem quadrupole mass spectrometer (Waters TQD Operator's Manuel). Samples are introduced at atmospheric pressure into the ionization source and pass through the sample cone into the vacuum system. The ions pass through the transfer optics to the first quadrupole where they are filtered according to their mass to charge ratio (m/z). The mass-separated ions pass into the T-Wave™ collision cell where they either undergo collision-induced dissociation (CID) or pass to the second quadrupole. Any fragment ions are then mass-analyzed by the second quadrupole and the transmitted ions are detected by the photomultiplier detection system.

Both positive ionization (Chen *et al.*, 2007; Pelilio *et al.*, 2004; Shen *et al.*, 2006; Zeeb *et al.*, 2000) and negative ionization (Del Rio *et al.*, 2004; Dou *et al.*, 2007; Masukawa *et al.*, 2006; Wang *et al.*, 2008) have been used in the mass spectrometric analysis of catechins. The characteristic fragment ions obtained by LC/ESI-MS and LC/APCI-MS (atmospheric pressure chemical ionization-MS) in positive or negative ion mode have been used to quantify 6 major catechins. These characteristic fragment ions are listed in Table 3.1.

Table 3.1. Assignment of molecular and fragment ions to specific compounds present in green tea after LC/MS and LC/MS/MS analysis using positive and negative ionization.

POSITIVE IONIZATION[a,b]						
Compound	Molecular weight	MS1 [M + H]⁺ (*m/z*)	MS2 Fragment ions (*m/z*)			
(+)-catechin	290	291	273	139		
(-)-epicatechin	290	291	273	139		
(-)-gallocatechin	306	307	289	139		
(-)-epigallocatechin	306	307	289	139		
(-)-epicatechin gallate	442	443	273	139		
(-)-gallocatechin gallate	458	459	289	139		
(-)-epigallocatechin gallate	458	459	289	139		
NEGATIVE IONIZATION[c]						
Compound	Molecular weight	MS1 [M - H]⁻ (*m/z*)	MS2 Fragment ions (*m/z*)			
(+)-catechin	290	298	203	159	123	
(-)-epicatechin	290	298	203	159	123	
(-)-gallocatechin	306	305	219	137	125	
(-)-epigallocatechin	306	305	219	137	125	
(-)-epicatechin gallate	442	441	289	245	169	125
(-)-gallocatechin gallate	458	457	305	193	169	125
(-)-epigallocatechin gallate	458	457	305	193	169	125

[a] Shen *et al.*, 2006 [b] Zeeb *et al.*, 2000 [c] Wang *et al.*, 2008

Besides the 6 major catechins low abundance catechins have also been successfully detected with MS/MS. Using LC/APCI-MS/MS in positive ion mode, Zeeb *et al.* (2000) detected low abundance catechins, (-)-epicatechin-3-(3"-O-methyl) gallate (ECmetG) and (-)-epigallocatechin-3-(3"-O-methyl) gallate (EGCmetG), in both green and black tea extracts. During the first MS stage (MS1), molecular ions were detected at *m/z* 457 for ECmetG and *m/z* 473 for EGCMetG. During MS2, fragment ions were detected at *m/z* 273 and 139 for ECmetG and *m/z* 289 and 139 for

EGCmetG. Dou *et al.* (2007) identified methylated catechins in oolong tea using a ion-trap ESI-MS/MS system under negative ion mode. According to their results, EGCmetG had a molecular ion of *m/z* 455 with fragment ions of *m/z* 289 and 183. A molecular ion of *m/z* 471 was obtained for ECmetG with fragment ions of *m/z* 305 and 183. Later, in 2008, Wang *et al.* used a triple quadrupole ESI-MS/MS system to identify 2 methylated and 2 digallated catechins in several tea cultivars from Japan and Taiwan (the chosen cultivars are mainly used for making oolong tea). They were able to quantify EGCmetG and (-)-epigallocatechin-3,5-digallate (EGCgg) by reference to (-)-gallocatchin-3-gallate (GCg). ECmetG and (-)-epicatechin-3,5-digallate (ECgg) were not quantified due to being present at levels below the limit of quantification. The molecular and fragmentation ions obtain for these compounds are listed in Table 3.2.

Table 3.2. Assignment of molecular and fragment ions to digallated and methylated catechins present in tea after LC/MS and LC/MS/MS analysis using positive and negative ionization.

POSITIVE IONIZATION						
Compound	**Molecular weight**	**MS1 [M + H]+ (*m/z*)**	**MS2 Fragment ions (*m/z*)**			
(-)-epicatechin-3-(3"-O-methyl) gallate[a]	456	457	273	139		
(-)-epigallocatechin-3-(3"-O-methyl) gallate[a]	472	473	289	139		
NEGATIVE IONIZATION						
Compound	**Molecular weight**	**MS1 [M - H]- (*m/z*)**	**MS2 Fragment ions (*m/z*)**			
(-)-epicatechin-3-(3"-O-methyl) gallate[b,c]	456	455	289	245	183	125
(-)-epigallocatechin-3-(3"-O-methyl) gallate[b,c]	472	471	305	219	183	125
(-)-epicatechin-3,5-digallate[c]	594	441	289	169	125	
(-)-epigallocatechin-3,5-digallate[c]	610	457	305	169	125	

[a] Zeeb *et al.*, 2000 [b] Dou *et al.*, 2007 [c] Wang *et al.*, 2008

This study aimed to identify digallated and methylated catechins, that are not commercially available to use as standards during the analysis of tea samples, within the elution profile of a tea sample. Examination of the general utility of UPLC/MS/MS for identification of these minor catechin components in a crude tea infusion was the specific aim of this study.

3.2 Materials and Methods

3.2.1 Materials

Green tea extract (instant green tea), produced from tea leaves originating from Sri-Lanka, was donated by Mitsui Norin (Japan). Minisart hydrophilic syringe filters, 0.2 μm, were obtained from Sartorius (Hannover, Germany). (-)-Epigallocatechin gallate (EGCg) was purchased from Sigma Chemical Company (Steinheim, Germany). Glacial acetic acid (Ultra ≥ 99.5%) was bought from Merck (Dramstads, Germany) and acetonitrile (Ultra purity solvent) was obtained from Romuel (Dramstads, Germany). All solutions and dilutions were prepared with double distilled de-ionized water (dddwater), produced by a MilliQ water purification system from Milipore Corp (Bedford, MA, USA). All reagents were of analytical grade unless otherwise indicated.

3.2.2 Sample Preparation

Green tea liquors were prepared from the instant green tea powder as 5% (w/v) tea solutions. Five millilitres water were added to 250 mg instant green tea powder. The tea solutions were filtered through 0.2 μm syringe filters to remove particulate matter.

3.2.3 Instrumentation

Analyses were performed on a Waters ACQUITY UPLC® system (Milford, MA, USA) which included a binary solvent system manager, sample manager, column manager and a TUV (tuneable ultraviolet) optical detector. The system was coupled to a

Waters ACQUITY TQ detector (Waters Corporation, Micromass UK Ltd, Manchester, UK), a tandem quadrupole, atmospheric pressure ionization (API) mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source. Waters Empower 2 software (Build nr 2154) was used for data acquisition and processing.

3.2.4 Chromatographic Conditions

Reverse phase chromatographic separations were carried out on an ACQUITY UPLC® BEH phenyl column (150 x 2.1 mm, 1.7 μm) obtained from Waters (Milford, MA, USA). The column temperature was maintained at 30 °C. Mobile phase A consisted of dddwater/acetonitrile/acetic acid (97:1:2 v/v/v) and mobile phase B consisted of dddwater/acetonitrile/acetic acid (18:80:2 v/v/v). The following gradient was used: 0 - 31.3 minutes, 0 - 13 % B; 31.3 - 33 minutes, 17% B; 33 - 37.5 minutes, 17 - 24% B; 37.5 - 41 minutes, 24 - 31% B; 41 - 42 minutes, 31 - 0% B; 42 - 45.5 minutes, 0% B. The flow rate of the mobile phase was 0.3 ml/min. The injection volume was 5 μl. Components of interest were monitored at 270 nm.

3.2.5 Spectroscopic Conditions

Mass spectrometer conditions were optimized using an EGCg standard solution. The source and the desolvation temperatures were maintained at 118 and 348 °C, respectively. The probe voltage (capillary voltage), cone voltage and extractor voltage were set at 2.71 kV, 50 V and 2 V, respectively. Nitrogen was used as desolvation (599 L/h) and drying gas (51 L/h). A full MS scan from 100.00 – 700.00 Da (*m/z*) was done. Mass spectra were acquired in positive ion mode ([M + H]$^+$). Selective ion monitoring was used to detect specific fragment ions. Mass spectrums

were scanned for the molecular ions of (-)-epigallocatechin gallate (*m/z* 459), (-)-epicatechin-3-(O-3"-methyl) gallate (*m/z* 457), (-)-epigallocatechin-(O-3"-methyl) gallate (*m/z* 473), (-)-epicatechin-3,5-digallate (*m/z* 595) and (-)-epigallocatechin-3,5-digallate (*m/z* 611).

3.3 Results and Discussion

3.3.1 Chromatographic Conditions

The chromatographic conditions used for the UPLC analysis was similar to a HPLC method developed in our research laboratory. This method was specifically optimized for the separation of low abundant catechin components in tea. A phenyl bonded column was used that provided additional selectivity over reverse phase materials resulting in improved separation of catechins. However, standards are not available for the catechins of interest (digallated and methylated) and identification of these catechins can not be based on their individual UV spectra alone due to the presence of other phenolic compounds with similar spectra. LC/MS/MS was performed to determine the HPLC elution times of digallated and methylated catechins. The search was narrowed down previously (Maliepaard, 2007) to a few potential HPLC retention times for 2 digallated catechins but the results were inconclusive and further investigation was required. Continuing this research with an UPLC/MS/MS system allowed shortening of the analysis time up to 4-fold compared to the HPLC system using 5 μm packed analytical column. Increased resolution was also observed resulting in a decreased number of compounds co-eluting. Furthermore, approximately 80% less organic solvent was used.

3.3.2 Identification of Minor Catechin Components by UPLC/MS/MS in a Tea Sample

Selective ion monitoring was used to detect specific fragment ions. Mass spectrums were scanned for the molecular ions, $[M + H]^+$, of (-)-epigallocatechin gallate (m/z 459), (-)-epicatechin-3-(O-3"-methyl) gallate (m/z 457), (-)-epigallocatechin-(O-3"-

methyl) gallate (*m/z* 473), (-)-epicatechin-3,5-digallate (*m/z* 595) and (-)-epigallocatechin-3,5-digallate (*m/z* 611). A typical UPLC-UV chromatogram indicating the retention times of the peaks tentatively identified as the compounds in question are shown in Figure 3.3. The retention times of 2 peaks tentatively identified as O-diglycosyl flavonoids are also indicated.

The molecular ion ([M + H]$^+$, *m/z* 459) of EGCg was detected at 22.31 minutes (see Figure 3.3) with characteristic fragment ions of *m/z* 289 and *m/z* 139 (Figure 3.4). These detected fragment ions for EGCg corresponds to the fragments observed by Shen *et al.* (2006) and Zeeb *et al.* (2000). The *m/z* 139 ion arises from the Retro-Diels-Alder (RDA) fragmentation of the non-variable portion of the catechin ring as shown in Figure 3.2 (Wu *et al.*, 2003). The fragment ion detected at m/z 289 arises from the loss of the gallic acid moiety [M + H − 169]$^+$ of EGCg.
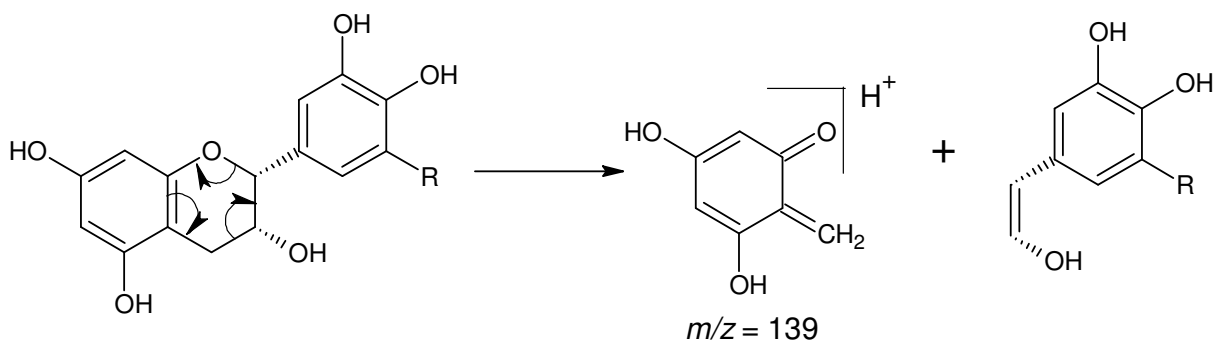
Figure 3.2. The Retro-Diels-Alder fragmentation of the non-variable portion of the catechin ring (adapted from Wu *et al.*, 2003). A characteristic *m/z* 139 product ion is observed when performing mass spectrometry under positive ion mode for non-gallated catechins. This fragment ion is also observed as a minor fragment component of mono-gallated catechins.
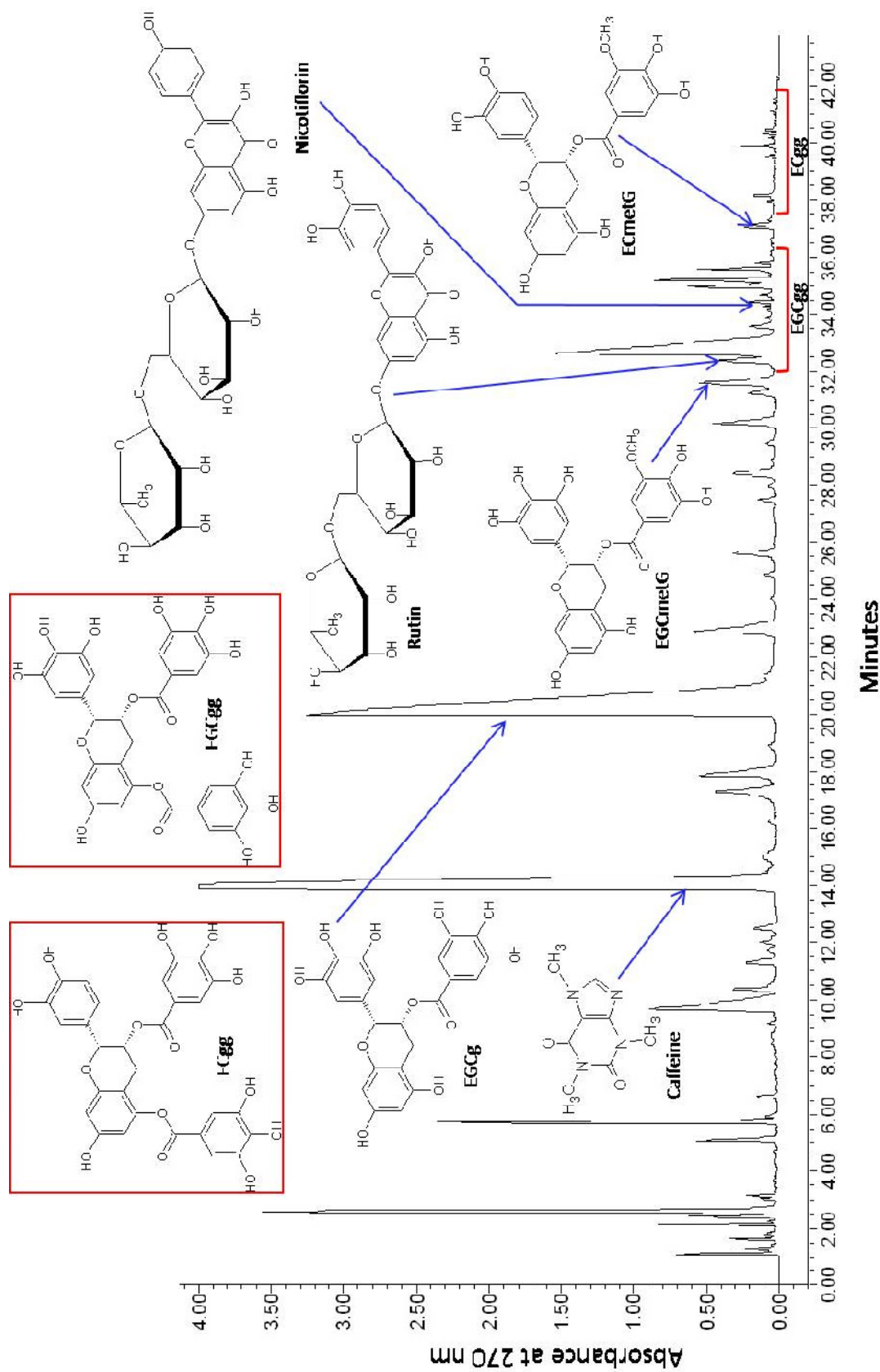
Figure 3.3. An UPLC-UV chromatogram of 5% instant green tea from Sri-Lanka indicating tentative retention times for digallated and methylated catechins. The retention times of rutin and nicotiflorin, 2 O-diglycosyl flavonoids tentatively identified in this study, are also indicated. The retention time windows in which EGCgg and ECgg were expected to elute are indicated in red (these windows are based on the previous study done by Maliepaard [2007]).

MW_{EGCg} = 458 g/mol
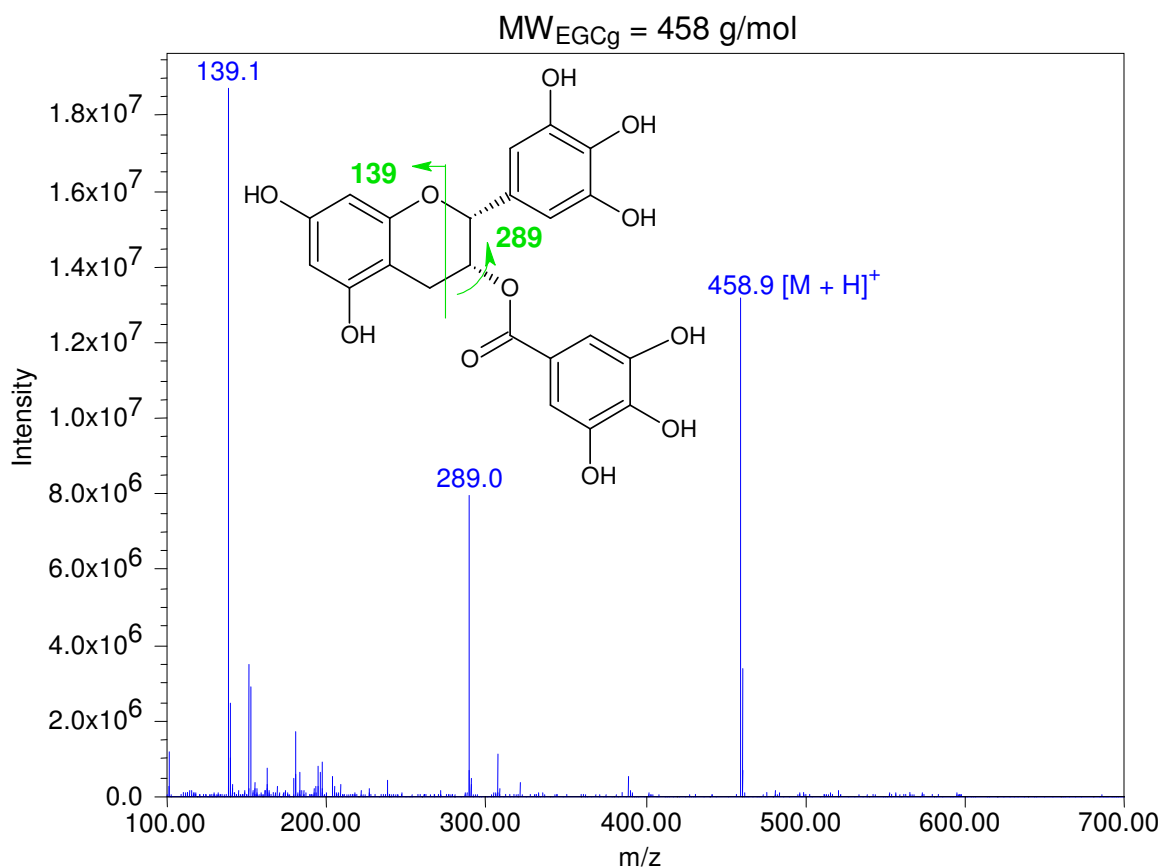
Figure 3.4. The mass spectrum of EGCg ($[M + H]^+$ = 459 m/z).

The peak eluting at 31.88 minutes was tentatively identified as epigallocatechin-(O-3"-methyl) gallate (EGCmetG), since it had a molecular ion of m/z 473 with fragment ions of m/z 289 and 139 (Figure 3.5). The presence of the m/z 139 established that the compound was structurally related to a catechin and that the A ring was unmodified (Zeeb *et al.*, 2000). The presence of the m/z 289 fragment confirmed the relation of this compound to EGCg and indicated that the A and B rings were both unmodified, leaving the gallic acid moiety as the site of methylation. Methylation of EGCg can take place at the 3"- or 4"-hydroxyl group but previous studies have shown that an unmodified 4"-hydroxyl group is required for the loss of gallic acid from catechin gallates to form the characteristic m/z fragment of 289 (Miketova *et al.*,

2000). NMR studies of catechins extracted from tea leaves have also revealed that EGCg is methylated at position 3" (Davis *et al.*, 1996). This supports the assignment of EGCmetG to the peak eluting at 31.88 minutes. The observed fragmentation pattern of this peak also corresponds to the fragmentation pattern assigned by Zeeb *et al.* (2000) to EGCmetG.
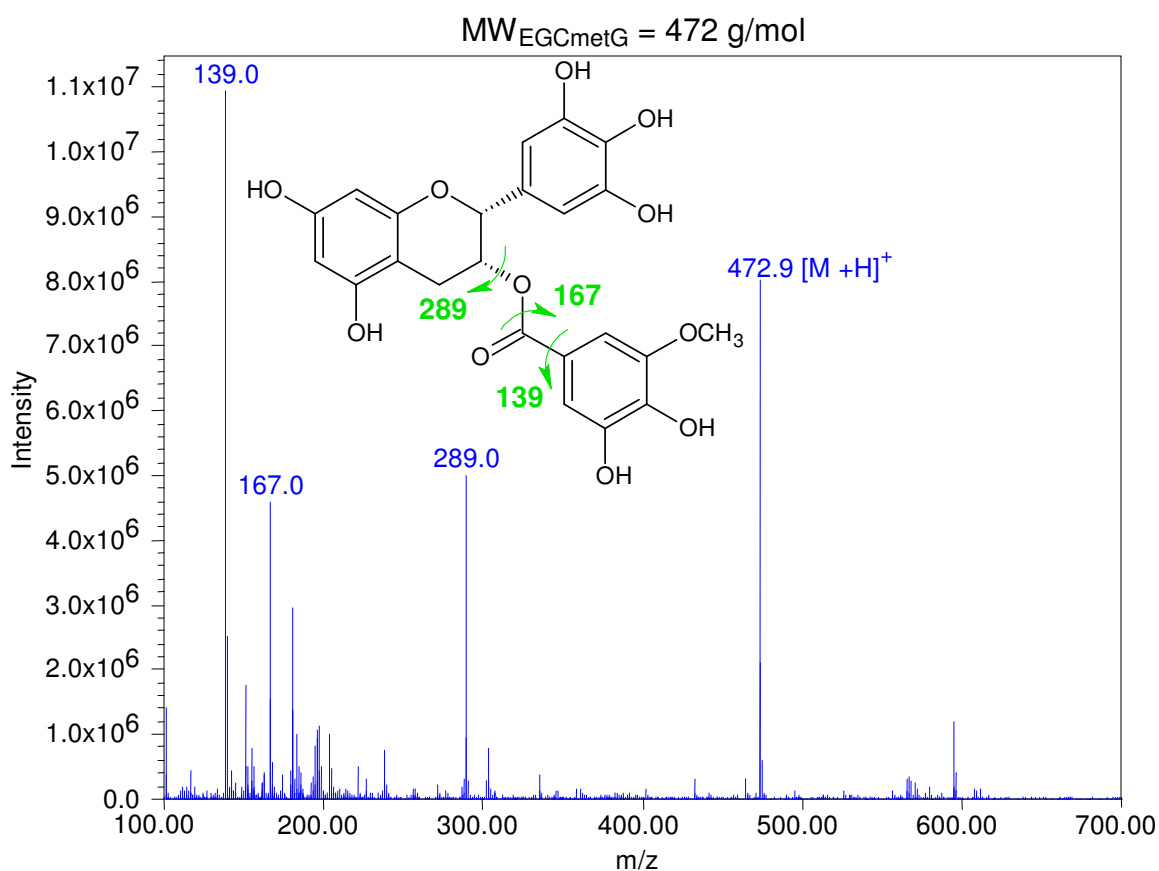
Figure 3.5. The mass spectrum of the peak at retention time 31.88 minutes, tentatively identified as EGCmetG ([M + H]$^+$ = 473 *m/z*).

The average mass spectrum recorded for the peak eluting at 37.47 minutes had a [M + H]$^+$ of *m/z* 457 and fragment ions of *m/z* 273 and 167, indicating that is was the methylated derivative of ECg, epicatechin-3-(O-3"-methyl) gallate, ECmetG (see Figure 3.6). The *m/z* 273 fragment ion confirmed the structural similarity to ECg

(Shen *et al.*, 2006) while the *m/z* 167 was consistent with the presence of a methylated gallic acid moiety. A similar mass spectrum was previously assigned to ECmetG by Zeeb *et al.* (2000).
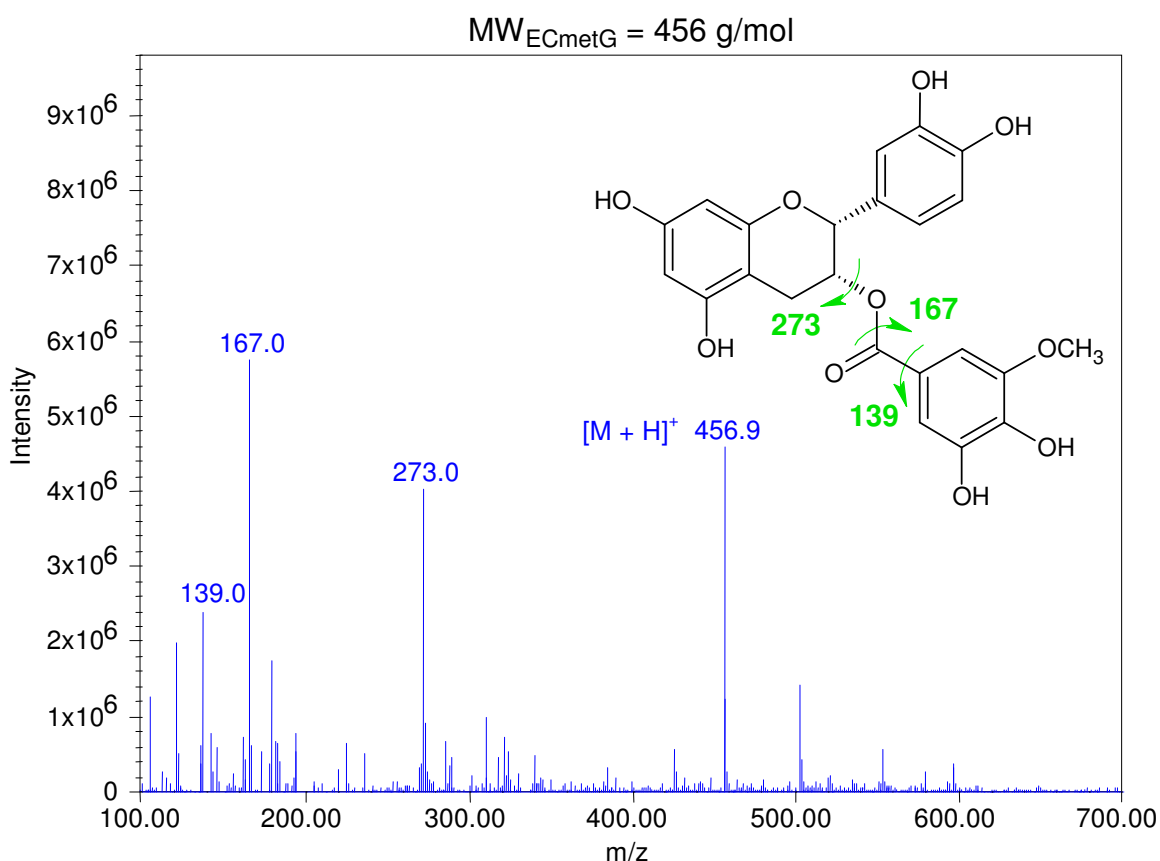
Figure 3.6. Mass spectrum of the peak at retention time 37.47 minutes, tentatively identified as ECmetG ([M + H]⁺ 457 *m/z*).

Several peaks with [M + H]⁺ of *m/z* of 595 or 611 were detected. None of the mass spectra recorded for these peaks could be matched to the expected mass spectra of the digallated catechins (see Figure 3.7).
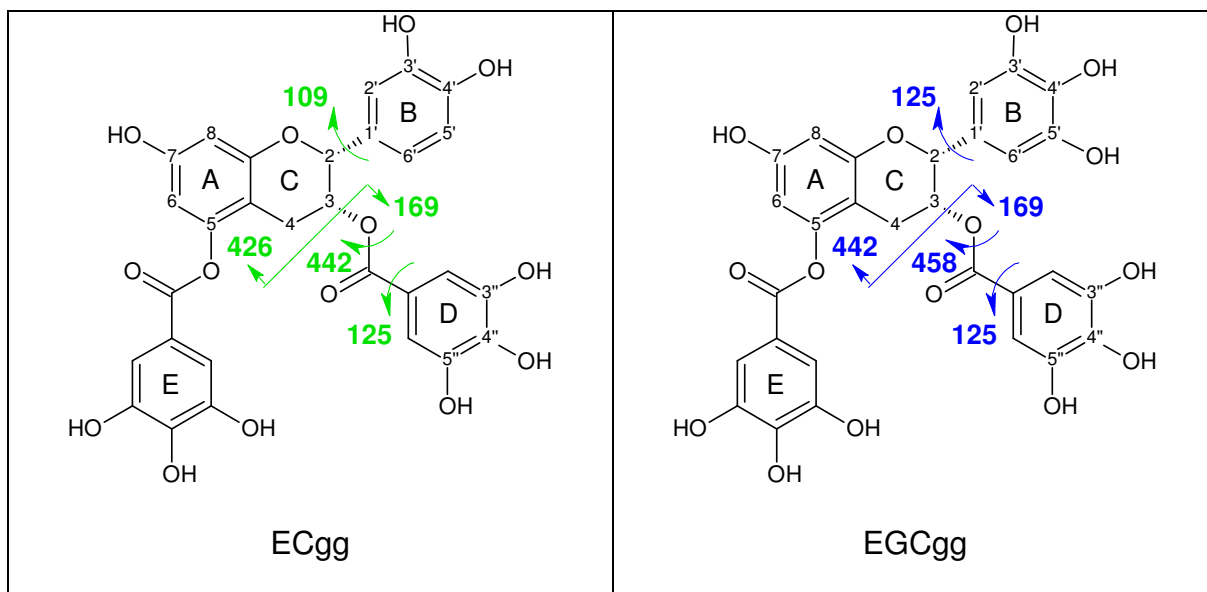
Figure 3.7. Possible mass fragmentation of ECgg (MW = 954 g/mol) and EGCgg (MW = 610 g/mol), using positive ionization (Adapted from Wang *et al.*, 2008).

However, the mass spectrum of the $[M + H]^+$ (*m/z* 611) of the peak eluting at 33.26 minutes matched the mass spectrum of rutin (quercetin-3-O-rutinoside), an O-glycosidic flavonoid. The 2 major fragment ions at *m/z* 287 and 449, shown in Figure 3.8, arise from cleavage at 2 glycosidic bonds that provide the glycone sequence (Ma *et al.*, 2000). The loss of 146 Da points to a terminal rhamnose unit and yields the fragment ion at *m/z* 465, while an additional loss of 162 Da (hexose) or the direct loss of the rutinose residue (308 Da) results in the aglycone fragment ion at *m/z* 303 (Cuyckens *et al.*, 2004). These results are consistent with the MS data of other studies that investigated O-glycosidic flavonoids (Cuyckens *et al.*, 2004; Ma *et al.*, 2000; Vickics *et al.*, 2008).
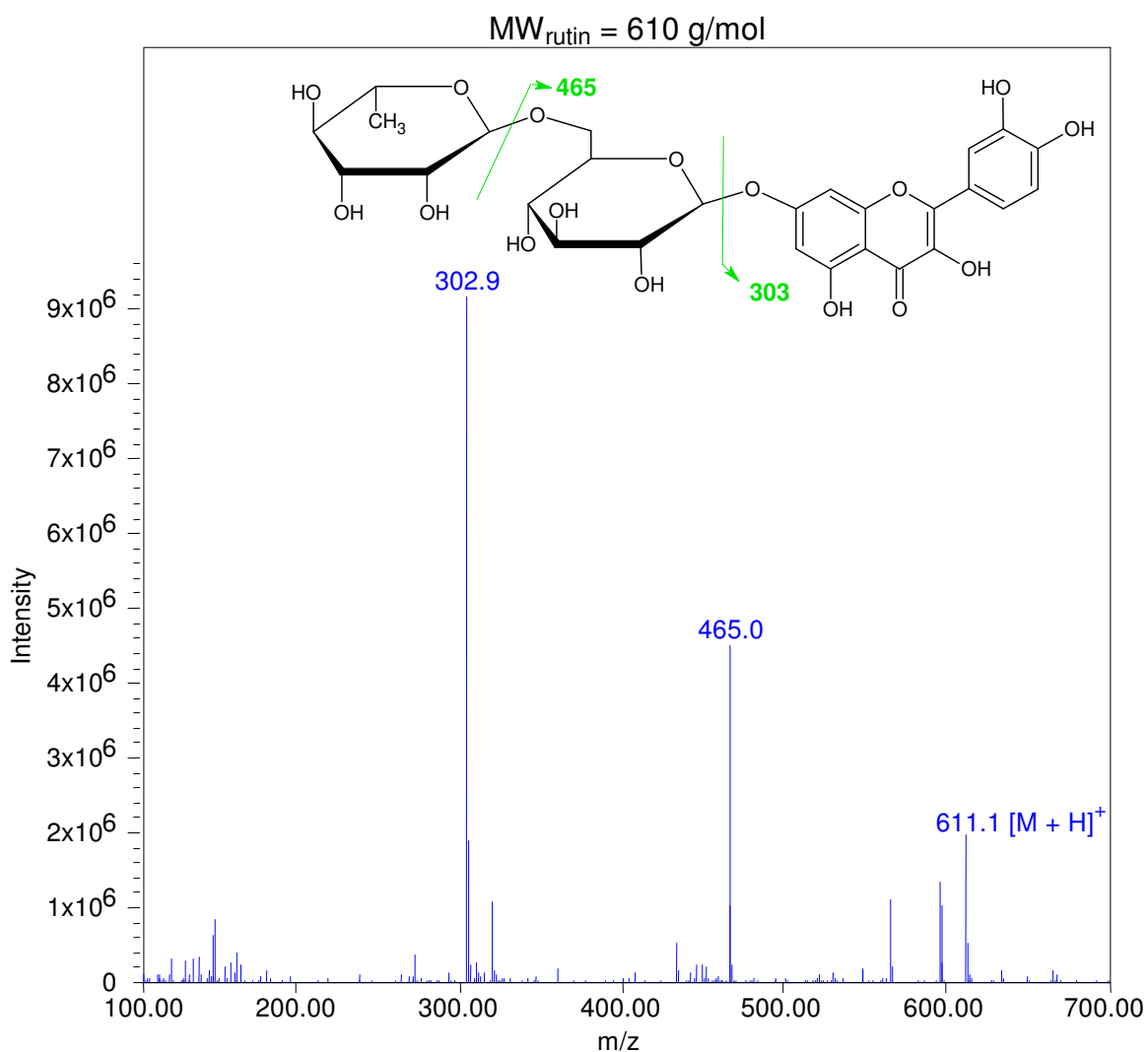
Figure 3.8. The mass spectrum of the peak at retention time 33.26 minutes, tentatively identified as rutin ([M + H]$^+$ = 611 m/z).

Nicotiflorin (kaempferol 3-O-rutinoside), another O-glycosidic flavonoid, was tentatively identified as the peak eluting at 34.94 minutes ([M + H]$^+$ m/z 595). Similar to the mass fractionation of rutin, cleavage of the glycosidic bonds yields 2 major fragment ions observed at m/z 287 and 449 (Figure 3.9). The fragment ion at m/z 449 corresponds to the loss of a terminal rhamnose unit [M + H - 146]$^+$, while the additional loss of hexose ([M + H − 146 - 162]$^+$) or the direct loss of the rutinose residue ([M + H − 308]$^+$) yields the aglycone fragment ion observed at m/z 287 (Cuyckens et al., 2004; Ma et al., 2000; Vickics et al., 2008).
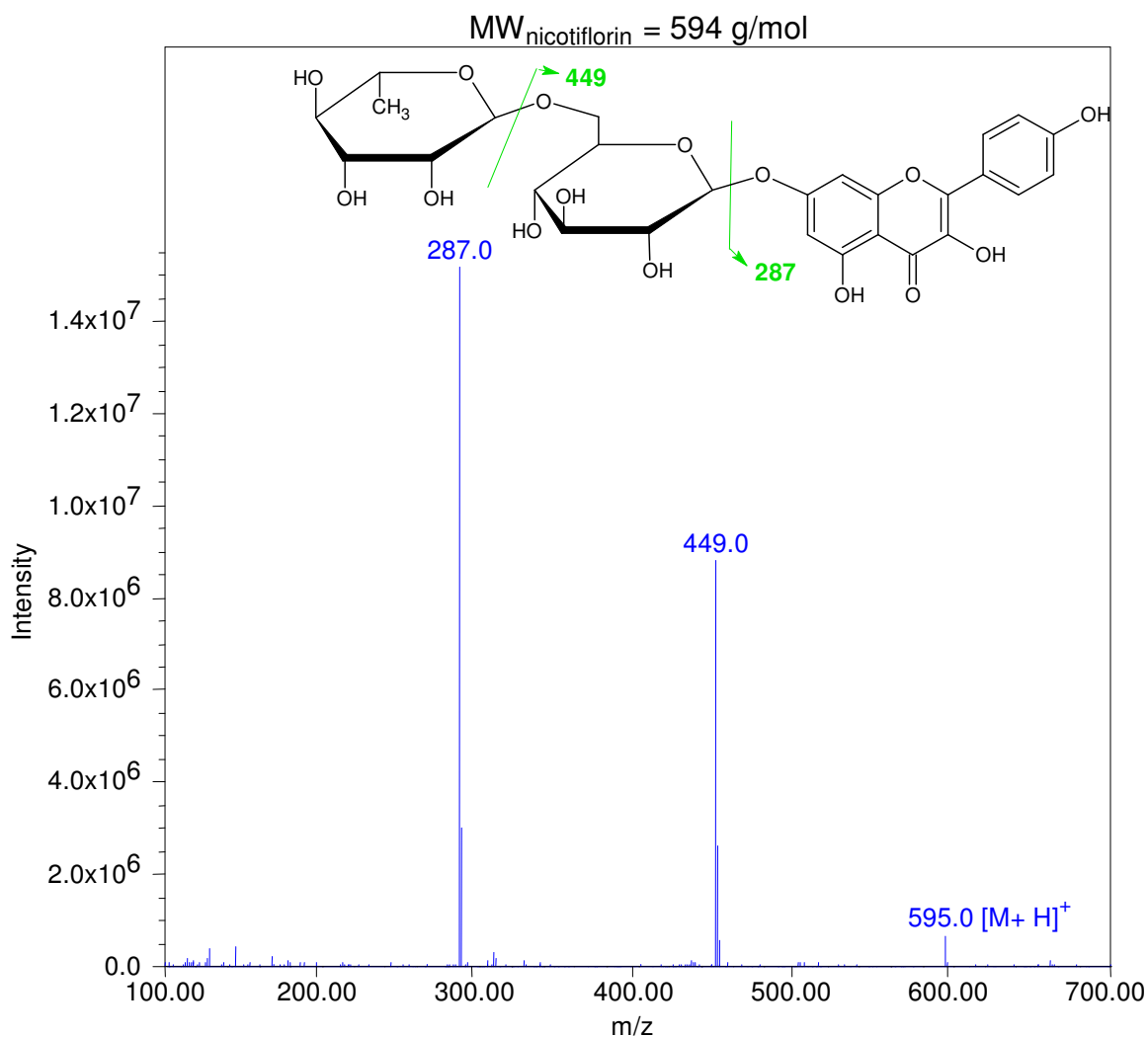
Figure 3.9. The mass spectrum of the peak at retention time 34.94 minutes, tentatively identified as nicotiflorin ($[M + H]^+$ = 595 *m/z*).

The previous study done in our laboratory by Maliepaard (2007) utilized HPLC/MS/MS and could tentatively identify several possible retention times of digallated catechins but failed to detect the methylated catechins. This current study utilizing UPLC/MS/MS succeeded where the previous study failed by tentatively identifying both methylated EGCgg and ECgg, but failed to identify the digallated catechins. A possible explanation is that the tea sample analyzed lacked these catechin components or that they were present at a level below the limit of detection. Both the previous and current study identified peak positions for rutin and nicotiflorin.

(Table 3.3 summarizes and Figure 3.3 illustrates the peaks tentatively identified in

this study).

Table 3.3. Assignment of specific molecular and fragment ions obtained to compounds present in 5% instant green tea solution from Sri-Lanka.

Retention Time (minutes)	[M + H]$^+$ (*m/z*)	Fragment Ions (*m/z*)			Tentative Identification
22.31	459	289	139		EGCg
31.88	473	289	167	139	EGCmetG
33.26	611	465	303		Rutin
34.94	595	449	287		Nicotiflorin
37.47	457	273	167		ECmetG

3.4 Conclusion

UPLC/MS/MS was effectively applied to investigate the presence of the digallated and methylated catechins in a crude tea extract. Peak positions for low abundance catechins (-)-epigallocatechin-(O-3"-methyl) gallate (*m/z* 473) and (-)-epicatechin-3-(O-3"-methyl) gallate (*m/z* 457) were successfully assigned at 31.88 and 37.47 minutes, respectively. The 2 digallated catechins investigated could not be detected, possibly due to being present at concentrations below the limit of detection. Two O-glycosidic flavonoids, rutin (m/z 611) and nicotiflorin (m/z 595), were tentatively identified at 33.26 and 34.94 minutes, respectively.

UPLC has proven to be one of the most promising developments in the area of high speed chromatographic separations with increased sensitivity and resolution. This study demonstrated that UPLC/MS/MS can be effectively used for the analysis of low abundance catechins present in tea infusions. Increased resolution, approximately 80% less use of organic solvent and significantly reduced analysis times are some of the advantages observed compared to conventional HPLC/MS/MS.

Chapter 4

Concluding Discussion

Tea (*Camellia sinensis*) is one of the most popular beverages consumed in the world. According to the UN Food and Agriculture Organization (FAO), global tea production amounted to 3.6 million tons in 2006. Tea is cultivated in approximately 52 countries worldwide and is generally consumed in the form of black (78%), green (20%) or oolong (2%) tea. Green tea is preferred in oriental countries, including a number of countries in North Africa and the Middle East, while black tea is the drink of choice in Western countries (Cooper *et al.*, 2005). Oolong tea is the preferred beverage in southern China and Taiwan.

The antioxidant activity and other health benefits of tea can largely be attributed to catechins. They are polyphenolic compounds classified as flavanols, a subclass of flavonoids. Tea catechins mainly act as antioxidants by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metals in the lipophilic and aqueous phase of the cell (Burda and Oleszek, 2001; Dugas *et al.*, 2000; Haenen *et al.*, 1997; Jain *et al.*, 2006; Ryan *et al.*, 2007; Salah *et al.*, 1995; Sang *et al.*, 2002; Schroeter *et al.*, 2002). The most profound antioxidative activity among catechins is attributed to EGCg, generally the most abundant catechin in green tea. Due to the health-benefiting effects from the catechins in green tea, including antioxidative, anti-cancer, anti-inflammatory, anti-aging, antibiotic, and antiviral effects, the consumption of green tea is rapidly increasing, as is the consumption of a variety of green tea products, including beverages, ice cream and cosmetics.

Theanine, the free amino acids found almost exclusively in tea plants, also contributes to the wide array of physiological functions of green tea extract. Several studies support the hypothesis that theanine may be a possible neuroprotective, cognitive enhancing and anxiolytic agent. Even though it is used extensively in supplements and beverages due to its relaxation effect, the pharmacology of theanine is still largely unknown.

Rapid Analysis of Theanine in Tea Samples.

The quality of tea is mainly assessed through its appearance (colour, colour intensity and clarity), its taste (astringency, bitterness and sweetness) and its aroma (floral, sweet, grassy, etc.). The most important chemical constituents that influence the flavour and taste of tea infusions are catechins, polyphenols, caffeine, sugars, organic acids, amino acids and volatile aroma compounds. However, the components that influence the quality of green and black teas differ. Black tea quality depends on the contents of theaflavin, thearubigen, catechin and caffeine, while the quality of green tea is more dependant on amino acid (especially theanine), catechin and caffeine contents (Le Gall *et al.*, 2004; Liang *et al.*, 2003; Nakagawa, 1975).

Many characteristics are taken into account to evaluate tea quality (Paul *et al.*, 2008). Teas of different origin have their own distinctive characteristics but in general high quality green teas are described as delicate or sweet (Nakagawa, 1975). The sweetness is attributed to amino acids, especially theanine which has a sweet, brothy or unami taste. Since theanine accounts for more than 50% of the amino acid contents in tea, it is considered an important flavour component that influences the

quality of green tea (Nakagawa, 1975). Other important components are caffeine and catechins which contribute to the characteristic astringency and bitterness of green tea.

The quality and price of tea is established through the judgement of professional tea tasters, but there is growing need to assess the quality of tea by some form of analytical measurement. Standard international analysis methods for the quantification of caffeine and catechins already exist but there is no standard analysis method for the important flavour component theanine (Liang *et al.*, 2008). Several methods with different advantages and disadvantages have been reported but most of them are unsuitable for routine quality control analysis in commercial tea samples.

The objective of this study was to develop a simple reverse phase HPLC method for the routine analysis of theanine in tea leaves/commercial tea samples with evaporative light scattering (ELS) detection, eliminating the need for any time consuming derivatization processes or expensive detection techniques such as mass spectrometry.

The chromatographic separation of theanine was achieved by reverse phase HPLC using a phenyl-hexyl column, isocratic water elution and ELS detection in commercial tea samples in less than 8 minutes. The new method requires no sample derivatization and includes simple sample clean-up. Applying the same chromatographic conditions to a widely available C_{18} column, separation of theanine was achieved within 10 minutes. The successful application of the C_{18} column facilitates the wide application of this method in quality control laboratories worldwide.

To yield reliable results that could be properly interpreted, the new methods using the phenyl-hexyl and C_{18} column were validated according to Reviewer Guidance Validation of Chromatographic Methods promulgated by the Centre for Drug Evaluation and Research (CDER) at the Food and Drug Administration (FDA), as well as the EU guideline for the Validation of Analytical Procedures Methodology as adopted by the Therapeutic Goods Administration (a division of the commonwealth Department of Health and Ageing) in 2002. The following parameters were examined: selectivity, precision, linearity, limit of detection and quantification, accuracy and sample stability.

The new method was found to be linear over several orders of magnitude and the LOD were 11.53 and 10.83 µg/ml and the LOQ were 39.44 and 35.10 µg/ml for the phenyl-hexyl and C_{18} column, respectively. The method was validated as selective, precise and accurate. Extracted tea samples were found to be stable up to 4 days if stored at 4 or -20 °C.

Using ELS detection for theanine analysis is a relatively novel approach. A method reported by Li Y *et al*. (2005) is the only other published method found for the determination of theanine in tea samples utilizing an ELSD. Both methods use reverse phase HPLC and were validated as simple, accurate and selective. The LODs and LOQs are similar but the new method, using a later model ELSD, has a wider linear range.

The new method is also better suited for high sample throughput due to the analysis time that is only a third of the analysis time of the Li Y *et al*. method. Approximately 40 samples can be prepared in an 8 hour day with overnight analysis by HPLC-ELSD

compared to approximately 16 samples that can be analysed overnight (8 hours) using the previously reported method of Li Y *et al.* (2005). The shorter analysis time was achieved by including a simple, fast but effective sample clean-up procedure utilizing polyvinylpolypyrrolidone (PVPP) and using a simple isocratic one solvent system. In comparison, the Li Y *et al.* method has no sample clean-up procedure and uses a gradient two solvent system.

The method reported in this study was demonstrated to be applicable and practical for the quantification of theanine in tea extracts as it was successfully used to determine theanine content in a variety of tea samples. The sensitivity and simplicity of this method renders it suitable for use in routine theanine analysis in quality control laboratories. This method is a prime candidate to be accepted as the standard international analysis method of theanine.

Future prospects include the provisional acceptance of this method by the ISO, followed by the official acceptance after a method evaluation, which includes a ring test, has been performed.

Identification of digallated and methylated catechins.

Recently, an accumulating body of evidence is showing that methylated forms of catechins have potent inhibitory activities to allergies (Chiu and Lin, 2005; Fujimara *et al.*, 2007; Maeda-Yamamoto *et al.*, 2004; Maeda-Yamamoto *et al.*, 2007). However, knowledge of the pharmacology of these catechins is still limited. These methylated catechins have been detected as minor components in Chinese, Vietnamese and Indian green teas (Saijo and Takeda, 1999).

In addition to the methylated catechins, another relative novel group of catechins exists, namely digallated catechins. Digallated catechins were first discovered in green tea in 1959 by Vuataz *et al.* Since then very little research has been done on these specific flavanols, possibly as they are usually present in very small amounts, if at all. The medicinal value of these compounds is not yet known, but it is reasonable to speculate that the antioxidant capacity of these compounds will be superior to that of epigallocatechin gallate (EGCg) due to the 3 additional hydroxyl groups provided by the second gallic acid moiety (the antioxidant activity of catechins are governed by the number and location of hydroxyl groups on the flavonoid ring system).

In order to detect digallated and methylated catechins present in green tea extracts for which standards are not available, separation and detection techniques are required that provide sufficient structural information to confirm the identity of these compounds.

In this study the general utility of UPLC/MS/MS for the identification of minor catechin components in a crude tea extract was examined. Ultimately, this will allow for the future identification of tea cultivars grown in southern Africa that have high contents of digallated and methylated catechins, which can be used in the production of high quality value added tea products.

UPLC/ESI-MS/MS was effectively applied to investigate the presence of digallated and methylated catechins in crude tea extracts. Compared to conventional HPLC/ESI-MS/MS, increased resolution and sensitivity, 80% less use of solvent and significantly reduced analysis times were observed.

The low abundance catechins (-)-epigallocatechin-(O-3"-methyl) gallate (EGCmetG) and (-)-epicatechin-3-(O-3"-methyl) gallate (ECmetG) were detected at 31.88 and 37.47 minutes, respectively. The peak eluting at 31.88 minutes that was tentatively identified as EGCmetG had a molecular ion ([M + H]$^+$) of m/z 473 with fragment ions of m/z 289 and 139. ECmetG had a [M + H]$^+$ at m/z 457 and fragment ions of m/z 273 and 167. These mass spectra are similar to mass spectra previously assigned to EGCmetG and ECmetG by Shen et $al.$ (2006) and Zeeb et $al.$ (2000). The 2 digallated catechins investigated could not be detected, possibly due to being present at concentrations below the limit of detection. However, 2 O-glycosidic flavonoids with similar molecular ions as the digallated catechins were tentatively identified. Rutin ([M + H]$^+$ of m/z 611) eluted at 33.26 minutes and nicotiflorin ([M + H]$^+$ of m/z 595) eluted at 34.94 minutes.

As mentioned before, this study is the first step that will ultimately allow for the future identification of tea cultivars grown in southern Africa that have high contents of digallated and methylated catechins, which can be used in the production of high quality value added tea products. Although screening the individual tea cultivars to find those with above average quantities of digallated and methylated catechins seems logical and practical, the risks involved should be understood and taken into account. The specific physiological reasons and mechanisms involved in catechins biosynthesis are still largely unknown. Furthermore, numerous horticultural and biological factors play a role in catechin synthesis. For example, it has been shown that small changes in small micro-ecological factors or geographical (localities) changes, changes the chemical composition of tea (Owuor et $al.$, 1987). The presence or absence of any of these mentioned factors at the time of sampling may falsely indicate tea cultivars with high or low novel digallated and/or methylated

catechin abundances. It is therefore important that the promising tea trees be closely monitored (environmental impact, genetic variance and possible pathology) and compared to surrounding tea trees over an extended period of time instead of performing once-off testing.

In conclusion, Hypothesis I states that reverse phase HPLC with evaporative light scattering detection will be a simple, fast and reliable method for analysis of theanine in tea samples. The newly developed HPLC-ELSD method was validated as selective, precise, accurate and practical for the quantification of theanine in tea extracts. The results therefore support Hypothesis I. The sensitivity and simplicity of this method renders it suitable for use in routine theanine analysis in quality control laboratories worldwide. This method is therefore an ideal candidate to be accepted by the ISO as the international standard analysis method of theanine in tea samples.

Hypothesis II, stating that the application of UPLC/MS/MS will aid in the identification of digallated and methylated catechins in a crude tea extract, is supported by the successful detection of low abundance catechins. (-)-Epicatechin-3-(O-3"-methyl) gallate and (-)-epigallocatechin-(O-3"-methyl) gallate were successfully detected and identified in a crude tea extract using UPLC/MS/MS in positive ionization mode. The effective application of UPLC/MS/MS facilitates future large scale isolation of low abundance catechins from fresh tea leaves.

"One cup does all disorders cure

With two, your troubles will be fewer

Thrice, to bone more vigour gives

With four, forever you will live

As young as on your day of birth."

Old Chinese poem
(Jain *et al.,* 2006)

Summary

Theanine is a free, non-protein amino acid found in *Camellia sinensis* (tea) leaves. It is the main amino acid component in tea and contributes 1 to 2% of the dry weight of tea leaves. The content of amino acids is a key green tea quality parameter. High grade teas contain high amounts of theanine. It is responsible for the sweet, brothy umami taste of green tea. To date there is no international standard method for the analysis of theanine in tea samples. Several methods with different advantages and disadvantages have been reported but most of them are unsuitable for routine quality control analysis in commercial tea samples.

In this study, a high performance liquid chromatography (HPLC) method with evaporative light scattering detection for the determination of theanine in tea samples has been developed. Chromatographic separation of theanine was achieved by reverse phase HPLC using a phenyl-hexyl column and isocratic water elution within 8 minutes. The method requires no sample derivatization and includes simple sample clean-up. Applying the same chromatographic conditions to a widely available C_{18} column, separation of theanine was achieved within 10 minutes. The successful application of the C_{18} column renders this method widely applicable. The method is linear over several orders of magnitude and the LOD are 11.53 and 10.83 µg/ml and the LOQ are 39.44 and 35.10 µg/ml for the phenyl-hexyl and C_{18} column, respectively. Simple but effective sample preparation and polyvinylpolypyrrolidone pre-treatment, along with the short analysis time facilitates high sample throughput (~40 samples can be prepared in an 8 hour day with overnight analysis by HPLC-ELSD). The method is selective, precise, accurate and practical for the quantification

of theanine in tea extracts and was successfully used to determine theanine content in a variety of tea samples. The sensitivity and simplicity of this method renders it suitable for use in routine theanine analysis in quality control laboratories.

Green tea, a simple refreshing beverage, has been believed to have therapeutic uses for centuries. Scientists have recognized that the wide range of physiological functions of green tea is due to the presence of catechins. An increasing body of evidence is showing that methylated forms of catechins have potent inhibitory activities to allergies. In addition to the methylated catechins, another relative novel group of catechins exists, namely digallated catechins. The medicinal value of these compounds is not yet known, but it is reasonable to speculate that the antioxidant capacity of digallated catechins will be superior to that of epigallocatechin gallate due to the 3 additional hydroxyl groups provided by the second gallic acid moiety.

In this study reverse phase UPLC/ESI-MS/MS was effectively applied to investigate the presence of digallated and methylated catechins in crude tea extract. Low abundance catechins (-)-epigallocatechin-(O-3"-methyl) gallate ([M + H]$^+$, *m/z* 473) and (-)-epicatechin-3-(O-3"-methyl) gallate ([M + H]$^+$, *m/z* 457) were successfully detected at 31.9 and 37.5 minutes, respectively. The 2 digallated catechins investigated could not be detected, possibly due to being present at concentrations below the limit of detection. Compared to conventional HPLC/ESI-MS/MS, increased resolution and sensitivity, 80% less use of solvent and significantly reduced analysis times were observed.

References

Alcàzar A, Ballesteros O, Jurado JM, Pablos F and Martiän MJ. (2007). Differentiation of green, white, black, oolong and pu-erh teas according to their free amino acids content. Journal of Agricultural and Food Chemistry, 55, 5960 – 5965.

Alpert AJ. (1999). Hydrophilic-interaction chromatography for the separation of peptides, nuclei acids and other polar compounds. Journal of chromatography, 499, 177 – 196.

Amarowicz R, Maryniak A and Shahidi F. (2005). TLC separation of methylated (-)-epigallocatechin-3-gallate. Czech Journal of Food Science, 23, 36 – 39.

Amarowicz R and Shahidi F. (1996). A rapid chromatographic method for separation of individual catechins from green tea. Food Research International, 29, 71 – 76.

Amić D, Davidovic-Amić D, Bašlo D, Rastija V. Luči B and Trinajstić N. (2007). SAR and QSAR of the antioxidant activity of flavonoids. Current Medicinal Chemistry, 14, 827 – 845.

Adhami VM, Malik A, Zaman N, Sarfaraz S, Siddiqui IA, Syed DN, Afaq F, Pasha FS and Mukhtar SMH. (2007). Combined inhibitory effects of green tea polyphenols and selective cyclooxygenase-2 inhibitors on the growth of human prostate cancer cells both *in vitro* and *in vivo*. Clinical Cancer Research, 13, 1611 – 1619.

Anderson RA and Polansky MM. (2002). Tea enhances insulin activity. Journal of Agricultural and Food Chemistry, 50, 7182 – 7186.

ACQUITY UPLC System Operator's Guide, 71500082502/Revision D, Copyright Waters Corporation 2004–2008.

Astill C, Birch MR, Dacombe C, Humphrey PG and Martin PT. (2001). Factors affecting the caffeine and polyphenol contents of black and green tea infusions. Journal of Agricultural and Food Chemistry, 49, 5340 – 5347.

Aucamp JP, Hara Y and Apostolides Z. (2000). Simultaneous analysis of tea catechins, caffeine, gallic acid theanine and ascorbic acid by micellar electrokinetic capillary chromatography. Journal of Chromatography A, 876, 235 – 242.

Baumann D, Adler S and Hamburger M. (2001). A simple isolation method for the major catechins in green tea using high speed countercurrent chromatography. Journal of Natural Products, 64, 353 – 355.

Bazinet L, David Labbè D and Trembla A. (2007). Production of green tea EGC- and EGCG-enriched fractions by a two-step extraction procedure. Separation and Purification Technology, 56, 53 – 56.

Bonoli M, Pelillo M, Toschi TG and Lercker G. (2003). Analysis of green tea catechins: comparative study between HPLC and HPCE. Food Chemistry, 81, 631 – 638.

Borzelleca JF, Peters D and Hall W. (2006). A 13-week dietary toxicity and toxicokinetic study with L-theanine in rats. Food and Chemical Toxicology, 44, 1158 – 1166.

Burda S and Oleszek W. (2001). Antioxidant and antiradical activities of flavonoids. Journal of Agricultural and Food Chemistry, 49, 2774 – 2779.

Camel V, Thiebault M, Caude M and Dreux M. (1992). Packed column subcritical fluid chromatography of underivatized amino acids. Journal of Chromatography, 605, 95 – 101.

Cao G, Sofic E and Prior RL. (1997). Antioxidant and pro-oxidant behaviour of flavonoids: structure-activity relationships. Free Radical Biology and Medicine, 22, 5, 749 – 760.

Cao X, Tian Y, Zhang T and Ito Y. (2001). Separation and purification of three individual catechins from tea polyphenol mixture by CCC. Journal of Liquid Chromatography and Related Technologies, 24, 1723 – 1732.

Casimir J, Jadot J and Renard M. (1960). Separation and characterization of N-ethyl-γ-glutamine in *Xerocomus badius* (Boletus ladius). Biochimica Biophysica Acta, 39, 462 -468.

Centre for Drug Evaluation and Research (CDER), Food and Drug Administration. (1994). Reviewer guidance: validation of chromatographic methods. www.fda.gov/cder/cmc3.pdf

Chang CJ, Chiu K, Chen Y and Chang C. (2000). Separation of catechins from green tea using carbon dioxide extraction. Food Chemistry, 68, 109 – 113.

Charlesworth JM. (1978). Evaporative analyzer as a mass detector for liquid chromatography. Analytical Chemistry, 50, 1414 – 1420.

Chavedes-das-Neves HJ, Braya-Morrais Z. (1997). HPLC assay of underivatized free amino acids with column switching and evaporative light-scattering detection. Journal of High Resolution Chromatography, 20, 115 – 118.

Chen C, Liang C, Lai J, Tsai Y, Tsay J and Lin J. (2003). Capillary electrophoretic determination of theanine, caffeine and catechins in fresh tea leaves and Oolong tea and their effects on rat neurosphere adhesion and migration. Journal of Agricultural and Food Chemistry, 2003, 51, 7495 – 7503.

Chen H, Liang H, Ding J, Lai J. Huan Y and Qiao X. (2007). Rapid differentiation of tea products by surface desorption atmospheric pressure chemical ionization mass spectrometry. Journal of Agricultural and Food Chemistry, 55, 10093 – 10100.

Chiu FL and Lin JK. (2005). HPLC analysis of naturally occurring methylated catechins, 3″- and 4″-methyl-epigallocatechin gallate, in various fresh tea leaves and commercial teas and their potent inhibitory effects on inducible nitric oxide synthase in macrophages. Journal of Agricultural and Food Chemistry, 53, 7035 – 7042.

Cho H, Kim S, Lee S, Park J, Kim S-J and Chun H. (2008). Protective effect of the green tea component, L-theanine on environmental toxins-induced neuronal cell death. NeuroToxicology, 29, 656 – 662.

Christen P and Veuthey J.L. (2001). New trend in extraction, identification and quantification of Artemisinin and its derivatives. Current Medicinal Chemistry, 88, 1827 – 1839.

Cooper R, Morrè J and Morrè DM. (2005). Medicinal benefits of green tea: part 1. Review of non-cancer health benefits. The Journal of Alternative and Complementary Medicine, 11, 521 – 528.

Coxon DT, Holmes A, Ollis WD, Vora VC, Grant MS and Tee JL. (1972). Flavanol digallates in green tea leaf. Tetrahedron, 28, 2819 – 2826.

Coyle CH, Philips BJ, Morrisroe SN, Chancellor MB and Yoshimura N. (2008). Antioxidant effects of green tea and its polyphenols on bladder cells. Life Sciences, 83, 12–18.

Cuyckens F and Claeys M. (2004). Special feature tutorial :Mass spectrometry in the structural analysis of flavonoids. Journal of Mass Spectrometry, 39, 1 – 15.

Dalluge JJ and Nelson BC. (2000). Determination of tea catechins. Review. Journal of Chromatography A, 881, 411 – 424.

Davis AL, Cai Y, Davies AP and Lewis JR. (1996). 1H and 13C NMR assignments of some green tea polypenols. Magnetic Resonance in Chemistry, 34, 887 – 890.

Del Rio D, Stewart AJ, Mullen W, Burns J, Lean MEJ, Brihgenti F and Crozier A. (2004). HPLC-MS Analysis of phenolic compound and purine alkaloids in green and black tea. Journal of Agricultural and Food Chemistry, 52, 2807 – 2815.

Deng W, Ogita S and Ashihara H. (2008). Biosynthesis of theanine (γ-ethylamino-L-glutamic acid) in seedlings of Camellia sinensis. Phytochemistry Letters, 1, 115 – 119.

Desai MJ and Armstrong DA. (2004). Analysis of derivatized and underivatized theanine enantiomers by high performance liquid chromatography/ atmospheric pressure ionization-mass spectrometry. Rapid Communications in Mass Spectrometry, 18, 251 – 256.

Ding Y, Yu H, Mou S. (2002). Direct determination of free amino acids and sugars in green tea by anion-exchange chromatography with integrated pulsed amperometric detection. Journal of Chromatography A, 982, 237 – 244.

Doner LW, Bécard G, Irwin PL. (1993). Binding of flavonoids by polyvinylpolypyrrolidone. Journal of Agricultural and Food Chemistry, 41, 753 – 757.

Dou J, Lee VS, Tzen JTC and Lee M. (2007). Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying process. Journal of Agricultural and Food Chemistry, 55, 7462 – 7466.

Dugas AJ, Castaňeda-Acosta J, Bonin GC, Price KL, Fischer NH and Winston GW. (2000). Evaluation of the peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. Journal of Natural Products, 63, 327 – 331.

Egashira N, Hayakawa K, Mishima K, Kimura H, Iwasaki K and Fujiwara M. (2004). Neuroprotective effect of gamma-glutamylethylamide (theanine) on cerebral infraction in mice. Neuroscience Letters, 363, 58 – 61.

Ekborg-Ott KH, Taylor A and Armstrong DW. (1997). Varietal differences in the total and enantiomeric composition of theanine in tea. Journal of Agricultural and Food Chemistry, 45, 353 – 363.

European Union (EU) Guideline – As adopted in Australia by the Therapeutic Goods Administration (TGA). (2002). Validation of analytical Procedures: Methodology, Eudralex 1998, volume 3A – 3AQ13A, 107 - 117. www.tga.gov.au/docs/html/euguide/euad_qual.htm

Rice-Evans C. (2001). Flavonoid Antioxidants. Current Medicinal Chemistry, 8, 797 – 807.

Rice-Evans CA, Miller NJ and Paganga G. (1996). Review article: Structure-anitoxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology & Medicine, 20, 933 – 956.

Frei B and Higdon JV. (2003). Antioxidant activity of tea polyphenols *in vivo*: Evidence from animal studies. The Journal of Nutrition, 133, 10, 3275S – 3284S.

Friedman M, Mackey BE, Kim H, Lee I, Lee K, Kozukue E and Kozukue N. (2007). Structure-activity relationships of tea compounds against human cancer cells. Journal of Agricultural and Food Chemistry, 55, 243 – 253.

Fujiwara M, Ando I and Arifuku K. (2006). Multivariate analysis for 1H-MNR spectra of two hundred kinds of tea in the word. Analytical Science, 22, 1370 – 1314.

Fujimura Y, Umeda D, Yano S, Maeda-Yamamoto M, Yamada K and Tachibana H. (2007). The 67 kDa laminin receptor as a primary determinant of anti-allergic effects of *O*-methylated EGCG. Biochemical and Biophysical Research Communications, 364, 79 – 85.

Gomez-Ramirez M, Higgings BA, Rycroft JA, Owen GN, Mahoney J, Shpaner M and Foxe JJ. (2007). The deployment of intersensory selective attention: a high-density electrical mapping study of the effects of theanine. Clinical Neuropharmacology, 30, 25 – 38.

Graziano A, Leggio G, Mandolesi L, Neri P, Molinari M and Petrosini L. (2002). Learning power of single behavioural units in acquisition of a complex spatial behavior: An observational learning study in cerebellar-lesioned rats. Behavioral Neuroscience, 116, 116 – 125.

Gruz J, Novak O, Strnad M. (2008). Rapid analysis of phenolic acids in beverages by UPLC–MS/MS. Food Chemistry, 111, 789 – 794.

Gulati A., Rawat R., Singh B., & Ravindranath S.D. (2003). Application of microwave energy in the manufacture of enhanced-quality green tea. Journal of Agriculture and Food Chemistry, 51, 4764 – 4768.

Guo Q, Zhao B, Shen S, Hou J, Hu J and Xin W. (1999) ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. Biochimica et Biophysica Acta, 1427, 13 – 23.

Haenen GRMM, Paquay JBG, Korthouwer REM and Bast A. (1997). Peroxynitrite scavenging by flavonoids. Biochemical and Biophysical Research Communications, 236, 591 – 593.

Hamer M. (2007). The beneficial effects of tea on immune function and inflammation: a review of evidence from *in vitro*, animal and human research. Nutrition Research, 27, 373–379.

Haskell CF, Kennedy DO, Milne AL, Wesnes KA and Scholey AB. (2008). The effects of L-theanine, caffeine and their combination on cognition and mood. Biological Psychology, 77, 113 – 122.

Horie H and Kohata K. (1998). Application of capillary electrophoresis to tea quality estimation. Journal of Chromatography A, 802, 219 – 223.

Horie H, Mukai T and Kohata K. (1997). Simultaneous determination of qualitatively important components in green tea infusions using capillary electrophoresis. Journal of Chromatography A, 758, 332 – 335.

Horie H, Nesumi A, Ujihara T and Kohata K. (2002). Rapid determination of caffeine in tea leaves. Journal of Chromatography A, 942, 271 – 273.

Hsieh M and Chen S. (2007). Determination of amino acids in tea leaves and beverages using capillary electrophoresis with light-emitting diode-induced fluorescence detection. Talanta, 73, 326 – 331.

ISO/DIS 14502-1. (2005). Tea: Methods for determination of substances characteristic of green and black tea. Part 1: Content of total polyphenols in tea – colorimetric method using Folin-Ciocalteu reagents. Geneva, Switzerland.

ISO/DIS 14502-2. (2003). Tea: Methods for determination of substances characteristic of green and black tea. Part 2: Determination of catechins in green tea – method using high performance liquid chromatography. Geneva, Switzerland.

Jain NK, Siddiqi MA and Weisburger JH. (2006). Protective effects of tea on human health; volume II, CAB International 2006, Cromwell Press, Trowbridge. ISBN-10: 1-84593-112-2

Juneja LR, Chu D, Okubo T, Nagato Y and Yokogoshi H. (1999). L-theanine a unique amino acid of green tea and its relaxation effect in humans. Trends in Food Science and Technology, 10, 199 – 204.

Kakuda T. (2002). Review: Neuroprotective effects of the green tea components theanine and catechins. Biological and Pharmaceutical Bulletin, 25, 1513 – 1518.

Kakuda T, Hinoi E, Abe A, Nozawa A, Ogura M and Yoneda Y. (2008). Theanine an ingredient of green tea, inhibits [^{3}H]Glutamine transport in neurons and astrogila in rat brain. Journal of Neuroscience Research, 86, 1846 – 1856.

Kakuda T, Nosawa A, Sugimoto A and Niiono N. (2002). Inhibition by theanine of binding of [^{3}H]AMPA, [^{3}H]Kainate and [^{3}H]MDL to glutamate receptors. Bioscience, Biotechnology and Biochemistry, 66, 2683 – 2686.

Kakuda T, Nosawa A, Unno T, Okamura N and Okai O. (2000a). Inhibiting effects of theanine on caffeine stimulation evaluated by EEG in the rat. Bioscience, Biotechnology and Biochemistry, 64, 287 – 293.

Kakuda T, Yanase H, Utsunomiya K, Nozawa A, Unno T and Kataoka K. (2000b). Protective effect of γ-glutamylethylamide (theanine) on ischemic delayed neuronal death in gerbils. Neuroscience Letters, 289, 189 – 192.

Kaneko S, Kumazawa K, Masuda H, Henze A and Hofmann T. (2006). Molecular and sensory studies on the unami taste of Japanese green tea. Journal of Agricultural and Food Chemistry, 54, 2688 – 2694.

Kato M, Gyoten Y, Sakai-Kato K and Toyo'oka T. (2003). Rapid analysis of amino acids in Japanese green tea by microchip and fluorescence detection. Journal of Chromatography A, 1013, 183 – 189.

Kelly SP, Gomez-Ramirez M, Montesi JL and Foxe JJ. (2008). L-Theanine and caffeine in combination affect human cognition as evidence by oscillatory alpha band activity and attention task performance. The Journal of Nutrition, 138, 1572S – 1577S.

Khan N and Mukhtar H. (2007). Tea polyphenols for health promotion. Life Sciences, 81, 519 – 533.

Kim W, Kim J-D, Kim J, Oh S and Lee Y. (2008). Selective caffeine removal from green tea using supercritical carbon dioxide extraction. Journal of Food Engineering, 89, 303 – 309.

Kito H, Kokura J, Isaki K and Sasaoka. (1968). Theanine, a precursor of the phloroglucinol nucleus of catechins in tea plants. Phytochemistry, 7, 599 – 603.

Kumara K, Ozeki M, Juneja LR and Ohira H. (2007). L-theanine reduces psychological and physiological stress responses. Biological Psychology, 74, 39 – 45.

Kumar NS and Rajapaksha M. (2005). Separation of catechin constituents from five tea cultivars using high speed counter-current chromatography. Journal of Chromatography A, 1083, 223 – 228.

Kvansnička F and Krátká J. (2006). Isotachophoretic determination of theanine. Central European Journal of Chemistry, 4, 216 – 222.

Labbè D, Tremblay A and Bazinet L. (2006). Effect of brewing temperature and duration on green tea catechin solubilization: Basis for production of EGC and EGCG-enriched fractions. Separation and Purification technology, 49, 1 – 9.

Lachova´ M, Lehotay L, Karasova´ G and Skacˇa´ni I. (2007). Isolation of L-theanine from plant material using a molecularly imprinted polymer. Journal of Liquid Chromatography & Related Technologies, 30, 2045 – 2058.

Larger PJ, Jones AD and Dacombe C. (1998). Separation of tea polyphenols using micellar electrokinetic chromatography with diode array detection. Journal of Chromatography A, 799, 309 – 320.

Lee B and Ong C. (2000). Comparative analysis of tea catechins and theaflavins by high performance liquid chromatography and capillary electrophoresis. Journal of Chromatography A, 881, 439 – 447.

Le Gall G, Colquhoun IJ and Defernez M. (2004). Metabolite profiling using ^{1}H NMR spectroscopy for quality assessment of green tea, *Camellia sinensis* (L.). Journal of Agricultural and Food Chemistry, 52, 692 – 700.

Li P, Wan X, Zhang Z, Li J and Shen Z. (2005). A novel assay method for theanine synthetase activity by capillary electrophoresis. Journal of chromatography B, 819, 81 – 84.

Li X, Xiong Z, Ying X, Cui L, Zhu W and Li F. (2006). A rapid ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometric method for the

qualitative and quantitative analysis of the constituents of the flower of *Trollius ledibouri* Reichb. Analytica Chimica Acta, 580, 170 – 180.

Li Y, Liu Z, Huang J and Gong Y. (2005). Determination of theanine in tea by reverse phase high performance liquid chromatography-evaporative light scattering detector. Journal of Tea Science, 25, 225 – 228.

Liang C, Fulian Y, Ming-zhe Y, Bo L, Kun Y and Yaun-yuan D. (2008). Preparation of the UPOV Guidelines for the Conduct of test for distinctness, uniformity and stability – Tea plant [*Camellia sinensis* (L.) O. Kuntze]. Agricultural Sciences in China, 7,224 – 231.

Liang Y, Lu J, Zhang L, Wu S and Wu Y. (2003). Estimation of black tea quality by analysis of chemical composition and colour difference of tea infusions. Food Chemistry, 80, 283 – 290.

Lien JE, Ren S, Bui H and Wang R. (1999). Quantitative structure activity relationship analysis of phenolic antioxidants. Free Radical Biology and Medicine, 26, 285 – 294.

Lin S, Liu E and Mau J. (2008). Effect of different brewing methods on antioxidant properties of steaming green tea. Food Science and Technology, 41, 1616-1623.

Lin Z, Tan J, Lu H, Yang Y, Yin J and Chen H. (2004). The extraction and purification of theanine. International Journal of Tea Science, 3, 157 – 161.

Lu K, Gray MA, Oliver C, Liley DT, Harrison BJ, Bartholomeusz CF, Phan KL and Nathan PJ. (2004). The acute effects of L-theaine in comparison with alprazolam on anticipatory anxiety in humans. Human Psychopharmacology: Clinical and Experimental, 19, 457 – 465.

Lun Su Y, Leung LK, Huang Y and Chen Z. (2003). Stability of tea theaflavins and catechins. Food Chemistry, 83, 189 – 195.

Ma Y, Vedernikova I, Van den Heuvel H and Claeys M. (2000). Internal glucose residue loss in protonated *O*-diglycosyl flavonoids upon low-energy collision-induced dissociation. Journal of the American Society for Mass Spectrometry, 11, 136 – 144.

Maeda-Yamamoto M, Ema K and Shibuichi I. (2007). *In vitro* and *in vivo* anti-allergic effects of 'benifuuki' green tea containing O-methylated catechin and ginger extract enhancement. Cytotechnology, 55, 135 – 142.

Maeda-Yamamoto M, Inagaki N, Kitaura J, Chikumoto T, Kawahara H, Kawakami Y, Sano M, Miyase T, Tachibana H, Nagai H, and Kawakami T. (2004). O-methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. Journal of Immunology, 172, 4486 – 4492.

Maliepaard R. (2007). Inactivation of polyphenol oxidase in *Camellia sinensis* for the production of high quality instant green tea - Chapter 3: Identification of novel flavan-3-ols by LC-ESI/MS/MS. M.Sc dissertation, University of Pretoria, South Africa.

Masukawa Y, Matsui Y, Shimizu, Kondou N, Endou H, Kuzukawa M and Hase T. (2006). Determination of green tea catechins in human plasma using liquid chromatography-electrospray ionization mass spectrometry. Journal of Chromatography B, 834, 26 – 34.

Meldrum BS. (2000). Glutamate as a neurotransmitter in the brain: review of physiology and pathology. Journal of Nutrition, 130, 1007S – 1015S.

McMurrough I, Madigan D and Smyth MR. (1995). Adsorption by polyvinylpyrrolidone of catechins and proanthocyanidins from beer. Journal of Agricultural and Food Chemistry, 43, 2687 – 2691.

Miketova P, Schram KH, Whitney J, Li M, Huang R, Kerns E, Valcic S, Timmermann BN, Rourick R and Klohr S. (2000). Tandem mass spectrometry studies of green tea catechins. Identification of three minor components in the polyphenolic extract of green tea. Journal of Mass Spectrometry, 35, 860 – 869.

Moghaddam B, Gruen RJ, Roth RH, Bunney BS and Adams RN. (1990). Effect of L-glutamate on the release of striatal dopamine: *In vivo* dialysis and electrochemical studies. Brain Research, 518, 55 – 60.

Nagasawa K, Akoi H, Yasuda E, Nagai K, Shimohama S and Fujimoto S. (2004). Possible involvement of group I mGluRs in neuroprotective effect of theanine. Biochemical and Biophysical Research Communications, 320, 116 – 122.

Nagatsu T and Sawada M. (2007). Biochemistry of post-mortem brains in Parkinson's disease: historical overview and future prospects. Journal of Neural Transmission, 72, 113 – 120.

Nakagawa M. (1970). Constituents in tea leaf and their contribution to the taste of green tea liquor. JARQ, 5, 43 – 46.

Nakagawa M. (1975). Chemical components and taste of green tea. JARQ, 9, 156 –160.

Nakakuki H, Horie H, Yamauchi Y and Kohata K. (1999). Rapid analysis of methylated xanthines in teas by an improved high-performance liquid chromatographic method using a polyvinylpolypyrrolidone pre-column. Journal of Chromatography A, 848, 523 – 527.

Neilson AP, Green RJ, Wood KV and Ferrussi MG. (2006). High-throughput analysis of catechins and theaflavins by high performance liquid chromatography with diode array detection. Journal of Chromatography A, 1132, 132 – 140.

Nishitani E and Sagesaka YM. (2004). Simultaneous determination of catechins, caffeine and other phenolic compounds in tea using new HPLC method. Journal of Food Composition and Analysis. 17, 675 – 685.

Owuor PO and Chavanji AM. (1986). Caffeine contents of clonal tea; seasonal variations and effects of plucking standards under Kenyan conditions. Food Chemistry, 20, 225 – 233.

Park HS, Choi H, Lee SJ, Park KW, Choi S and Kim KH. (2007). Effect of mass transfer on the removal of caffeine from green tea by supercritical carbon dioxide. Journal of Supercritical Fluids, 42, 205 – 211.

Paul SK. (2008). Predictability of price of tea from sensory assessments and biochemical information using data-mining techniques. Journal of the Science of Food and Agriculture, 88, 1354 – 1362.

Pelillo M, Bonoli M, Biguzzi B, Bendini A, Toschi TG and Lercker G. (2004). An investigation in the use of HPLC with UV and MS-electrospry detection for the quantification of tea catechins. Food Chemistry, 87, 465 – 470.

Peng L, Song X, Shi X, Li J and Ye C. (2008). An improved HPLC method for simultaneous determination of phenolic compounds, purine alkaloids and theanine in *Camellia* species. Journal of Food Composition and Analysis, 21, 559 – 563.

Perva-Usunalić, Škerget M, Knez Z, Weinreich B, Otto F and Grüner S. (2006). Extraction of active ingredient from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. Food Chemistry, 96, 597 – 605.

Piñeiro Z, Palma M and Barroso CG. (2004). Determination of catechins by means of extraction with pressurized liquids. Journal of Chromatography A, 1026, 19 - 23.

Petritis KN, Chaimbault P, Elfakir C, Dreux M. (1999). Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using per fluorinated carboxylic acids as ion pairing agent. Journal of Chromatography A, 833, 147 – 155.

Pirker KF, Severino JF, Reichenauer TG and Goodma BA. (2007). Free radical processes in green tea polyphenols (GTP) investigated by electron paramagnetic resonance (EPR) spectroscopy. Biotechnology Annual Review, 14, 349 – 401.

Poon GK. (1998). Analysis of catechins in tea extracts by liquid chromatography-electrospray ionization mass spectrometry. Journal of Chromatography A, 794, 63 – 74.

Rogers PJ, Smith JE, Heatherley SV and Pleydell-Pearce CW. (2008). Time for tea: mood, blood pressure and cognitive performance effects of caffeine and theanine administered alone and together. Psychopharmacology, 195, 569 – 577.

Row HK and Jin Y. (2006). Recovery of catechin compounds from Korean tea by solvent extraction. Biosresource Technology, 97, 790 – 793.

Rusak G, Komes D, Likic´ S, Horzˇic´ D and Kovacˇ M. (2008). Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chemistry, 110, 852 – 858.

Ryan P and Hynes MJ. (2007). The kinetics and mechanisms of the complex formation and antioxidant behaviour of the polyphenols EGCg and ECG with iron(III). Journal of Inorganic Biochemistry, 101, 585 – 593.

Sadzuka Y, Inoue C, Hirooka S, Sugiyama T, Umegaki K and Sonobe T. (2005). Effects of theanine on alcohol metabolism and hepatic toxicity. Biological and Pharmaceutical Bulletin, 28, 1720 – 1706.

Sadzuka Y, Sugiyama T, Nagamine M, Umegaki K, Sonobe T. (2006). Efficacy of theanine is connected with theanine metabolism by any enzyme, not only drug metabolizing enzymes. Food and Chemical Toxicology, 44, 286 – 292.

Sadzuka Y, Sugiyama T, Sonobe T. (2000a). Efficacies of tea components on doxorubicin induced antitumor activity and reversal of multi-drug resistance. Toxicology Letters, 114, 155 – 162.

Sadzuka Y, Sugiyama T, Sonobe T. (2000b). Improvement of idarubicin induced antitumor activity and bone marrow suppression by theanine, a component of tea. Cancer Letters, 158, 119 – 124.

Saijo R and Takeda Y. (1999). HPLC analysis of catechins in various kinds of green tea produced in Japan and abroad. Nippon Shokuhin Kagaku Kogaku Kaishi, 46, 138 – 147.

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP and Rice-Evans C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radical and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics, 322, 2, 339 – 346.

Sano M, Suzuki M, Miyase T, Yoshino K, Maeda-Yamamoto M. (1999). Novel antiallergic catechin derivatives isolated from oolong tea. Journal of Agricultural and Food Chemistry, 47, 1906 – 1910.

Sang S, Cheng X, Stark RE, Rosen RT, Yang CS and Ho C. (2002). Chemical studies on antioxidant mechanism of tea catechins: Analysis of radical reaction products of catechin and epicatechin with 2,2-Diphenyl-1-picrylhydrazyl. Bio-organic and Medicinal Chemistry, 10, 2233 – 2237.

Sarioğlu K. (2007). Synthesis of octadecyl acrylate-co-ethylene glycol dimethacrylate resin for the removal of dark-colour compound from apple juice and comparison with polyvinylpolypyrrolidone (PVPP). European Food, 225, 443 – 449.

Schroeter H, Boyd C, Spencer JPE, Williams RJ, Candenas E and Rice-Evans C. (2002). MAPK signalling in neurodegenaration: influences of flavonoids and of nitric oxide. Neurobiology of Aging, 23, 861 – 880.

Shadkami F, Estevez S and Helleur R. (2008). Analysis of catechins and condensed tannins by thermally assisted hydrolysis/methylation-GC/MS and by a novel two step methylation. Journal of Analytical and Applied Pyrolysis, doi:10.1016/j.jaap.2008.09.001

Sharma V, Gulati A and Ravindranath SD. (2005). Extractability of tea catechins as a function of manufacture procedure and temperature of infusion. Food Chemistry, 93, 141 – 148.

Sharma V, Gulati A and Ravindranath SD and Kumar V. (2005). A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. Journal of Food Composition and Analysis, 18, 583 – 594

Shen D, Wu Q, Wang M, Yang Y, Lavoie EJ and Simon JE. (2006). Determination of the predominant catechins in *Acacia catechu* by liquid chromatography/ electrospray ionization–mass spectrometry. Journal of Agricultural and Food Chemistry, 54, 3219 – 3224.

Sherer TB, Richardson JR, Testa CM, Seo BB, Panov AV and Yagi T. (2007). Mechanisms of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. Journal of Neurochemistry, 100, 1469 – 1479.

Singh C, Ahmad I and Kumar A. (2007). Pesticides and metals induced Parkinson's disease: involvement of free radicals and oxidative stress. Cellular and Molecular Biology, 53, 19 – 28.

Spiro M and Price WE. (1986). Determination of theaflavins in tea solution using the Flavognost complexation method. Analyst, 3, 331 – 333.

Stolyhwo A, Colin H and Guiochon G. (1983). Use of the light scattering as a detector as a detector principle in liquid chromatography. Journal of Chromatography, 265, 1 – 18.

Stolyhwo A, Colin H, Martin M and Guiochon G. (1984). Study of the qualitative and quantitative properties of the light scattering detector. Journal of Chromatography, 288, 253 – 215.

Sugiyama T and Sadzuka Y. (2003). Theanine and glutamate transporter inhibitors enhance the antitumor efficacy of chemotherapeutic agents. Biochemica et Biophysica Acta, 1653, 47 – 59.

Sugiyama T and Sadzuka Y. (2004). Theanine, a specific glutamate derivative in green tea, reduces the adverse reactions of doxorubicin by changing the glutathione level. Cancer Letters 212, 177 – 184.

Suzuki M, Yoshino K, Maeda-Yamamoto M, Miyase T, Sano M. (2000). Inhibitory effects of tea catechins and O-methylated derivatives of (-)-epigallocatechin-3- O-gallate on mouse type IV allergy, Journal of Agricultural and Food Chemistry, 48, 5649 – 5653.

Syu K, Chih-Li L, Huang H and Lin J. (2008). Determination of theanine, GABA and other amino acids in green, oolong, black and pu-erh teas with dabsylation and High-Performance Liquid Chromatography. Journal of Agricultural and Food Chemistry, 56, 7637 – 7643.

Thippeswamy R, Mallikarjun Gouda KG, Rao D, Martin S and Gowda LR. .(2006). Determination of theanine in commercial tea by liquid chromatography with fluorescence and diode array ultraviolet detection. Journal of Agricultural and Food Chemistry, 54, 7014 – 7019.

Terashima T, Takido J and Yokogoshi H. (1999). Time-dependent changes of amino acids in the serum, liver, brain and urine of rats administrated with theanine. Bioscience, Biotechnology and Biochemistry, 63, 165 – 168.

Thompson M, Stewart GG and McKeown IP. (2003). A comparison of the selective removal of beer polyphenols by Lucilite TR and Polyvinylpolypyrrolidone from All-Malt Lager. Master Brewers Association of the Americas TQ, 40, 17 – 19.

Vaher M and Koek M. (2003). Separation of polyphenolic compound extracted from plant matrices using capillary electrophoresis. Journal of Chromatography A, 990, 225 – 230.

Valko M, Rhodes CJ, Monocol J, Izakovic M and Mazur M. (2006). Free radicals, metals and antioxidants in oxidative-induced cancer. Chemico-Biological interactions, 160, 1 – 40.

Van der Meeren P, Vanderdeelen J, and Baert L. (1992). Simulation of the mass response of the evaporative light scattering detector. Analytical Chemistry, 64, 1056 – 1062.

Vuataz L. Brandenberger H and Egli RH. (1959). Plant phenols: Separation of the tea leaf polyphenols by cellulose column chromatography. Journal of Chromatography, 2, 173 – 187.

Vukics V, Ringer T, Kery A, Bonn GK, Guttman A. (2008). Analysis of heartsease (*Viola tricolor* L.) flavonoid glycosides by micro-liquid chromatography coupled to multistage mass spectrometry. Journal of Chromatography A, 1206, 11 – 20.

Wang D, Lu J, Miao A, Xie Z and Yang D. (2008). HPLC-DAD-ESI-MS/MS analysis of polyphenols and purine alkaloids in leaves of 22 tea cultivars in China. Journal of Food Composition and Analysis, 21, 361 – 369.

Wang H and Helliwell K (2000). Epimerization of catechins in green tea infusions. Food Chemistry, 70, 337 – 344.

Wang R, Zhou W and Wen RH. (2006). Kinetic study of the thermal stability of tea catechins in aqueous system using a microwave reactor. Journal of Agricultural and Food Chemistry, 53, 5924 – 5932.

Waters TQ Detector Operator's Guide, 71500126802/Revision B, Copyright Waters Corporation 2007.

Wörth CCT, Wiebler M and Schmitz OJ. (2000). Analysis of catechins and caffeine in tea extracts by micellar electrokinetic chromatography. Electrophoresis, 21, 3634 – 3638.

Wu Q, Wang M and Simon JE. (2005). Determination of proanthocyanidins in fresh grapes and grape products using liquid chromatography with mass spectrometric detection, 19, 2062 – 2068.

Yamada T, Nishimura Y, Sakurai T, Terashima T, Okuba T, Juneja L and Yokogoshi H. (2008). Administration of theanine, a unique amino acid in tea leaves, changed feeding-related components in serum and feeding behaviour in rats. Bioscience, Biotechnology and Biochemistry, 72, 1352 – 1355.

Yamada T, Terashima T, Kawano S, Furuno R, Okubo T, Juneja R and Yokogoshi H. (2009). Theanine, γ-glutamylethylamide, unique amino acid in tea leaves, modulate neurotransmitter concentration in the brain striatum interstitium in conscious rats. Amino Acids, 36, 21 – 27.

Yamada T, Terashima T, Okubo T, Juneja LR and Yokogoshi H. (2005). Effects of theanine, r-glutamylethylamide, on neurotransmitter release and its relationship with glutamic acid neurotransmission. Nutritional Neuroscience, 8, 219 – 226.

Yanagida A, Shoji A, Shibusawa Y, Shindo H, Tagashira M, Mo Y. (2006). Analytical separation of tea catechins and food-related polyphenols by high speed counter-current chromatography. Journal of Chromatography A, 1112, 195 – 201.

Yang XR, Ye CX, Xu JK and Jiang YM. (2007). Simultaneous analysis of purine alkaloids and catechins in *Camellia sinensis, Camellia ptilophylla* and *Camellia assamica* var. *kuch* by HPLC. Food Chemistry, 100, 1132 – 1136.

Yao L, Jiang Y, Datta N, Singanusong R, Liu X, Duan J, Raymont K, Lisle A and Xu Y. (2004). HPLC analysis of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. Food Chemistry, 84, 253 – 263.

Ye JH, Jin J, Liang HL, Lu JL, Du YY, Zheng XQ and Liang YR. (2009). Using tea stalk lignocellulose as an adsorbent for separating decaffeinated tea catechins Bioresource Technology, 100, 622 – 628.

Ying Y, Ho JW, Chen ZY, Wang J. (2005). Analysis of theanine in tea leaves by HPLC with fluorescence detection. Journal of Liquid Chromatography and Related Technologies, 28, 727 – 737.

Yoshida Y, Kiso M and Goto T. (1999). Efficiency of the extraction of catechins from green tea. Food Chemistry, 67, 429 – 433.

Yokogoshi H, Kobayashi M, Mochizuki M and Terashima T. (1998a). Effect of theanine, r-glutamyethylamide, on brain monoamines and striatal dopamine release in conscious rats. Neurochemistry Research, 23, 667 – 673.

Yokogoshi H, Mochizuki M and Saitoh K. (1998b). Theanine-induced reduction of brain serotonin concentration in rats. Bioscience, Biotechnology and Biochemistry, 62, 816 – 817.

Yokogoshi H and Kobayashi M. (1998). Hypotensive effects of γ-glutamylmethylaminde in spontaneously hypertensive rats. Life Sciences, 62, 1065 – 1068.

Yokogoshi H, Terashima T. 2000. Effect of theanine, γ-glutamylethylamide, on brain monoamines, striatal dopamine release and some kinds of behaviour in rats. Nutrition, 16, 776 – 777.

Young CS and Dolan JW. (2003). Success with evaporative light scattering detection. LCGC North America, 21, (2) 120 – 128.

Yu K, Little D, Plumb R and Smith B. (2006). High-throughput quantification for a drug mixture in rat plasma – a comparison of Ultra Performance TM liquid chromatography/tandem mass spectrometry with high-performance liquid chromatography/tandem mass spectrometry. Rapid Communication in Mass Spectrometry, 20, 544 – 552.

Zaveri NT. (2006). Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications. Life Sciences, 78, 2073 – 2080.

Zeeb DJ, Nelson BC, Albert K, Dalluge JJ. (2000). Separation and identification of twelve catechins in tea using liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry. Analytical Chemistry, 72, 5020 – 5026.

Zhang R, Xiao W, wang X, Wu X and Tian W. (2006). Novel inhibitors of fatty-acid synthase from green tea (*Camellia sinensis* Xihu Longjing) with high activity and new reacting site. Biotechnology and Applied Biochemistry, 43, 1 – 7.

Zhang Y, Chen B, Huang Z and Shi Z. (2004). Preparative isolation and purification of L-theanine by HPLC. Journal of Liquid Chromatography and Related Technologies, 27, 875 – 884.

Zhen M, Wang Q, Huang X, Cao L ,Chen X, Sun K, Liu Y, Li W and Zhang L. (2007). Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride–induced hepatic fibrosis. Journal of Nutritional Biochemistry, 18, 795 – 805.

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

Zuo Y, Chen H and Deng Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta, 57, 307 – 316.