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INACTIVATION OF POLYPHENOL OXIDASE IN *Camellia sinensis* FOR THE PRODUCTION OF HIGH QUALITY INSTANT GREEN TEA

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Declaration

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TABLE OF CONTENTS

page

Acknowledgements…………………………………………………………………… i
Table of Content……………………………………………………………………… ii
List of Tables…………………………………………………………………………….iv
List of Figures………………………………………………………………………... v
List of Abbreviations…………………………………………………………………..viii

CHAPTER 1……………………………………………..……………….1

INTRODUCTION

1.1. The role of polyphenol oxidase (PPO) in tea (*Camellia sinensis*)……………6
1.1.1. Characterization of PPO…………………………………………………..…6
1.1.2. Morphological distribution and abundance of PPO in *Camellia sinensis*……………………………………..………...........................9
1.2. Inactivation of PPO during green tea manufacture.……………………..…..10
1.2.1. The use of chemical inhibitors……………………………………...…..10
1.2.2. The use of mechanical methods……………………………………..….13
1.3. Determining green tea quality…………………………………………...…..15
1.3.1. Composition of tea leaves……………………………………………15
1.3.2. Biochemical quality parameters of green tea and green tea extracts……………………………………………………………….20
1.4. Applications of green tea extracts…………………………………..….25
1.5. Aim………………………………………………………………………..26
1.6. Hypotheses……………………………………………………………..26

CHAPTER 2…………………………………………………………27

QUALITY EVALUATION FOR GREEN TEA LEAF SAMPLES PRODUCEDE USING VARIOUS PPO INACTIVATION METHODS

2.1 Introduction……………………………………………………………...…27
2.2 Materials and Methods……………………………………………….....38
2.2.1 Materials…………………………………………………………...38

page

2.2.2 PPO inactivation methods for the manufacture of green tea leaf samples……………………………………………….…..39

2.2.3 Sample extraction……………………………………………………43

2.2.4 Theaflavin content (Flavognost method)……………………….……43

2.2.5 Total free amino acid content (Ninhydrin method)…………………..44

2.2.6 Caffeine content (Lead acetate method)…………………………...…45

2.2.7 Colour measurement (HunterLab MiniScan L*a*b* values)…..……47

2.2.8 Total polyphenol content (Folin-Ciocalteu method)…………………47

2.2.9 Organoleptic analysis……………………………………..……48

2.2.10 Freeze drying………………………………………………………....48

2.2.11 HPLC analysis………………………………………………………..49

2.3 Results………………………………………………………………..……50

2.4 Discussion……………………………………………………………..…..72

CHAPTER 3……………………………………...………………80

IDENTIFICATION OF NOVEL FLAVAN-3-OLS BY LC-ESI/MS/MS

3.1 Introduction……………………………………………………………….80

3.2 Materials and Methods…………………………………………...…..87

3.2.1 Materials…………………………………………………………...…87

3.2.2 LC-ESI/MS/MS analysis……………………………………..……87

3.2.2.1 Instrumentation………………………………………………...87

3.2.2.2 Chromatographic conditions………………………………..……87

3.2.2.3 Spectroscopic conditions………………………………………….88

3.3 Results……………………………………………………………………..89

3.4 Discussion…………………………………………………………………94

CHAPTER 4……………………………………………………………97

CONCLUDING DISCUSSION

SUMMARY………………………………………...…………103

REFERENCES…………………………………………………….…105

LIST OF TABLES

page

Table 2.1 Observations made for each PPO inactivation method used……………… 52

Table 2.2 Data summary of biochemical quality analysis for green tea samples using 6 different PPO inactivation methods……………………………………….57

Table 2.3 Average HunterLab L* a* b* colour values for the 4 independent experiments, performed in duplicate………………………………………………62

Table 2.4 Tea taster's results score sheet for selected tea samples from batch 25/2 using Freshpack green tea as reference……………………………………………63

Table 2.5 Steps involved for the different manufacturing methods followed during optimization of the manufacturing process…………………………………...69

Table 2.6 Specifications for the individual manufacturing steps used during the optimization of the manufacturing process………………………………69

Table 2.7 Total polyphenol content for tea samples (PC108) prepared using 4 different manufacturing processes…………………………………………………69

Table 2.8 Percentages of individual catechins, caffeine and total polyphenols of fresh leaf for 40 different TRF cultivars, calculated from molar amounts…………...70

Table 3.1 Assignment of molecular- and fragment ions to specific compounds present in green tea after LC/MS and LC/MS/MS analysis under positive ion mode……………………………………………………………………………….83

Table 3.2 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from Sri-Lanka (5mg/ml) after LC/MS analysis under positive ion mode…………………………………………………………...90

Table 3.3 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from Kenya (5mg/ml) after LC/MS analysis under positive ion mode……………………………………………………………….90

Table 3.4 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from China (5mg/ml) after LC/MS analysis under positive ion mode……………………………………………………………….90

Table 3.5 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from India (5mg/ml) after LC/MS analysis under positive ion mode……………………………………………………………….90

LIST OF FIGURES

page

Figure 1.1: Structures of flavan-3-ols found in *Camellia sinensis*…………………...2

Figure 1.2: Diagrammatic presentation of the manufacturing of different classes of tea……………………………………………………………………...3

Figure 1.3: Formation of theaflavins in the presence of polyphenol oxidase………...4

Figure 1.4: Structures of theaflavins found in fermented *Camellia sinensis* leaves…..4

Figure 1.5: Proposed formation of thearubigens in *Camellia sinensis* by polyphenol oxidase in the presence of oxygen……………………………………...5

Figure 1.6: Cresolase activity by polyphenol oxidase…………………………………7

Figure 1.7: Catecholase activity by polyphenol oxidase………………………………8

Figure 1.8: Structures of compounds known to inhibit polyphenol oxidase………...12

Figure 1.9: Chart indicating a representative composition of fresh green tea (*Camellia sinensis*) leaves…………………………………………………………16

Figure 1.10: Typical HPLC chromatograms measured at 270 nm for black tea, oolong tea and green tea. ……………………………………………………………20

Figure 1.11: Typical HPLC chromatograms measured at 270 nm for three different ice teas……………………………………………………………………...20

Figure 2.1: Natural hydrolysis of theanine (γ-glutamylethylamide) to form glutamic acid and ethylamine in *Camellia sinensis*………………………………….31

Figure 2.2: Formation of Rahmann's purple from the reaction between an α-amino acid and ninhydrin…………………………………………………………….32

Figure 2.3: Simplified schematic representation of steaming apparatus set-up……...39

Figure 2.4: Simplified schematic representation of blanching apparatus set-up…..…39

Figure 2.5: Simplified schematic representation of fluid bed drying apparatus set-up………………………………………………………………………40

Figure 2.6: Simplified schematic representation of pan-frying apparatus set-up……………………………………………………………………………....41

Figure 2.7: Simplified schematic representation of indirect grilling apparatus set-up………………………………………………………………………41

page

Figure 2.8: Simplified schematic representation of direct grilling apparatus set-up……………………………………………………………………………………42

Figure 2.9: Graph to indicate the change in theaflavin content (µmol/g dry weight) in tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied……………………………………53

Figure 2.10: Graph to indicate the change in caffeine content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods……………………………………………………………………………54

Figure 2.11: Graph to indicate the change in total free amino acid content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied………………………………56

Figure 2.12: Graph to indicate the change in flavan-3-ol content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied……..…………………………………………………..58

Figure 2.13: Graphs to indicate L*a*b* values for green tea leaf infusions………...60

Figure 2.14: Overlay of DAD-HPLC chromatograms measured at 270 nm to indicate loss of caffeine observed when blanching (samples from 25/1 batch)……...65

Figure 2.15: Graph indicating the reduction of caffeine after PPO inactivation using the blanching method by RP-HPLC analysis……………………………………66

Figure 2.16: Overlay of DAD-HPLC chromatograms measured at 270 nm to indicate the difference in flavan-3-ol profiles between 4 foreign IGTs and IGT produced from a tea sample assessed as being of highest quality (1.5 min blanching) with respect to the measured quality parameters……………….67

Figure 2.17: Graph for RP-HPLC comparison of the flavan-3-ol and caffeine content determined of 4 IGTs, with a different country of origin, against IGT produced from th e tea samples assessed as being of highest quality (1.5 min blanching)……………………………………………………………………………68

Figure 3.1: Representation of a triple quadrupole mass spectrophotometer with electrospray ionization source……………………………………………………..81

Figure 3.2: Formation of ionization fragments via the retro-Diels-Alder reaction (RDA)………………………………………………………………………………...82

Figure 3.3 Total wave count (TWC) of DAD spectral data for IGT from Sri-Lanka (5mg/ml), indicating tentative HPLC retention times for novel Catechins……………………………………………………………………………..91

Figure 3.4: ESI-MS spectrum obtained for EGCg………………………………..92

Figure 3.5: Tentative identification of EC-digallate from ESI-MS spectrum……….93

LIST OF ABBREVIATIONS

AMP	Adenosine 5’-Monophosphate
AOAC	Association of Official Analytical Chemists
C	(+)-catechin
C_2H_3N	Acetonitrile
CE	Capillary Electrophoresis
CHS	Chalcone Synthase
CUR	Curtain Gas
CV	Coefficient of Variance
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
DC	Direct Current
$dddH_2O$	Double Distilled De-ionized Water
DNA	Deoxyribonucleic Acid
DP	Declustering Potential
DPV	Differential Pulse Voltammetry
E	Free Enzyme
EC	(-)-epicatechin
ECg	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCg	(-)-epigallocatechin gallate
EP	Entrance Potential
ES	Enzyme-substrate Complex
ESI	Electrospray Ionization Mass Spectrometry
EtOH	Ethanol
FBD	Fluid Bed Dry
FC	Folin Ciocalteu
FDA	US Food and Drug Administration
FTIR	Fourier Transform Infrared Spectrometry
GC	(+)-gallocatechin
GC	Gas Chromatography
GCg	(+)-gallocatechin gallate

GLM	General Linear Model
GRAS	Generally Recognized As Safe
GS1	Nebulizer Gas Pressure
GS2	Heater Gas Pressure
GTE	Green Tea Extract
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPV	Human Papilloma Virus
HSCCC	High-speed Countercurrent Chromatography
IBMK	Isobutylmethylketone
IGT	Instant Green Tea
IR	Infrared Spectrometry
ISO	International Standards Organization
K_I	Inhibition Constant
LC	Liquid Chromatography
LIT	Linear Ion Trap
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI-TOF	Matrix-assisted Laser Desorption Ionization-Time Of Flight
MC	Moisture Content
MEKC	Micellar Electrokinetic Capillary Chromatography
MI	Multiple Ion
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MT	Migration time
NACE	Non-aqueous Capillary Electrophoresis
$(NH_4)H_2PO4$	Ammonium Dihydrogen Phosphate
NI	Neutral Loss
NIRS	Near Infrared Reflectance Spectroscopy
NMR	Nuclear Magnetic Resonance
PET	Polyethylene terephthalate
PDMS	Poly(dimethylsiloxane)
PI	Product Ion
POD	Peroxidase

PPO	Polyphenol oxidase
PTFE	Polytetraflouroethylene
PVDF	Polyvinyldifluoride
PVPP	Polyvinylpolypyrrolidone
Q1	1st Quadrupole
Q2	Collision Cell
Q3	2nd Quadrupole
RAPD	Rapid Amplified Polymorphic DNA
RC	Regenerated Cellulose
RF	Radio Frequency
RP-HPLC	Reverse phase high performance liquid chromatography
RTD	Ready To Drink
SARS	Severe Acute Respiratory Syndrome
SAS	Statistical Analysis Software
SS	Soluble Solids
TEM	Temperature
TF	Theaflavin
TIC	Total Ion Count
TR	Thearubigen
t_R	Retention Time
TWC	Total Wavelength Chromatogram
UV	Ultraviolet
V_0	Total Volume of Tea Solution
W	Dry Weight of Tea Sample
XIC	Extracted Ion Count

CHAPTER 1

Introduction

Tea (*Camellia sinensis*) is the beverage most consumed worldwide, excluding water. It originated in the year 2737 BC and is becoming increasingly popular not only for its taste and aroma, but is also consumed as a functional food due to its medicinal benefits. Tea is currently grown in at least 30 countries throughout the world, which produce a total of more than 3.4 million tons of tea annually (FAO, 2005). The per capita worldwide consumption of tea is estimated at 120 ml/day with black tea comprising about 78%, green tea 20% and oolong tea 2% of the consumption. Between different countries, consumption may vary from no tea up to 20 cups of tea per day at variable strength. Cup size, however, differs between countries where for Saudi Arabia a typical cup equals 80 ml, in Japan 100 ml, Italy 135 ml, 150 ml in England and 240 ml in the USA (Jain *et al*, 2006). Ice tea is typically presented in larger volumes of 340 ml or 500 ml, but is usually diluted. The average amount of tea consumed in the UK is 3.5 to 4 cups per day. Drinking 1 to 2 cups (250 ml per cup, 1% $^{w}/_{v}$) of tea provides a radical scavenging capacity equivalent to 400 mg vitamin C, 5 fruit portions or 12 potions of vegetables (Du Toit *et al*, 2001).

Through scientific research, tea has been proven to reduce cholesterol (Bursill *et al*, 2001), have anti-tumour or anti-carcinogenic properties (Thangapazham *et al*, 2007; Gupta *et al*, 1999), prevent loss of cartilage in arthritis by inhibiting production of matrix-degrading enzymes (Ahmed *et al*, 2004), promote weight reduction (Tian *et al*, 2004), protects skin against UV damage (Katiyar *et al*, 2001), block oxidative DNA damage to the liver (Hasegawa *et al*, 1995), aid in the prevention of tooth decay and oral cancer (Lee *et al*, 2004), lower the risk of developing hypertension (Yang *et al*, 2004), act as an antimicrobial (Bandyopadhyay *et al*, 2005), have anti-inflammatory properties (Sang *et al*, 2004), lower plasma glucose levels of type 2 diabetes subjects (Hosoda *et al*, 2003), possibly prevent the development and progression of Alzheimer's disease (Lee *et al*, 2005), reduce the probability of renal stone formation (Itoh *et al*, 2005) and can potentially prevent type IV allergy (Suzuki *et al*, 2000). Tea has also been found to inhibit HIV-1 entry into target cells by preventing glycoprotein-mediated membrane fusion through inhibition of gp41 six-helix bundle formation (Liu *et al*, 2005). HIV-1 reproduction is also inhibited by tea where gp120-

CD4 interaction is blocked through binding of specific tea constituents to CD4 (Liu *et al*, 2005). Most of these health properties listed above have been assigned to the presence of polyphenolic compounds, more specifically the flavan-3-ols, in tea (*see* fig. 1.1).

Tea leaves are generally processed in one of three different ways to produce black-, oolong- or green tea (Graham *et al*, 1992). Although there are several biochemical changes taking place during processing, the most important event is enzymatic oxidation. This enzymatic oxidation, also known as “enzymatic browning” in the food industry, is catalysed by polyphenol oxidase (PPO). In the tea industry this process is termed “fermentation”. However, the meaning of “fermentation” is not

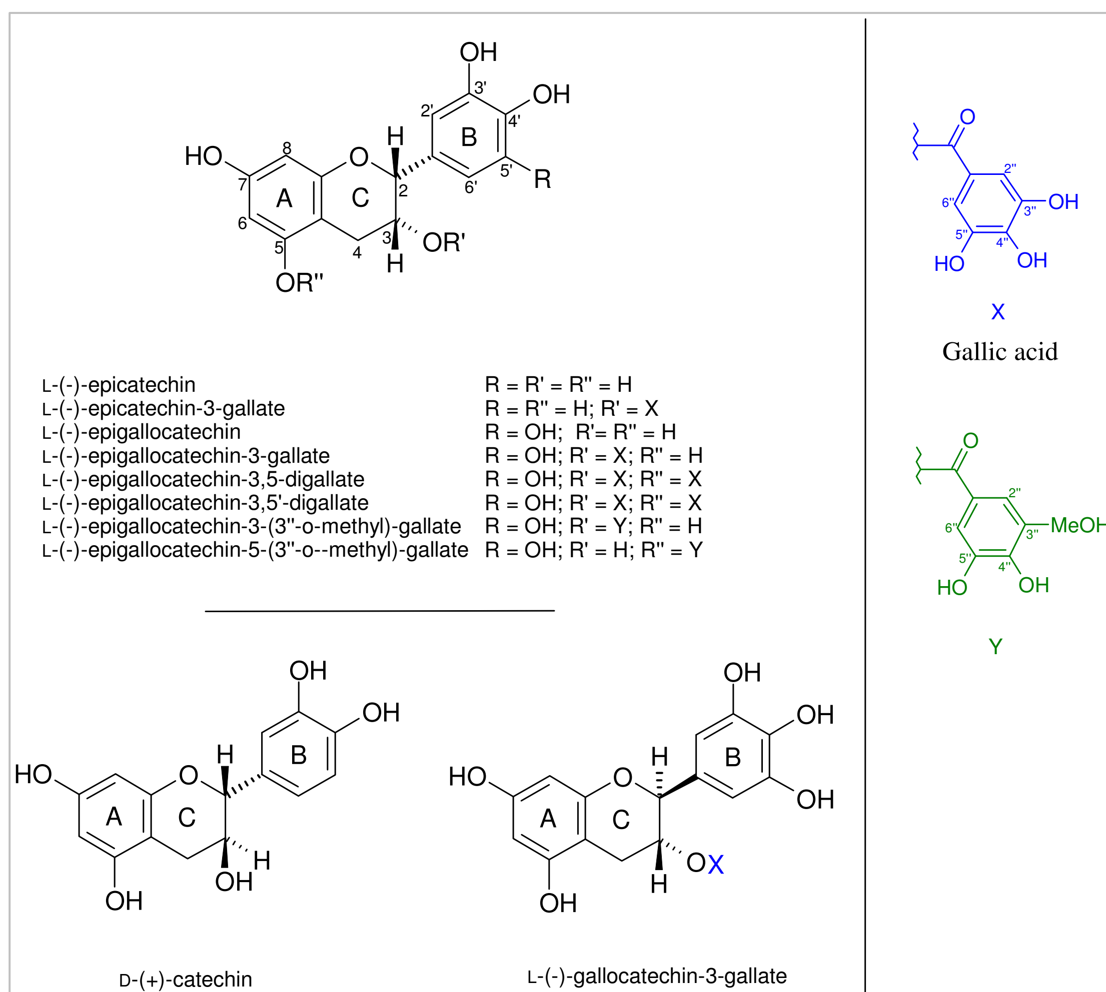

Figure 1.1: Structures of flavan-3-ols found in *Camellia sinensis*.

true to the process involved as the browning reaction occurs in the presence of oxygen and does not lead to the concomitant formation of ethanol, as would be expected

during anaerobic fermentation. Hence, whenever used in the tea industry, the term 'fermentation' refers to aerobic enzymatic oxidation, the latter being a better word choice in that it prevents any confusion.

During aerobic enzymatic oxidation flavan-3-ols dimerise to form yellow-orange theaflavins in a reaction catalyzed by polyphenol oxidase in the presence of oxygen (*see* fig. 1.3 & 1.4). Structures of flavan-3-ols normally found in *Camellia sinensis* are presented in figure 1.1. Subsequent polymerization, which may also involve peroxidase enzymes, brings about the formation of red-brown thearubigens (*see* fig. 1.5).

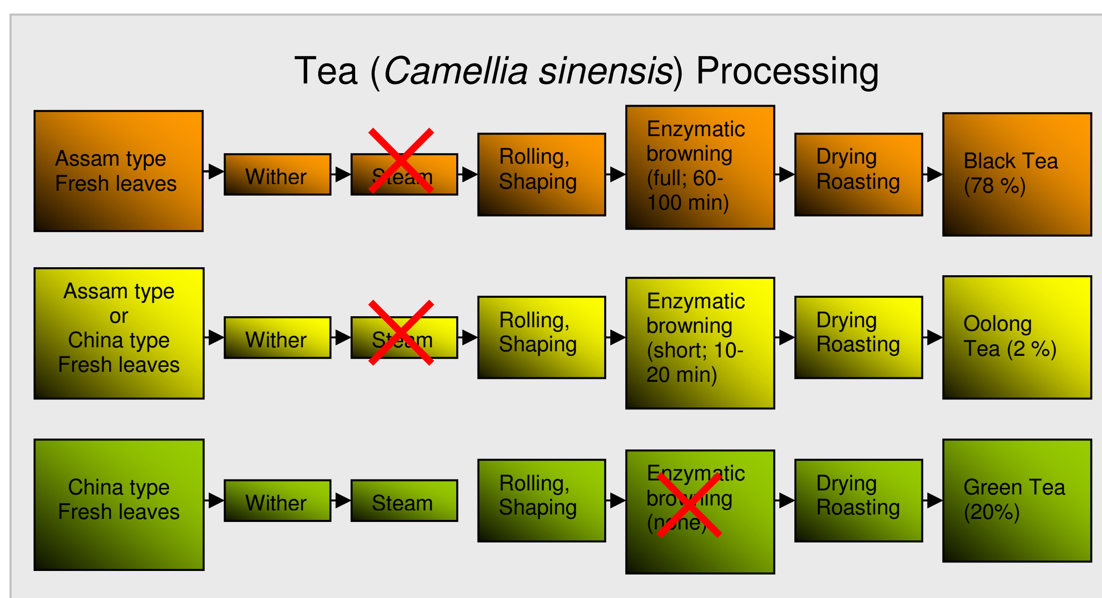

Figure 1.2: Diagrammatic presentation of the manufacturing of different classes of tea.

The difference in colour and taste between black-, oolong- and green tea is mainly attributed to the degree of enzymatic oxidation. Black tea (oxidized leaves) typically contains high levels of theaflavins and thearubigens, whereas oolong tea (partially oxidized leaves) contains only a small amount and green tea (non-oxidized leaves) barely any (*see* fig. 1.2).

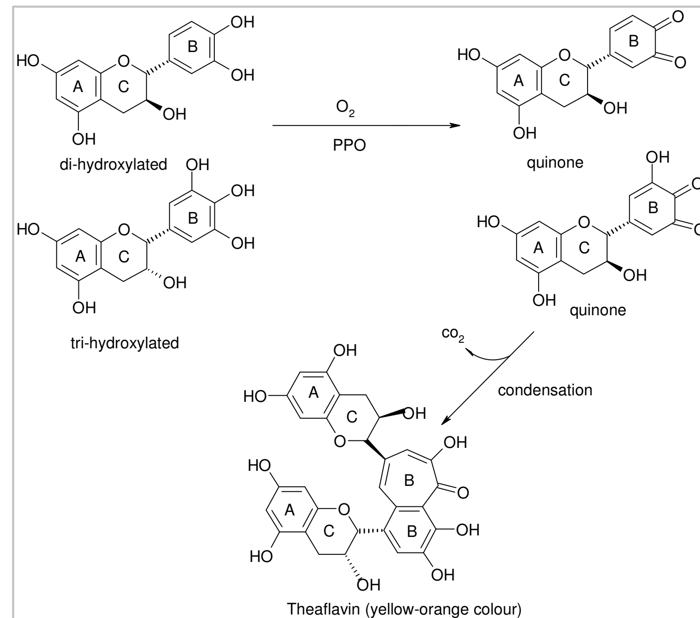

Figure 1.3: Formation of theaflavins in the presence of polyphenol oxidase.

All of the teas mentioned above are manufactured using plucking standards of between two to four leaves and a bud. Apart from black-, oolong- and green tea, white tea is also produced in a similar fashion than green tea, but from tea buds alone excluding leaves.

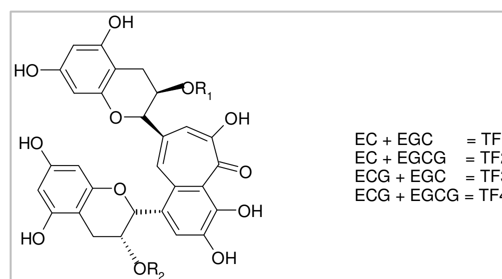

Figure 1.4: Structures of theaflavins found in fermented *Camellia sinensis* leaves. Theaflavin (TF1) is a dimer of epicatechin (EC) and epigallocatechin (EGC): R_1, R_2 = H; Theaflavin-3-gallate (TF 2) is a dimer of EC and epigallocatechin gallate (EGCG): R_1 = H, R_2 = galloyl; Theaflavin-3'-gallate (TF 3) is a dimer of epicatechin gallate (ECG) and EGC: R_1 = galloyl, R_2 = H; Theaflavin-3,3'-digallate (TF 4) is a dimer of ECG and EGCG: R_1, R_2 = galloyl. (Subramanian *et al*, 1999)

Keeping up with the trends and lifestyles of today, tea may also be bought as an instant tea or tea extract. These instant tea markets are focussed more on green tea than oolong- or black tea. The production of instant green tea powder requires an additional manufacture procedure in that the made tea is extracted with hot water followed by spray-drying to remove the water. The soluble solids originally present in the water extract becomes a powder as the water is vaporized yielding the final

product, instant green tea powder. The preparation method of the hot water extract is of cardinal importance as this will determine the final composition of the instant green tea. Further refinement steps may be included, such as removal of caffeine by supercritical CO_2 extraction. Green tea extracts, consisting of the individual flavan-3-ols for commercial purposes, can be obtained through purification steps with the use of organic solvents. It is important to optimize the initial extraction process in such a way as to minimize loss or decay of these compounds.

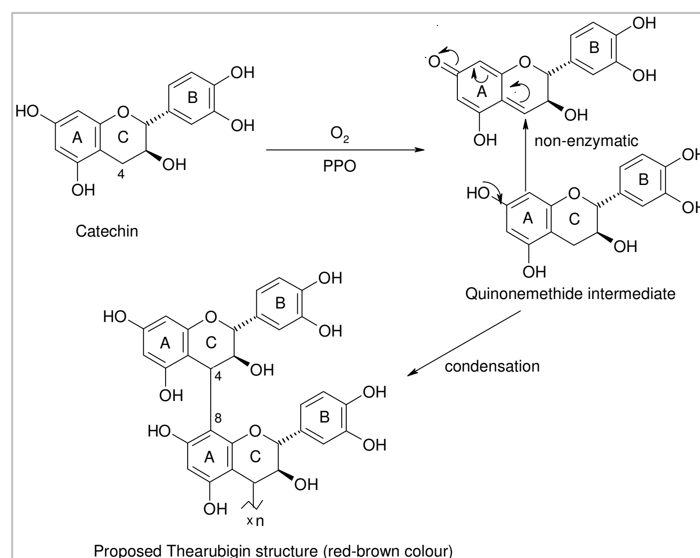

Figure 1.5: Proposed formation of thearubigens in *Camellia sinensis* by polyphenol oxidase in the presence of oxygen.

Unlike made tea, the market value of tea extracts are often determined by the percentage flavan-3-ols, caffeine or theanine as these extracts are commonly used in supplements or other products with applications other than beverages at present. Extractions of this kind may be sold for well over US $200/kg, which is more or less a 100 times above the selling price of loose tea per kilogram. Considering that 1 kg of green tea extract at US $200 may originate from 10 kg loose tea at US $2/kg, the selling price is increased 10 fold following further refinement.

Recent changes in the tea market, which also include high labour costs, led to a significant decrease in profitability on tea farms in South Africa producing conventional black teas. This caused several tea estates to shut down in southern Africa. For these tea estates and others to regain their economical viability, consideration of alternative end products with an increased market value is of great importance. Keeping in mind, the difference in market price between various tea

products as mentioned above, one possible outcome would be to shift their focus from producing only black tea for tea bags to producing instant green tea extracts. Several factors and/or parameters involved that should be kept in mind, when developing such a method, will be discussed.

Firstly, the PPO enzyme involved in the browning of the leaf needs to be understood as the prevention of enzymatic browning of the tea leaves is the most important aspect when producing green tea.

1.1 The role of polyphenol oxidase (PPO) in tea (*Camellia sinensis*)

Enzymatic browning is a common phenomenon observed upon harvesting, cutting, bruising or storage of fruits and vegetables. The enzyme responsible for this browning is polyphenol oxidase (PPO), a copper containing enzyme that is also present in tea leaves (Halder *et al*, 1998). PPO plays an integral role in the manufacture of black tea as it catalyses several biochemical reactions from which quality determining compounds (theaflavins and thearubigens) are formed. However, for the manufacture of green tea, the opposite applies - PPO present in the freshly harvested tea leaves needs to be inactivated as rapidly as possible to prevent any browning of the leaves to occur.

1.1.1. Characterization of PPO

Polyphenol oxidase (E.C.1.10.3.1), an *o*-diphenol:oxygen oxidoreductase, is also called cresolase, catecholase, catechol oxidase, tyrosinase or polyphenolase (Coggon *et al*, 1973; Burton and Kirchmann, 1997). PPO is localised in the chloroplasts and can either be membrane bound or unbound and soluble (Halder *et al*, 1998). Research by Mayer and Harel, 1979, also suggests the enzymes' presence in mitochondria and peroxisomes of tea leaves.

According to Coggon *et al,* 1973, up to 6 isozymes may exist for PPO and the main catechol oxidase fraction isolated by them had a pI of 4.1. Halder *et al*, 1998, mentioned a maximum of three isoenzymes, but that the active enzyme had a molecular weight of 72kDa with *o*-diphenols as its only substrate. More recently, Subramanian *et al*, 1999, concluded that there are three isozymes consisting of a

75kDa- and 100kDa homodimer and a 75kDa monomer with pI values of 5.0, 7.5 and 9.6 independently. Any of the 3 isomers has the capability to form theaflavins from flavan-3-ols (Subramanian *et al*, 1999).

The reaction catalysed by polyphenol oxidase follows typical Michaelis-Menten enzyme kinetics and the amount of oxidised flavanols produced by PPO is dependent on the amount of oxygen available for the reaction to occur (Coggon *et al*, 1973). Polyphenol oxidase exerts both cresolase- and catecholase activity (*see* fig 1.6 & 1.7). During cresolase activity, oxygen is inserted into the ortho position from an existing hydroxyl group. This is followed by the oxidation of the *o*-diphenol to quinone (Mayer and Harel, 1979). When PPO catalyses this type of reaction (monophenol to diphenol to quinone) PPO may also be referred to as tyrosinase as this reaction will form a quinone from the monophenolic group present in tyrosine.

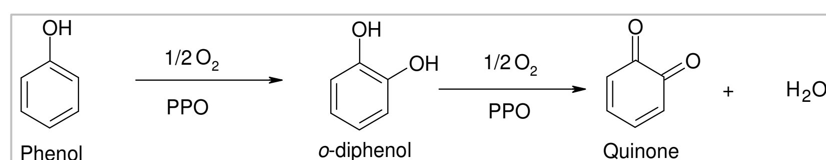

Figure 1.6: Cresolase activity by polyphenol oxidase. Oxygen is inserted into the ortho position followed by oxidation of the diphenol. (Reaction obtained from Mayer and Harel, 1979)

This cresolase activity may be induced by hormones, trypsin proteolysis and exposure to acidic conditions or urea for a short time (Mayer and Harel, 1979). A sharp decrease is observed in cresolase activity when comparing activity observed in the young leaves (bud) with the older leaves (5th leaf). According to a recent study by Baruah, 2003, PPO does not exhibit any tyrosinase activity (ability to catalyze the formation of quinone from monophenolic tyrosine) in *Camellia sinensis*. This is an indication of the specificity of PPO towards its substrate. In the same study, another enzyme, peroxidase (POD), however, was found to be able to oxidize tyrosine. Purity of enzyme extracts may have played a role in previous observations that PPO exerts cresolase activity, but further research is needed before excluding the possibility.

Alternatively, also in the presence of oxygen, PPO may exert catecholase activity whereby *o*-diphenols are oxidized with the release of hydrogen (Mayer and Harel, 1979). Tea PPO is very substrate specific and oxidizes the 3’, 4’-*o*- dihydroxy phenyl B-ring to form *o*-quinones (fig. 1.7). It should be noted that *o*-quinones are powerful

electrophiles which can also suffer nucleophilic attack by water, other polyphenols, amino acids, peptides and proteins, leading to Michael-type addition products (Kim and Uyama, 2005). Typical K_m values for the different PPO substrates usually range between 0.2 and 2.3 mM at 25°C (K_m = 2.3 mM for EGCG). Halder *et al*, 1998, reported a K_m of 0.49 and 0.81 mM for catechin and epicatechin repectively, the preferred substrates, and 19.33 mM for gallic acid. The K_m value with catechol as substrate is 0.2 mM (Coggon *et al*, 1973). For epicatechin and catechin, V_{max} values of 25 and 15 µmol/min have been reported respectively for substrate concentrations of 0-5 mM (Coggon *et al*, 1973).

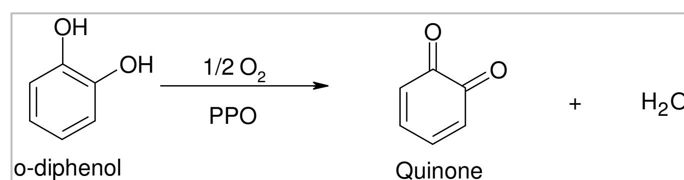

Figure 1.7: Catecholase activity by polyphenol oxidase. An *o*-diphenol is oxidised during which hydrogen is removed from the hydroxyl groups to form a quinone in the presence of PPO. (Reaction obtained from Mayer and Harel, 1979)

Polyphenol oxidase promotes epimerization of flavanols at the C-2 position, but the C-3 position remains unaltered (Coggon *et al*, 1973). Oxidative degallation where gallate groups are removed from gallated catechins under aerobic conditions may occur in the presence of PPO (Coggon *et al*, 1973). PPO may also lead to the formation of de-esterified gallated flavanols in small amounts (Coggon *et al*, 1973).

PPO has an optimum temperature of 40°C (Subramanian *et al*, 1999) with a notable decrease in PPO activity being observed at temperatures above 40°C (Baruah, 2003). Rapeanu *et al*, 2006, determined that PPO from Victoria grapes (*Vitis vinifera* ssp. *Sativa*) has an optimum temperature of 25°C and that total thermal inactivation occurs after a 10 minute incubation period at 70°C. Importantly, Rapeanu found that PPO enzyme is very stable at -85 and -35°C, but 25% activity is lost after 10 days at 4°C. Also, in Chinese cabbage (*Brassica rapa* L.) a 100% loss of PPO activity was observed after a 14 day storage period at 4°C (Nagai and Suzuki, 2001). This implies that withering at ±4°C will be beneficial during green tea manufacture as PPO activity is reduced significantly under moderately cold conditions. PPO and POD, exhibit their maximum activities between 12 and 16 hours of withering (Baruah, 2003).

During fermentation, a steep increase in PPO and POD activity is observed for up to 75 minutes where after PPO activity decreases linearly while POD remains active (Baruah, 2003). PPO activity decreases due to the decrease in cell leaf sap pH from 5.5 to 3.8 during fermentation. POD also inhibits PPO which affects the formation of quinones and semiquinones. This results in a loss of theaflavins and flavanol glycosides by producing secondary products such as the thearubigens (Baruah, 2003). POD activity also leads to the enhanced formation of phenol-protein complexes, deteriorating tea quality (Baruah, 2003).

The pH optimum for PPO with catechol as substrate is 5.5 (Subramanian *et al*, 1999; Baruah, 2003) although values of 5.7 (Coggon *et al*, 1973) and 5.0 (Halder *et al* 1998) have also been documented. Fermentation at a pH of 4.5 instead of 5.5 leads to an increase of theaflavins by 30-40% with a simultaneous decrease in thearubigens when using a crude PPO enzyme preparation. However, when using purified PPO this is not observed. PPO activity causes the formation of theaflavins and H_2O_2, where the latter product is required by peroxidase (POD) to be active. Peroxidase has a pI value of 9.6 (Coggon *et al*, 1973). POD also utilise the theaflavins to form thearubigens. The conclusion is thus made that at pH 5.5 PPO has a higher activity yielding higher amounts of H_2O_2, leading to a decrease in theaflavins with an increase in thearubigens caused by POD.

PPO enzymes from different origins display different pressure stabilities where differences can range from PPO being pressure activated to PPO being pressure inactivated (Rapeanu *et al*, 2006). Though, the effect of pressure on PPO originating from *Camellia sinensis* is not yet known. Pressure inactivation behaviour of PPO is dependent on enzyme source, immersion medium, pH, temperature and time (Rapeanu *et al*, 2006).

1.1.2. Morphological distribution and abundance of PPO in *Camellia sinensis*

The PPO enzyme may be bound to the chloroplast lamellae and grana, but the strength to which it binds depends on the plant tissue where it is present, whether or not the plant is under stress and age (Mayer and Harel, 1979). The use of a detergent is required to solubilize these membrane bound PPO enzymes (Mayer and Harel, 1979).

Baruah, 2003, found that the highest PPO and POD activity is found in the internodes followed by the 1st leaf, 2nd leaf, bud and 3rd leaf, in that order. On the other hand, protease activity is highest in the 2nd leaf > 1st leaf > apical bud > stem (Baruah, 2003). Inspection of the influence of seasonal variation revealed that PPO and POD activity were the greatest during the 2nd flush, during which mellow sunshine and relatively cold weather is experienced (Baruah, 2003). Second greatest PPO and POD activity were detected for the 1st flush followed by the autumn flush and rain flush (Baruah, 2003).

The level of PPO varies significantly at different growth stages and under different growth conditions (Mayer and Harel, 1979). Differences in PPO levels are also observed between different cultivars (Mayer and Harel, 1979). Cultivars containing high amounts of PPO are known as fast fermenters, where leaves rapidly turn brown after rupturing of their cell membranes. These cultivars are ideal for black tea production, but for green tea production the degree of oxidative fermentation, occurring from harvesting till after PPO inactivation, needs to be limited. Thus, slow fermenters will be favourable for green tea production. To distinguish between fast and slow fermenters, a rapid field test, the chloroform test, can be performed where leaves are incubated in a tube containing a piece of cotton wool dipped in chloroform. Fast fermenters will turn brown faster than slow fermenters (Sanderson, 1963).

1.2. Inactivation of PPO during green tea manufacture

To prevent browning of the tea leaves during green tea manufacture, PPO can be inactivated or inhibited either mechanically or chemically or both. Chemical inactivation will be discussed first in the following section. PPO activity is also decreased over time, by lowered moisture content of the leaves and by altering pH (Baruah, 2003).

1.2.1. The use of chemical inhibitors

PPO is a copper containing enzyme because tropolone, a known Cu^{2+} chelator, inhibits this enzyme (Halder *et al*, 1998). Tropolone (2-hydroxy-2,4,6-cyclohepatriene), which may also act as an *o*-diphenol structure analogue, is one of the most potent PPO inhibitors currently known (Kim and Uyama, 2005). Several

other inhibitors (*see* fig. 1.8) as mentioned by Mayer and Harel, 1979, include cyanide, benzoic acid, 2,3-naphtalenediol, phenylthiourea and 3,4-dichlorophenylserine. These inhibitors mainly function by either blocking the PPO active site or by reacting with Cu^{2+} in the enzyme, but there can also be other 'inhibitor sites'. CO_2 inhibit catechol oxidase where inhibition is not reversed by light. Polyvinylpolypyrrolidone (PVPP) adsorbs to phenols thereby inhibiting PPO.

Mushroom PPO is reversibly inhibited by alkylbenzoic acids (Huang *et al*, 2006). These *p*-alkylbenzoic acids are generally uncompetitive inhibitors (Huang *et al*, 2006). The most potent alkylbenzoic acid inhibitor is *p*-octylbenzoic acid, which contains a long hydrophobic eight carbon chain (Huang et al, 2006). Polyphenol oxidase may be inhibited by *p*-octylbenzoic acid by insertion of the alkyl group into a hydrophobic pocket present in the enzyme although many differences are observed in inhibition mechanism between different alkylbenzoic acid analogues (Huang et al, 2006). Mixed-type inhibitors, such as *o*-toluic and *m*-toluic acid, bind competitively to free enzyme (E) with a specific inhibition constant (K_I), but can also bind uncompetitively to enzyme-substrate complexes (ES) with a different inhibition constant (K_{IS}) (Huang *et al*, 2006). Methimazole is another mixed-type inhibitor of mushroom PPO that can either conjugate with *o*-quinones or chelate copper atoms from the enzyme active site (Kim and Uyama, 2005).

Excellent inhibition of Victoria grape PPO was observed using vitamin C (up to 99%), L-cysteine (up to 100%) and sodium metabisulfite (up to 100%) at 5mM inhibitor concentrations. Inhibition by ascorbic acid is more likely to be due to its antioxidant activity whereby quinone intermediates formed by PPO are reduced back to diphenols. Though, irreversible enzyme inhibition is another possible mechanism (Rapeanu *et al*, 2006). Less potent inhibitors at the same concentration include benzoic acid, citric acid, EDTA, glutathione and NaCl which inhibit PPO by 56, 26, 24, 27 and 24% respectively (Rapeanu *et al*, 2006).

PPO originating from Chinese cabbage (*Brassica rapa* L.) is inhibited by 98% with 2-mercaptoethanol, 75% with ascorbic acid and 67% with glutathione at 8 mM inhibitor concentrations (Nagai and Suzuki, 2001). Also, PPO from medlar fruit (*Mespilus*

germanica L., Rosaceae) is completely inhibited by cysteine, sodium metabisulfite, benzoic acid and sodium azide (Dincer *et al*, 2002).

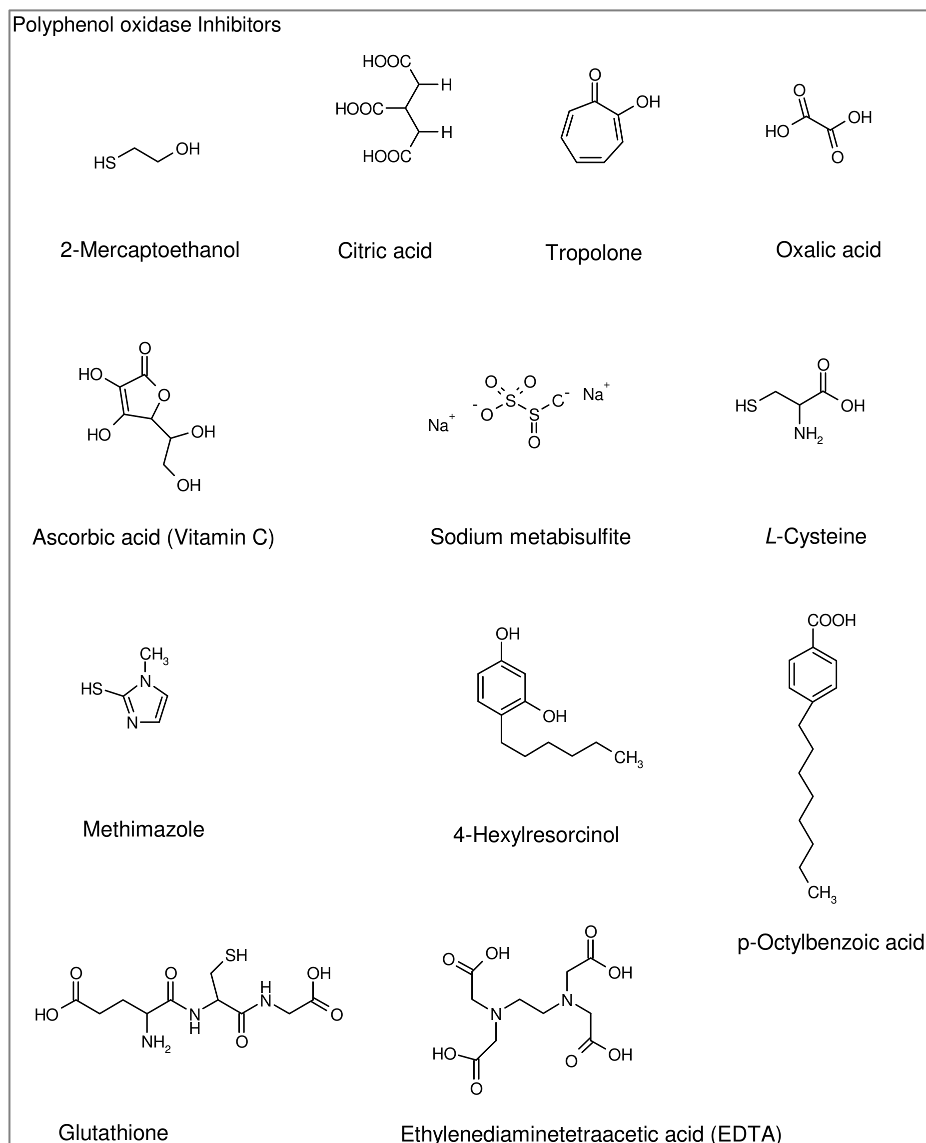

Figure 1.8: Structures of compounds known to inhibit polyphenol oxidase.

In the food industry, 4-hexylresorcinol is alleged to be the most efficient PPO inhibitor as it is water soluble, stable, non-toxic, non-mutagenic and non-carcinogenic (Kim and Uyama, 2005). It has also been recognised as safe for use as anti-browning agent of hot air dried apple slices (Kim and Uyama, 2005). To select an appropriate PPO inhibitor for use in green tea manufacture is however complicated by the likelihood of the formation of off-flavours or off-odours, possible toxicity and economic feasibility. Consumers may also encompass a negative connotation towards chemicals used for PPO inhibition in that it may be perceived as being an adulteration

of the tea leaves. Most, if not all, of these factors can be eliminated with the sole use of mechanical inactivation methods. Hence, it is very likely that the confined use of mechanical methods of PPO inactivation alone will lead to the production of higher quality green tea with a higher market value when compared to methods that include the use of chemical inhibitors.

1.2.2. The use of mechanical methods

Several different manufacturing methods are currently used to produce green tea. The steps involved to produce orthodox green tea are: 1.) Plucking. Normally plucking two leaves and the bud or 'two and a bud' is desirable to produce high quality green tea, although plucking standards of up to four and a bud is often used which gives a higher yield per hectare of tea trees. 2.) Withering. During the withering step, water content of the tea leaves is reduced to about 50% by spreading the leaves thinly onto large troughs. These troughs may be fitted with fans that will blow air, which may be at ambient temperature or heated, from below to speed up the withering process. Alternatively, drum driers or similar equipment can also be used to shorten the required withering time. Soluble proteins tend to decrease by up to 30% during the withering stage, while total free amino acids increase with up to 20% (Baruah, 2003). 3.) Enzyme inactivation. To prevent fermentation, PPO is inactivated by either steaming of the leaves for a short period (20-60 seconds) or by pan firing or roasting. Long, rotating heated cylinders are also used. The major interest lies with this production step for this project where several alternative methods are explored for their effectiveness. 4.) Rolling. During the rolling procedure, the cells are broken and their contents released and mixed during which characteristic flavours develop. Lately, rolling or twisting of the tea leaves is achieved within 2 hours with the use of rolling machinery (Ekborg-Ott *et al*, 1997). Rolling also improves appearance. In the absence of the enzyme inactivation procedure, disruption of the cells when rolling will cause the release of oxidoreductase enzymes leading to the browning of the tea leaves 5.) Drying. The moisture content is further reduced to 3-6% moisture content with the use of hot air at 100-120°C. This is typically achieved with the use of a fluid bed drier (FBD) or similar equipment. Unique aromatic compounds are formed during this drying process that may differ depending on the specific methods used to perform the latter and preceding steps (Graham *et al*, 1992). Caution should be taken not to allow the tea particles to reach temperatures above 110°C when drying as this

will have a deleterious effect. An inlet or initial stage drying temperature of 140°C is acceptable since the wet tea particles will be at ±40°C due to evaporative cooling. The final stage temperature should be reduced to between 90 and 100°C to prevent a burnt taste and loss of quality as the temperature of the now dry tea particles will near the outlet temperature. 6.) Sorting, grading & blending. Finally, the tea is sorted and graded (usually by particle size and preparation method and not by quality). There are different grades of green tea available where appearance is used in part to describe the specific green tea grade. Gunpowder tea consists of small tight balls of young- and medium aged leaves. Sencha tea consists of 0.5 inch long, straight, twisted leaves of different age. Imperial tea is similar to gunpowder tea but it is not as tight and consists of older large leaves. Lastly, Hyson tea consists of long twisted leaves of different age (Ekborg-Ott *et al*, 1997). Tea manufacturers will buy tea, produced as explained above, at an auction and blend it with up to 30 other teas to create their own tea brand that is consistent in aroma, taste, quality and price, which can be entered into the retail market (Ekborg-Ott *et al*, 1997). The market value of the tea usually depends on the quality of the brewed tea liquor as evaluated by a professional tea taster.

Implementation of a specific enzyme inactivation method will require the black tea production lines already installed on the South African tea estates to be converted for green tea production. The conversion should be realized at the lowest possible cost to assure economical viability. At the same time, the specific method of PPO inactivation should allow for high quality green tea extract to be obtained from the further processing of the green tea. Therefore, a low technology yet efficient method needs to be developed that can be implemented by various tea estates.

Utilization of such a PPO inactivation method will allow for year round production of green tea extracts. Green tea leaf produced during the plucking seasons can be dried and sent off to a central processing station where it can be stored until further use. The green tea leaf will typically be extracted with hot water and spray-dried resulting in instant green tea powder. Individual or groups of constituents, such as theanine, caffeine or catechins, can be obtained through further refinement of the extracts by applying separation methods that involve the use of organic solvents, super critical CO_2 or water based processes.

1.3. Determining green tea quality

When determining green tea quality, it should be realized that the quality of any tea is influenced by both horticultural factors and manufacturing practice. A weakness in either of these will result in a tea of lower quality. The initial concern would therefore be to obtain high quality fresh tea leaf from the best cultivar grown under optimum growth conditions. This is then followed by applying the manufacturing method that will provide the desired end product.

1.3.1. Composition of tea leaves

Apart from flavan-3-ols, which varies greatly from <15% ($^w/_w$) to >20% ($^w/_w$), fresh tea leaves also contain methylxanthines (3.5%), amino acids (4%), volatiles (<0.1%), and others (*see* fig. 1.9).

Today a number of different varieties are recognized for the genus *Camellia*. Five taxa namely *C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica*, *C. sinensis* var. *dehungensis*, *C. talinensis* and *C. crassicolumna* have been compared by their caffeine content. Inter taxa caffeine content comparison had a coefficient of variance (CV) of 22.7% and intra taxa comparison of selections gave a CV of up to 31.2% (Chen *et al*, 2005). Also, earlier research has indicated that *C. sinensis* var. *assamica* is higher in caffeine (4.09% w/w) than *C. sinsensis* var. *sinensis* (3.11% w/w) (Astill *et al*, 2001). For this reason the *assamica* variety is usually preferred in black tea production as the caffeine plays a role in creaming and quality of black tea. Creaming does not occur in green tea due to the absence of thearubigens (no fermentation) and since caffeine contributes to the bitterness of tea, the *sinensis* variety is preferred for green tea production, which will have a less bitter taste. It is also important to mention that from a historical view, the commercial tea clones that are available today are higher in caffeine than Indian hybrid material, as it was considered of good quality when a tea cultivar contained high amounts of caffeine. During the selection process, those cultivars containing low caffeine levels would have been removed from the breeding programme (Cloughley, 1982).

The composition of the tea leaves can be manipulated by alteration of specific horticultural factors. In 1987, HPLC results from the studies of Owuor *et al* (1987)

indicated how climate and geographical localities affected caffeine biosynthesis. Rainfall, average air temperature, sunshine hours and cloud cover all affected caffeine biosynthesis, but altitude had no significant effect.

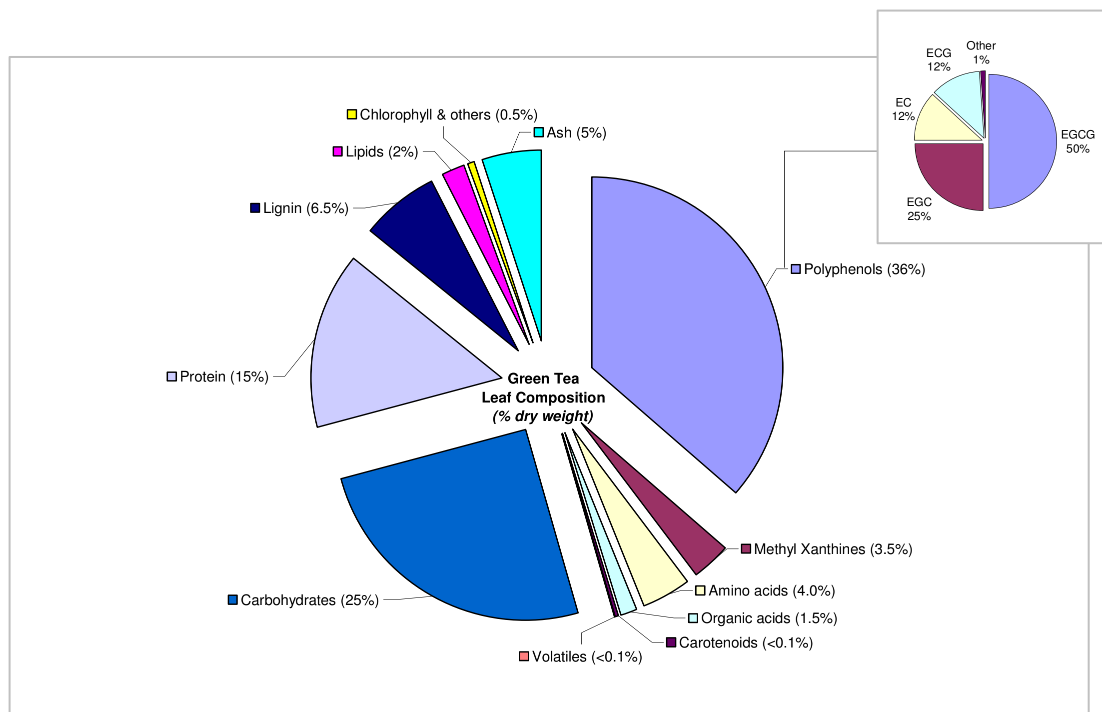

Figure 1.9: Chart indicating a representative composition of fresh green tea (*Camellia sinensis*) leaves. (Percentages obtained from Graham *et al,* 1992) *Insert:* Relative percentages of the 4 major flavan-3-ols present in dried fresh tea leaf. (Percentages obtained from Nagai *et al*, 2005).

Seasonal variation where caffeine levels are up to 50% higher during the plucking season (or in season) than during the winter months have been detected in southern Malawi (Cloughley, 1982). In Kenya, where seasonal temperature fluctuations are less than in Malawi, 24-30% w/w variation in caffeine levels was detected (Owuor *et al*, 1986). Seasonal variations are also observed for the individual catechins that fluctuate between different flushes. The highest amount of total catechins is obtained from the 2nd flush followed by the autumn flush, 1st flush and rain flush (Baruah, 2003). A study to determine the necessity of light for caffeine biosynthesis to occur was performed by Koshiishi *et al*, 2000. Changes in growth rate, caffeine content, caffeine synthase activity and overall metabolism were compared between plants grown in the dark and plants growing under normal light conditions after a seven day period. Under light deprived conditions a decrease in caffeine synthesis is observed, but this decrease is mainly attributed to the greatly reduced growth rate. Hence it was

concluded that light is not essential for caffeine synthase activity to produce caffeine (Koshiishi *et al*, 2000).

Application of fertilizers also has a large impact on caffeine contents as is the case for nitrogen addition where caffeine levels are 35% w/w higher after administering 315 kg/ha nitrogen fertilizer instead of 45 kg/ha. Amino acid content can also be significantly increased pre-harvest by combined application of K and Mg in the sulphate form (Ruan *et al*, 1998). Unfortunately, addition of K and Mg also increases caffeine content slightly (Ruan *et al*, 1999). Applying 70kg/ha potassium fertilizer increase caffeine content significantly (P=0.05) when compared to tea plants where the fertilizer was absent (Cloughley, 1982). Magnesium fertilizer increases chlorophyll which is negatively correlated with polyphenol production (Ruan *et al*, 1999). Though, the results presented by Ruan *et al*, 1999 that propose a decrease in polyphenols is unclear and do not seem to be significant.

The effect of pruning on caffeine biosynthesis has been investigated and it was found that caffeine levels increase with age after pruning over four years (Thomas *et al*, 2005). According to the authors, this may be caused by the impairment of adenine nucleotide biosynthesis, the formation of caffeine from 7-methylxanthine *via* theobromine or generation of adenosine by S-adenosyl-L-homocysteine (SAH) in the S-adenosyl-L-methionine (SAM) cycle.

The effect of plucking standards on caffeine content in tea is determined by analysis of the distribution of caffeine in the tea plant. The highest quantities of caffeine can be found in the buds (± 6.3% w/w) from where a decrease is observed to the first (± 4.6% w/w), second (± 3.6% w/w), third (± 3.1% w/w) and fourth (2.7% w/w) leaf. The same is observed for flavan-3-ols where the highest amount is found in the bud >1st leaf >2nd leaf > 3rd leaf > stem (Baruah, 2003). For Malawi teas, a 50% w/w decrease in caffeine occurs between the bud and the third leave, whereas in the case of teas from Kenya the same is observed between the bud and the second leave. Caffeine content in the stems (± 2% w/w) is significantly lower than in the leaves (Cloughley, 1982; Owuor *et al*, 1986). With this information at hand it is obvious how fine (two and a bud) vs. coarse plucking standards will affect the caffeine

contents in tea. Poor control over the plucking standards used to produce a specific tea will thus lead to potentially large variations in quality.

A significant difference in catechin content and composition can be observed between different tea cultivars (Baruah, 2003). Some cultivars also include methylated catechins such as epicatechin-3-O-(3-methyl) gallate, which have been identified for their strong anti-allergic properties by inhibition of histamine release. These methylated catechins are present in specific tea varieties which include 'Benifuuki', 'Benifuji', and 'Seishin-taipan' (Nagai *et al*, 2005). The presence of these methylated flavan-3-ols in high percentages in tea leaves may thus also raise the quality of the tea produced. Yet another novel group of flavan-3-ols include the digallated catechins. Very little research has been done on this group due to their low abundance in most tea cultivars. During the processing of the fresh leaves, the composition ratios of these compounds are altered depending on the specific processing method

When taking a closer look at the manufacturing process of tea, many variables also exist in this area. For black tea, a linear increase in caffeine of up to 20.6% over 30h can occur during the withering stage. The increase is temperature dependant and is at a maximum at ambient temperature. Temperatures above 35°C will cause the necessary enzymes to denature keeping the caffeine content relatively constant (Cloughley, 1983). During production of green tea, inclusion of a withering phase causes a general increase in free amino acids (Roberts and Wood, 1951). Total free amino acids increase from 2.6 to 3.5% ($^{w}/_{w}$) due to hydrolysis of proteins by proteases. Caffeine and polyphenols also increase slightly during the withering phase (Baruah, 2003).

The next stage is fermentation, during which the caffeine content decreases (5.5-14% w/w) again depending on temperature. Furthermore, a decrease in amino acids is observed due to their conversion to volatile carbonyls which includes heptanal, benzaldehyde and phenylacetaldehyde. Catalyzed by polyphenol oxidase, complexion of individual catechins forms theaflavins and further complexion forms the thearubigens. Caffeine interacts with the latter during fermentation and this caffeine-thearubigen complex gives black tea its tangy, astringent taste. A further

reduction of caffeine is observed during the drying stage which is performed at high temperatures, but this is less than during the fermentation stage (Cloughley, 1983). Temple *et al*, 2001, also found that caffeine decrease with an increase in drying temperatures from 60 to 140°C, but concluded after HPLC analysis that 100°C is best to use as temperatures of 110°C and above cause a decline in other quality factors which results in a loss of value. This deleterious effect is confirmed by Wang *et al*, 2000, regarding the quality of green tea. These high temperatures cause the caffeine to sublimate or evaporate and the extent to which this occurs is also affected by the type of drying system used and time spent in the drying system. Following storage for six months a slight increase in caffeine is observed again which is due to the breakdown of the caffeine-thearubigen complex to yield free caffeine again. This goes hand in hand with the increase in bitter taste over time. At the end of the manufacturing process a net effect of 7.0 – 15.6% increase in caffeine can occur depending on the tea cultivar used as raw material (Cloughley, 1983). For green tea, caffeine content is relatively stable during process and storage and the authors concluded that in the case of green tea the changes in taste with storage is due to changes in phenolic compounds and not caffeine (Wang *et al*, 2000). Astill *et al*, 2001 however, reported that a short withering stage during green tea production has a larger initial caffeine increase but no significant loss during shaping and firing stages. This means that green tea will have higher caffeine content than black tea when the same raw material is used. A comparison of typical HPLC chromatograms, measured at 270 nm, of the three main types of tea clearly indicates the differences in polyphenolic- and caffeine content (*see* fig.1.10).

Lastly, particle size of the processed tea leaves, infusion time, infusion temperature, whether or not the tea leaves are contained in a teabag, material used for the teabag and size of the teabag are all factors which should be kept in mind by the consumer (Astill *et al*, 2001). The latter also applies to polyphenol content when making a cup of tea. Most of the factors mentioned above should be kept in mind when producing an extract from the green tea leaves for the production of instant green tea, as this will significantly affect the quality of the instant green tea powder.

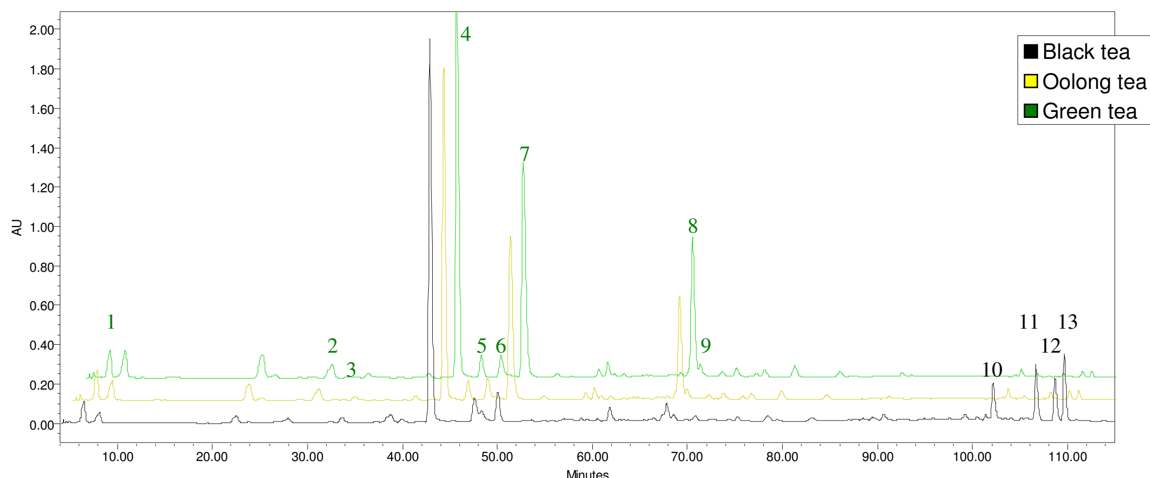

Figure 1.10: Typical HPLC chromatograms measured at 270 nm for black tea, oolong tea and green tea. Phenolic compounds were separated on a phenyl-hexyl HPLC column using (A) 1% acetonitrile, 2% acetic acid and (B) 80% acetonitrile, 2% acetic acid as mobile phases. 1: gallic acid; 2: EGC; 3: C; 4: caffeine; 5: EC; 6: ethyl gallate (internal standard); 7: EGCG; 8: ECG; 9 CG; 10-13: theaflavins. An increase in theaflavins is observed where black- > oolong- > green tea. The reverse is observed for flavan-3-ols where green- > oolong- > black tea. Caffeine content also usually follow the trend green- > oolong- > black tea when using the same cultivar. (Maliepaard, unpublished data)

The composition of ice teas vary greatly as can be seen in the comparison of HPLC chromatograms of different ice tea brands (*see* fig. 1.11). Unfortunately, as minimum amounts are not regulated, these extremely weak tea beverages also find their way onto the shelves where quality to the consumer is determined by taste.

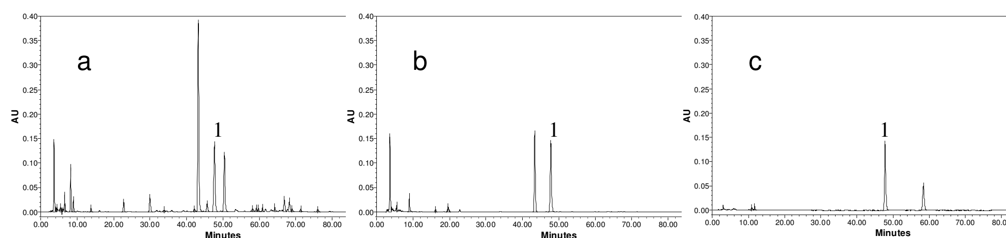

Figure 1.11: Typical HPLC chromatograms measured at 270 nm for three different ice teas. Complexity of the phenolic profiles varies from highly complex (a), corresponding to normal tea chromatograms, to very ordinary (c). 1: Internal standard (ethyl gallate). (a) Lipton Ice tea; (b) Nestea Ice tea; (c) Pick ‘n Pay Ice tea. (Maliepaard, unpublished data)

The health benefits of tea should thus rather be promoted through the use of alternative tea products such as supplements and not by drinking ice tea.

1.3.2. Biochemical quality parameters of green tea and green tea extracts

Tea drinking is a practice that has literally been ongoing for thousands of years. To determine the quality or value of the infusion, tea tasters played a critical role in the organoleptic analysis thereof. Today still, with tea being consumed at such high volumes worldwide, tea tasters are indispensable.

With regard to the health benefits of tea, increased consumer expectations are currently experienced (Collier, 2005). The use of green tea and green tea extracts for applications other than beverages, which includes dietary supplements and cosmetic products, are also becoming increasingly popular, requiring additional quality measures. This means advancing from organoleptic evaluation to biochemical evaluation by quantification of individual biochemical quality markers. Adulteration of tea is a negative element which also occurs in the tea industry where unethical attempts are made to improve tea quality. Tea manufacturers may, for instance, add monosodium glutamate to improve green tea taste (Horie and Kohata, 2000). Green tea may also be treated with ammonium hydrogencarbonate to enhance colour (Horie and Kohata, 2000). The use of biochemical markers will thus also aid to establish the integrity of a specific tea.

Relevant biochemical quality markers include:

1. *Fluoride content*. Tea plants tend to accumulate fluoride (F), which may serve as quality marker where high amounts of F present in tea is negatively correlated to green tea quality (Lu *et al*, 2004). Abnormally low amounts of fluoride, however, may imply possible adulteration of the tea or tea extract involved.

2. *Theanine content*. Theanine is primarily responsible for the calming effect of tea. This amino acid may directly provide neuroprotection against focal cerebral ischemia and may be clinically useful for preventing cerebral infarction (Egashira *et al*, 2004). Recent studies also indicated that theanine is effective against alcoholic liver injury (Sadzuka *et al*, 2005). This amino acid also contributes to the umami (meaty or brothy) taste of tea. Although all amino acids present in tea play an important role in the taste and/or the volatiles formed during tea manufacture, tea extracts may be sold purely by their theanine content. Theanine can be further isolated from high theanine containing extracts to be sold as pure theanine. High amounts of theanine present in tea are thus positively related to green tea quality.

2. *Caffeine content*. Caffeine or 1,3,7-trimethylxanthine (a mild central nervous stimulant that may cause flushing, chills, irritability, loss of appetite, anxiety, and

shivering at high dosages) is rated as one of the most important 'health quality markers'. In extreme cases where an overdose of caffeine is consumed (although this is exceptional) arrhythmia, tachycardia, vomiting, convulsions, coma and death may follow (Kerrigan and Lindsey, 2005). In a report by Eteng *et al*, 1997 it was stated that the physiological effects experienced by caffeine consumption is due to a build up of cyclic AMP caused by inhibition of phosphodiesterase. Caffeine also has the ability to cross the placental barrier causing impaired foetal development in rats; therefore, women are advised to reduce their consumption of caffeine containing beverages during pregnancy. On average, 200-300 mg of caffeine or 463 mg for moderate to heavy drinkers is consumed daily and the lowest reported toxic dose for caffeine in humans is 2000-3000 mg or 57 mg/kg body weight (Eteng *et al*, 1997). A cup of tea (250 ml), when containing 4.5% w/w of caffeine, brewed as a 1% w/v solution, provides 112.5 mg of caffeine (Graham *et al*, 1992, Aucamp *et al*, 2000). This means that the average caffeine consumption is reached by consuming only 2-3 cups of green tea per day. Graham *et al*, 1992, reported that in some populations up to 20 cups of tea may be consumed on a daily basis, but that this should still be tolerable physiologically as the total caffeine amounts to less than 1 g. This, however, is not the case should 20 cups of tea containing 4.5% w/w caffeine prepared as above be consumed for the benefit of its high polyphenol content. The latter will amount to 2.25 g of caffeine being consumed daily, which is in the range of the reported 2 to 3 g toxic dose. Some people may also experience insomnia or irritation of the gastrointestinal tract. Thus, even though a high caffeine content is typically associated with a high quality tea based on organoleptic evaluation and its role in creaming (complex formation with thearubigens) in black tea (Owuor *et al*, 1986), the above reasons (where caffeine is considered a biochemical 'health' marker) calls for the production of tea containing very little or no caffeine at all. This may be achieved either by removal of caffeine during the tea manufacturing process, by producing cultivars or clones where caffeine synthesis is absent or minimized or a combination of the two.

In contrast to the above, tea containing above normal quantities of caffeine may also be sought after for use in the food or pharmaceutical industry as natural source of caffeine since caffeine has been classified as a GRAS (Generally Recognized As Safe) compound by the US Food and Drug Administration in 2001 (FDA) (Uefuji *et*

al, 2005). Conventional extraction methods fail in significantly reducing the cost of natural caffeine ($500-800/kg), but increasing biosynthesis of caffeine in the plant itself may prove different (Wang *et al*, 2005). Smit *et al*, 2005, concluded that caffeine and theobromine (a caffeine precursor) may play an important role in obtaining an 'acquired taste' for dark chocolate because of the psychoactive effect exerted by the methylxanthines on the subjects used in their study. Similarly, the presence of methylxanthines may also aid to attain a liking for green tea.

Thus, the quality of a specific selection of green tea, with regard to caffeine content, is very dependent on the specific application of the green tea as well as the marketing strategy.

4. *Total free amino acid content and composition*. Amino acids contribute to both taste and colour of tea infusions. Amino acids play an integral role in the aroma and flavour of green tea, although other volatile compounds are also involved (Ekborg-Ott *et al*, 1997). Sweet, sour, salty, bitter and umami are the predominant taste sensations caused by amino acids in humans (Ekborg-Ott *et al*, 1997). Specifically in green tea, amino acids contribute to the brothy, sweet, umami taste (Ekborg-Ott *et al*, 1997). High free amino acid content correlates positively towards green tea quality.

5. *Volatile content and composition*. Taste and aroma is determined by the specific volatile composition of a tea, which may vary greatly between teas originating from different production methods. Some volatiles may be favoured above others. With regard to detection methods, gas chromatography (GC) or GC-Mass spectrometry (GC-MS) is usually used to analyze volatiles or aroma compounds in tea (Horie and Kohata, 2000). Dutta *et al*, 2003, have developed a method to discriminate between differences in the volatile composition between different tea samples, as would be recognized with a sense of smell, with the use of a metal oxide sensor based electronic nose. An optical tongue is also being developed by Edelmann and Lendl, 2002, where flow-through sensing of tannin-protein interactions is performed based on Fourier transform infrared (FTIR) spectroscopy.

By comparison of the relative amount of 11 volatiles from 15 tea samples, Kato *et al*, 2001, concluded that high levels of linalool and hexanal is associated with high

quality green tea, whilst high levels of geraniol is associated with low quality green tea. Also, in general, high quality green teas contain higher levels of volatiles (Kato *et al*, 2001).

6. *Flavan-3-ol content and composition*. Lower polyphenol content is desirable for green tea beverage to be less astringent and to have a fresh taste (Ruan *et al*, 1999). For instant green tea, however, polyphenol content must be higher as it may be used for alternative applications than drinking. Also, most of the health properties of tea known to date are attributed to the presence of flavan-3-ols. Hence, these astringent polyphenolic compounds are positively related to quality for most applications of green tea or green tea extracts. Similarly, Wright *et al*, 2000, has indicated a correlation between flavan-3-ol composition of fresh tea leaves and the quality of black tea where they concluded that fresh leaves containing higher amounts of flavan-3-ols give rise to a higher value black tea.

7. *Theaflavin* content. Wright *et al*, 2002, also found that the sum of individual theaflavins or total theaflavins in black tea can be used to predict its value, where the presence of high quantities of theaflavins reflects good quality. Conversely, as theaflavins are a product of oxidative fermentation, a step which is excluded from green tea manufacture, the presence of this compound is negatively correlated to green tea quality. Theaflavins also contribute negatively to colour of the final green tea product. Pre-harvest damage control and effective post-harvest processing is required to keep the theaflavin content at a minimum.

8. *Colour*. In a consumer survey by Zellner and Durlach, 2002, to establish which products are associated by the public as being refreshing, 90% responded that water is refreshing and 49% responded that iced tea is refreshing as their 3rd choice. Also, 41% of the 86 students responded that, by colour, a clear beverage is more refreshing (Zellner and Durlach, 2002). Although colour is determined by the quantity of the individual compounds, including several quality parameters, colour measurement is also performed to determine visual quality of tea as a beverage. Colour thus serves as an indirect quality parameter where yellow to brown infusion colours are associated with low quality green tea or green tea extracts. Measurement of colour also gives an indication of the extent to which fermentation took place. Liang and his colleagues,

2005, expressed infusion colour in terms of ΔL, Δa and Δb values, where tea infusions were compared to a distilled water control. They found that ΔL decreased, while Δa and Δb increased with the degree of fermentation. The ΔL or L* value is positively correlated to green tea quality whereas Δa or a* and Δb or b* are negatively correlated to green tea quality (Liang *et al*, 2005).

1.4 Applications for green tea extracts

Apart from using tea as a beverage (hot or cold), ample alternative applications exists for green tea extracts that are mainly attributed to the array of health properties possessed by tea. These applications vary from dietary supplements to cosmetics. Green tea extracts have even found their way into products such as toothpaste and chewing gum. In November, 2006, the FDA approved a green tea extract (Polyphenon® E) produced by Mitsui Norin Co., Ltd. as a safe and effective prescription drug for the topical treatment of genital warts caused by the human papilloma virus (HPV). Mitsui Norin Co., Ltd., who provided funding for this current research project as well, has also developed air filters together with Panasonic that contained amongst others, catechins from green tea as anti-bacterial and anti-viral agents. The air purifiers produced, using this technology, have proven to be highly effective in inactivation of the severe acute respiratory syndrome (SARS) virus.

With the seemingly endless scope for products that incorporate green tea extract, the demand for green tea extract is destined to increase as new products are developed.

1.5 Aim

The goal of this study is to discover a method to economically produce an Instant Green Tea (IGT) that features enhanced quality properties when compared to conventionally produced IGTs. Different polyphenol oxidase inactivation methods are expected to result in IGTs with a difference in quality with regard to flavan-3-ol, caffeine, theaflavin and total free amino acid content, taste and colour. Discovery of such a method may lead to implementation of the method on tea estates in Africa to add value to their products.

1.6 Hypotheses

Hypothesis I: The six polyphenol oxidase inactivation methods investigated will produce instant green tea with different catechin to caffeine ratios.

Hypothesis II: Application of LC-MS will aid in the identification of HPLC retention times of compounds (novel catechins) from a crude extract.

CHAPTER 2

Quality evaluation of Green Tea Leaf samples produced using various PPO inactivation methods

2.1 Introduction

The inactivation of polyphenol oxidase in *Camellia sinensis* is of primary importance during the production of green tea leaf. Orthodox methods of PPO inactivation usually include the use of steam or pan-frying. The choice of PPO inactivation method affects the taste and appearance of the final product. These differences are the result of a repertoire of biochemical changes taking place during the manufacturing process. To produce a high quality green tea, a manufacturing method should be applied that favours the desired biochemical reactions whilst preventing or minimizing undesirable reactions to occur. Several green tea manufacturing methods have been explored for their potential to bring about a quality green tea. Though, little research has been done towards the comparison of the biochemical composition as quality was mainly determined by expert tea tasters.

Here, six manufacturing methods were investigated and the quality of their end products was compared at biochemical level. This project is focussed on a specific application where certain quality parameters are more important than others. However, this study may also provide valuable information towards green tea manufacture for alternative applications. The methods under investigation are:

1. Steaming (method used by several tea factories in the Far East to inactivate PPO, which can easily be implemented on African tea estates);
2. Blanching (previously found to cause a decrease in caffeine content);
3. Fluid bed drying (most African tea factories already make use of this technology to dry black tea);
4. Pan-frying (another Far East method commonly applied during green tea manufacture);
5. Grilling using direct heat (additional method to be explored);
6. Grilling using indirect heat (additional method to be explored).

Before evaluation of green tea leaf samples for quality, the concerning quality determinants need to be identified. At present, a variety of biochemical quality markers exist, although not all of these carry the same weight with regard to their importance in determining the quality of a specific tea. For the purpose of instant green tea production the most important quality is the percentage of catechins, where the higher the percentage, the better the quality. Hand in hand with the percentage of catechins, is the amount of caffeine present in the manufactured tea or ultimately in the green tea extract. The opposite, meaning a low percentage caffeine, is favourable in high quality green tea extracts. Hence, an increase in the catechin to caffeine ratio equals an increase in quality. As scientific knowledge increase on the differences in health related activities of the individual catechins, preference may be given to specific catechins in future, influencing quality. Other quality parameters investigated in this project include theaflavin content, total free amino acid content, theanine content, infusion colour and taste.

Quantitative and qualitative caffeine analysis

Caffeine is a mild central nervous stimulant that contributes to the bitterness of tea infusions. The quality determining status of caffeine in tea depends on the application of the tea product. This project is aimed at producing an instant green tea that expresses an above average catechin to caffeine ratio. Low caffeine content is thus desirable for high quality. Removal of caffeine to increase the catechin to caffeine ratio also improves quality. Importantly, caffeine removal should be, unlike current known methods, inexpensive and not leave any solvent residues.

Various methods have been developed for the detection of caffeine in *Camellia sinensis*. In 2000, Aucamp *et al* published a method developed to analyse catechins, caffeine, gallic acid and theanine simultaneously using micellar electrokinetic capillary chromatography (MEKC). Although previous MEKC methods exist, an increased resolution was obtained with a limit of detection (LOD) of 1.0-20 mg/L and a limit of quantification (LOQ) of 2.0-12.0 mg/L. Sample analysis can be performed within 13 minutes to give a full separation for samples of fresh leaves or tea liquor. They also reported the use of capillary zone electrophoresis (CZE) for caffeine detection, but a higher resolution is observed when utilizing the MEKC method. This

method was further improved by Bonoli *et al*, 2003, where the LOD was reduced to 1.9 μg/L and sample analysis time was 12 minutes.

A near infrared reflectance spectroscopy (NIRS) method for caffeine detection in green tea has been developed by Shulz *et al*, 1999. NIRS is integrated with analysis software that uses a partial least-squares algorithm. Time of analysis is less than 1 minute and a very good correlation ($R^2 = 0.97$ for caffeine, $R^2 > 0.85$ for individual flavan-3-ols) between NIRS predictions and HPLC values for caffeine and polyphenols is obtained. This method can be used to determine caffeine, theobromine, total polyphenols, individual catechins and dry matter contents for green tea at a low cost compared to HPLC, where expensive equipment is required.

Ohnsmann *et al*, 2002, were able to determine caffeine content using Fourier transform infrared spectrometry (FTIR) with a LOD value of 1 mg/L and a LOQ value of 3.4 mg/L. This method only requires 15 minutes for analysis and when compared to the Association of Official Analytical Chemists (AOAC) method it correlates well, but FTIR results are slightly higher. This method does not require the use of diethyl ether or Celite and is therefore more environmentally friendly. It is also more cost efficient as less reagents are used and because of the short analysis times (Ohnsmann *et al*, 2002).

A new method using poly(dimethylsiloxane) (PDMS) microchannel electrophoresis with electrochemical detection has been developed by Zhang *et al*, 2005, to separate caffeine and theophylline in urine samples within 40 seconds. Future work includes online sample pre-treatment and as this method has the advantage of being inexpensive, simple and giving good resolution, it may very well be developed further to include analysis of tea samples as well (Zhang *et al,* 2005).

Various spectrophotometric methods are available for the detection and quantification of caffeine. This includes the UV-spectrophotometric method provided by the AOAC where caffeine is extracted with the use of $CHCl_3$ and caffeine absorbance is measured at 276 nm against a $CHCl_3$ blank. Another method by the AOAC makes use of the same extraction method, followed by gas chromatography (GC), the latter

being more time consuming than the spectrophotometric method (AOAC, 1995). The national standard method for caffeine determination in China is also a UV-spectrophotometric method where polyphenols and other impurities are removed by addition of lead acetate, followed by addition of H_2SO_4 and absorbance is read at 274 nm. This method, however gives higher results when compared to HPLC methods, but a recent study by Xiaogang *et al*, 2005, indicated that there is a 97% correlation between these two methods. Multiplying by a correction factor of 0.7606, when using this spectrophotometric method, will be much more accurate in that it accounts for other xanthines that are present in tea (Xiaogang *et al*, 2005). This method has the advantage over HPLC in that expensive equipment and a technique specialist is not required. HPLC does however also allow for rapid analysis (within 10 minutes) by a method described by Nakakuki *et al*, 1999, where a polyvinylpyrrolidone (PVPP) pre-column is used to purify the tea extract from polyphenols etc. before the sample enters the reverse phase column. HPLC is also more sensitive and may allow for the other xanthines to be detected as well. As indicated by the ISO/CD 14502-2 reverse phase HPLC method, currently under review by the International Standards Organization (ISO), simultaneous analysis of individual catechins, another quality marker in tea, can also be achieved when performing RP-HPLC (ISO/CD 14502-2, 2002). This can be further enhanced when subsequent electrospray ionization mass spectrometry (ESI-MS) analysis is performed (Del Rio *et al*, 2004). To obtain full separation of three methylxanthines (caffeine, theobromine and theophylline) from tea, coffee or urine samples, De Aragão *et al*, 2005, have designed a RP-HPLC method where, in contrast to other HPLC methods, ethanol is included in the mobile phase. This method has a LOD value of 0.1 mg/L and a LOQ value of 0.33 mg/L for caffeine and a separation time of less than 6 minutes. The results correlated well with literature values (De Aragão *et al,* 2005).

Theanine and total free amino acids

The predominant amino acid present in tea is theanine (γ-glutamylethylamide), which may comprise >50% of the total free amino acids or 1-2% of the dry weight of tea (Ekborg-Ott *et al*, 1997). This amino acid is known to play a role in quality and characteristics of green tea (Ekborg-Ott *et al*, 1997). Apart from being present in tea, another source has been identified namely the mushroom, *Xerocomus badius*. Theanine has a similar structure to glutamine and is broken down to glutamic acid and

ethylamine upon hydrolysis (*see* fig. 2.1). L-Theanine and glutamic acid have been found to contribute the most to the umami taste of green tea and in addition, theanine also provides a sweet taste without any bitter after taste (Ekborg-Ott *et al*, 1997).

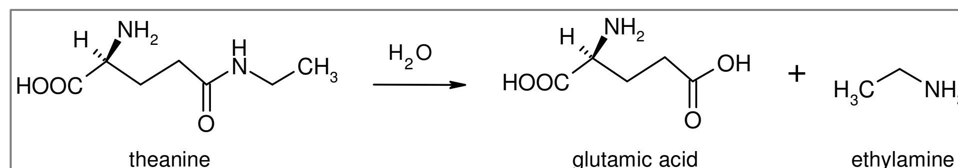

Figure 2.1: Natural hydrolysis of theanine (γ-glutamylethylamide) to form glutamic acid and ethylamine in *Camellia sinensis*.

Theanine does not only contribute towards the taste of tea, but some of the health properties of tea have also been ascribed to the presence of this amino acid. For instance, theanine is known for its calming effect and to reduce the possibility of developing hypertension.

As a whole, amino acid content plays an important role as quality parameter in green tea with regard to taste and colour (Ruan *et al*, 1999; Yao *et al*, 2006). A polyphenol to amino acid ratio of 8:1 is considered to be the ideal for tea beverage (Ruan *et al*, 1999). The best method of PPO inactivation would therefore be a method that will not cause a significant decrease in total free amino acids. Even though the main focus of this project is not to produce high quality instant green tea specifically for beverage applications, it is still considered to be a very important quality determinant due to the health properties provided by theanine. It was therefore deemed essential to include quantification of total free amino acids in this project.

Total free amino acids are determined using a spectrophotometric ninhydrin (triketohydrindene hydrate) method. The mechanism is based on the reaction between ninhydrin and α-amino acids which yields the coloured product, Rahmann's purple (diketohydrindylidene-diketohydrindamine) (*see* fig. 2.2). The optimum absorption wavelength of Rahmann's purple is at 570 nm (Moore and Stein, 1948).

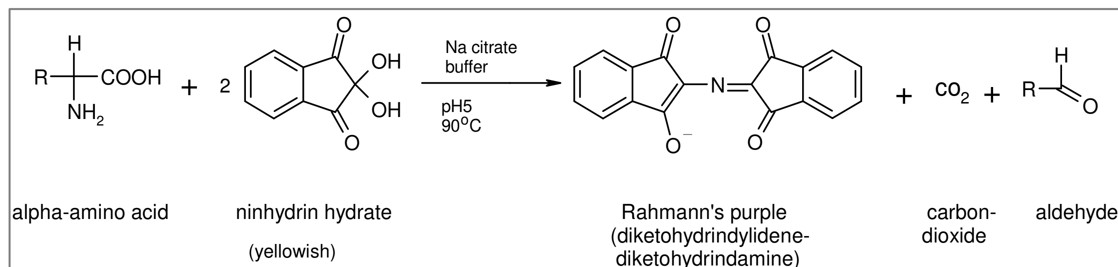

Figure 2.2: Formation of Rahmann's purple from the reaction between an α-amino acid and ninhydrin. (adopted from Moore and Stein, 1948 and Friedman, 2004).

Application of a non-specific spectrophotometric amino acid assay is used to quantify total free amino acids. However, as theanine is known to comprise >50% of the total amino acids present in tea, this assay also allows for a rough estimate to be made on the theanine content.

Organoleptic analysis

Taste quality is one of the key criteria used by professional tea tasters to describe quality of tea beverages. Amino acids contribute 70% of umami taste intensity in green tea (Kaneko *et al*, 2006). Nucleotides such as adenosine 5'-monophosphate (AMP) are also umami-tasting and enhance the taste of monosodium L-glutamate (Kaneko *et al*, 2006). Conversely, nucleic acid contribution is negligible due to its low concentration in green tea leaf infusions. L-theanine, succinic acid and theogallin also enhance umami taste (Kaneko *et al*, 2006).

The bitter taste is due to the presence of caffeine and flavan-3-ols (Lesschaeve and Noble, 2005). Flavan-3-ols also provide an astringent (dry mouth feeling) taste to tea (Lesschaeve and Noble, 2005). It has been found that epicatechin is more bitter and astringent than catechin.

In contrast to the belief that the polyphenols are the main contributors to astringency, Scharbert *et al*, 2004, identified 14 flavon-3-ol glycosides as being the major astringency contributors in black tea. These compounds have taste thresholds up to 190 000 times lower than that measured for epigallocatechin gallate (EGCG) (Scharbert *et al*, 2004).

Theaflavin analysis

Theaflavins originate from the condensation of one di-hydroxylated with one tri-hydroxylated flavan-3-ol (*see* fig. 1.3). In the case where two tri-hydroxylated flavan-3-ols condense, a colourless theasinensin (bisflavanol) is formed. Bisflavanols, however, are only present in very small quantities in black tea (Graham *et al*, 1992) and will not be discussed further. There are four major theaflavins usually present in black tea (TF1, TF2, TF3 and TF4) (*see* fig. 1.4). A comparative study by Leung *et al*, 2001, on the antioxidant activities of theaflavins *versus* catechins by means of a LDL oxidation assay indicated that TF4>ECG>EGCG≥TF3≥TF2>TF1≥EC>EGC. The results were compared on a mole per mole basis, which explains the high antioxidant activity obtained for TF4. Between cultivars there are cultivars regarded as fast fermenters due to the fact that they contain, on average, a higher amount of PPO. These are ideal for black tea production, but slow fermenters are preferred for green tea production as leaf browning will occur at a slower rate. Geographical location also cause significant variation in theaflavin content found in black teas due to differences in rainfall, average temperature, cloud cover and sunshine hours (Owuor *et al*, 1987). A decrease in theaflavins is caused by oxidative breakdown by POD enzymes and subsequent generation of thearubigens (Haslam, 2003). Thearubigens are usually retained on a HPLC column and elute as an unresolved Gaussian shaped hump (Haslam, 2003).

In nature, theaflavins form in response to plant cells being damaged, ruptured or injured by predators. The presence of theaflavins leads to a decrease in free flavan-3-ols as these are the building blocks of theaflavins. The formation of theaflavins from flavan-3-ols is catalyzed by PPO, which is inactivated during green tea manufacture. The presence of theaflavins will have a deleterious effect on green tea quality. High quality green tea should thus contain very little if any theaflavins at all. Hence, theaflavin analysis is performed not only to determine the degree of PPO inactivation, but also to establish quality. Theaflavins in green tea are markers for low quality.

The Flavognost assay was used to quantify theaflavins in the tea samples. Factors that have a significant influence on the outcome of the results includes particle size of the test material, grinding method used, infusion temperature and the infusion time

(Robertson and Hall, 1989). An infusion time of more than 10 minutes causes theaflavin breakdown leading to a reduced level of quantification (Robertson and Hall, 1989). It is therefore crucial that all of the above mentioned variables be kept constant for the duration of sample analysis. Proper practice will allow for accurate inter- and intra experimental comparative statistical analysis to be performed on results. Other variables with regard to the solvent extraction of theaflavins from the sample extract include the use of hot versus cold sample extracts. Allowing the sample extract to cool down prevents the formation of an emulsion as seen when using a hot sample extract (Robertson and Hall, 1989). The incubation temperature used determines the rate of colour formation and it was found that at 37 °C a plateau is reached after ±10 minutes (Robertson and Hall, 1989). A downscaled version of the Flavognost method was created and optimized to improve productivity and repeatability. Aluminium salts are known to react with theaflavins to give a red colour (Reeves *et al*, 1985). For this reason, aluminium chloride has also been investigated for use in the theaflavin assay. Despite the use of aluminium chloride being cheaper, easily available and yielding comparable results to using Flavognost reagent, the Flavognost method was preferred as this is currently the internationally accepted method. According to Spiro and Price, 1986, only 85% of theaflavins are observed as the green Flavognost complex, which is quantified spectrophotometrically. The green colour is formed when Flavognost reagent, a diphenyl-substituted boron compound, complexes with the *cis*- 1,2-di- or 3,4,5-tri hydroxybenzoyl groups of theaflavins (Spiro and Price, 1986). Although theaflavins were not extracted 100%, consistent proportions were extracted, which meets the requirements of an international method for theaflavin analysis as stated by Reeves *et al*, 1985.

Analytical methods using sophisticated techniques, such as HPLC and CE, have been developed for quantification of individual theaflavins. During HPLC analysis theaflavins, detected at 205 nm with a diode array detector, are separated from flavan-3-ols by means of gradient elution (Lee *et al*, 2000). Capillary electrophoresis was 3 times faster than HPLC analysis, but HPLC analysis was 5 times more sensitive and had a LOD of 0.5 µg/ml (Lee and Ong, 2000). A rapid non-aqueous CE (NACE) method was developed by Wright *et al*, 2001, but their method lacks the ability to

simultaneously quantify theaflavins and flavan-3-ols, as can be accomplished using HPLC. MALDI-TOF mass spectrometry has been used to partly characterize the polymerized and oxidized structures of theaflavins and thearubigens (Menet *et al*, 2004).

Colour profiling

Roberts and Smith, 1963, used optical density at 460 nm of tea infusions to calculate total colour and brightness as an indication of quality. Interestingly, they found that the water source can have a significant effect on brightness of the infusion. When using distilled water, much brighter infusions (>25%) were obtained as opposed to using mains water exerting a temporary hardness that gave an infusion with increased pH. Theaflavins give an orange-red colour to tea infusions and thearubigens give a red-brown colour (Graham *et al*, 1992). Darkening of the leaf, and subsequently also the infusion, is observed with increased chlorophyll content as the latter is converted to pheophytins and pheophorbide (Graham *et al*, 1992). Reverse phase HPLC methods have been developed to detect both chlorophylls and their metabolites (Horie and Kohata, 2000).

Liang *et al*, 2005, found that colour measurements performed on tea infusions correlate significantly to total quality scores awarded by professional tea tasters for green, oolong and black tea. Both green and black teas give a positive Δb or b* value (yellowness > 0 > blueness) which denotes both teas as being yellow, although black tea has a much higher Δb or b* value than green tea (Liang *et al*, 2005). Tea colour usually decreases with aging of the tea. Huang *et al*, 2005, indicated that colour decay due to storage can be significantly reduced by selenium (Se)-enrichment. Selenium-enrichment also increases aroma, sweetness and vitamin C content, while total polyphenol- and chlorophyll content remains unchanged and bitterness decreases (Huang *et al*, 2005). The increased aroma and sweetness may be due to increased amino acid synthesis, specifically theanine, which will also suppress the bitter taste.

Advanced qualitative and quantitative flavan-3-ol analysis

Several advanced techniques have been investigated for their ability to separate phenolic substances from tea. These include near infrared reflectance spectroscopy (NIRS) and electromigration methods such as capillary zone electrophoresis (CZE)

and micellar electrokinetic capillary chromatography (MECK). MECK is more sensitive than HPLC for polyphenol detection, but repeatability of quantification is lower. High-speed countercurrent chromatography (HSCCC) can be used to isolate catechins on a preparative scale in gram amounts (Degenhardt *et al*, 2000).

Electrochemical methods, such as differential pulse voltammetry (DPV) or a tyrosinase-biosensor using screen-printed graphite electrodes, can also be used but is less accurate than HPLC (Romani *et al*, 2000). DPV and biosensor do pose greater sensitivity than HPLC, but still requires further optimization (Romani *et al*, 2000). False positives are not obtained using HPLC, hence HPLC can be used for qualitative and quantitative analysis of polyphenols from complex mixtures (Romani *et al*, 2000).

Diode array UV detectors are most commonly used, although electrochemical or chemiluminescence detectors may also be used, which may provide 50 times higher sensitivity.

Sano *et al*, 2001, managed to separate 12 catechins using HPLC with an electrochemical detector. The four major catechins, their epimers and four methylated catechins were detected. The LOD for this method was 10-40 pmol/ml when applying 600 mV. Different solvent systems have been utilized for the separation of polyphenolic compounds. The mobile phase used by Sano *et al*, 2001, to separate 12 catechins consisted of 0.1 M NaH_2PO_4-acetonitrile (87:13) with 0.1 mM EDTA.2Na. Kumamoto *et al*, 2000, claimed that they could achieve enhanced catechin separation using mixed-solvents of water, acetonitrile and ethyl acetate as opposed to gradient elution using only water and acetonitrile. Their conclusions were made from using only 4 catechins for which a shift in retention times was observed with the addition of ethyl acetate. If indeed the separation of all catechins are enhanced and not caused to overlap with one another or other components by the addition of ethyl acetate, still requires further investigation.

Catechins dissolve more readily in acetonitrile than in water and will therefore elute as the acetonitrile gradient is increased. Acid is required in the mobile phase to prevent tailing, to give better peak resolution and also function to stabilize the catechins, preventing epimerization. Absence of any salts, such as NaH_2PO_4,

prevents complications when performing LC-MS where salts may interfere with ionization. Partition of catechins according to previously published methods was not reproducible, a problem also encountered by Dalluge *et al*, 1998, and methods had to be altered to obtain the desired separations. Column selection is also of great importance as the degree of separation varies when using different types of columns. Dalluge *et al*, 1998, indicated that catechin separation is column-dependent where deactivated monomeric C_{18} columns outperform non-deactivated monomeric or polymeric C_{18} columns. Phenylhexyl columns have been developed specifically for the separation of phenolic substances, giving advanced separations of catechins above other columns.

The filters used during sample preparation before HPLC analysis, can have a significant effect on the accuracy of individual flavan-3-ol quantification. Gallated catechins tend to be retained the most by polyvinyldifluoride (PVDF) filters where the recovery for ECg is <10% (Yoshida *et al*, 1999). This is followed by regenerated cellulose (RC) with 87% recovery for ECg and thirdly, cellulose acetate with 92% ECg recovery (Yoshida *et al*, 1999). Polytetraflouroethylene (PTFE) filters with a hydrophilic coating proved best for the application having catechin recoveries of >99.0% (Yoshida *et al*, 1999).

2.2 Materials and methods

2.2.1 Materials

Fresh tea leaves (*Camellia Sinensis*), specifically the PC 108 cultivar, were obtained from SAPECO tea estate in Tzaneen, South Africa, on four different occasions during the mid summer peak production period (17/2/2006, 25/2/2006, 1/3/2006 & 10/3/2006). The leaves were plucked in the early morning and transported at room temperature for about 4 hours to the University of Pretoria, South Africa, where it was stored at 4 °C until processed. PPO inactivation methods were performed within two days. Two-and-a-bud plucking standard for the tea leaves was used for all experimental purposes. Double distilled deionised water (Milli-Q system, Millipore, USA) was used in all experiments.

All chemicals were of analytical grade unless otherwise indicated. Citric acid, tri-sodium citrate dehydrate, glycine, propanol, stannous chloride ($SnCl_2$), glycine, lead bi-acetate, isobutylmethylketone (IBMK), Folin-Ciocalteu reagent and anhydrous sodium carbonate were obtained from Merck (Darmstadt, Germany). Ninhydrin was obtained from Pierce Chemical Company (Illonois, USA). Methyl cellosolve (2-methoxyethanol; chemically pure grade), 32% hydrochloric acid, 98% sulphuric acid and absolute ethanol were purchased from Saarchem (Muldersdrift, South Africa). Caffeine, diphenylboric acid-2-aminoethyl ester (2-aminoethyl-diphenyl borate/ Flavognost), were obtained from Sigma.

Minisart hydrophilic syringe filters, 0.20 µm, were obtained from Sartorius (Hannover, Germany). Acetonitrile E CHROMASOLV® (HPLC grade), methanol CHROMASOLV® HPLC grade, (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechingallate (EGCG) and gallic acid monohydrate were purchased from Sigma Chemical Company (Steinheim, Germany). Acetic acid (Ultra ≥ 99.5%) and ethyl gallate were purchased from Fluka (Steinheim, Germany). Filter paper circles 595, ∅ 125mm, were obtained from Schleicher & Schuell Microscience (Dassel, Germany).

2.2.2 PPO inactivation methods for the manufacture of green tea leaf samples

Green tea leaf samples originating from 100 g (fresh weight) tea leaves were required for biochemical analysis. PPO inactivation methods that were limited to processing 50 g fresh leaf at a time was performed in duplicate. Four independent repeats of all experiments were performed.

2.2.2.1 Steaming

Apparatus was set up as shown in fig. 2.3. When fully steaming, 100 ±1 g fresh tea leaves were added and the lid replaced as quick as possible to prevent significant cooling.

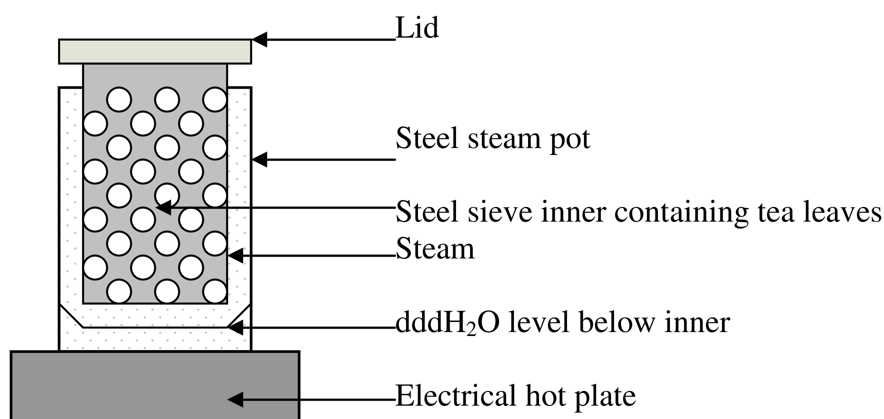

Figure 2.3: Simplified schematic representation of steaming apparatus set-up.

This was performed once for 30, 60, 90, 120, 150, 180, 210 and 240 seconds (as determined by exploratory experiments) independently. At the end of each incubation time, leaves were spread out on paper towel and allowed to air-dry.

2.2.2.2 Blanching

Apparatus was set up as shown in fig. 2.4. The Heidolph MR-82 hotplate was used on maximum heat setting to bring the dddH$_2$O to boiling point. Once boiling point was reached, 50 ±0.5 g fresh tea leaves was added, a perforated lid placed on top of the leaves and the lid replaced as quick as possible to prevent significant cooling. This was performed for 30, 60, 90, 120, 180 and 240 seconds (as determined by exploratory experiments) independently in duplicate where after samples were pooled together. At the end of each PPO inactivation time, the leaves were centrifuged using a commercial food centrifuge to remove excess surface water and then air dried.

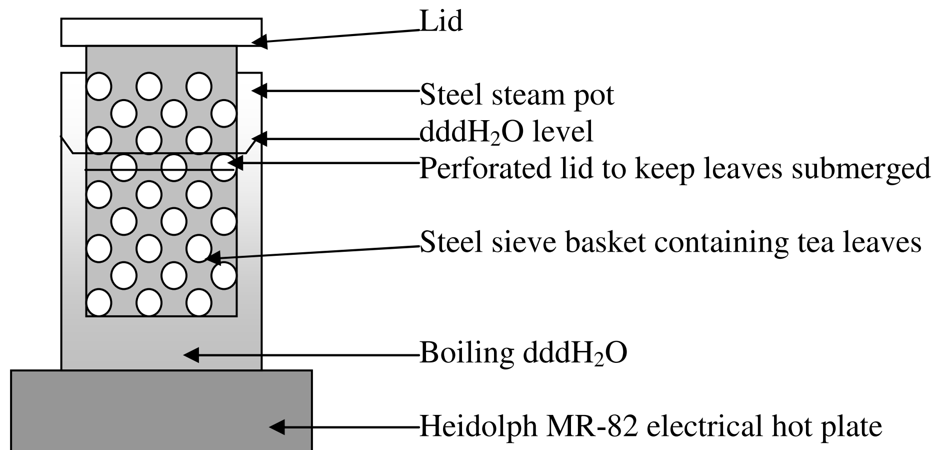

Figure 2.4: Simplified schematic representation of blanching apparatus set-up

2.2.2.3 Fluid bed drying (FBD)

Apparatus was set up as shown in fig. 2.5. The Fluid bed dryer (Sherwood Scientific, Mark II, England) was pre-heated to 120°C.

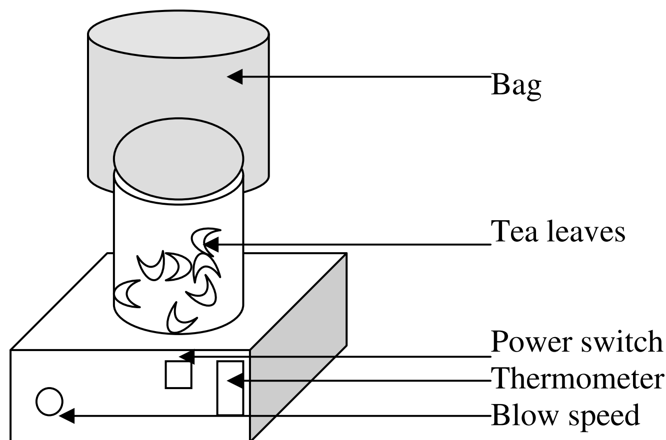

Figure 2.5: Simplified schematic representation of fluid bed drying apparatus set-up.

Fresh tea leaves (50 ±0.5 g) were added and blow speed was set to 100% for the duration of this PPO inactivation procedure. This was performed in duplicate for 4, 8, 12, 17, 20 and 30 minutes (as determined by exploratory experiments), independently in duplicate, where after samples were pooled together.

2.2.2.4 Pan-frying

Apparatus was set up as shown in fig. 2.6. The tray was pre-heated (medium heat) and then 50 ±0.5 g fresh tea leaves were added and stirred continuously for the

duration of the treatment. This was performed for 30, 60, 90, 120, 180 and 240 seconds (as determined by exploratory experiments), independently in duplicate, where after samples were pooled together.

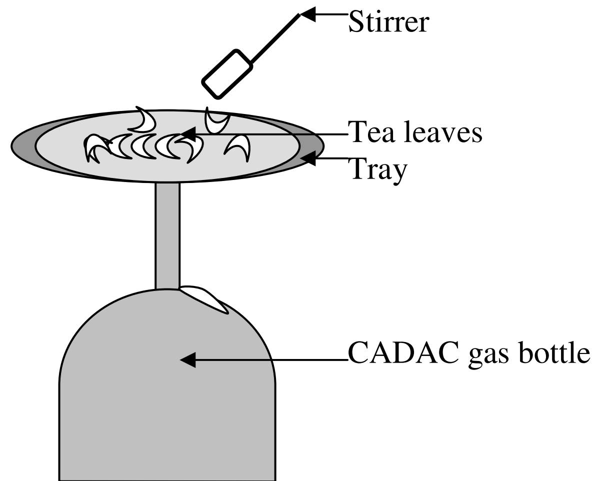

Figure 2.6: Simplified schematic representation of pan-frying apparatus set-up.

2.2.2.5 Grilling – Indirect heat

Apparatus was set up as shown in fig. 2.7. The fresh tea leaves (50 ±0.5 g) were placed in the grill, where after it was positioned on the pre-heated tray (medium heat).

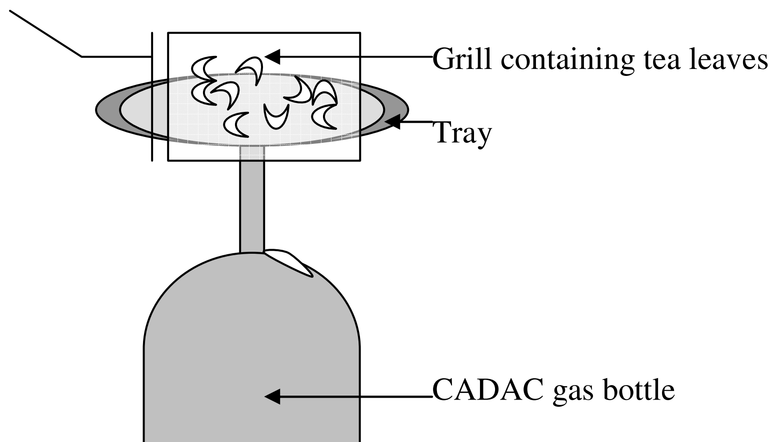

Figure 2.7: Simplified schematic representation of indirect grilling apparatus set-up.

The grill was turned over every 15 seconds. This was performed for 1, 2, 3, 4, 5 and 6 minutes (as determined by exploratory experiments), independently in duplicate, where after samples were pooled together.

2.2.2.6 Grilling – Direct heat

Apparatus was set up as shown in fig.2.8. Fresh tea leaves (50 ±0.5 g) were placed in the grill and placed on the pre-heated tray (medium heat). The grill was turned over every 15 seconds to prevent burning of the leaves.

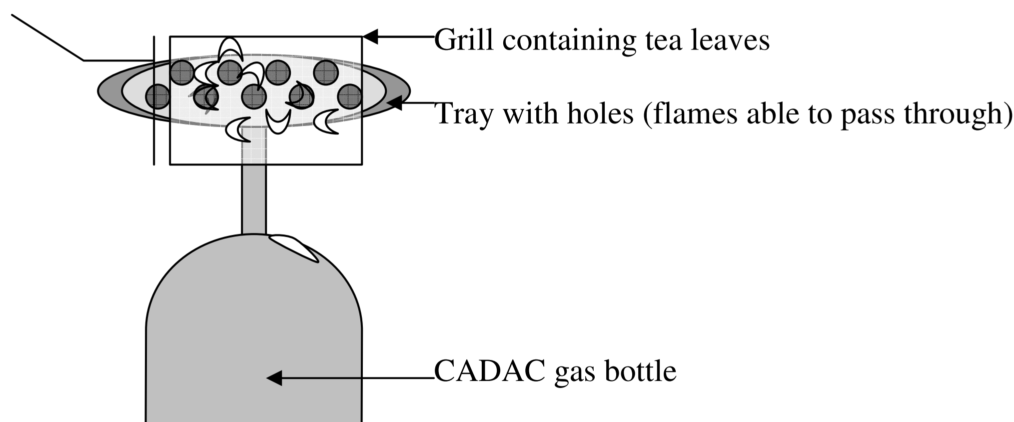

Figure 2.8: Simplified schematic representation of direct grilling apparatus set-up.

This was performed for 1, 2, 3, 4, 5 and 6 minutes (as determined by exploratory experiments), independently in duplicate, where after samples where pooled together.

For each PPO inactivation method where duplicate treatments were performed, it was done in opposite process time directions (high to low and low to high). All samples where stored at 4 °C overnight, where after samples were further dried in the fluid bed drier set at 100 °C, 100% blow speed for 15-60 minutes, depending on moisture content of the individual samples after applying the initial PPO inactivation step. The dried tea leaves where ground using a FRITSCH pulverisette (Type 02.102, 220 Volt, No 3786). Ground tea sample was sieved using a Labotec test sieve (particle size < 355 µm) and transferred to 50 ml centrifuge tubes, which were sealed in Ziploc bags and stored at -20 °C till further analysis was done. Before any sample was opened for further analysis, it was allowed to equilibrate to room temperature for 1 hour.

Moisture content was determined for each sample as described in the ISO 1573 method for determination of loss in mass at 103°C.

2.2.3 Sample extraction

The sample extract required to determine theaflavin content, total free amino acid content, caffeine content and to perform HunterLab colour measurements was prepared as follows: A flask containing double distilled deionised water (dddH_2O), used for sample extraction, was equilibrated for 20 minutes in a pre-warmed waterbath at 90 ±1 °C. Glass tubes (10 ml) containing 0.1 ±0.0001 g ground tea sample were placed into the same waterbath and 5 ml equilibrated dddH_2O added to each tube. Each tube was sealed with a rubber stopper. The samples were vortexed for 2 seconds at time zero, 5 minutes and at the end of the extraction time (10 minutes). The samples were then centrifuged at 3500 rpm (2000 g) for 10 minutes using a BHG Hermle Z320 centrifuge to pellet the insoluble material where after the supernatant (1st fraction) was decanted into a 10 ml volumetric flask. A 2nd extract fraction was obtained by following the extraction procedure as above on the pelleted sample material to which 5.5 ml equilibrated dddH_2O was added. The two fractions where pooled together and made up to 10 ml using dddH_2O to give 10 ml of a 1% ($^w/_v$) tea sample extract. The tea samples, prepared as described in section 2.2.2, were extracted and analyzed at random.

2.2.4 Theaflavin content (Flavognost method)

The downscaled method used to determine theaflavin content was based on the improved Flavognost method presented by Robertson and Hall, 1989. Theaflavins were extracted from 600 µl 1% ($^w/_v$) sample extract with an equal volume of isobutylmethylketone (IBMK) by adding these together, followed by 4 vortex pulses lasting for 30 seconds each

The IBMK and H_2O layers were allowed to separate where after 100 µl of the top IBMK layer was transferred to a new Eppendorph tube in duplicate. Then, 200 µl Flavognost reagent (1% ($^w/_v$) diphenylboric acid-2-aminoethyl ester / EtOH) was added, the tubes vortexed for 5 seconds and incubated at 37 ±1 °C for 12 ±1 minutes. Absorbance was then read at 625 nm in a Shimadzu UV/visible spectrophotometer using IBMK/EtOH (1:2 $^v/_v$) as blank.

The amount of theaflavins contained in the dried tea leave samples were calculated using the following equation:

Amount of Theaflavin (µmol/g dry tea) = E_{625nm} * 47.9*2.4 / (% dry matter / 100)

Where

E_{625nm} = Absorbance at 625 nm.

47.9 = micro molar absorbance coefficient for theaflavin-Flavognost complex at 625 nm.

2.4 = extraction dilution factor

2.2.5 Total free amino acid content (Ninhydrin method)

The method used to determine total free amino acid content was adopted from Moore and Stein, 1948. A stannous chloride ($SnCl_2$) solution was prepared first by dissolving 0.08 ±0.0001 g $SnCl_2$ in 50 ml 0.1 M Citric acid ($C_6H_8O_7.H_2O$) / Tri-Sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) buffer solution at pH 5. Ninhydrin solution was then prepared by dissolving 2 ±0.01 g ninhydrin in 40 ml methyl cellosolve, followed by addition of the $SnCl_2$ solution, where after the ninhydrin solution was made up to 100 ml with methyl cellosolve. All flasks used to prepare the ninhydrin solution were flushed with $N_{2\,(g)}$ to prevent precipitation of hydrindantin.

In duplicate, 200 µl ninhydrin solution was added to 60 µl of each 1% tea sample extract in a 1.5 ml Eppendorph tube. The same was done with a glycine standard dilution range prepared from a 0.200 mg/ml ($^{w}/_{v}$) glycine stock solution (prepared daily). The concentrations of the glycine standards used were 0.000, 0.033, 0.067, 0.100, 0.133 and 0.167 mg glycine/ml dddH_2O. The reaction mixtures were then vortexed for 10 seconds followed by incubation at 90 ±1°C for 20 minutes in a water bath. After incubation, the tubes were allowed to cool down to room temperature and 1240 µl 20% propanol/dddH_2O diluent solution was added. The tubes were then vortexed for 10 seconds and the absorbance read at 570 nm in a Shimadzu UV/visible spectrophotometer. The results were used to draw up a standard curve for glycine concentration against absorbance at 570 nm. Although theanine accounts for about 50% of free amino acids in tea leaves, pure theanine is not readily available. Hence theanine was only used to construct a standard curve (same as for glycine) initially to determine a correlation factor to glycine. Colour intensity of 1.0000g glycine =

colour intensity of 2.480g theanine. Total free amino acid content could subsequently be determined for the individual tea samples.

To keep hydrindantin in solution, ninhydrin is dissolved in methyl cellosolve, an organic solvent (Moore and Stein, 1948). Proline and hydroxyproline is excluded from the quantification as these amino acids form a yellowish red product, which absorbs at 440 nm and cannot be measured in the presence of other amino acids. Propanol/H_2O is used instead of pure water as diluent to keep the Rahmann's purple in suspension. Conversely, a 1:1 propanol/H_2O ratio is too viscous to perform accurate measurements on a micro scale, thus 20% propanol was used instead.

The total amount of free amino acids contained in the dried tea leaf samples was calculated using the following equation:

% Total Free Amino Acids ($^{w}/_{w}$) = E x V_0 x 25 x 100 / 1000 / (W-(W x MC/100)) x 2.480

Where

E	= mg amino acids obtained from standard curve.
V_0	= total volume of tea solution (10 ml)
x 25	= dilution factor
/ 1000	= mg to g conversion
W	= g dry weight of tea sample used
MC	= % moisture content
2.480	= glycine-theanine correction factor

The correction factor calculated by molar ratio can be determined as follows:

MW_{Gly}	= 75.07 g/mol;
$MW_{Theanine}$	= 174.20 g/mol
Molar ratio	= (2.480/174.20) / (1/75.07)
	= 1.07 (colour intensity is 94% similar on molar basis)

2.2.6 Caffeine content (Lead acetate method)

The method to determine the caffeine content of the dried tea leaf samples was adopted from Yao *et al*, 2006 and Pan *et al*, 2003. Briefly, 500 µl of the 1% ($^{w}/_{v}$) sample extracts prepared from the dried samples were first diluted 50:50 with

dddH_2O. Then, 100 µl of the now 0.5% sample extract was transferred to a 1.5 ml Eppendorph tube in duplicate. Also, 50 µl 0.032% HCl solution (analytical grade) and 10 µl 0.5 g/ml lead acetate ($(CH_3COO)_2Pb$) / dddH_2O solution was added. This reaction mixture was then vortexed for 5 seconds and allowed to stand for 10 minutes. At this stage, a caffeine dilution range was included and treated the same as the sample extracts. The caffeine dilution range consisted of 0, 100, 200, 300, 400 and 500 µl of 0.05 mg/ml caffeine solutions, each containing 40 µl of 0.032% hydrochloric acid, made up to a final volume of 1 ml with dddH_2O. Thereafter 840 µl dddH_2O was added to each tube and vortexed for 10 seconds. The tubes were then centrifuged in an Eppendorph 5414S centrifuge for 30 seconds at 10000 g and 500 µl of the supernatant was transferred to a new 1.5 ml Eppendorph tube. 10 µl of 16.3% sulphuric acid was added, the reaction mixture vortexed for 5 seconds and allowed to stand for 5 minutes. The reaction mixtures were made up to 1 ml by addition of 490 µl dddH_2O. After vortexing for 10 seconds and centrifuging for 30 seconds at 10000 g, the supernatants' absorbance was measured at 274 nm using a Shimadzu UV/visible spectrophotometer. The blank used in the spectrophotometer consisted of 970 µl dddH_2O, 20 µl 0.032% HCl and 10 µl 16.3% H_2SO_4.

The amount of caffeine contained in the dried tea leaf samples was calculated using the following equation:

$$\% \text{ Caffeine } (^{w}/_{w}) = (E/1000) \times V_0 \times (100/V_1) \times (1/0.5)/(W - (W \times MC/100))$$

Where

E	= mg caffeine obtained from standard curve
/1000	= conversion from mg to g
V_0	= total volume of tea solution
V_1	= volume of tea used for measurement
100/	= dilution of tea sample solution
1/0.5	= dilution factor (500 µl tea solution diluted to 1 ml)
W	= g dry weight of tea sample used
MC	= % moisture content

2.2.7 Colour measurement (Hunter Lab MiniScan L*a*b* values)

A 1% ($^{w}/_{v}$) extract was made from every sample as described earlier. Of this extract, 8 ml was transferred to a glass sample cup with a radius of 30mm and a height of 25mm. The white calibration tile was used as background. Four colour readings were made for each sample extract, using a Hunter Lab MiniScan light meter followed by statistical analysis. This was done in duplicate. Double distilled deionised water was used as blank. The colour measurements were expressed as L*a*b* values where L* indicates lightness (0 = black, 100 = white), a* indicates greenness when < 0, redness when > 0, grey when = 0 and b* indicates blueness when < 0, yellowness when > 0 and grey when = 0.

Absorbance readings were also acquired spectrophotometrically from 400-700 nm (data not shown). As mentioned by Huang *et al*, 2007, the higher the reading at 420 nm, the better the value of the green tea.

2.2.8 Total polyphenol content (Folin-Ciocalteu method)

The Folin-Ciocalteu method was applied to determine the total polyphenol content for each tea sample. Sample extracts were prepared slightly different from the previously mentioned method (*see* section 2.2.3). A flask, containing 70% ($^{v}/_{v}$) methanol extraction solvent, was equilibrated for 20 minutes in a pre-warmed water bath at 70 ±1 °C. Glass tubes (10 ml) containing 0.2 ±0.0001 g ground (< 355 µm particle size) tea sample were then placed into the same water bath and 5 ml equilibrated 70% ($^{v}/_{v}$) methanol was added to every tube. The samples were vortexed for 2 seconds at time zero, after 5 minutes and at the end of the extraction time (10 minutes). The samples were then centrifuged at 2000 g for 10 minutes using a BHG Hermle Z320 centrifuge to pellet the insoluble material where after the supernatant (1st fraction) was decanted into a 10 ml volumetric flask. A 2nd extract fraction was obtained by following the extraction procedure as above on the pelleted sample material to which 6 ml equilibrated 70% ($^{v}/_{v}$) methanol was added. The two fractions were pooled together and made up to 10 ml using dddH_2O to give 10 ml of a 2% ($^{w}/_{v}$) tea sample extract.

Then, 1.0 ml sample extract was transferred into a 100 ml volumetric flask and made up to the mark with dddH_2O. After dilution of the sample extract, 1.0 ml was pipetted

into a 10 ml tube in duplicate. The same was done using dddH_2O (blank) and a gallic acid dilution range (0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 mg/ml) prepared from a 0.1% ($^w/_v$) gallic acid stock solution. This was followed by addition of 5.0 ml Folin-Ciocalteu (FC)/dddH_2O (1:10 ($^v/_v$)) reagent and subsequent addition of 4.0 ml 7.5% ($^w/_v$) anhydrous sodium carbonate to each tube. The tubes were vortexed and allowed to stand for 30 minutes where after it was vortexed again and allowed to stand a further 30 minutes for the colour reaction to complete. The tubes were then vortexed again and optical density was measured at 765 nm using a Unico spectrophotometer.

2.2.9 Organoleptic analysis

Organoleptic analysis was performed by a professional tea taster, Mr. D. Wishart at WM Cahn's (Johannesburg, South Africa). Performing organoleptic analysis on large numbers of samples may cause obscured or inaccurate characterization and comparison of the taste observed between different tea samples due to desensitization. For this reason, nineteen samples were selected for tasting. The selection criteria being that each PPO inactivation method was to be represented by at least one sample. Furthermore, the samples used had to include the first time point where PPO has been successfully inhibited by the applied method. Lastly, samples from the same time points and method had to be selected from at least two of the four independent experiments. The green- positive control and black tea negative control sample was also included. Freshpack green tea, a readily commercially available green tea, served as reference sample. For each sample, 1 g was weighed off and transferred to a tea tasters sample cup. After addition of 100 ml boiled water, the tea samples were allowed to draw for 5 minutes, followed by decanting of the tea liquor into a tea sample cup for tasting.

2.2.10 Freeze drying

A 2.5% ($^w/_v$) tea extract was obtained by first infusing 5 ± 0.001g of the samples with 190 ml dddH_2O for 20 minutes in a water bath at 85°C. Tea infusions were then filtered under vacuum followed by a 10 ml H_2O wash step. The filtrates were decanted into pre-weighted round bottom flasks. The filtrates were snap freezed in a Fryka-Kältetechnik KB 300 methanol/water bath with a Thermomix 1441 thermostat set at minus 15°C. The frozen filtrates were then freeze dried until dry (2 days) using

a Virtis Bench top 2K freeze drier (United Scientific, Gauteng, S.A.), fitted with an Edwards RV5 (A653-01-903) vacuum pump.

2.2.11 HPLC Analysis

Instrumentation:

Reverse phased chromatographic separation was performed on a Waters 600E HPLC system using a Phenomenex, 250 x 4.6 mm i.d. Luna 5 µm, Phenyl-Hexyl column with a Phenomenex Phenyl Guard Column, 4.0 x 3.0 mm. The HPLC system was fitted with a Waters 996 photodiode array UV/VIS detector and a Waters 712 WISP auto-sampler. Data analysis of results was done using Waters Millennium[32] Chromatography Manager Software. A sonication bath (7.2 L Ultrasonic Cleaner, 40 KHz operating frequency, Optima Scientific, South Africa) was used to degas the mobile phases under vacuum, prior to using it on the HPLC system.

Chromatographic conditions:

Based on the International Standards Organization HPLC method (ISO 14502-2), mobile phase A consisted of dddH_2O/acetonitrile/acetic acid (97:1:2 v/v/v) and mobile phase B consisted of dddH_2O/acetonitrile/acetic acid (18:80:2 v/v/v). The following gradient was used: 0-8 minutes, 0% B; 8-60 minutes, 0-17% B; 60-70 minutes, 17% B; 70-130 minutes, 17-45% B; 130-135 minutes, 45-100% B; 135-138 minutes, 100% B; 138-142 minutes, 100-0% B; 142-150 minutes, 0% B. HPLC was performed at 30 °C with a flow rate of 1 ml/min and both mobile phases were sparged with helium gas at 20 ml/min. Components of interest were monitored at 270 nm, although UV/VIS spectral data was collected from 210-400 nm to verify compounds by means of their individual UV spectra.

2.3 Results

Green tea leaf sample manufacture

The PPO inactivation experiments were performed in quadruplicate (run 1-4) using fresh tea leaves harvested on 4 different days about 10 days apart during the mid summer. Both a positive (freeze dried green tea) and negative (bench dried black tea) control sample were prepared during each run. Freeze drying was selected to be suitable for creating a positive control for which biochemical changes were to be minimized. The positive control had a very distinguishable bright green colour after freeze drying. In contrast, the negative control was produced by incubating tea leaves at room temperature for 4 days, allowing natural biochemical reactions to occur. The negative control turned dark brown with a typical black tea aroma.

The observations as listed in table 2.1 were similar for run 1-4 with the following exceptions: For run 2 & 4, the tea leaves were not fully dry after fluid bed drying for 30 minutes. In run 3, after pan-frying for 1 minute some leaves were burned, after pan-frying for 3 minutes many leaves were burned and most of the leaves were burned after pan-frying for 4 minutes. In general, pan-fried and grilled (direct & indirect) tea leaves were more burned during run 2, 3 and 4 than in run 1. Fluid bed drying times varied slightly between the 4 independent experiments.

Significant differences were detected with regard to colour, aroma and leave texture following the different PPO inactivation methods. When steaming or blanching for extended periods, loss of chlorophyll seems apparent judged by change in colour from green to yellow. Both these methods also cause the leaves to soften. The initial fresh and leafy aroma is lost after prolonged blanching, which may be due to loss of volatile components in the water used to perform blanching or other components which may be lost due to loss of cell integrity. This effect is significantly less when leaves are steamed, hence the fresh leafy aroma was retained. Browning still occurred in tea leaves steamed for less than 90 seconds, making it a more time expensive method to inactivate PPO compared to blanching. For blanching, 30 seconds were sufficient to prevent browning. A drawback of the 2 ‘wet’ inactivation methods, namely steaming or blanching, is that leaves may take up to 42% longer to dry compared to the ‘dry’

PPO inactivation methods. Fluid bed drying of tea leaves produced dry dark green leaves with a pleasant aroma.

Table 2.1 Observations made for each PPO inactivation method used

PPO inactivation method	Treatment time (min)	Tea leaf property (observations after treatment before fluid bed drying)				FBD time (min)			*
		Colour	Aroma	Tea leave texture	Other	Run 1	Run 2	Run 3	Run 4
Steaming	0.5	bright green	fresh, leafy	firm	turns brown after 10 min	55	55	55	60
	1	green	fresh, leafy	firm	some browning after 10 min	56	55	55	60
	1.5	greenish	fresh, leafy	softening		58	55	55	60
	2	green-yellow	fresh, leafy	soft		58	55	65	60
	3	yellowish	fresh, leafy	soft		57	55	55	60
	4	very yellowish	fresh, leafy	soft		55	55	55	60
Blanching	0.5	bright green	fresh, leafy	soft		55-60	55	56	60
	1	green-yellow	leafy	soft		55-60	55	60	60
	1.5	yellow-green	leafy	soft		55-60	55	55	60
	2	yellowish	vague leafy	soft		55-60	55	57	60
	3	yellowish	vague leafy	soft		55-60	55	55	60
	4	very yellowish	vague leafy	soft		55-60	55	57	60
FBD	4	green	leafy	partially crisp leaves	stems wet	50	50	50	50
	8	green	leafy	most leaves crisp		45	45	50	50
	12	darker green	leafy	all leaves crisp	stems moderately wet	35	35	30	40
	17	dark green	rich	leaves crisp	stems partially crisp	30	30	25	35
	20	dark green	heavy, rich	leaves crisp	most stems dry	15	15	25	20
	30	dark green	pleasant aroma	stems + leaves crisp	leaves not fully dry for run 2 & 4	none	10	none	10
Pan-frying	0.5	green	leafy	soft		50	55	55	55
	1	green	slight roasted	soft		50	55	55	55
	1.5	green	roasted	soft, starting to dry		50	55	55	55
	2	green	slight burned roasted	leaves partially dried	stems wet	50	55	50	50
	3	green, some burned	burned roasted	hard	leaves partially dried, stems wet	45	55	45	45
	4	green, many burned	burned roasted	crisp, dry leaves	stems still wet	50	55	40	45
Grill (Indirect)	1	bright green	leafy	soft	browning occurs	50	52	50	55
	2	bright green	leafy	some leaves dry	some browning	50	48	50	55
	3	bright green	leafy	some crispy leaves		50	42	48	50
	4	green	leafy	leaves partially dried		45	37	47	50
	5	green	mild grilled, leafy	leaves partially dried		45	35	45	40
	6	yellow-green	mild grilled	leaves partially dried	some burned leaves	48	35	40	40
Grilled (direct)	1	bright green	fresh, leafy	soft	browning occurs	50	50	50	50
	2	bright green	fresh, leafy	soft	some browning	50	50	50	50
	3	bright green, some burned	mild roast, leafy	some crispy leaves		45	45	50	40
	4	green	roasted	partially crispy leaves		45	38	45	40
	5	green, some burned	strong roast	partially crispy leaves	partially burned leaves	45	38	40	35
	6	brown, green, burned	burned roasted	most leaves crisp		45	38	40	35
Positive control (Freeze dried)	2 days	bright light green	slight leafy	leaves + stems crisp		0	0	0	0
Negative control (Bench dried)	4 days	dark brown	typical black tea	leaves + stems crisp		0	0	0	0

Theaflavin content

Quantification of theaflavins using the Flavognost method, indicated an increase from 2.11 ±0.41 µmol/g dry tea (data not shown), for the freeze dried tea leaf positive control, to 15.69 ±2.77 µmol/g dry tea for the bench dried negative tea leaf control (fig. 2.3.1). Conversely, as theaflavins consists of condensed flavan-3-ols, a subsequent decrease in flavan-3-ols was observed that corresponds with the increase in theaflavins (fig. 2.3.4). The bench dried negative control that has undergone prolonged fermentation, represents 'the worst quality green tea' due to long-lasting exposure to active PPO enzymes. As expected a very high theaflavin content was measured for this control since it is in effect a black tea sample. This value also correlates very well with the literature value of ±16 µmol/g dry weight for black tea (Robertson and Hall, 1989). Also included, was a Lipton Yellow label black tea external control, which have a theaflavin content of 20.86 ±0.56 µmol/g dry tea (data not shown). The reproducibility of the results obtained for the external control assured consistent precision throughout the course of applying the Flavognost method and between the 4 independent experiments.

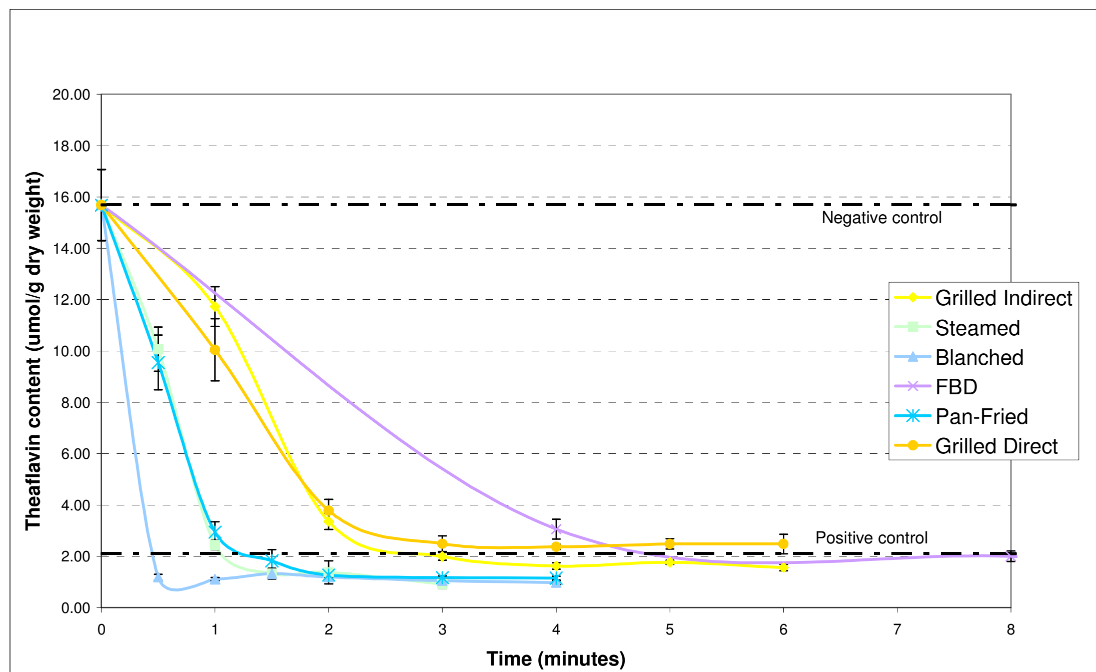

Figure 2.9: Graph to indicate the change in theaflavin content (µmol/g dry weight) in tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied. As treatment time progresses, PPO inactivation is indicated with the decrease in theaflavin content. Symbols represent the mean of the 4 independent experiments. Error bars indicate standard error of the mean. The negative control sample was used for time zero, which has the highest value.

The results obtained from theaflavin analysis indicate that as little as 30 seconds of treatment (blanching) time was required to accomplish a 100% PPO inactivation (fig. 2.9). From figure 2.9 it can be seen that, in general, applying dry heat increases the time required for PPO to be fully inactivated as opposed to using hot water or steam. Thus, PPO initially remains active, but activity is lost by heat denaturation as treatment time progresses rendering the enzyme unable to catalyze any further formation of theaflavins.

Caffeine content

Quantification of caffeine content to determine quality, led to the discovery that blanching significantly reduces the amount of caffeine. Compared to the positive control, a 54% decrease from 2.55 ±0.11 to 1.17 ±0.02% ($^{w}/_{w}$) occurred when

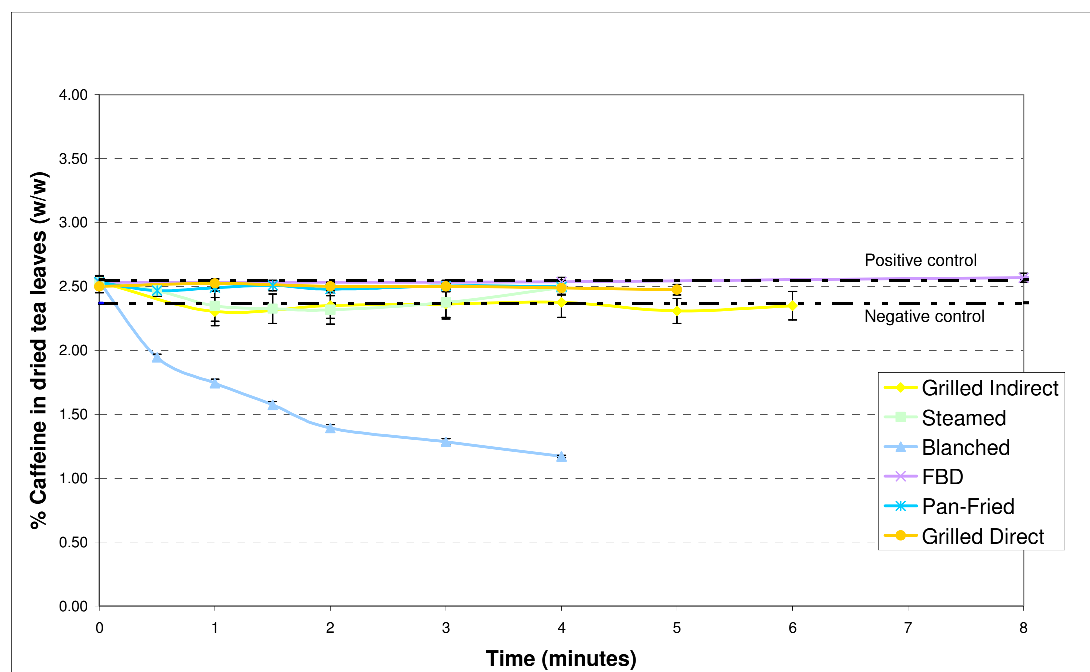

Figure 2.10: Graph to indicate the change in caffeine content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods. PPO inactivation by means of blanching causes a parabolic decrease in caffeine content with an increase in treatment time. All other PPO inactivation methods were more or less equal to the positive control and thus had no statistically significant change in caffeine content. Symbols represent the mean of the 4 independent experiments, analysed in duplicate. The positive control was used as zero time point. Error bars indicate standard error of the mean.

blanching for 4 minutes. This is in accordance with results published by Liang *et al*, 2007. The caffeine content of the 5 remaining methods did not deliver any significant differences and varied between 2.30 and 2.58% ($^{w}/_{w}$). Corresponding with previous reports (Graham *et al*, 1992, and others), a slight decrease of 7% in caffeine content is

observed between the 'green' positive control and the 'black' negative control, having an abundance of 2.55 ±0.11 and 2.37 ±0.13% ($^w/_w$) respectively. Baring in mind the role of caffeine for the purpose of this project, blanching stands out considerably from the other PPO inactivation methods due to this drastic decrease in caffeine. Should flavan-3-ol content also have been reduced to a similar extend, blanching would have given rise to a low quality green tea. The external control (Lipton Yellow Label black tea), included to assure consistency, had an average caffeine content of 1.67 ±0.01% ($^w/_w$).

Total free amino acid content

The obvious choice of amino acid standard to be used for total free amino acid analysis is theanine, as it is the most abundant free amino acid in tea. Since theanine is not readily available, glycine was used instead. Not all amino acids give the same intensity signal on a per mole basis when performing the ninhydrin method (Moore and Stein, 1948). Thus, the colour intensity difference between theanine and glycine had to be determined first, before substituting theanine with glycine as standard. The glycine-theanine correction factor was determined by comparing the difference in slope for their individual standard curves, constructed using 5 different concentrations. The R^2-value for each standard curve, used to determine the correction factor, was ≥0.999. Data from 2 independent experiments were used to calculate the average correction factor of 2.480 ±0.003. This means that the colour intensity obtained for 1.000g of glycine represents or equals 2.480g theanine. Stannous chloride is included in the reaction, which serves as a reducing agent causing reduction of ninhydrin to water insoluble hydrindantin.

The percentage of total free amino acids quantified, was between 1.5 and 2.0% ($^w/_w$) for all treatments (fig. 2.3.3). A value of 1.81 ±0.36% and 2.26 ±0.62% ($^w/_w$) was obtained for the positive and negative control, respectively. The positive control typically has a lower free amino acid content (20% less) than the bench dried negative control.

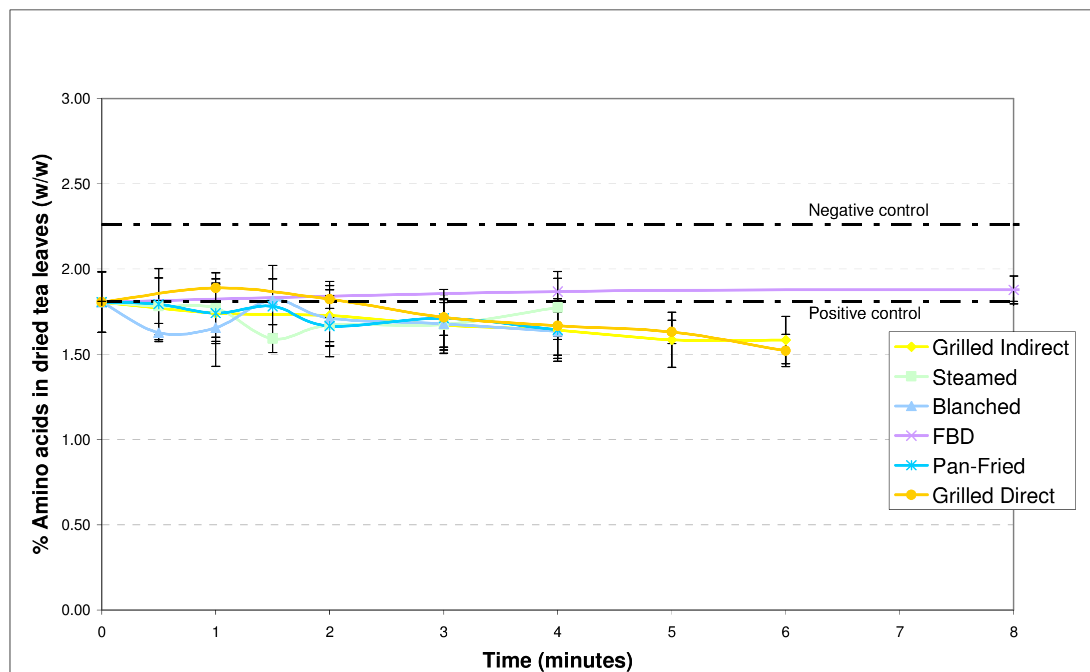

Figure 2.11: Graph to indicate the change in total free amino acid content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied. Total free amino acid content fluctuated between 1.50 and 2.00% ($^{w}/_{w}$) with the dry treatments following a slight decreasing trend over increased treatment time. The positive control was used as zero time point. Symbols represent the mean of the 4 independent experiments. Error bars indicate standard error of the mean.

In the negative control, proteolytic breakdown of proteins could occur, causing the free amino acid content to rise. Pan-frying, grilling direct and grilling indirectly indicate a slight decrease in amino acid content as treatment time increases. This decrease may be due to deterioration caused by burning of the leaves according to observations made for the different treatment times. Also, the Maillard reaction, a non-enzymatic browning reaction, may occur during which the nucleophilic amino groups from amino acids bind covalently to carbonyl groups from sugars. Applying the fluid bed drying method gave constant results over the range of time points.

Table 2.2 Data summary of biochemical quality analysis for green tea samples using 6 different PPO inactivation methods. (Average of 4 independent experiments performed in duplicate) Letters indicate Duncan multiple level test as determined by General Linear Model (GLM) analysis. The same small letters indicate no statistical significant difference between time points within a PPO inactivation method, while the same capital letter indicates no significant difference between the best time points for different treatments for a specific parameter.

Treatment	Timepoint (minutes)	Biochemical green tea quality parameter															
		Theaflavin content				Caffeine content				Total free amino acid content				Flavan-3-ol content			
		[Theaflavin] (umol/g dw)	Standard deviation	Coeff. Of Variance	Std error of mean	Caffeine (%)	Standard deviation	Coeff. Of Variance	Std error of mean	AA (%)	Standard deviation	Coeff. Of Variance	Std error of mean	Flavanol (%)	Standard deviation	Coeff. Of Variance	Std error of mean
Steamed	0.5	10.07 a	1.73	17.18	0.87	2.47 a AB	0.09	3.61	0.04	1.78 a A	0.33	18.67	0.17	20.72 a	0.66	3.16	0.33
	1	2.44 b	0.36	14.66	0.18	2.35 a	0.24	10.27	0.12	1.77 a	0.34	19.36	0.17	22.56 b A	0.98	4.33	0.49
	1.5	1.33 bc B	0.43	32.00	0.21	2.33 a	0.23	9.92	0.12	1.59 a	0.16	10.19	0.08	23.14 b	1.00	4.30	0.50
	2	1.38 bc	0.89	64.92	0.45	2.32 a	0.22	9.60	0.11	1.67 a	0.19	11.63	0.10	23.15 b	0.86	3.72	0.43
	3	0.92 c	0.34	36.85	0.17	2.37 a	0.23	9.81	0.12	1.68 a	0.30	18.05	0.15	23.01 b	0.83	3.60	0.41
	4	1.13 bc	0.37	32.74	0.18	2.49 a	0.11	4.59	0.06	1.77 a	0.34	19.33	0.17	23.11 b	1.15	4.97	0.57
Blanched	0.5	1.19 a B	0.23	19.07	0.11	1.94 a	0.05	2.73	0.03	1.63 a A	0.11	6.48	0.05	23.25 a A	1.41	6.07	0.71
	1	1.11 a	0.12	11.10	0.06	1.74 b	0.06	3.48	0.03	1.66 a	0.45	27.43	0.23	23.33 a	1.02	4.39	0.51
	1.5	1.32 a	0.14	10.70	0.07	1.57 c	0.06	3.56	0.03	1.82 a	0.40	22.24	0.20	23.05 a	1.35	5.85	0.67
	2	1.20 a	0.19	16.20	0.10	1.39 d	0.06	3.96	0.03	1.71 a	0.33	19.44	0.17	22.83 a	1.28	5.62	0.64
	3	1.06 a	0.31	29.27	0.15	1.28 e	0.05	3.92	0.03	1.68 a	0.34	20.43	0.17	22.05 a	1.27	5.76	0.64
	4	0.98 a	0.15	14.97	0.07	1.17 f C	0.02	1.40	0.01	1.63 a	0.32	19.53	0.16	21.75 a	1.12	5.17	0.56
FBD	4	3.06 a	0.77	25.23	0.39	2.54 a A	0.07	2.83	0.04	1.87 a A	0.24	12.69	0.12	22.73 a A	1.27	5.60	0.64
	8	2.00 b B	0.41	20.37	0.20	2.57 a	0.07	2.80	0.04	1.88 a	0.16	8.68	0.08	23.33 a	1.10	4.70	0.55
	12	1.92 b	0.47	24.27	0.23	2.58 a	0.07	2.70	0.03	1.87 a	0.23	12.47	0.12	23.08 a	1.08	4.68	0.54
	17	1.94 b	0.45	22.96	0.22	2.55 a	0.11	4.18	0.05	1.89 a	0.26	13.84	0.13	23.14 a	1.12	4.85	0.56
	20	2.04 b	0.16	8.03	0.08	2.53 a	0.09	3.74	0.05	1.88 a	0.31	16.27	0.15	23.33 a	0.89	3.80	0.44
	30	2.05 b	0.37	18.08	0.19	2.56 a	0.10	3.79	0.05	1.80 a	0.20	11.00	0.10	23.27 a	1.27	5.47	0.64
Pan-Fried	0.5	9.55 a	2.13	22.34	1.07	2.47 a AB	0.09	3.49	0.04	1.79 a A	0.42	23.23	0.21	20.28 a	0.87	4.28	0.43
	1	2.94 b	0.82	28.01	0.41	2.49 a	0.06	2.45	0.03	1.74 a	0.35	20.39	0.18	21.91 b A	0.87	3.95	0.43
	1.5	1.83 bc B	0.86	46.88	0.43	2.51 a	0.08	3.37	0.04	1.78 a	0.32	18.00	0.16	22.31 b	0.51	2.30	0.26
	2	1.28 bc	0.17	13.12	0.08	2.48 a	0.10	4.13	0.05	1.66 a	0.36	21.51	0.18	22.47 b	0.51	2.26	0.25
	3	1.18 c	0.14	12.09	0.07	2.50 a	0.09	3.64	0.05	1.71 a	0.34	19.82	0.17	22.28 b	0.51	2.31	0.26
	4	1.15 c	0.15	13.33	0.08	2.50 a	0.10	3.92	0.05	1.64 a	0.37	22.25	0.18	22.37 b	0.25	1.10	0.12
Grilled Direct	1	10.05 a	2.41	24.01	1.21	2.50 a AB	0.07	2.85	0.04	1.89 a A	0.18	9.47	0.09	20.15 a	0.31	1.52	0.15
	2	3.78 b	0.89	23.45	0.44	2.52 a	0.07	2.64	0.03	1.82 a	0.21	11.48	0.10	22.38 b A	0.72	3.23	0.36
	3	2.49 c B	0.60	24.06	0.30	2.50 a	0.04	1.77	0.02	1.72 a	0.21	12.13	0.10	22.82 b	0.34	1.50	0.17
	4	2.37 c	0.22	9.10	0.11	2.50 a	0.04	1.61	0.02	1.67 a	0.16	9.52	0.08	22.97 b	0.61	2.66	0.31
	5	2.49 c	0.40	16.19	0.20	2.49 a	0.08	3.35	0.04	1.63 a	0.14	8.47	0.07	22.86 b	0.66	2.87	0.33
	6	2.48 c	0.76	30.60	1.46	2.47 a	0.04	1.69	0.02	1.52 a	0.19	12.41	0.09	22.40 b	0.49	2.18	0.24
Grilled Indirect	1	11.73 a	1.54	13.11	0.77	2.30 a B	0.22	9.60	0.11	1.74 a A	0.33	19.15	0.17	21.27 a	0.25	1.19	0.13
	2	3.36 b A	0.64	19.01	0.32	2.35 a	0.20	8.58	0.10	1.73 a	0.35	20.25	0.17	22.67 b A	0.54	2.36	0.27
	3	2.00 b	0.30	15.04	0.15	2.36 a	0.23	9.86	0.12	1.67 a	0.26	15.68	0.13	23.20 b	1.00	4.32	0.50
	4	1.63 b	0.24	14.98	0.12	2.38 a	0.24	9.97	0.12	1.64 a	0.29	17.80	0.15	23.02 b	0.64	2.78	0.32
	5	1.77 b	0.16	9.24	0.08	2.31 a	0.19	8.42	0.10	1.59 a	0.32	20.42	0.16	22.85 b	0.89	3.88	0.44
	6	1.56 b	0.24	15.59	0.12	2.35 a	0.22	9.56	0.11	1.58 a	0.28	17.57	0.14	22.73 b	0.61	2.70	0.31
+ control (green)		2.11	0.41	19.63	0.21	2.55	0.11	4.14	0.05	1.81	0.36	19.68	0.18	23.61	0.70	2.95	0.35
- control (black)		15.69	2.77	17.65	1.38	2.37	0.13	5.59	0.07	2.26	0.62	27.44	0.31	15.16	1.12	7.38	0.56

Flavan-3-ol content

In contrast to the theaflavin content, flavan-3-ol content is lower for the first time points. Flavan-3-ol content increases until the PPO enzymes are fully inactivated, preventing any flavan-3-ol loss due to condensation to form theaflavins.

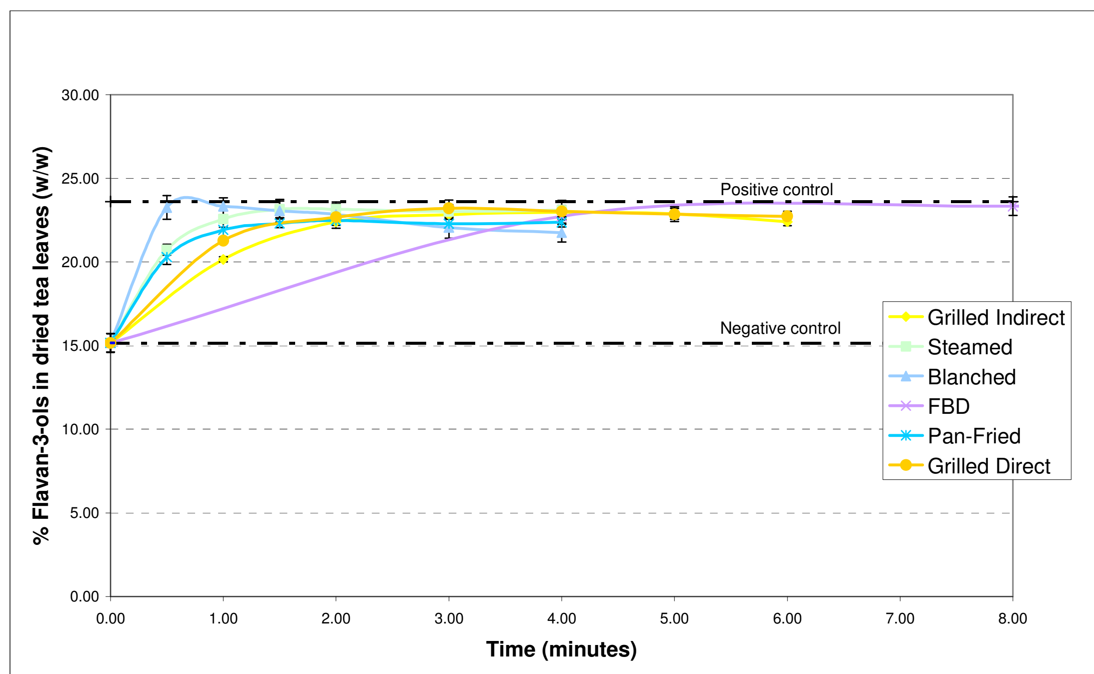

Figure 2.12: Graph to indicate the change in flavan-3-ol content (% $^{w}/_{w}$) in dried tea leaves with an increase in PPO inactivation time for the six different inactivation methods applied. Flavan-3-ols increase and reach a plateau once PPO is fully inactivated. The negative control was used as zero time point. Symbols represent the mean of the 4 independent experiments. Error bars indicate standard error of the mean.

A plateau is then reached which, after prolonged treatment, is followed by a decrease again. This loss is either due to degradation caused by excessive heat exposure or by leaching from the plant cells. The latter is expected for the wet treatments, while the former is expected to occur when applying the dry treatments.

At the zero time point, represented by the negative control, a low flavan-3-ol content of 15.16 ±1.12% ($^{w}/_{w}$) was measured. The positive control gave a flavan-3-ol content measurement of 23.61 ±0.70% ($^{w}/_{w}$) (table 2.2). The maximum flavan-3-ol content for all treatments were 23% ($^{w}/_{w}$) except for pan-frying, which gave a maximum of 22% ($^{w}/_{w}$), although this difference in not statistically significant. The highest decrease in flavan-3-ols after reaching a plateau, was identified for blanching, which reduced from 23.33 ±1.02% to 21.75 ±1.12% ($^{w}/_{w}$) after 4 minutes. When taking the

standard deviation into consideration, this decrease is not significant, although the difference may be decreased following process optimization.

Colour measurement

L* a* b* colour results obtained (*see* table 2.3), correlates with work done by Liang *et al*, 2005. The L* value for the negative control is much lower at 24.67 ±2.59 than the green tea leaf test samples. This is to be expected since the L* value depicts the lightness of the infusions. The Lipton Yellow Label black tea infusion has a slightly higher *L value of 35.53 ±0.88. The L* values varied between 42 and 57 for the green tea leaf infusions. Comparing these results to the theaflavin content results indicates how an increase in theaflavins causes a decrease in lightness of an infusion. The rapid inactivation using the blanching method is noticed again by its invariable colour results. Judging by the change in L* values during increased processing time, PPO is inactivated after 30 seconds when blanching, 1.5 minutes when steaming, 1.5-2 minutes when pan-frying, 3 minutes when grilling over direct or indirect heat and 8 minutes when fluid bed drying. Infusions from blanched and steamed green tea leaf samples are slightly lighter than the positive control, which may be due to a loss in chlorophyll and/or theaflavins present in the starting material.

Opposite to the L* values, a* values decreased with increased treatment time. Positive a* values indicate redness, while greenness is depicted by negative a* values. As PPO inactivation progresses there are less and less theaflavins forming, hence less reddish thearubigens form resulting in lower a* values. Grilling and pan-frying infusion a* values start to increase again at the longer PPO inactivation times. This may be solely because the green colour is decreased by the burning of the leaves. Hence, this observation is not made for the wet treatments.

A profile similar to that of the a* values was obtained for the b* values. One difference however, is the lower b* value for the negative control compared to the test samples. During the production of black tea, fermentation is usually ended by the drying step, which involves high temperatures that will inactivate PPO and POD enzymes. A quality black tea contains a high amount of theaflavins, but a relatively low amount of thearubigens. Fermentation should thus be ended before significant

polymerization of theaflavins take place. Such a tea sample will therefore be more yellowish than reddish. This practice is also to be expected for the production of the external black tea control.

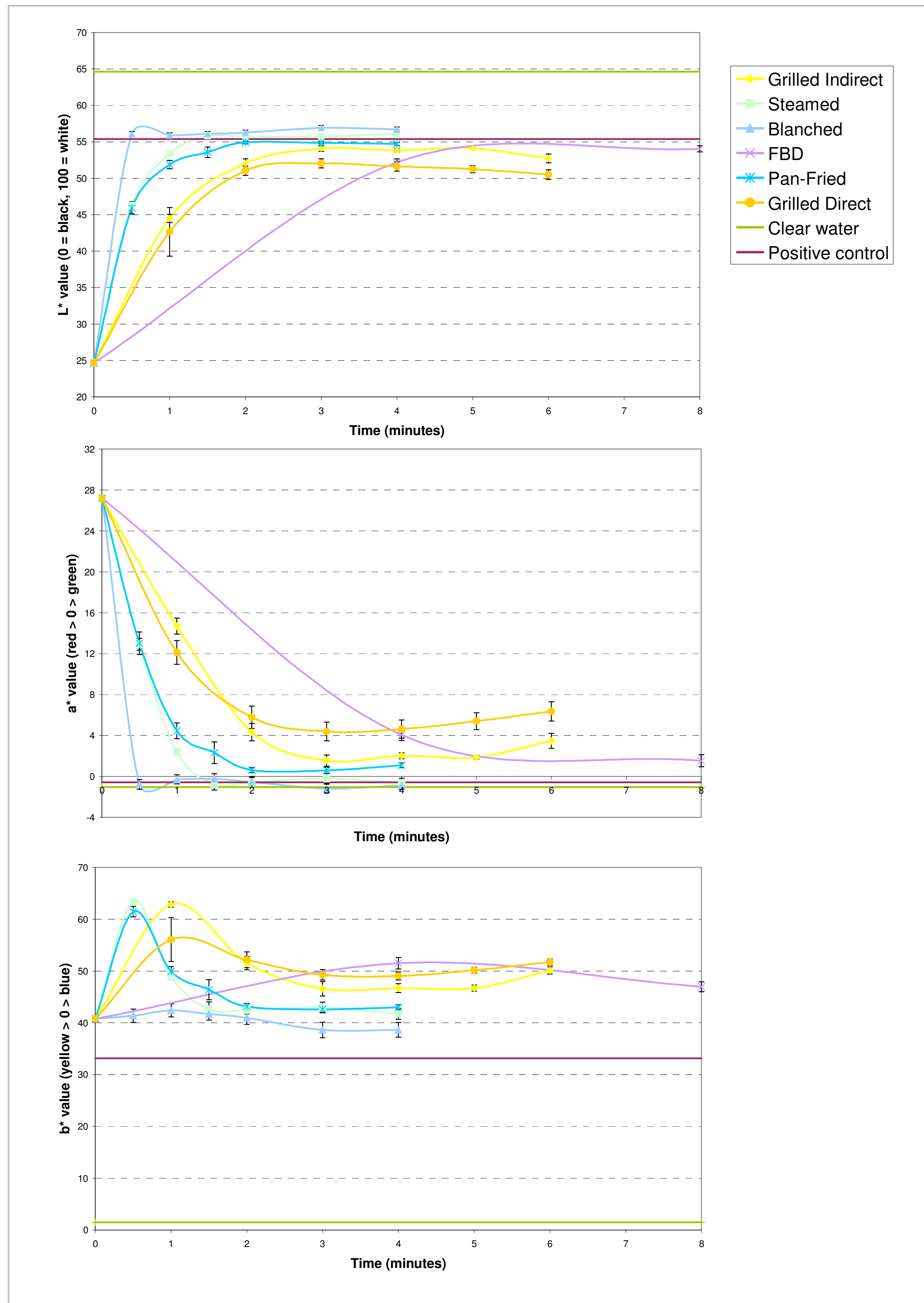

Figure 2.13: Graphs to indicate L*a*b* values for green tea leaf infusions. Negative control (bench dried black tea) was used as zero time point.

The internal negative control however, was bench dried over four days, meaning that extensive theaflavin polymerization could occur that will give rise to a darker, more reddish infusion due to the thearubigens forming. Hence, the negative control's infusion has a very high a* value of 27.19 ±0.57, but a relatively low b* value of 40.76 ±4.11 when compared to test samples. The external control has a slightly lower a* value of 26.02 ±0.75 and, as would be expected under the manufacture conditions mentioned above, the b* value is significantly higher than the negative control at 56.32 ±3.20.

Obtaining L* a* b* values from tea infusions may therefore serve as a rapid test for quality for both green and black tea when compared to a reference sample. With reference to figure 2.3.1, L* a* b* values corresponded well with theaflavin content. The higher the theaflavin content the lower the L* value and the higher the a* and b* value. Infusions from blanched green tea leaf infusions compared the best to the positive control for all three colour values (L*, a* & b*). Lipton Yellow Label black tea, used as external control, gave consistent results with a coefficient of variance of less than 6%. This confirmed precision of the analytical method between the 4 independent experiments and also for inter day sample analysis.

Table 2.3 Average HunterLab L* a* b* colour values for the 4 independent experiments, performed in duplicate. Letters indicate Duncan multiple level test as determined by GLM analysis. The same letters indicate no statistical significant difference between time points within a PPO inactivation method.

PPO inactivation method	Time (minutes)	Averages of L* a* b* data obtained for green tea leaf test samples and control samples														
		L*					a*					b*				
		Average		Standard deviation	Coefficient of varience	Error of mean	Average		Standard deviation	Coefficient of varience	Error of mean	Average		Standard deviation	Coefficient of varience	Error of mean
Grilled Indirect	1	44.49	a	1.08	2.44	0.54	14.70	a	1.57	10.65	0.78	62.85	a	1.05	1.66	0.52
Grilled Indirect	2	52.03	b	1.24	2.38	0.62	4.34	b	1.71	39.35	0.85	51.53	b	2.62	5.08	1.31
Grilled Indirect	3	54.08	c	0.78	1.44	0.39	1.55	c	1.11	71.58	0.56	46.58	c	2.79	5.98	1.39
Grilled Indirect	4	53.82	c	0.41	0.76	0.21	2.02	c	0.59	29.00	0.29	46.69	c	1.68	3.61	0.84
Grilled Indirect	5	54.10	c	0.23	0.43	0.12	1.88	c	0.36	18.91	0.18	46.70	c	1.23	2.64	0.62
Grilled Indirect	6	52.73	bc	1.22	2.31	0.61	3.48	bc	1.48	42.47	0.74	50.10	bc	1.38	2.75	0.69
Steamed	0.5	46.34	a	0.70	1.50	0.35	12.92	a	1.16	8.95	0.58	63.46	a	0.74	1.16	0.37
Steamed	1	53.40	b	0.30	0.57	0.15	2.42	b	0.47	19.27	0.23	48.91	b	1.26	2.58	0.63
Steamed	1.5	55.79	c	0.66	1.17	0.33	-0.76	c	1.14	150.25	0.57	42.62	c	2.82	6.61	1.41
Steamed	2	55.74	c	0.48	0.86	0.24	-0.54	c	0.92	168.15	0.46	42.45	c	1.47	3.47	0.74
Steamed	3	55.73	c	0.66	1.19	0.33	-0.20	c	0.99	486.13	0.50	42.99	c	1.93	4.50	0.97
Steamed	4	56.08	c	0.73	1.30	0.36	-0.65	c	0.96	148.20	0.48	41.73	c	2.10	5.03	1.05
Blanched	0.5	56.07	a	0.67	1.19	0.33	-0.78	a	0.95	121.70	0.47	41.37	a	2.56	6.19	1.28
Blanched	1	55.89	a	0.66	1.18	0.33	-0.29	a	0.89	307.39	0.44	42.39	a	2.52	5.95	1.26
Blanched	1.5	56.11	a	0.59	1.04	0.29	-0.24	a	1.03	429.10	0.51	41.69	a	2.30	5.53	1.15
Blanched	2	56.26	a	0.70	1.24	0.35	-0.53	a	1.07	203.45	0.53	40.98	a	2.59	6.33	1.30
Blanched	3	56.90	a	0.67	1.18	0.34	-1.17	a	0.87	74.44	0.44	38.61	a	2.99	7.74	1.49
Blanched	4	56.72	a	0.66	1.16	0.33	-0.86	a	0.84	97.71	0.42	38.62	a	2.84	7.37	1.42
FBD	4	52.18	a	0.95	1.82	0.48	4.07	a	1.13	27.90	0.57	51.51	a	2.22	4.31	1.11
FBD	8	54.05	b	0.84	1.55	0.42	1.54	b	1.16	75.53	0.58	46.96	c	1.91	4.06	0.95
FBD	12	54.00	b	0.72	1.34	0.36	1.74	b	0.88	50.63	0.44	47.52	bc	1.63	3.44	0.82
FBD	17	53.83	b	0.57	1.06	0.29	1.76	b	0.77	43.94	0.39	47.81	bc	1.25	2.62	0.63
FBD	20	54.22	b	0.26	0.48	0.13	1.58	b	0.33	20.85	0.16	48.04	bc	1.02	2.13	0.51
FBD	30	53.34	b	0.29	0.54	0.15	2.73	ab	0.38	13.83	0.19	50.14	ab	0.73	1.46	0.37
Pan-Fried	0.5	45.95	a	1.69	3.67	0.84	13.02	a	2.18	16.72	1.09	61.45	a	2.01	3.28	1.01
Pan-Fried	1	51.88	b	1.15	2.21	0.57	4.46	b	1.53	34.27	0.76	49.93	b	1.89	3.78	0.94
Pan-Fried	1.5	53.57	bc	1.42	2.64	0.71	2.32	bc	2.15	92.51	1.07	46.40	bc	3.85	8.29	1.92
Pan-Fried	2	54.92	c	0.36	0.66	0.18	0.62	c	0.52	82.67	0.26	43.13	c	1.18	2.73	0.59
Pan-Fried	3	54.86	c	0.32	0.58	0.16	0.59	c	0.61	102.92	0.30	42.62	c	1.37	3.22	0.69
Pan-Fried	4	54.71	c	0.42	0.76	0.21	1.10	c	0.43	38.72	0.21	43.00	c	0.94	2.19	0.47
Grilled Direct	1	42.65	a	6.69	15.68	3.34	12.12	a	2.32	19.12	1.16	56.05	a	8.49	15.14	4.24
Grilled Direct	2	51.04	b	1.31	2.56	0.65	5.79	b	2.19	37.83	1.09	52.18	a	3.10	5.93	1.55
Grilled Direct	3	52.04	b	1.23	2.36	0.61	4.40	b	1.84	41.85	0.92	49.27	a	2.03	4.12	1.02
Grilled Direct	4	51.62	b	1.30	2.52	0.65	4.64	b	1.78	38.31	0.89	49.04	a	1.56	3.19	0.78
Grilled Direct	5	51.26	b	0.99	1.93	0.50	5.41	b	1.65	30.54	0.83	50.16	a	1.17	2.33	0.59
Grilled Direct	6	50.50	b	1.32	2.61	0.66	6.37	b	1.88	29.56	0.94	51.75	a	1.22	2.36	0.61
+ control (green)		55.38		0.61	1.11	0.31	-0.56		1.02	182.50	0.51	33.16		1.95	5.88	0.98
- control (black)		24.67		2.59	10.48	1.29	27.19		0.57	2.10	0.29	40.76		4.11	10.08	2.05
External control		35.53		0.88	2.47	0.18	26.02		0.75	2.87	0.16	56.32		3.20	5.68	0.67

Organoleptic analysis

The selection criteria for samples to undergo organoleptic analysis were based on the time required to inactivate most of the PPO, as judged by TF and flavan-3-ol content and colour analysis. According to the tea taster's results, the positive control sample had a brighter colour than the Freshpack green tea reference.

Table 2.4 Tea taster's results score sheet for selected tea samples from batch 25/2 using Freshpack green tea as reference. The reference was assigned a score of 10/20 for both colour and briskness. Any score below 10/20 denotes a negative value with regard to the reference and any score above 10/20 denotes a positive value compared to the reference. Similar results were obtained for batch 17/2 (data not shown).

Sample	Colour	Briskness	Comment
+ control (freeze dried)	12	8	Thin
- control (bench dried)	0	-	Revolting, wet earth
Fluid bed dried 8 min	11	15	Thick as reference, green, bitter
Steamed 1.5 min	12	12	Brighter yellow, light, astringent
Blanched 1 min	12	10	Body as reference, green, astringent
Blanched 4 min	12	8	Lacks character, flat
Grilled Directly 3 min	9	10	Similar to reference in body + aroma
Grilled Indirectly 3 min	10	12	Same as reference in body, astringent
Pan-Fried 1.5 min	9	9	Lack body, slightly astringent

However, the positive control had a thin taste with a lower briskness compared to the reference. This may be expected as the positive control samples did not undergo any heat treatment, which enhances taste by the formation of volatiles. As could be expected, the negative control had a very revolting, muddy taste and performed extremely poorly against the reference. Fluid bed drying alone gives a bright and thick brew, but very bitter tasting. The grilled samples performed very similar to the reference sample. Two time points of the blanched samples were included. Analysis of these two samples indicates the effect of blanching time on taste. Prolonged blanching decreases the strength of the brew and the decrease in caffeine is also evident in the reduced briskness from 10 to 8. Colour valuation by the tea taster corresponded with L^* a^* b^* values where PPO inactivation methods involving wet treatments were lighter or brighter compared to the dry treatments.

Not all leaves had undergone pre-experimental fermentation to the same extent in the starting material of the four independent experiments performed. Reasonable variation was therefore expected within all assays due to the difference in starting material used to prepare each sample. Nonetheless, a general trend was clearly observed during the analysis of each biochemical parameter for each of the six PPO

inactivation methods. With TF and flavan-3-ol content, variation in the values obtained for the initial time points, where PPO is not fully inactivated was also expected as inconsistent and uncontrolled fermentation was still taking place. Also, as the negative control was subjected to uncontrolled natural biochemical changes, the final composition of these controls varied slightly more between individual experiments.

Optimum method identification

By comparing results obtained for the six PPO inactivation methods on the basis of theaflavin-, caffeine-, amino acid- and flavan-3-ol content, as well as colour and taste, blanching was decided upon as being the best method. The catechin to caffeine ratio carries the most weight of all quality parameters analyzed. All methods of PPO inactivation resulted in similar analytical profiles except for the blanching technique. The sharp decrease in caffeine observed when blanching, out performs all other PPO inactivation methods by far. Also, blanching was the most rapid method of PPO inactivation by comparison of TF content. The TF content was less than that of the positive control after 30 seconds (first time point) of blanching. To strengthen conclusions, statistical analysis indicated that there is no significant difference between the best time points of each PPO inactivation method with regard to flavanol, amino acid and TF content (*see* table 2.2). Conversely, GLM analysis indicated that blanching caused a statistically significant decrease in caffeine, while caffeine content for the other PPO inactivation methods did not vary significantly. Comparing the colour for the brews of the green tea originating from the different PPO inactivation methods, blanched tea leaves gave the lightest and greenest brew. Taste may however be affected negatively by long blanching times (*see* table 2.4), but this variable may be corrected by manipulation of the manufacturing procedures to follow, such as the drying method. Thus, blanched green tea leaf samples, as well as green tea extract prepared from these blanched green tea leaf samples, were further analysed by HPLC.

HPLC analysis

HPLC analysis clearly indicated a loss in caffeine content as blanching time was increased, while retaining most of the flavan-3-ols (fig. 2.14). This also confirms earlier analysis using the lead acetate and Folin-Ciocalteu methods, which gave similar results.

The greatest loss in flavan-3-ols was observed for EC which decreased from 3.32 ±0.08 to 2.61 ±0.06% $(^{w}/_{w})$ and EGC decreasing from 7.31 ±0.09 to 6.51 ±0.13% $(^{w}/_{w})$. This amounts to a loss of 21% in EC and 11% for EGC. The total amount of flavan-3-ols, however, only decreased from 21.32 to 19.38% $(^{w}/_{w})$, which is a decrease of less than 10%. Conversely, caffeine decreased by 66% from 3.48 ±0.03 to 1.17 ±0.04% $(^{w}/_{w})$ (*see* fig. 2.15). Subsequently, a 66% improvement or increase of catechin to caffeine ratio from 5.94 to 16.60 was observed (*see* fig. 2.15 insert). For all purposes of this project, the quality of green tea increases with an increase of catechin to caffeine ratio.

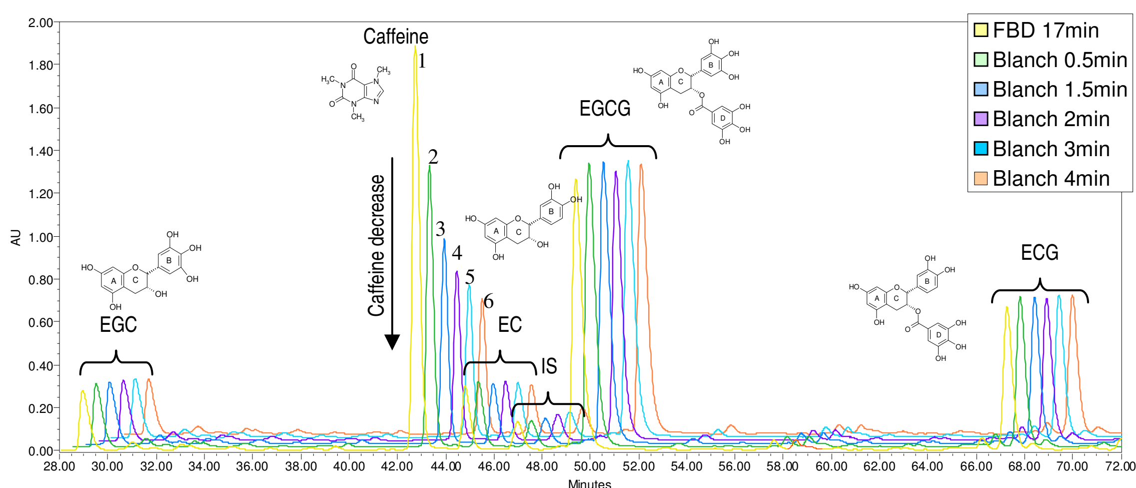

Figure 2.14: Overlay of DAD-HPLC chromatograms measured at 270 nm to indicate loss of caffeine observed when blanching (samples from 25/1 batch). 1: fluid bed dry 17 min; 2: blanch 0.5 min; 3: blanch 1.5 min; 4: blanch 2 min; 5: blanch 3 min; 6: blanch 4 min. Comparison of peak hight indicate that most of the four main catechins have been retained in the tea leaves whereas a steep decrease in peak hight is observed for caffeine. Internal standard (IS) = ethyl gallate.

Blanching thus greatly contributed to the production of high quality green tea. The 17 minute fluid bed dried samples were used as positive control instead of the freeze dried samples, to cancel out any changes brought about by fluid bed drying. Ethyl gallate was the preferred choice of internal standard, as it does not interfere with analysis, eluting between EC and EGCG. The amount of ethyl gallate quantified for each sample remained constant and peak areas corresponded with that from the standards cocktail, confirming the precision of the individual runs.

A phenyl bonded column was used, which gives additional selectivity over reversed phase materials resulting in improved separation of catechins. Following HPLC

analysis of the blanched tea leaf samples, freeze dried extracts were prepared from the 1.5 minute blanched tea leaf samples.

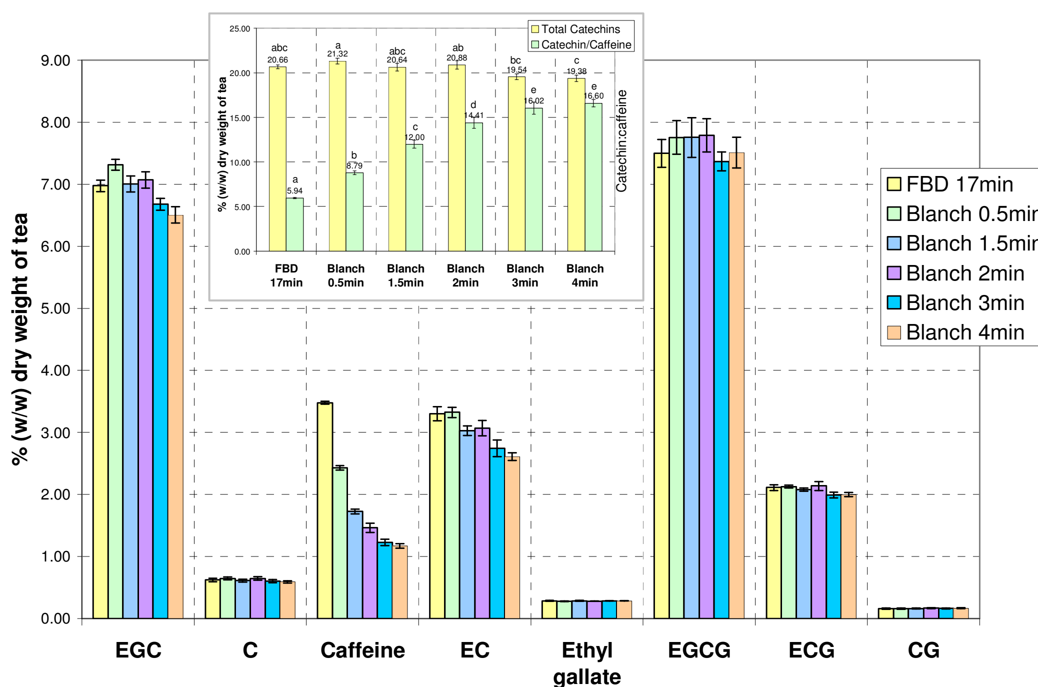

Figure 2.15: Graph indicating the reduction of caffeine after PPO inactivation using the blanching method by RP-HPLC analysis. These results indicate a statistically significant loss of caffeine. A loss of 53.8% caffeine is observed between 17 min FBD and 4 min blanching. No significant loss of (+)-catechin, (+)-catechin gallate, (-)-epicatechin gallate or (-)-epigallocatechin gallate were detected. A minor decrease in (-)-epicatechin and (-)-epigallo catechin was observed. Ethyl gallate is the internal standard used during HPLC analysis. *Insert:* A three fold increase (5.94-16.60) in total catechin to caffeine ratio is observed between the 17 min FBD tea samples (serving as control) and the 4 min blanched samples, due to the decrease in caffeine. Letters indicate Duncan Grouping as determined by SAS analysis. Catechin/caffeine ratios with the same letters have no statistical significant difference. The total catechins did not indicate a statistically significant decrease/increase compared to the FBD sample. Error bars indicate the error of the mean of the results obtained for the 4 independent sets of leaves analyzed for each treatment (main graph + insert).

These samples represented instant green tea extracts, which is usually prepared by spray-drying in the industry. To determine quality, instant green tea samples originating from Sri-Lanka, Kenya, China and India were also analysed for comparison. The most prominent difference between these samples observed in an overlay of their HPLC chromatograms (*see* fig 2.16), is the large EGC peak and rather small caffeine peak detected for the blanched IGT sample. Also, the China IGT sample had a very large caffeine and ECG peak with a below average size EGCG peak.

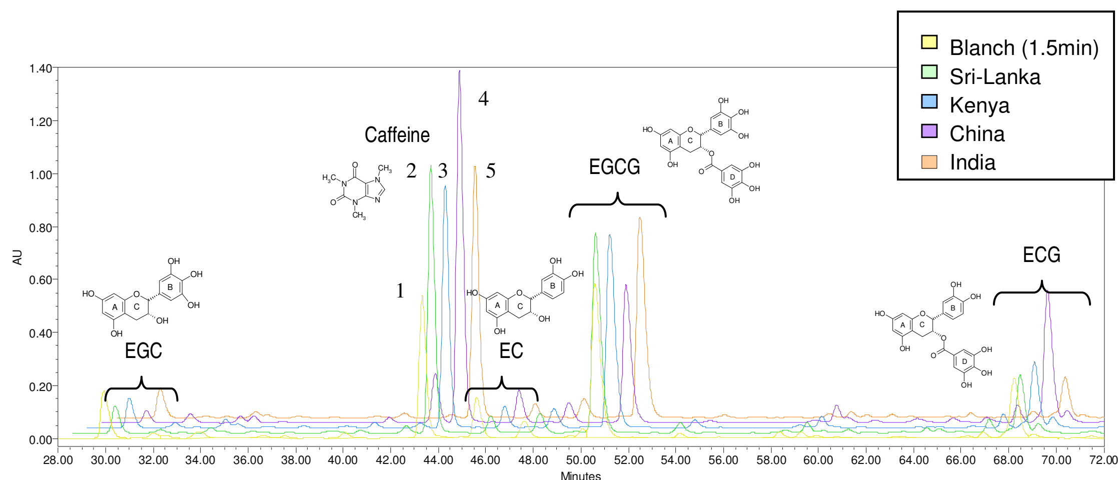

Figure 2.16: Overlay of DAD-HPLC chromatograms measured at 270 nm to indicate the difference in flavan-3-ol profiles between 4 foreign IGTs and IGT produced from a tea sample assessed as being of highest quality (1.5 min blanching) with respect to the measured quality parameters. 1: IGT from 1.5 min blanch tea sample; 2: IGT origin Sri-Lanca; 3: IGT origin Kenya; 4: IGT origin China; 5: IGT origin India.

Quantification of the individual peaks indicated an EGC content of 13.51 ±0.57% ($^{w}/_{w}$) for the blanched IGT sample, which is 39% higher than the highest value of the competitor samples (*see* fig 2.17). The caffeine content was significantly lower with a value of 3.60 ±0.19% ($^{w}/_{w}$) compared to the foreign IGTs, where up to 7.95 ±0.12% ($^{w}/_{w}$) was detected for the China IGT. This is a difference of 55% that also corresponds with the caffeine loss compared with the green tea leaf samples of the 5 alternative methods of PPO inactivation. The sum of the individual flavan-3-ols is 32.66 ±0.68% ($^{w}/_{w}$) while a catechin:caffeine ratio of 9.06:1 was obtained for the blanched IGT. The highest total flavan-3-ol content of between the foreign IGTs is 26.78% for the Kenya IGT. The highest catechin to caffeine ratio between the foreign IGTs is 5.02, again for the Kenya IGT. This means that an enhanced quality IGT can be produced from PC108 green tea leaf manufactured by means of blanching for 1.5 minutes, followed by fluid bed drying, grinding, sieving and spray drying. Such an IGT can have a total catechin content that is >31% higher than that of existing IGTs. Also, such an IGT can have a catechin to caffeine ratio that is as much as 69% higher than existing IGTs.

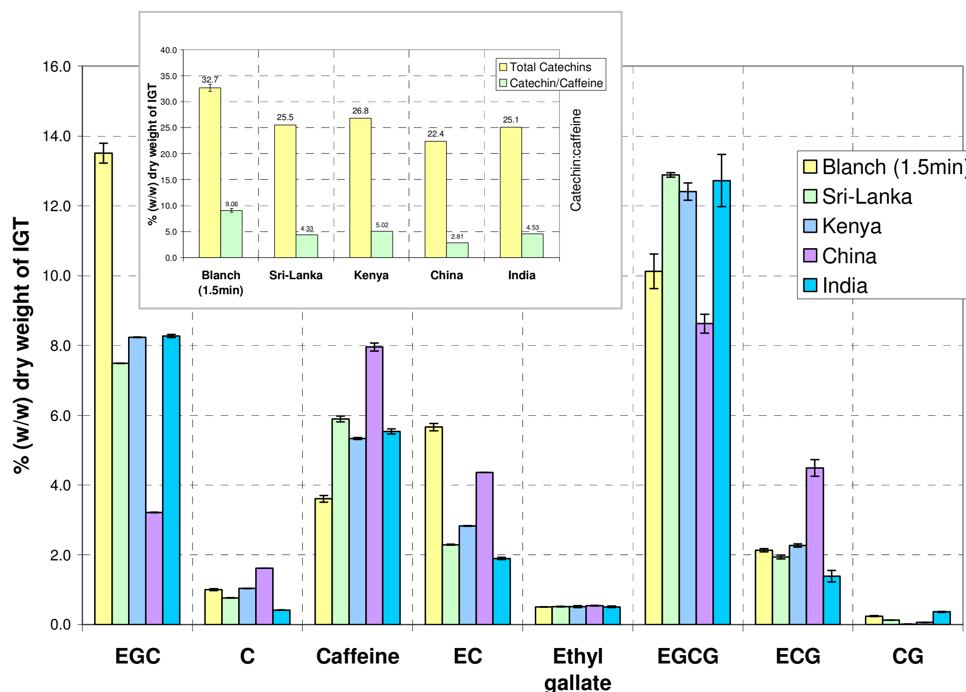

Figure 2.17: Graph for RP-HPLC comparison of the flavan-3-ol and caffeine content determined of 4 IGTs, with a different country of origin, against IGT produced from the tea samples assessed as being of highest quality (1.5 min blanching). These results indicate that the caffeine content of the foreign IGTs is statistically significantly higher than the caffeine content of the IGT from the 1.5 min blanched tea. For the foreign IGT samples, error bars indicate the error of the mean of the duplicate results obtained after RP-HPLC analysis. *Insert:* The total catechin to caffeine ratio observed for the 1.5 min blanched IGT sample (9.08) is more than twice the quantity than what is observed for the foreign IGTs (Kenya = 5.02). A difference of up to 10.3% in total catechins is observed in favour of the 1.5 min blanched IGT. Error bars indicate the error of the mean of the results obtained for IGTs produced from the 4 independent sets of 1.5 min blanched tea samples (main graph + insert).

Introduction to manufacturing method optimization

Two green tea manufacture steps, leaf withering and cutting, were investigated to determine the effect that these variables have on green tea polyphenol content of the made tea (*see* tables 2.5, 2.6 & 2.7). FC analysis of green tea samples, produced on a pilot scale in the Tingamera and Ratelshoek factory of the Tanganda tea estate in Zimbabwe (Africa), indicated that there is no statistically significant difference in polyphenol content between 4 hour withered and non-withered green teas. Green tea originating from 4 hour withered leaves does, however, have slightly lower polyphenol content. Due to oxidation, this difference may become significant when increasing withering time. Conversely, inclusion of the cutting step had a more detrimental effect on polyphenol content (1% loss), although the difference was found not to reach statistical significance due to the limited number of samples. A

statistically significant difference was obtained when comparing the average of the combined results obtained for method 1 and 3 (21.51%) with that obtained for method 2 and 4 (20.40%). Cutting of the leaves speeds up the oxidation process as plant cell walls are broken and substrate and catalytic enzymes are brought together. The PPO inactivation method therefore needs to follow rapidly after cutting to keep the level of oxidation to a minimum.

Table 2.5 Steps involved for the different manufacturing methods followed during optimization of the manufacturing process.

Treatment	Manufacturing steps				
	1	2	3	4	5
	Wither	Cut	Dry	Crush	Sort
Method 1	x	x	√	√	√
Method 2	x	√	√	x	√
Method 3	√	x	√	√	√
Method 4	√	√	√	x	√

Table 2.6 Specifications for the individual manufacturing steps used during the optimization of the manufacturing process.

Manufacturing step		Repeat	Specifications
Step	Description		
1	Wither	1-3	4 hours in trough, ambient temperature
2	Cut	1	Rotor vein (RV)
		2	Laurie tea processor (LTP)
		3	Laurie tea processor (LTP) (RV, LTP + CTC was used for Method 4)
3	Dry	1	Fluid bed dry (FBD) at Tingamera factory
		2	Fluid bed dry (FBD) at Ratelshoek factory
		3	Fluid bed dry (FBD) at Ratelshoek factory
4	Crush	1-3	Cut, tear, curl (CTC), wide gap for dried leaf
5	Sort	1-3	

Table 2.7 Total polyphenol content for tea samples (PC108) prepared using 4 different manufacturing processes. (Means with the same letter are not significantly different as determined by GLM analysis applying the Duncan multiple level test)

Treatment	Total Polyphenol content (% w/w)	Standard deviation	Coeff. of variation (%)
Method 1	21.520 a	1.089	5.062
Method 2	20.549 a	0.279	1.358
Method 3	21.502 a	0.272	1.264
Method 4	20.257 a	0.789	3.896

Another critical factor determining the quality of a made tea or IGT is the starting material used to produce the tea. As previously mentioned, flavanol and caffeine content varies between different tea cultivars. All experiments performed in this

study were based on cultivar PC108 fresh leaves. In previous work by Wright, 2005, total polyphenol and caffeine content of fresh leaf for 40 different Tea Research Foundation (TRF) cultivars were determined (*see* table 2.8). PC108 was ranked 17th, from low to high, with regard to its total polyphenol:caffeine ratio of 5.40.

Table 2.8 Percentages of individual catechins, caffeine and total polyphenols of fresh leaf for 40 different TRF cultivars, calculated from molar amounts. (data obtained from the thesis of L.P. Wright, 2005). Cultivars are arranged from lowest to highest total polyphenol to caffeine ratio.

No.	TRF Cultivar	C % ($^w/_w$) DL	EGC % ($^w/_w$)	EGCg % ($^w/_w$)	EC % ($^w/_w$)	ECg % ($^w/_w$)	Caffeine % ($^w/_w$)	Total Polyphenols % (w/w)	Total Polyphenols/ Caffeine
1	PC169	0.23	1.19	7.60	0.61	3.21	3.95	12.70	3.21
2	PC192	0.26	5.62	10.38	2.37	4.09	4.51	14.65	3.25
3	PC200	0.17	3.65	10.06	1.40	2.95	3.68	13.34	3.62
4	PC186	0.13	3.01	10.83	1.00	2.60	2.72	10.40	3.83
5	88/110-4	0.14	0.86	10.58	0.47	3.10	3.83	14.77	3.85
6	PC213	0.28	4.96	9.18	1.84	2.85	3.28	13.13	4.01
7	88/54-11	0.18	3.62	10.22	1.23	3.02	3.75	15.73	4.19
8	NKW30	0.19	2.33	10.64	0.68	2.52	3.41	15.24	4.47
9	NKW20	0.10	2.20	11.31	0.50	1.91	3.47	15.57	4.49
10	88/54-14	0.19	3.54	10.50	1.03	2.26	2.72	12.91	4.75
11	88/5-2	0.24	3.07	10.56	1.16	3.10	3.29	16.05	4.89
12	11M-3	0.15	4.16	10.46	1.62	3.02	3.39	16.84	4.96
13	88/79-1	0.12	2.14	10.09	0.74	2.27	3.05	15.47	5.07
14	15M-1	0.33	3.77	11.86	1.88	4.42	3.54	18.04	5.10
15	88/79-2	0.11	2.69	11.81	0.78	2.65	3.26	16.65	5.10
16	88/61-11	0.20	1.20	12.78	0.63	3.63	4.11	21.51	5.24
17	**PC108**	**0.25**	**5.41**	**11.53**	**2.00**	**3.55**	**3.60**	**19.45**	**5.40**
18	15M-18	0.21	4.28	8.63	1.50	2.29	3.18	17.99	5.67
19	PC104	0.34	5.79	12.47	3.33	5.73	2.76	16.27	5.88
20	PC211	0.21	3.01	13.28	0.78	2.94	3.19	18.88	5.91
21	PC194	0.15	3.74	10.39	1.16	2.38	3.16	19.04	6.02
22	NKW44	0.26	1.52	10.68	0.78	2.22	2.98	18.02	6.05
23	88/119-1	0.25	3.75	12.40	1.11	2.97	3.42	20.85	6.10
24	NKW51	0.34	4.06	10.32	1.59	2.99	3.29	20.58	6.25
25	15M-58	0.23	4.31	10.63	1.66	3.17	3.15	19.82	6.28
26	88/50-5	0.20	3.29	10.15	1.32	3.19	2.73	18.07	6.62
27	PC168	0.29	2.67	11.04	1.93	5.80	3.49	23.41	6.70
28	PC117	0.39	4.31	11.20	1.97	3.94	3.27	21.98	6.73
29	88/60-9	0.16	3.77	11.39	1.15	2.77	3.07	20.78	6.77
30	88/3-3	0.20	1.90	11.15	0.72	3.39	3.41	23.08	6.77
31	33/10-47	0.34	4.93	8.37	2.03	2.43	3.06	21.95	7.17
32	15M-39	0.13	3.99	11.17	1.07	2.42	2.80	21.19	7.58
33	PC118	0.19	5.86	10.13	2.71	3.93	3.32	25.36	7.63
34	PC1	0.29	7.67	8.01	2.64	2.57	2.85	22.50	7.89
35	SFS204	0.27	2.39	10.78	0.96	3.46	2.63	20.82	7.90
36	SFS42	0.36	4.21	10.71	1.40	3.29	2.42	20.38	8.44
37	PC206	0.33	3.61	13.47	1.02	3.29	2.60	23.38	9.00
38	PC190	0.17	3.32	15.08	1.18	4.39	2.89	27.61	9.57
39	PC119	0.26	6.49	10.41	2.22	3.27	2.45	23.84	9.71
40	CL12	0.07	4.17	11.62	1.38	2.94	2.21	29.01	13.15

Another cultivar, CL12, indicated a total polyphenol to caffeine ratio of 13.15:1, which is 2.44 times higher than that obtained for PC108 fresh leaf. This means that although an enhanced quality IGT, when compared to existing or foreign IGTs, was produced by applying the blanching PPO inactivation method, the quality of the IGT can still be further increased substantially by cultivar selection.

2.4 Discussion:

Barely a decade ago, the notion that the market value of tea depended solely on the quality of the brewed tea liquor and the texture or appearance of the made tea, dominated. Consequently, tea tasters played a decisive role in identifying high quality teas. This practice is still employed to a large extend today, although there is a growing need to establish quality at a biochemical level. Several different PPO inactivation methods have been investigated for their potential for use in production of a high quality instant green tea for implementation on African tea estates.

Green tea leaf sample manufacture

In general, green tea leaf colour (as determined by L*a*b* values) and appearance varies greatly according to the method used to produce the tea. Pan-frying and grilling using direct or indirect heat need to be performed under tightly regulated conditions. Under-treatment will lead to the browning of the leaves, whereas over-treatment causes the leaves to burn which will present off-notes in the flavour of the brewed tea. To inactivate PPO effectively in the shortest possible time, an even distribution of the applied heat is required, which is complicated by the shape of the tea leaves. The tender buds dry comparatively fast and subsequently burn very easily compared to the more hardy stems and older leaves. The temperature of the more rigid stems also increase slower compared to the softer leaves, meaning that it will require a longer heat exposure for PPO to be fully inactivated. Hence, for optimal conditions, a fine balance needs to be found where the treatment is terminated immediately after PPO inactivation in the stems before any burning takes place. The heat source needs to be very constant, as this will greatly influence the necessary treatment time. Due to the nature of the experimental setup, this variable could not be kept constant for all 6 treatments between the 4 independent experiments. It is therefore suggested that an alternative heat source be used during possible follow-up research when optimizing these methods.

In contrast to the belief that enzyme inactivation at 100°C simply occurs faster than at 60°C, recent research indicated that in addition to denaturation, irreversible reactions such as deamination and peptide bond cleavage also occur at temperatures of 100°C and above (Daniel *et al*, 1996). This also includes the Maillard reaction whereby

proteins are irreversibly changed after reacting with sugars. These changes may also affect volatile composition. This should be kept in mind when optimizing a specific green tea production method, as this will allow the manufacturer to confer unique characteristics to their tea brand.

Theaflavin content

As mentioned earlier, polyphenol oxidase is responsible for catalyzing the condensation reaction between a di-hydroxylated- and tri-hydroxilated flavan-3-ol in the presence of oxygen to form TF. Inactivation of PPO will thus prevent the formation of TF's. Moreover, TF content provides a means of detection of retained PPO activity after applying a specific PPO inactivation method for a specific time. These compounds start to form immediately after harvesting of the fresh tea leaves and continue to form during the withering stage, as PPO comes into contact with its substrate, flavan-3-ols. For these experiments, tea leaves were withered for 24 hours. Hence, a low level of TF's is expected to be present in all leaves after treatment, even though PPO has been fully inactivated. The possibility also exists that for pan-frying and the grilling methods PPO was only fully inactivated in the stems after the fluid bed drying step. Theaflavin formation could thus have occurred, but at a much reduced rate giving rise to only a minor increase in TF content. Optimization of these methods individually, will thus require an enzyme activity assay to be performed immediately after each treatment time to determine the degree of PPO inactivation.

Caffeine content

Against our previous knowledge, a Chinese publication by Tsushida and Murai, 1985, mentioned an optimal loss of 71% caffeine, <10% catechins and <20% amino acids after blanching for 1 minute. In a recent study by Liang *et al*, 2007, this loss of caffeine phenomenon was once again investigated. Liang and colleagues found that leaf to water ratio influenced efficiency of caffeine removal and that optimal caffeine loss occurs at a 1:20 leaf to water ratio. Extraction time and extraction temperature also plays a significant role. Optimally, 83% caffeine could be removed with a 5% catechin loss after a 3 minute extraction at 100°C. For this project, not having undergone any optimization steps, a 66% caffeine reduction, a loss of <10% catechins and no statistically significant loss in amino acids was observed after blanching for 4 minutes at ±94°C.

Of all the PPO inactivation methods investigated, blanching is the only method where one component is selectively removed without affecting other components. Several possibilities exist as to why this phenomenon occurs, where caffeine is removed without a noteworthy loss in catechins. One reason being a difference in solubility. Caffeine has a solubility of 21.7g/L, while the most abundant catechin, EGCG, has a solubility of only 5g/L (Liang *et al*, 2007). An increased diffusion rate from cells due to the smaller molecular size of caffeine (MW=194 g/mol) compared to catechins (MW=290-610 g/mol) may also play a role. Considering that catechins are localized in vacuoles, but there is not yet certainty about the localization of caffeine in fresh tea leaf, a difference in compartmentalization may also cause caffeine to be removed more readily by blanching. Other factors, which may be investigated to further optimize caffeine removal by means of hot water extraction or blanching include plucking standard, withering time, water pH, salt concentration and extent of agitation. This manner of caffeine removal is however restricted to fresh leaf decaffeination. This process is only suitable for green tea manufacture because blanching inactivates the PPO enzyme, which is required in the active form to catalyze oxidative browning reactions during black tea manufacture. Also, a significant catechin loss occurs when attempting to remove caffeine from rolled or dry leaf by means of hot water extraction (Liang *et al*, 2007).

Total free amino acid content

Sample analysis indicated no significant changes between any of the six PPO inactivation methods under investigation. Total free amino acid content in green tea is therefore not as susceptible to variation due to differences in the manufacturing method. On the contrary, changes in amino acid content during black tea manufacture will play a considerable role. This can also be concluded from the 20% difference in free amino acid content between the positive- and negative control samples. It should be noted that the ninhydrin method is not specific for amino acids only, but will give colour reactions for peptides, primary amines and ammonia as well (Moore and Stein, 1948).

Flavan-3-ol content

From the results obtained, it is evident that a decrease in flavan-3-ols is caused by fermentation. Any reduction in flavan-3-ols affects quality negatively. The simultaneous loss of caffeine observed when blanching, however, is far greater and compensates for this loss in quality.

Colour measurement

Measuring infusion colour using a HunterLab colour meter gives a very accurate semi-quantitative indication of green tea quality, where a resultant effect is caused by a combination of individual biochemical markers. The presence of theaflavins and thearubigens has a great effect on the infusion colour. Amino acid content and/or composition and chlorophyll content also have a significant impact on infusion colour. Comparing L*, a* and b* values with suitable controls, external (such as a made tea known to have a high/low market value) and internal (representing onsite best and worst quality tea), may serve as a rapid indication towards the efficiency and consistency of a manufacturing procedure. Further optimization of a specific manufacturing procedure can also be achieved quicker by performing colour scans during initial stages (stage monitoring). Typically, poor quality green tea will have lower L* values and higher a* and b* due to the presence of theaflavins and thearubigens, which darkens the infusion colour.

Interestingly, Wang *et al*, 2000, found that extracts made from steamed tea leaves were more stable to colour and taste changes during heat processing and storage than extracts made from roasted tea leaves. This may also suggest that blanching will result in a more stable green tea extract than will the dry treatments. This will strengthen the viability and feasibility of the blanching method, already proving to be an unsurpassed method of PPO inactivation for the purposes of this project.

Organoleptic analysis

Generally, bitter beverages such as tea, coffee or beer are not very popular amongst children and astringency is often considered to make a dissatisfying contribution towards taste (Lesschaeve and Noble, 2005). However, individuals illustrate variation in salivary flow rates, causing them to experience bitterness or astringency different from one another (Lesschaeve and Noble, 2005). Evidently, individuals also display

vast differences in acceptance of a specific astringent product (Lesschaeve and Noble, 2005). Acceptance can also be overwhelmingly influenced by extrinsic factors such as health claims, price, appearance, brand and colour of the product (Lesschaeve and Noble, 2005). Even more determining are social factors, where for example the liking of the product by one individual is observed by another (Lesschaeve and Noble, 2005). Thus, even though an increased flavan-3-ol with a simultaneous decrease in caffeine content may greatly influence taste, adjusting social perception by addressing the above mentioned factors, may increase consumption of a healthier tea beverage sold at a higher price.

The occurrence of off-note flavours are more likely to be connected with dry treatments, where leaves are easily burned. Again, blanching to inactivate PPO may be preferred as dry heat is only applied during the drying stage, for which at least a 10 times higher safety timeframe exists. Increasing the treatment time was found to cause a loss in strength of the green tea leaf liquor. Instant green tea produced for beverages may thus be served at higher concentrations, increasing the potency of the beverage, without a considerable change in palatability.

With regard to the presence of pesticide residues, it is reasonable to suggest that a greater reduction in pesticide deposits will be observed when blanching, as opposed to using 'dry' PPO inactivation methods. Although, pesticide residues are usually greatly reduced by a combination of factors, which includes rainfall, dew, evaporation photolysis, biodegradation, growth dilution and the withholding time between application and harvesting (Sood *et al*, 2004). Sood and his colleagues indicated that pesticide residues are also greatly reduced by vaporization and decomposition caused by the high temperatures used during PPO inactivation and drying stages of green tea manufacture.

Optimum method identification

Judged by experimental observations before biochemical sample analysis, fluid bed drying seems to be the most convenient way of PPO inactivation. This method could be easily implemented in existing black tea factories, since no additional equipment needs to be installed. There are also fewer steps in the manufacturing process, which simplifies the tea manufacture process and saves on energy input. Ranking of the

different PPO inactivation methods based on observations as presented in table 2.1 for each green tea, the placement is as follows: fluid bed dry > blanching > steaming > grilled > pan-frying. The above only serve as an indication of which method might be the best for PPO inactivation and a comprehensive biochemical analysis was performed to determine the correctness of this ranking. After this comprehensive analysis (*see* table 2.2, 2.3 & 2.4), the ranking order of the manufacturing methods changed to blanching > fluid bed drying > steaming > pan-frying > grilled indirect > grilled direct, from best to worst.

Applying the fluid bed drying treatment alone is the most economical way to produce green tea leaf. Factories where FBD facilities are already installed and which are not able to upgrade their factories to implement the blanching method should thus be advised to rather optimize the FBD method. Unfortunately, this means catering for a different instant green tea market that, in the long run, may yield a lower profit compared to blanching. None the less, fluid bed dried green tea still offers a tea with a high quality biochemical composition. The same applies to steaming, although steamed tea is produced at a higher cost and has an initial setup or installation cost which is also disadvantageous.

Pan-frying and grilling is suitable for conventional tea beverages, but is not recommended for the production of high quality instant green tea, where quality is determined by biochemical markers such as TF, caffeine and flavan-3-ol content.

HPLC analysis

Confirmation of spectrophotometric methods was achieved by HPLC analysis. A clear reduction in caffeine was observed on the chromatograms for the consecutive time points using the blanching PPO inactivation method. An above average quality instant green tea, when compared to IGTs from 4 other countries, was produced by freeze drying of a 1.5 minute blanched green tea leaf infusion.

Introduction to manufacture method optimization

The next step to follow this study is the optimization of the manufacturing method that incorporates the use of blanching as PPO inactivation method. Firstly, cultivar selection needs to be performed to ensure that only those cultivars that have a natural

high catechin to caffeine ratio is used for IGT production. If the manufacturer also considers taste to carry a significant weight towards value, steps that promote volatile formation should be evaluated thoroughly as many of the volatiles or aroma compounds may be lost during the blanching procedure. This loss may lead to production of a flat tasting IGT or an IGT without character. One solution to better the taste may be to perform the blanching technique based on the same principal as the SWISS water extraction technique. This technique is in use for the production of naturally decaffeinated coffee. In short, coffee beans are soaked in hot water to extract the caffeine. Aroma compounds are extracted with the caffeine. To prevent this, a caffeine trap is incorporated into the system which removes caffeine from the hot water, but not aroma compounds. By soaking new coffee beans in this decaffeinated, aroma rich hot water, caffeine is removed by means of differentiation, but since there is no concentration differential to drive diffusion of the aroma compounds, flavour is retained. Based on the same principle, refinement of the blanching technique will therefore produce a decaffeinated, aroma rich IGT where, at the same time, the caffeine can be salvaged to be sold separately.

Volatile composition may also be altered by altering the drying step i.e. FBD to roasting, or drying temperature. Using data along with its statistical analysis similar to that found in table 2.2 may also aid during the optimization steps with regard to treatment time by assigning a specific weight to each of the different quality parameters. A significant change in one quality parameter does not necessarily denote a significant change in another.

To conclude, the objective to inactivate polyphenol oxidase in freshly plucked *Camellia sinensis* leaves for the production of instant green tea, can be met by applying one of several different methods. By measuring theaflavin and flavan-3-ol content it was possible to conclude that all six PPO inactivation methods explored in this study were successful in that respect. However, when the objective shifts from the mere inactivation of PPO enzyme to producing a high quality instant green tea, only few of these methods meet the criteria. The best method to inactivate PPO enzyme is therefore a method that generates tea leaves of high biochemical quality, which is economically viable and meets consumer requirements.

The method best suited to achieve this goal is the blanching method, by which an instant green tea can be produced having up to 69% higher catechin to caffeine ratio than existing instant green teas. As a result it was concluded that indeed the biochemical composition is affected by the method of PPO inactivation selected for the manufacture of the instant green tea. To integrate the blanching method into existing tea factories in Africa will require low technology modifications. This can be achieved at a low cost, thus making blanching an economical method for IGT production following PPO inactivation.

CHAPTER 3

Identification of novel flavan-3-ols by HPLC-ESI/MS/MS

3.1 Introduction

In order to confirm the identity and presence of novel catechins present in green tea extracts for which standards are not available, additional techniques are required that will provide sufficient structural information. For this reason high performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC-ESI/MS) may be performed. Firstly, all the different catechins present in the complex samples (tea extract) are separated from one another by means of HPLC. This is followed by ESI-MS/MS performed on each individual peak obtained during HPLC analysis, which allows for structural verification based on the specific ionization patterns obtained. Ultimately, a compound is given a tentative identity according to its HPLC elution time, ultra violet (UV) absorption spectrum as per diode array detector (DAD) recognition and ionization pattern obtained via MS analysis.

For this study, RP-HPLC-ESI/MS/MS was performed using a quadropole linear ion trap mass spectrophotometer, although other alternatives do exist. Since the basic principles are very similar, but the technical details vary significantly, attention will only be give to the working of the quadrupole system (*see* fig. 3.1).

When performing LC –ESI/MS/MS, phenolic components from the crude extracts are separated by selective interaction of these molecules with the non-polar stationary phase (silica with alkyl and phenyl groups) of the column. The analytes are eluted sequentially by decreasing the polarity of the mobile phase from a polar (water) to non-polar (organic solvent) using a gradient. The more lipophilic analytes have longer retention times since these molecules have stronger hydrophobic interactions with the stationary phase. The eluent from the HPLC then enters the ESI source where it is sprayed into a fine mist from an electrically charged capillary into a heated compartment (*see* fig. 3.1 (2)). Nitrogen gas acts as a neutral carrier gas and is used as nebulizer that flows around the capillary, promoting evaporation of the HPLC eluent containing the analytes. A curtain gas of nitrogen serves to prevent entry of uncharged compounds that may cause contamination of the ion optics into the mass

analyser. The positively charged droplets decrease in size as evaporation occurs, which forces the molecules closer together. Eventually the repulsive Coulomb forces between the charged molecules inside each droplet will start to exceed the surface tension of the droplet, causing the droplet to split. This process continues until the analytes are free from solvent, giving rise to ions that can enter the mass analyser.

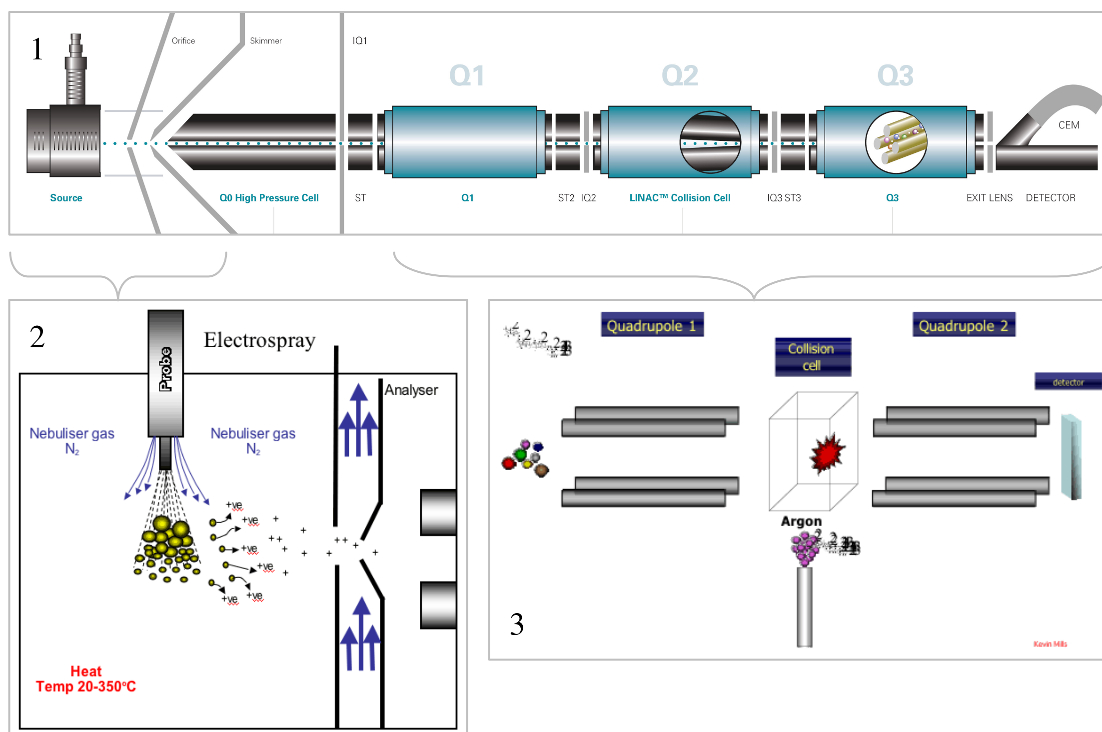

Figure 3.1: Representation of a triple quadrupole mass spectrophotometer with electrospray ionization source. (Image 1 obtained from Applied Biosystems Brochure, and 2 & 3 from UCL Institute of Child Health, London, with permission from Dr. Kevin Mills)

A skimmer and charged cone breaks hydrogen bonds between ion/solvent clusters, which, if present, may give rise to artefact masses entering the first mass analyser. In the 1st quadrupole (Q1) or MS1 of the mass analyzer, introduced ions are sequentially ejected by employing a swept and oscillating direct current (DC) and a superimposed radio frequency (RF). As the ions progress through Q1, they are filtered and separated according to the mass to charge ratio. The separated ions then exit Q1 into a collision cell (Q2) where the high speed ions may collide with introduced argon (Ar) or nitrogen gas molecules, causing the ions either to fragment or to pass through the collision cell into Q3 without fragmenting (*see* fig. 3.1 (2)).

In Q3, fragment and/or precursor ions can then be filtered and separated again by their mass to charge ratio (m/z) before detection, using a channel electron multiplier (CEM)

detector that amplifies ion signals by producing an amplified cascade of electrons that can be accurately quantified.

Infrared spectrometry, time of flight (TOF) MS and nuclear magnetic resonance (NMR) have also been utilized as alternative methods to obtain structural information for polyphenolic compounds. Recently, agarose gel electrophoresis has been applied to isolate pure individual catechins from a polyphenolic tea fraction (John *et al*, 2006).

When applying MS, the Retro-Diels-Alder reaction gives a major *m/z* 139 ionization fragment observed in non-gallated catechins and also as minor fragment for mono-gallated catechins (*see* fig. 3.2). Characteristic fragment ions obtained by Wu *et al*, 2003, for C, EC, Cg and ECg by LC/ESI-MS/MS (Agilent 1100 series, Agilent technologies, Waldbronn, Germany) in positive ion mode, were used to quantify these compounds with a LOQ ranging between 20 and 45 ng/mL in grape products. A linearity of 99% was obtained for standards. Operating conditions employed by Wu *et al* were as follows: 20% collision energy, 3.5 kV needle voltage, desolvation by 12 L/min nitrogen gas with 350°C heater temperature, helium nebulizer gas at 60 psi. During the first MS stage (MS1), molecular ions were detected at *m/z* 291 for C and EC, *m/z* 443 for Cg and ECg, *m/z* 307 for EGC and *m/z* 459 for EGCg.

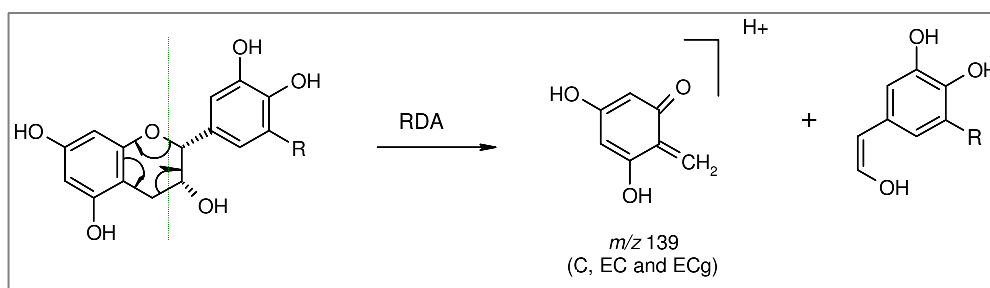

Figure 3.2: Formation of ionization fragments via the retro-Diels-Alder reaction (RDA). This reaction is mainly observed for non-gallated catechins (C and EC) and to a lesser extent in mono-gallated catechins (ECg). A characteristic m/z 139 product ion is observed when performing mass spectrometry under positive ion mode. (Adapted from Wu *et al*, 2003)

During MS2, fragment ions were detected at *m/z* 139 for C, EC and EGC *m/z* 273 for Cg and ECg, *m/z* 289 for EGCg. Later, in 2006 the same method was applied (using Agilent 1100 series, Agilent technologies, Waldbronn, Germany) to green tea for which seven catechins were detected and identified by their molecular- and fragment ions (Shen *et al*, 2006) (*see* 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 under positive ion mode. (Adapted from Shen *et al*, 2006)

Compound Number	$[M+H]^+$ MS (*m/z*)	Fragment ion MS/MS (*m/z*)		Tentative Identification
1	195	138	-	caffeine*
2	291	273	139	(+)-catechin*
3	291	273	139	(-)-epicatechin*
4	307	289	139	(-)-gallocatechin
5	307	289	139	(-)-epigallocatechin*
6	443	273	139	(-)-epicatechin gallate*
7	459	289	139	(-)-gallocatechin gallate
8	459	289	139	(-)-epigallocatechin gallate*

*Compared with standard.

In earlier work, performed by Poon, 1998, direct infusions of tea extract into an ESI-MS (quadrupole) system at 10 µl/min were made to observe the different catechins. This was done under negative ion mode, which gave better sensitivity and selectivity. Following ESI-MS, tandem mass spectrometry was done using the product- and precursor-ion scans of the characteristic fragment ions of catechins to obtain additional structural confirmation. The molecular ion of quinic acid (*m/z* 191) gave a strong signal and fragment ions were detected for EGCg at *m/z* 305, *m/z* 169 (from release of gallic acid) and *m/z* 125. For ECg, fragment ions were detected at *m/z* 289, m/z 169 and *m/z* 125.

Amarowicz *et al*, 2003, identified two different methylated catechins in green tea using a quadrupole ESI-MS/MS system under positive- and negative ion mode and further structural elucidation was obtained by NMR analysis. According to their results, fragment ions of *m/z* 183 and *m/z* 125 was obtained during MS2 under negative ion mode for (-)-epigallocatechin-3-(3″-*o*-methyl)-gallate. Additionally, ^{1}H NMR studies also concluded that methylation of EGCg can occur at either C3 or C4 from the D-ring (galloyl ring), which was consistent with earlier results by Saijo in 1982, where infrared (IR) and ^{1}H NMR spectroscopy was used. The ESI-MS/MS results for both (-)-epigallocatechin-3-(3″-*o*-methyl)-gallate and (-)-epigallocatechin-3-(4″-*o*-methyl)-gallate are thus expected to yield the same major fragment ions. Saijo, 1982, found that ECg may also be methylated, existing as either (-)-epicatechin-

3-(3″-*o*-methyl)-gallate or (-)-epicatechin-3-(4″-*o*-methyl)-gallate. Both methylated EGCg and -ECg will give a MS2 fragment ion of *m/z* 183, but the latter will also give a *m/z* 109 instead of *m/z* 125 due to the absence of the 3rd OH-group in the B-ring. Whether the gallic acid moiety of the D-ring or the B-ring is methylated can be determined by observation of the fragment ions formed. Should methylation have occurred in the B-ring, the *m/z* 125 or *m/z* 109 fragments from methylated EGCg and ECg respectively would be absent. Also, instead of observing the *m/z* 183 fragment ion for the methylated gallic acid moiety, a fragment ion of *m/z* 169 will be observed for the non-methylated D-ring. To monitor column chromatography during preparation of standard methylated EGCg from crude tea extract, TLC using silica gel plates with chloroform-methanol-water (65:35:10) as mobile phase may be applied (Amarowicz *et al*, 2005).

Methylated EGCg has demonstrated good promise in cold-preservation of hepatic cells (Kagaya *et al*, 2003). Another novel application for methylated EGCg is its use against type I (hypersensitivity to pollens, mold spores, dust mites etc.) and type IV (delayed onset hypersensitivity caused by metal, jewellery etc.) allergy (Suzuki *et al*, 2000; Sano *et al*, 1999; Maeda-Yamamoto *et al*, 2004; Fujimura *et al* 2002). The anti-allergy effect of EGCG has been previously identified, but the methylated EGCG proved to be more effective (Suzuki *et al*, 2000).

In addition to the methylated catechins, another novel group of catechins exists, namely digallated catechins. The first discovery of digallated catechins in green tea was as far back as 1959, by Vuataz and his colleagues. Though, at that stage the identity of the compound was still unknown. Later in 1972, Coxon and his colleagues repeated the work of Vuataz *et al* and identified the unknown compound as being 3,5-digallated EGC by isolation of individual compounds followed by UV spectra and NMR analysis. Simultaneously, they also detected the presence of 3,5-digallated EC. To date, not much follow-up research has been done on these specific flavan-3-ols, possibly as they are usually present in very small amounts if at all. This said, the medicinal value of these compounds is not yet known, but it is reasonable to believe that its antioxidant capacity will be superior to that of EGCG due to the 3 additional hydroxyl groups provided by the 2nd gallate moiety.

Catechin isomerization accelerates at temperatures above 80°C. Significant isomerization occurs when applying thermal treatment at 120°C for 30 min at pH 5 (Nagai *et al*, 2005). Prolonged heat exposure, 98°C for 15 minutes, may cause 15% degradation of green tea catechins, where after degradation tempo decrease so that a total of 20% is lost over 7 hours (Chen *et al*, 2001). This information should be kept in mind when producing ready to drink (RTD) tea beverages in cans or polyethylene terephthalate PET bottles, which usually undergo pasteurization. Also, at higher pH conditions (≥ pH 6.0) the major flavan-3-ols, EC, EGC, ECg and EGCG, tend to epimerize to form the minor flavan-3-ols, GC, C, GCg and Cg (Yoshida *et al*, 1999). The sum of the individual flavan-3-ols remains the same (Yoshida *et al*, 1999). Flavan-3-ols are also more susceptible to epimerization at higher temperatures with a 71% decrease in EGC being observed for a 20 minute extraction at 80°C and pH of 6 (Yoshida *et al*, 1999). Alteration of the extraction procedure may thus be explored to optimize yield of novel flavan-3-ols. HPLC quantification of individual catechins and caffeine indicated that the smaller non-gallated catechins generally have a faster infusion rate compared to the larger gallated catechins (Price and Spitzer, 1994). Caffeine infuses up to 3 times faster from green tea than from black tea of similar size and infusion rate is 2 times higher than that of the catechins (Price and Spitzer, 1994). Therefore, di-gallated catechins are likely to have even slower infusion rates than mono-gallated catechins. This information may imply that di-gallated catechins may be isolated preferentially by means of counter-current extraction or a selective, multiple extraction procedure. A novel inexpensive lignocellulose column prepared from sawdust as mentioned by Sakanaka, 2003, can be used to obtain a virtually caffeine free gallated catechin polyphenol fraction, since these catechins bind to the column and can be eluted with 60% ethanol. Pan and colleagues, 2003, found that a microwave-assisted extraction procedure can be applied to reduce extraction time and to improve polyphenol extraction. Similar research by Sharma *et al*, 2005, indicated that infusions made from green tea, where microwave-mediated PPO inactivation was used as opposed to steaming or hot air inactivation, contained more catechins and caffeine. Pre-leaching (soaking) of the tea leaves, liquid to solid ratio, extraction temperature and the type of solvent used to perform the extraction also play a significant role on the rate and degree of polyphenol extraction.

Although flavan-3-ols are expected to play a role in the plant defence system, the exact function or biosynthesis pathways for the individual flavan-3-ols are unknown. Some flavan-3-ols are likely to be either the precursors or the metabolites of others.

Once known, tea trees can be cultivated under specific conditions to maximize the yield of novel catechins on a weight per weight basis. To obtain all these answers to be able to manipulate flavan-3-ol production will entail many years of expensive research. A short term solution would involve screening of different cultivars to find those cultivars that, on average, contain higher amounts of the novel catechins. Within a specific cultivar, screening can also be performed on individual trees to find intra cultivar differences. This is also the aim of this chapter – to enable screening of individual tea trees for novel catechin content by identifying retention times of these novel catechins by means of LC-MS. Once found, these high novel catechin containing trees can be closely monitored to determine factors that influence the production of these catechins. Such cultivars can also be propagated to allow for large scale harvesting of novel catechins from the tea leaves. The saying: 'knowledge is power', is however very applicable, since a change in an unknown factor that causes a decrease in novel catechin synthesis may render the tea field useless for the specific application. Nonetheless, it is worthwhile investigating inter and intra cultivar flavan-3-ol distributions at a genetic and environmental level to gain insightful information on flavan-3-ol synthesis.

3.2 Materials and Methods

3.2.1 Materials

Green tea extract, produced from tea leaves originating from China, Kenya, Sri-Lanka and India, were donated by Mitsui Norin (Japan). Minisart hydrophilic syringe filters, 0.20µm, were obtained from Sartorius (Hannover, Germany). Acetonitrile E CHROMASOLV® (HPLC grade), methanol CHROMASOLV® HPLC grade, (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechingallate (EGCG) and gallic acid monohydrate were purchased from Sigma Chemical Company (Steinheim, Germany). Acetic acid (Ultra ≥ 99.5%) and ethyl gallate was purchased from Fluka (Steinheim, Germany).

3.2.2 LC-ES/MS/MS Analysis

Instrumentation

Reverse phased chromatographic separation was performed on an Agilent 1100 series HPLC system using a Phenomenex, 250 x 4.6 mm i.d. Luna 5 µm, Phenyl-Hexyl column with a Phenomenex Phenyl Guard Column, 4.0 x 3.0 mm. The HPLC system was fitted with an Agilent 1100 photodiode array UV/VIS detector (DAD), a column oven and an Agilent 1100 wellplate autosampler. The HPLC was connected to an Applied Biosystems/MDS SCIEX 4000 Q TRAP quadrupole mass spectrophotometer with linear ion trap (LIT) technology and electrospray ionization source. Dry air was used as nebulizer while $N_{2(g)}$ was used as collision gas. Data analysis of results was done using Analyst 1.4.1 software. A sonication bath (7.2 L Ultrasonic Cleaner, 40 KHz operating frequency, Optima Scientific, South Africa) was used to degas the mobile phases under vacuum prior to using it on the LC-MS/MS system.

Chromatographic conditions

Mobile phase A consisted of dddH_2O/Acetonitrile/Acetic acid (97:1:2 v/v/v) and mobile phase B consisted of dddH_2O/Acetonitrile/acetic acid (18:80:2 v/v/v). The following gradient was used: 0-8 minutes, 0% B; 8-60 minutes, 0-17% B; 60-70 minutes, 17% B; 70-130 minutes, 17-45% B; 130-135 minutes, 45-100% B; 135-138 minutes, 100% B; 138-142 minutes, 100-0% B; 142-150 minutes, 0% B. HPLC was performed at 30°C with a flow rate of 1 ml/min. Components of interest were

monitored at 270 nm although UV/VIS spectral data was collected from 210-400 nm to verify components by means of their individual UV spectra.

Spectroscopic conditions

MS and MS/MS experiments were conducted in positive mode. A curtain gas (CUR) of purified air at 20.00 psi was used. The nebulizer gas pressure (GS1) was maintained at 20 psi and the heater gas (GS2) at 30 psi with a temperature (TEM) of 450°C. Entrance potential (EP) was set at 10.00 V while declustering potential was set at 45.00 V. Ion energy for Q1 (IE1) was set at 0.8 V.

3.3 Results

Instant green tea extracts originating from 4 different countries were screened for novel flavan-3-ol content. Direct injection of standards was done to determine the optimal conditions to obtain the molecular ions of all the standards during the first MS stage (Q1). Selective ion monitoring mode was used to detect specific fragment ions. Mass spectrums were scanned for the molecular ions of (-)-epicatechin-3-(5'-o-methyl)-gallate (*m/z* 456), (-)-epigallocatechin-3,5'-digallate (*m/z* 473), (-)-epicatechin-3,5'-digallate (*m/z* 595) and (-)-epigallocatechin-3,5'-digallate (*m/z* 611). MS data for novel flavan-3-ols revealed very similar results for all 4 extracts (*see* tables 3.2-3.5).

Results obtained for Q1 experiments of the standards corresponded to those obtained by Shen *et al*, 2006, for the individual catechins (*see* table 3.1). Firstly, EGC eluted after 30.1 minutes and gave a Q1 peak at *m/z* 307 $[M+H]^+$. Isotopes with *m/z* 308 and 309 and corresponding fragment ions of *m/z* 291 and 139 were also detected. The EGC fragment ion with *m/z* 291 differs from literature where *m/z* 289 was reported. A Q1 peak at m/z 289 was, however, observed within the first half of the HPLC eluted peak for EGC. The 2nd flavan-3-ol that eluted was C with t_R 32.3 minutes. A *m/z* 291 $[M+H]^+$ with isotopes of *m/z* 292 and 293 as well as fragment ions of *m/z* 273 and 139 were detected for C. Caffeine was also included in the standard and gave a molecular ion of *m/z* 195 $[M+H]^+$, isotopes of *m/z* 196,197 and 198 with t_R 41.7 minutes, but no peak was observed at *m/z* 138.

Table 3.2 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from **Sri-Lanka** (5mg/ml) after LC/MS analysis under positive ion mode.

Compound Number	$[M+H]^+$ MS (*m/z*)	Fragment ion MS (*m/z*)				t_R (min)	Tentative Identification
1	459	289	139			50.1	(-)-epigallocatechin-3-*O*-gallate
2	595	459	391	159		51.7	(-)-epicatechin-3,5'-digallate
3	595	565	438	391		61.7	(-)-epicatechin-3,5'-digallate
4	595	617	449	195		70.7	(-)-epicatechin-3,5'-digallate
5	595	449	287	195		76.4	Kaempferol hexose-deoxyhexoside/ Kaempferol-3-rutinoside
6	611	465	433	303		65.7	Quercetin hexoside
7	611	579	473	303	289	64.6	(-)-epigallocatechin-3,5'-digallate
8	473	391				64.2	EGC 3,5 methylgallate

Table 3.3 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from **Kenya** (5mg/ml) after LC/MS analysis under positive ion mode.

Compound number	$[M+H]^+$ MS (*m/z*)	Fragment ion MS (*m/z*)				t_R (min)	Tentative Identification
1	459	289	139			50.1	(-)-epigallocatechin-3-*O*-gallate
2	595	435	391			62.0	(-)-epicatechin-3,5'-digallate
3	595	741	433	391	195	70.6	(-)-epicatechin-3,5'-digallate
4	595	449	391	287		76.6	Kaempferol hexose-deoxyhexoside/ Kaempferol-3-rutinoside
5	611	465	433	303		65.8	Quercetin hexoside
6	611	579	541	303	246	64.5	(-)-epigallocatechin-3,5'-digallate

Table 3.4 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from **China** (5mg/ml) after LC/MS analysis under positive ion mode.

Compound number	$[M+H]^+$ MS (*m/z*)	Fragment ion MS (*m/z*)				t_R (min)	Tentative Identification
1	459	289	139			50.2	(-)-epigallocatechin-3-*O*-gallate
2	595	441	435			62.1	(-)-epicatechin-3,5'-digallate
3	595	579	449	391	287	70.8	(-)-epicatechin-3,5'-digallate
4	595	449	391	287		76.9	Kaempferol hexose-deoxyhexoside/ Kaempferol-3-rutinoside
5	611	465	303			64.7	Quercetin hexoside
6	611	465	303			65.8	Quercetin hexoside
7	473	427	391			72.6	EGC 3,5 methylgallate

Table 3.5 Assignment of molecular- and fragment ions to novel flavan-3-ols present in green tea extract from **India** (5mg/ml) after LC/MS analysis under positive ion mode.

Compound Number	$[M+H]^+$ MS (*m/z*)	Fragment ion MS (*m/z*)					t_R (min)	Tentative Identification
1	459	289	139				50.1	(-)-epigallocatechin-3-*O*-gallate
2	595	435	391				62.2	(-)-epicatechin-3,5'-digallate
3	595	741	449	443	287		70.6	(-)-epicatechin-3,5'-digallate
4	595	449	391	287			76.9	Kaempferol hexose-deoxyhexoside/ Kaempferol-3-rutinoside
5	611	465	433	449	303	287	65.7	(-)-epigallocatechin-3,5'-digallate
6	611	579	473	303	289		64.7	(-)-epigallocatechin-3,5'-digallate
7	611	465	303				63.0	Quercetin hexoside

EC, with t_R 45.1 minutes, presented a *m/z* 291 $[M+H]^+$, *m/z* 139 and weak *m/z* 273 as well as EC isotopes of *m/z* 292, 293 and 294. For EGCG, eluting after 46.9 minutes, *m/z* 459 $[M+H]^+$, isotopes of *m/z* 460 and 461 and fragment ions of *m/z* 139 and 289

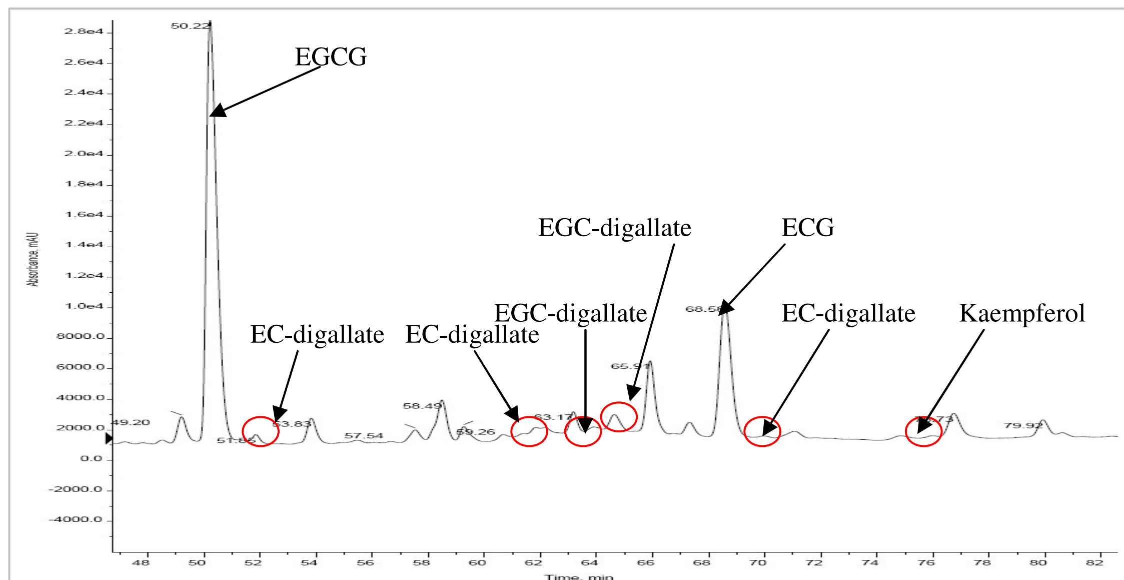

Figure 3.3 Total wave count (TWC) of DAD spectral data for IGT from Sri-Lanka (5mg/ml), indicating tentative HPLC retention times for novel catechins.

were detected. The elution time for ECG was 68.6 minutes and a Q1 *m/z* 443 $[M+H]^+$, isotopes of *m/z* 444, 445 and 446 and a fragment ion with *m/z* 273 was detected. No *m/z* 139 fragment ion was detected for ECG. Lastly, CG with t_R 70.2 minutes, presented a m/z 443 $[M+H]^+$, *m/z* 444, 445 and 446 isotopes and a *m/z* 273 fragmentation ion. The *m/z* 139 fragment ion was not detected for CG.

Screening of the foreign IGTs gave inconclusive results, although the search was narrowed down to only a few potential HPLC retention times for 2 digallated catechins (*see* fig. 3.3). The molecular ion ($[M+H]^+$) for EGCg with *m/z* 459 was detected at t_R 50 minutes for all samples analysed, as expected, with fragment ions of *m/z* 289 and *m/z* 139. This corresponds to the fragments as observed by Shen *et al*, 2006, and these are also the same fragments observed for the EGCg standard.

Figure 3.4: ESI-MS spectrum obtained for EGCg. (China GTE, 5mg/ml, t_R = 50.2 min, Positive mode, Q1, * =impurities). MRM indicated 4.4e[4] cps signal for Q1/Q3 masses of 459/125 amu.

The *m/z* 289 fragment is depicted (*see* fig. 3.4) to be [M+H-galloyl+H-H_2O]$^+$ according to Shen *et al*, 2006. From tables 3.2-5 it can be seen that the results indicate several retention times for analytes with the same nominal mass (*m/z* 595 and 611) and similar major fragmentation patterns. A possible mass spectrum representing EC-digallate is provided in figure 3.5. The molecular ion with *m/z* 595 [M+H]$^+$, detected for the HPLC retention time of 76 minutes, gave fragment ions (*m/z* 449 and 287) that correspond to those identified by Shen *et al*, 2006, and Del Rio *et al*, 2004, as being kaempferol hexose-deoxyhexoside or kaempferol-3-rutinoside respectively.

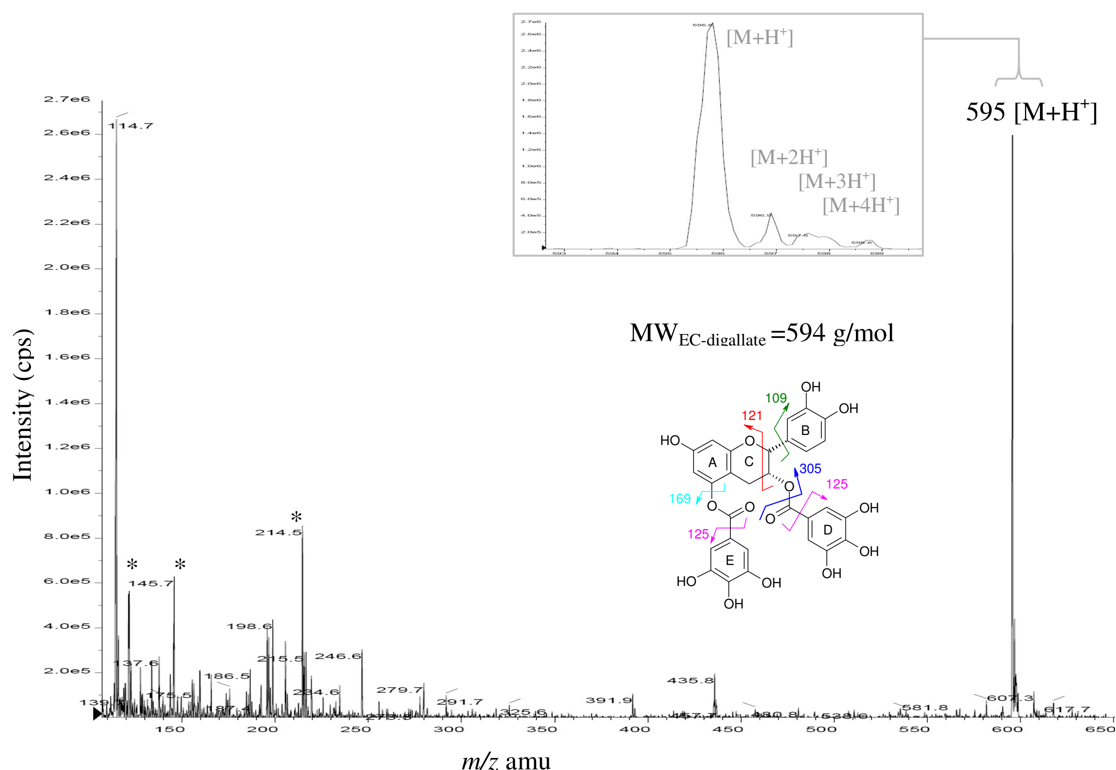

Figure 3.5: Tentative identification of EC-digallate from ESI-MS spectrum. (India GTE, 5mg/ml, t_R = 62.2 min, Positive mode, Q1, * = impurities). MRM signals for selected Q1/Q3 masses were insignificant. Insert indicate *m/z* 595 $[M+H]^+$ isoform peaks.

The accurate mass, where decimal point mass values are assigned instead of whole numbers, may be used to distinguish between digallated catechins and the above mentioned compounds. The accurate mass of EGCg is 458.4 g/mol and should give a peak with *m/z* 459.4 under positive mode, but results for the EGCG standard gave a peak at 459.7 *m/z*. For this reason, nominal masses only indicate potential HPLC retention times, that should be confirmed by NMR.

3.4 Discussion

The purpose of performing LC-MS on IGTs, originating from 4 different countries, was to determine the HPLC elution times of novel flavan-3-ols. As standards are not available for the catechins of interest (methylated & digallated), quantification of the identified catechins could not yet be performed. The identities of the novel catechins detected via ESI-MS/MS remain tentative until confirmation by another structural elucidating method such as NMR. Once retention times are known, standards can be generated by preparative HPLC fraction collection, concentration and purification followed by LC-MS/MS and NMR verification. From there, high throughput cultivar screening by HPLC will be possible for identification and quantification of novel flavan-3-ol 'super synthesisers'. The results obtained, however, are inconclusive with regard to the specific HPLC elution times of these novel compounds, although positive identification for (-)-epicatechin-3,5'-digallate and (-)-epigallocatechin-3,5'-digallate was achieved only tentatively. Possible HPLC retention times for EC-digallate were detected at t_R 62.0 ± 0.2, 70.7 ± 0.1 and 76.7 ± 0.2 minutes while t_R 65.8 ± 0.1 and 64.6 ± 0.1 minutes were detected for EGC-digallate.

From the data listed in tables 3.2-5, it can be seen that the expected molecular ions of a specific novel flavan-3-ol occurs more than once at different elution times. Exclusion of compounds was done by cross referencing in literature and by inspection of the specific MS2 fragment ions.

Due to limitations of the current LC-ESI/MS/MS system experienced, multiple reaction monitoring (MRM) mode had to be used to prevent data overload instead of product ion (PI) or neutral loss (NI) mode, which monitors the formation of all fragment ions. A MRM chromatogram displays the signal of a specified Q3 fragment ion originating from a specified Q1 precursor ion detected for a specified time. The intensities of the individual selected mass values can also be visualised together in an extracted ion chromatogram (XIC) against time. In other words, the XIC therefore indicates the summed intensities of the selected mass range as a function of time. This is similar to the total ion chromatogram (TIC), which indicates the sum of the intensities of all ions from a series of mass scans against time. In PI mode, however, the signal of a specified Q1 precursor ion and all the fragments from that ion from Q3

is detected. Furthermore, NI mode allows for all precursor ions from Q1 and all fragment ions from Q3 to be detected. Unfortunately, these studies to determine the minor fragmentation patterns were hindered by instrument failures resulting from contamination of the ion source, which caused a very high background that prevented accurate sample analysis.

HPLC chromatograms were visualised as total wavelength chromatograms (TWC) (typical HPLC chromatograms), which provides maximum signal strength (absorbance) for the duration of the HPLC run. A DAD chromatogram pane can also be selected for any selected time frame from the TWC to obtain DAD spectral data that provides the combined intensity for each wavelength over the selected time frame. Flavan-3-ols usually have a distinct DAD chromatogram that peaks at ± 270 nm, with slight differences within this group of compounds. These slight differences also aid in identification of specific flavan-3-ols although variation may lead to the incorrect identification of individual flavan-3-ols when analysing a crude extract. The task of identifying compounds by analysis of their mass spectrum is often complicated by the presence of impurities in the buffers, sample extract or ion source. It is of great importance that the analyst is aware of any such contamination, which may obscure or falsely present compounds of interest. Future studies should preferably list minor fragmentation patterns to prevent any discrepancies. Available compounds known to have the same nominal mass as the compound of interest, with unknown retention time, should be included in the standards to allow for their elimination from the chromatograms.

From the LC-MS results it is evident that the composition of teas or tea extracts having a different origin differs in flavan-3-ol composition. This is already a well known phenomenon, which prompts the screening of individual tea trees to identify ‘super synthesisers’ of novel flavan-3-ols. Tea trees containing higher amounts of novel catechins can then be propagated and harvested specifically for their novel catechins.

Although screening of individual tea trees to find those with above average quantities of novel catechins may seem logical and practical, the risk involved should be realized. Several horticultural and biological factors play a role in catechin synthesis.

This risk can also be highlighted from work done by Owuor *et al*, 1987, who concluded that while changes in small microecological factors or geographical (localities) exist, chemical composition of tea, hence quality, will change. Furthermore, the exact reason for synthesis or mechanisms involved during synthesis remains unknown. Presence or absence of any of these factors at the time of sampling may falsely indicate tea trees with high or low novel flavan-3-ol 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. Wachira *et al*, 1995, indicated an immense genetic diversity between different tea varieties. Rapid amplified polymorphic DNA (RAPD) analysis of different tea clones indicated polymorphism variations of 50.3 to 88.5% where the highest variation occurs within populations. Between tea trees of the same cultivar, however, this genetic variation may be less significant.

In-depth knowledge on the function of catechins in tea trees may allow for synthesis of novel catechins to be induced by design to optimize catechin yield. In a review of the biotechnological advances in the tea industry by Chen *et al*, 2006, it was mentioned that 3 genes, chalcone synthase 1 (CHS1), CHS2 and CHS3, responsible for flavonoid biosynthesis, have been cloned and characterised. It was also mentioned that flavonoids may play an important role in growth and development, and defence against microorganisms and pests. Takeuchi *et al*, 1994, isolated cDNA of the CHS gene and indicated sugar responsive expression of the gene. A breeding programme, employing a series of plant cell culture strategies, may be started to create new clones that contain high amounts of novel catechins while containing very low amounts of caffeine, but this may take up to 20 years or longer. Alternatively, it may be worth while for transgenic tea to be considered as an option for the production of such a tea, which is destined to have a much higher market value due to enhanced health properties. Transgenic modification may include the manipulation of flavonoid pathway genes, modification of expression of regulatory genes and by generating novel enzyme specificities, as suggested by Dragan *et al*, 2007. Another possibility mentioned by Dragon *et al*, to obtain high quantities of novel catechins, which may be a threat to the tea industry, is the *in vitro* chemical synthesis and also microbial or enzymatic transformation of *in vivo* synthesised EGCG (most abundant flavan-3-ol).

CHAPTER 4

Concluding Discussion

Tea (*Camellia sinensis*) is the beverage most consumed worldwide, excluding water. It originated some 2000 years ago and is becoming increasingly popular not only for its taste and aroma, but is also consumed as a functional food due to its medicinal benefits. Tea is currently grown in at least 30 countries throughout the world, which produce a total of more than 3.4 billion kilograms of tea annually (FAO, 2005). The per capita worldwide consumption of tea is estimated at 120 ml/day with black tea comprising about 78%, green tea 20% and oolong tea 2% of the consumption (Jain *et al*, 2006). Between different countries, consumption may vary from no tea up to 20 cups of tea per day at variable strength.

Through scientific research, tea has been proven to reduce cholesterol (Bursill *et al*, 2001), have anti-tumor or anti-carcinogenic properties (Thangapazham *et al*, 2007; Gupta *et al*, 1999), prevent loss of cartilage in arthritis by inhibiting production of matrix-degrading enzymes (Ahmed *et al*, 2004), promote weight reduction (Tian *et al*, 2004), protects skin against UV damage (Katiyar *et al*, 2001), block oxidative DNA damage to the liver (Hasegawa *et al*, 1995), aid in the prevention of tooth decay and oral cancer (Lee *et al*, 2004), lower the risk of developing hypertension (Yang *et al*, 2004), acts as an antimicrobial (Bandyopadhyay *et al*, 2005), have anti-inflammatory properties (Sang *et al*, 2004), lower plasma glucose levels of type 2 diabetes subjects (Hosoda *et al*, 2003), possibly prevent the development and progression of Alzheimer's disease (Lee *et al*, 2005), reduce the probability of renal stone formation (Itoh *et al*, 2005) and can potentially prevent type IV allergy (Suzuki *et al*, 2000). Tea has also been found to inhibit HIV-1 entry by preventing glycoprotein-mediated membrane fusion (Liu *et al*, 2005). Most of these health properties listed above have been assigned to the presence of polyphenolic compounds, more specifically the flavan-3-ols, in tea.

The aim of this study was to discover an economically viable PPO inactivation method for the production of an instant green tea (IGT) that features enhanced quality properties when compared to conventionally produced IGTs. Ultimately, the

discovery of such a method may lead to implementation of the method on tea estates in Africa to add value to this basic commodity.

Several quality parameters, such as theaflavin, caffeine, catechin and free amino acid content, as well as infusion colour and taste, were investigated for samples prepared using different PPO inactivation methods. Ideally, the best PPO inactivation method would be a method that leads to a higher catechin to caffeine ratio of the final IGT product.

Indeed, the results obtained for this project indicated that PPO inactivation by means of blanching is an effective method to produce a natural low caffeine containing tea. This finding, and also that no statistically significant decrease in flavan-3-ols is observed when blanching, is in accordance with literature (Liang *et al*, 2007; Tsushida *et al*, 1985). The variance in flavan-3-ol, caffeine and amino acid content between individual experiments performed with tea leaves obtained 1 week apart was ascribed to horticultural factors as described by Owuor *et al*, 1986 and 1987. Tsushida and Murai, 1985, mentioned an optimal loss of 71% caffeine, <10% catechins and <20% amino acids after blanching for 1 minute. The blanching method was not optimized for this study, having to affect a slightly lower caffeine loss than that achieved by Liang and colleagues. A 66% caffeine reduction, a loss of <10% catechins and no statistically significant loss in amino acids was observed after blanching for 4 minutes at ±94°C. According to Liang *et al*, 2007, optimal blanching conditions is at 100 °C for 3 minutes with a 1:20 ($^{w}/_{v}$) leaf to water ratio, which gives a 83% loss of caffeine and <5% catechins. From an economical point of view, blanching will be the best PPO inactivation technique for production of green tea or IGT as the market value will be higher due to the end product being naturally low in caffeine. Supercritical fluid extraction with CO_2, another chemical free technique used for decaffeinated coffee/tea production, is very expensive and the final product may disappoint consumers in that flavour and aroma is lost to a certain extend (Kato *et al*, 2000). The blanching method can be implemented and maintained at a significantly lower cost. In contrast, all other PPO inactivation methods investigated result in a green tea or IGT that mainly differs in taste. The major quality parameters, such as their catechin to caffeine ratio, remain more or less the same, not separating their end products from existing tea products. To eliminate competition by setting new product standards, it

would thus be beneficial to further optimize the blanching technique for implementation on African tea estates.

Through optimization, blanching of fresh tea leaves will serve to inactivate PPO and remove caffeine while maintaining the desired aroma content. Also, it would be recommended that the maximum withering time be used to assure the highest amount of free amino acids without compromising flavanol content to address the unwanted 'flat' taste obtained from blanching. Baruah, 2003, found that proteases, causing free amino acid content to rise by hydrolysis of proteins, exhibit their maximum activities after ± 12 hours of withering. They also found the maximum activity of PPO occurs at ± 15 hours of withering, which did not cause a significant decrease in total polyphenol content. Withering for 3 hours did not cause any significant decrease in total polyphol content during pilot scale tea manufacture in this study either. Cutting or rolling of the leaves, withered for extended periods where PPO has reached maximum activity, may cause faster browning compared to fresh leaves. In 1992, Mahanta and Baruah also concluded that the degree of wither and type of maceration will determine the rate of oxidation. When blanching, this would not be of concern though, as the withered whole leaves will be blanched, inactivating PPO, before cutting or rolling. The effect of withering on the efficiency of caffeine removal and percentage flavan-3-ol loss is not yet known and should be investigated first.

Following removal of excess water after blanching, the next step to be optimized will be the drying procedure. Interestingly, Cloughley, 1983, found that drying with a FBD, apparatus employed on most tea estates in Africa, causes an additional loss in caffeine due to evaporation caused by high operational temperatures. However, no statistically significant difference between 8 and 30 minutes FBD were observed under the conditions used in this study. Inlet and outlet temperatures will be the main variables to optimize during this stage, which will affect the formation of specific aroma compounds. In addition, manufacturing steps such as cutting, rolling or frying of the leaves may also be included for production of a unique tasting IGT. Inclusion of additional manufacturing steps may, however, be at the expense of novel flavan-3-ols present in the tea leaves. Although the origin of the raw material used to produce the 4 foreign IGTs screened for novel flavan-ols by LC-ESI/MS is not known; only

low quantities of the tentatively identified digallated catechins were detected, while methylated catechins appeared to be absent. Stability of these novel compounds should thus be determined for the various individual manufacturing processes before implementation. LC-MS results indicated possible HPLC retention times for EC-digallate at t_R 62.0 ± 0.2, 70.7 ± 0.1 and 76.7 ± 0.2 minutes while EGC-digallate was tentatively identified at t_R 64.6 ± 0.1 and 65.8 ± 0.1. Molecular and fragment ions obtained for standards in Q1 correspond to MS and MS/MS data from Wu *et al*, 2003, and Shen *et al*, 2006.

Closer investigation of the remainder of the PPO inactivation methods also indicated various differences. Infusions prepared from pan-fried and grilled tea leaves contained slightly less flavan-3-ols than the steamed leaves. Wang *et al* compared catechin content between steamed and roasted tea leaves and also found that catechin content is lower when roasting. They attributed this loss to the high temperatures used for roasting compared to steaming. In this study, it was found that catechin content from FBD tea leaves was slightly higher than that of the 'roasted' (pan-fry & grilling) tea leaves. This can be expected since the temperature (120°C) used during fluid bed drying was still lower than that of the roasting techniques. Also, when 'roasting', the leaves are in direct contact with the heat source/plate and flavan-3-ols may decay due to localized heat transfer. This is another event that will not be of concern when blanching.

Results for infusion colour, another quality parameter monitored during this study, expressed as L* a* b* values, are in accordance with the findings by Wang *et al*, 2003, who stated that a decrease in L* value and increases in a* and b* value indicated the development of a brown colour. It was found that the brown negative control as well as the black tea external sample had a very low L* value while a* and b* values where significantly higher than that of the test samples. Generally, over-treatment of the leaves using the dry PPO inactivation methods had the same effect on L* a* b* colour values where leaves start to darken and resulting infusions are more brown due to excessive heat exposure. This is evident in the slight decrease of L* and increase in a* and b* when grilling for more than 4 minutes, while these values do not differ significantly when steaming. Steamed and blanched tea samples had a brighter

colour (↑ L*, ↓ a*, ↓ b*) compared to the other PPO inactivation methods. Findings by Wang *et al* based on sensory analysis and effects of processing and storage suggested steaming, a wet treatment, to be a better PPO inactivation method than roasting, a dry treatment similar to pan-frying and grilling, for the production of tea beverages.

Although the volatile composition of the made tea has been neglected in this project, it should be noted that these compounds also play an important role in taste, hence quality of the final product. The different manufacturing techniques used to produce green tea causes different biochemical reactions to occur. Identification of the specific biochemical pathways (and how they are activated) causing the formation of volatiles that contribute positively to quality, will also facilitate control over the manufacturing process to produce a tea with a specific taste as required. Kumazawa *et al*, 2005, observed that one of the quality determining volatiles of Sencha tea, 4-mercapto-4-methyl-2-pentanone, varied under different roasting conditions. Therefore, once the best method of production has been determined for a specific tea conveying certain characteristics, a lot of effort is still required to optimize the method involved. Concurrently, further insight into how exactly these volatiles are being formed or synthesized will thus aid the optimization process substantially. In addition to flavan-3-ols, recent findings indicated that tea volatiles also exhibit anti-oxidant activities, but its contribution to the health benefits of tea requires further investigation (Yanagimoto *et al*, 2003).

Future studies may include the use of radioactive labelling, which is commonly used during feeding and/or *in vitro* studies where the goal is to obtain information with regard to the biosynthesis pathways in *Camellia sinensis*. Enzymes involved in the biosynthesis pathways of interest then need to be isolated, identified and characterized to be able to explain how exactly all these different pathways function as a whole. In turn, such studies will then provide astute information that may enable control or manipulation of individual quality parameters. This will also help to shed light as to why some tea trees produce high amounts of certain novel flavan-3-ols while others don't. Transgenic modification may, at a later stage, also come into play for the creation of a tea clone to fill all the different segments of the tea market (decaffeinated tea clones, high caffeine containing tea clones for extraction, high quality green tea

clones, high quality black tea clones etc.). The result of gene manipulation on the plant and its surroundings should however, be thoroughly investigated first.

SUMMARY

A concerning situation has developed over the past few years where several tea estates had to close down due to high labour costs and low profitability. Solutions are desperately required to save these estates from further regression and to prevent others from joining their ranks. One solution is to redirect the tea factories from the current production of black tea to producing a value added commodity such as a high quality green tea extract with an increased market value.

The aim of this study was to find an economically viable PPO inactivation method that can be implemented in existing tea factories for the production of high quality instant green tea. Further enhancement of quality may then be achieved by high throughput cultivar screening where those with a higher natural catechin to caffeine content will be favourable.

Six different PPO inactivation methods (steaming, blanching, fluid bed drying, pan-frying, grilling over direct heat and grilling over indirect heat) were explored. Four independent experiments were performed in duplicate with these six methods using fresh tea leaves donated by a tea estate in Tzaneen, South Africa. All samples were dried in a fluid bed drier and milled after PPO inactivation. Biochemical analysis of specific quality parameters followed where extractions from these green tea leaf samples were tested for theaflavin content, caffeine content, flavan-3-ol content, total free amino acid content, colour and taste.

The six PPO inactivation methods were compared by data evaluation of the individual quality parameters where certain quality parameters carried a higher weight than others. For the purpose of this project, the catechin to caffeine ratio was the most important quality determinant to yield a high value IGT. Also, to prove effectiveness of the PPO inactivation method, low TF content was compulsory. All PPO inactivation methods explored proved successful in rapidly inactivating PPO enzyme. As hypothesised, a significant difference in biochemical composition is brought about between green teas produced by employing different PPO inactivation methods. Blanching was found to be the most efficient PPO inactivation method as well as the

method resulting in the highest catechin to caffeine ratio (16.67:1 for 4 min blanch vs. 5.72:1 for 17 min FBD as determined by HPLC analysis). A freeze dried extract from a 1.5 minute blanched sample (IGT) was compared with foreign IGTs originating from Sri-Lanka, Kenya, China and India by HPLC analysis. A more than two fold greater catechin to caffeine ratio was obtained for the sample originating from the blanching method (9.08:1 vs. 2.81-5.02:1). A high quality, naturally low caffeine, instant green tea can therefore be produced by utilizing the blanching method of PPO inactivation.

To allow for inter and intra cultivar screening, tentative identification of novel catechins (digallated catechins) and their HPLC retention times was done using HPLC-ESI-MS/MS. Potential HPLC retention times for EC-digallate were detected at t_R 62.0 ± 0.2, 70.7 ± 0.1 and 76.7 ± 0.2 minutes while t_R 64.6 ± 0.1 and 65.8 ± 0.1 minutes were detected for EGC-digallate. With this information at hand, cultivars of a higher quality, hence increased economical potential, can be identified upon confirmation by NMR. HPLC-ESI-MS/MS screening coupled with NMR confirmation is to be continued to detect several other novel flavan-3-ols that could not be detected in the 4 IGTs of different origin used in this study.

This study gives an overview of the biochemical differences between green tea leaves prepared using six different PPO inactivation methods. The aim of this study was met by identification of the significant increase in quality brought about by PPO inactivation using the blanching method, which is also economical for use in Africa. Blanching of tea leaves caused a significant decrease in caffeine. Therefore, the 1st hypothesis, stating that the six polyphenol oxidase inactivation methods investigated will produce instant green teas with different catechin to caffeine ratios, is accepted. Also, a means to perform large scale screening of individual tea trees in Africa for their novel flavan-3-ol content was provided by tentative identification of these novel catechins by LC-MS. Thus, the 2nd hypothesis, stating that application of LC-MS will aid in the identification of HPLC retention times of compounds (novel catechins) from a crude extract, is also accepted.

REFERENCES

Ahmed, S., Wang, N., Lalonde, M., Goldberg, V.M., Haqqi, T.M. (2004). Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1β-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. *JPET,* **308**: 767-773.

Amarowicz, R., Maryniak, A., Shahidi, F. (2005). TLC separation of methylated (-)-epigallocatechin-3-gallate. *Czech J. Food Sci.*, **23** (1): 36-39.

Amarowicz, R., Shahidi, F. (2003). Presence of two forms of methylated (-)-epigallocatechin-3-gallate in green tea. *Nahrung/Food*, **47** (1): 21-23.

Astill, C., Birch, M.R., Dacombe, C., Humphrey, P.G., Martin, P.T. (2001). Factors affecting the caffeine and polyphenol contents of black and green tea infusions. *J. Agric. Food Chem.*, **49**: 5340-5347.

Aucamp, J.P., Hara, Y., Apostolides Z. (2000). Simultaneous analysis of tea catechins, caffeine, gallic acid, theanine and ascorbic acid by micellar electrokinetic capillary chromatography. *J. Chromatogr.*, **876**: 235-242.

Bandyopadhyay, D., Chatterjee, T.K., Dasgupta, A., Lourduraja, J., Dastidar, S.G. (2005). *In vitro* and *in vivo* antimicrobial action of tea: The commonest beverage of Asia. *Biol. Pharm. Bull.*, **28**(11): 2125-2127.

Baruah, A.M. (2003). Fermentation characteristics of some *Assamica* clones and process optimization of black tea manufacturing. *J. Agric. Food Chem.*, **51**: 6578-6588.

Bonoli, M., Colabufalo, P., Pelillo, M., Toschi, T.G., Lercker, G. (2003). Fast determination of Catechins and Xanthines in tea beverages by Micellar Electrokinetic Chromatography. *J. Agric. Food Chem.*, **51**: 1141-1147.

Bursill, C., Roach, P.D., Bottema, C.D.K., Pal, S. (2001). Green tea upregulates the low-density lipoprotein receptor through the sterol-regulated element binding protein in HepG2 liver cells. *J. Agric. Food Chem.*, **49**: 5639-5645.

Burton, S.G., Kirchmann, S. (1997). Optimised detergent-based method for extraction of a chloroplast membrane bound enzyme: polyphenol oxidase from tea (*Camellia sinensis*). *Biotech. Techniques*, **11** (9): 645-648.

Chen, J., Wang, P., Xia, Y., Xu, M., Pei, S. (2005). Genetic diversity and differentiation of *Camellia sinensis L.* (cultivated tea) and its wild relatives in

Yunnan province of China, revealed by morphology, biochemistry and allozyme studies. *Genetic Resourc. Crop Evolution*, **52**: 41-52.

Chen, L., Yao, M.Z., Zhao, L.P., Wang, X.C. (2006) Recent research progresses on molecular biology of tea plant (*Camellia sinensis*). *Floriculture, Ornamental and Plant Biotech,* vol. IV p 425-435.

Chen, Z., Zhu, Q.Y., Tsang, D., Huang, Y. (2001). Degradation of green tea catechins in tea drinks. *J. Agric. Food Chem.*, **49**: 477-482.

Cloughley, J.B. (1982). Factors influencing the caffeine content of black tea: Part 1 – The effect of field variables. *Food Chem.*, **9**: 269-276.

Cloughley, J.B. (1983). Factors influencing the caffeine content of black tea: Part 2 – The effect of production variables. *Food Chem.*, **10**: 25-34.

Coggon, P., Moss, G.A., Sanderson, G.W. (1973). Tea catechol oxidase: Isolation, purification and kinetic characterisation. *Phytochemistry*, **12**: 1947-1955.

Collier, P.D. (2005). ISO tea standards, International tea symposium, p66-74.

Coxon, D.T., Holmes, A., Ollis, W.D., Vora, V.C. (1972). Flavanol digallates in green tea leaf. *Tetrahedron*, **28**: 2819-2826.

Dalluge, J.J., Nelson, B.C., Brown Thomas, J., Sander, L.C. (1998). Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *J. Chromatogr.*, **793**: 265-274.

Daniel, R.M., Dines, M., Petach, H.H. (1996). The denaturation and degradation of stable enzymes at high temperatures. *Biochem. J.*, **317**: 1-11.

De Aragão, N.M., Veloso, M.C.C., Souza Bispo, M., Ferreira, S.L.C., De Andrade, B.J. (2005). Multivariate optimization of the experimental conditions for determination of three methylxanthines by reversed-phase high-performance liquid chromatography. *Talanta*, **67**: 1007-1013.

Degenhardt, A., Engelhardt,U.H., Lakenbrink, C., Winterhalter, P. (2000). Preparative separation of polyphenols from tea by high-speed countercurrent chromatography. *J. Agric. Food Chem.*, ***48***: 3425-3430.

Del Rio, D., Stewart, A., Mullen, W., Burns, J., Lean, M.E.J., Brighenti, F., Crozier, A. (2004). HPLC-MS of phenolic compounds and purine alkaloids in green and black tea. *J. Agric. Food Chem.*, **52**: 2807-2815.

Dincer, B., Colak, A., Aydin, N., Kadioglu, A., Güner, S. (2002) Characterization of polyphenoloxidase from medlar fruits (*Mespilus germania* L., Rosaceae). *Food Chem.*, **77**: 1-7.

Dragan, A., Dusanka, D., Drago, B., Vesna, R., Bono, L., Nenad, T. (2007). SAR and QSAR of the antioxidant activity of flavonoids. *Curr. Med. Chem.*, **14**: 827-845.

Du Toit, R., Volsteedt, Y., Apostolides, Z. (2001). Comparison of the antioxidant content of fuits, vegetables and teas measured as vitamin C equivalents. *Toxicology*, **166**: 63-69.

Dutta, R., Kashwan, K.R., Bhuyan, M., Hines, E.L., Gardner, J.W. (2003). Electronic nose based tea quality standardization. *Neural Networks*, **16**: 847-853.

Edelmann, A., Lendl, B. (2002). Toward the optical tongue: Flow-through sensing of tannin-protein interactions based on FTIR Spectroscopy. *J. Am. Chem. Society*, **124**: 14741-14747.

Egashira, N., Hayakawa, K., Mishima, K., Kimura, H., Iwasaki, K., Fujiwara, M. (2004). Neuroprotective effect of γ-glutamylethylamide (theanine) on cerebral infarction in mice. *Neuroscience letters*, **363**: 58-61.

Ekborg-Ott, K.H., Taylor, A., Armstrong, D.W., (1997). Varietal differences in the total and enantiomeric composition of theanine in tea. *J. Agric. Food Chem.* **45**:353-363.

Eteng, M.U., Eyong, E.U., Akpanyung, E.O., Agiang, M.A., Aremo, C.Y. (1997). Recent advances in caffeine and theobromine toxicities: a review. *Plant Foods Human Nutr.*, **51**: 231-243.

FAO. (2005). Core production data. http://faostat.fao.org/ (accessed December 2006).

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides and proteins to agricultural and biomedical sciences. *J. Agric. Food Chem.*, **52**: 385-406.

Fujimura, Y., Tachibana, H., Maeda-Yamamoto, M., Miyase, T., Sano, M., Yamada, K. (2002). Antiallergic tea catechin, (-)-epigallocatechin-3-o-(3-o-methyl)-gallate, suppresses FcεRI expression in human basophilic KU812 cells. *J. Agric. Food Chem.*, **50**: 5729-5734.

Graham, H.N. (1992). Green Tea composition, consumption, and polyphenol chemistry. *Preventive Medicine*, **21**: 334-350.

Gupta, S., Ahmad, N., Mohan, R.R., Husain, M.M., Mukhtar, H. (1999). Prostate cancer chemoprevention by green tea: In vitro and in vivo inhibition of testosterone-mediated induction of ornithine decarboxylase. *Cancer Research*, **59**: 2115-2120.

Halder, J., Tamuli, P., Bhaduri, A.N., (1998). Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis). J. Nutr. Biochem.*, **9**: 75-80.

Hasegawa, R., Chujo, T., Sai-Kato, K., Umemura, T., Tanimura, A., Kurokawa, Y. (1995). Preventive effects of green tea against liver oxidative DNA damage and hepatotoxicity in rats treated with 2-nitropropane. *Food Chem. Toxicol.*, **33** (11): 961-970.

Haslam, E. (2003). Thoughts on thearubigens. *Phytochem.*, **64**: 61-73.

Health benefits of green tea, green tea extract and EGCG: A systematic review of scientific evidence from human studies from 1998-2004. (2004). *Medical update Vitamin Information Centre*, **42**: October.

Horie, H., Kohata, K. (2000). Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. *J. Chromatogr.*, **881**: 425-438.

Hosoda, K., Wang, M., Liao, M., Chuang, C., Iha, M., Clevidence, B., Yamamoto, S. (2003). Antihyperglycemic effect of oolong tea in type 2 diabetes. *Diabetes Care*, **26**: 1714-1718.

Huang, X., Chen, Q., Wang, Q., Song, K., Wang, J., Sha, L., Guan, X. (2006). Inhibition of the activity of mushroom tyrosinase by alkylbenzoic acids. *Food Chem.*, **94**: 1-6.

Huang, Y., Sheng, J., Yang, F., Hu, Q. (2007). Effect of enzyme inactivation by microwave and oven heating on preservation quality of green tea. *J. Food Engineering*, **78**: 687-692.

Huang, Y., Xu, J., Hu, Q. (2005). Effect of selenium on preservation quality of green tea during autumn tea-processing season. *J. Agric. Food Chem.*, **53**: 7444-7447.

Itoh, Y., Yasui, T., Okada, A., Tozawa, K., Hayashi, Y., Kohri, K. (2005). Preventive effects of green tea on renal stone formation and the role of oxidative stress in nephrolithiasis. *J. Urol.*, **173** (1): 271-275.

Jain, N.K., Siddiqi, M.A., Weisburger, J.H. (2006). Protective effects of tea on human health. *CAB international* 2006. pp 60, 93, 121.

John, K.M.M., Vijayan, D., Rahul, P.R., Joshi, S.D., Kumar, R.R., Mandal, A.K.A. (2006). Electrofocusing of methanolic extracts for identification of individual flavonol biomolecules in *Camellia* species. *J. Agric. Food Chem.*, **54**: 2828-2831.

Kagaya, N., Hara, Y., Saijo, R., Kamiyoshi, A., Tagawa, Y., Kawase, M., Yagi, K. (2003). Novel function of rare catechin, epigallocatechin-3-(3"-o-methyl)gallate, against cold injury in primary rat hepatocytes. *J. Biosci. Bioeng.*, **96** (6): 559-563.

Kaneko, S., Kumazawa, K., Masuda, H., Henze, A., Hofmann, T. (2006). Molecular and sensory studies on the umami taste of Japanese green tea. *J. Agric. Food Chem.*, **54**: 2688-2694.

Katiyar, S.K., Afaq, F., Perez, A., Mukhtar, H. (2001). Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis*, **22**: 287-294.

Kato, M., Mizuno, K., Crozier, A., Fujimura, T., Ashihara, H. (2000). Caffeine synthase gene from tea leaves. *Nature*, **406**: 956-957.

Kato, M., Shibamoto, T. (2001). Variation of major volatile constituents in various green teas from Southeast Asia. *J. Agric. Food Chem.*, **49**: 1394-1396.

Kerrigan, S., Lindsey, T. (2005). Fatal caffeine overdose: Two case reports. *Forensic Sci. Intern.*, **153**: 67-69.

Kim, Y.J., Uyama, H. (2005). Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell. Mol. Life Sci.*, **62**: 1707-1723.

Koshiishi, C., Ito, E., Kato, A., Yoshida, Y., Crozier, A., Ashihara, H. (2000). Purine alkaloid biosynthesis in young leaves of Camellia sinensis in light and darkness. *J. Plant Res.*, **113**: 217-221.

Kumamoto, M., Sonda, T., Takedomi, K., Tabata, M. (2000). Enhanced separation and elution of catechins in HPLC using mixed-solvents of water, acetonitrile and ethyl acetate as the mobile phase. *Analyt. Sci.*, **16**: 139-144.

Kumazawa, K., Kubota, K., Masuda, H. (2005). Influence of manufacturing conditions and crop season on the formation of 4-mercapto-4-methyl-2-pentanone in Japanese green tea (Sencha). *J. Agric. Food Chem.*, **53**: 5390-5396.

Lee, B., Ong, C. (2000). Comparative analysis of tea catechins and theaflavins by high performance liquid chromatography and capillary electrophoresis. *J. Chromatogr.*, **881**: 439-447.

Lee, M., Lambert, J.D., Prabhu, S., Meng, X., Lu, H., Maliakal, P., Ho, C., Yang, C.S. (2004). Delivery of tea polyphenols to the oral cavity by green tea leaves and black tea extract. *Cancer Epidem. Biomark. Prevent.*, **13**: 132-137.

Lee, M., Prabhu, S., Meng, X., Li, C., Yang, C.S. (2000). An improved method for the determination of green and black tea polyphenols in biomatrices by High-Performance Liquid Chromatography with Coulometric Array Detection. *Analyt. Biochem.*, **279**: 164-169.

Lee, S.Y., Lee, J.W., Lee, H., Yoo, H.S., Yun, Y.P., Oh, K.W., Ha, T.Y., Hong, J.T. (2005). Inhibitory effect of green tea extract on β-amyloid-induced PC12 cell death by inhibition of the activation of NF-κB and ERK/p38 MAP kinase pathway through antioxidant mechanisms. *Mol. Brain Research*, **140**: 45-54.

Lesschaeve, I., Noble, A.C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *Am. J. Clin. Nutr.*, **8** (supl.): 330s-335s.

Leung, .K., Su, Y., Chen, R., Zhang, Z., Huang, Y., Chen, Z. (2001). Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr.*, **31**: 2248-2251.

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

Liang, Y., Lu, J., Zhang, L., Wu, S., Wu, Y. (2005). Estimation of tea quality by infusion colour difference analysis. *J. Agric. Food Chem.*, **85**: 286-292.

Liu, S., Lu, H., Zhao, Q., He, Y., Niu, J., Debnath, A.K., Wu, S., Jiang, S. (2005). Theaflavin derivatives in black tea and catechin derivatives in green tea inhibit HIV-1 entry by targeting gp41. *Biochimica et Biophysica Acta*, **1723**: 270-281.

Lu, Y., Guo, W., Yang, X. (2004). Fluoride content in tea and its relationship with tea quality. *J. Agric. Food Chem.*, **52**: 4472-4476.

Maeda-Yamamoto, M., Inagaki, N., Kitaura, J., Chikumoto, T., Kawahara, H., Kawakami, Y., Sano, M., Miyase, T., Tachibana, H., Nagai, H., Kawakami, T. (2004). O-Methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. *J. Immunol.*, **172**: 4486-4492.

Mahanta, P., Baruah, H. (1992). Theaflavin pigment formation and polyphenol oxidase activity as criteria of fermentation in orthodox and CTC teas. *J. Agric. Food Chem.*, **40**: 860-863.

Mayer, A.M., Harel, E. (1979). Polyphenol oxidases in plants. *Phytochem.*, **18**: 193-215.

Menet, M., Sang, S., Yang, C.S., Ho, C., Rosen, R.T. (2004). Analysis of theaflavins and thearubigens from black tea extract by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.*, **52**: 2455-2461.

Moore, S., Stein, W. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.*, **176**: 367-388.

Nagai, H., Maeda-Yamamoto, M., Suzuki, Y., Sato, K., Mitsuda, H. (2005). The development of a suitable manufacturing process for 'Benifuuki' green tea beverage with anti-allergic effects. *J. Sci. Food Agric.*, **85**: 1606-1612.

Nagai, T., Suzuki, N. (2001). Partial purification of polyphenol oxidase from Chinese cabbage *Brassica rapa* L. *J. Agric. Food Chem.*, **49**: 3922-3926.

Nakakuki, H., Horie, H., Yamauchi, Y., Kohata, K. (1999). Rapid analysis of methylated xanthines in teas by an improved high-performance liquid chromatographic method using a polyvinylpolypyrroridone pre-column, *J. Chromatogr.*, **848**: 523-527.

Official methods of analysis of AOAC international. (1995). by Cunniff P., 16th edition, AOAC method 969.15.

Ohnsmann, J., Quintás, G., Garrigues, S., De la Guardia, M. (2002). Determination of caffeine in tea samples by Fourier transform infrared spectrometry. *Anal. Bioanalyt. Chem.*, **374**: 561-565.

Owuor, P.O., Chavanji, A.M. (1986). Caffeine contents of clonal tea; Seasonal variations and effects of plucking standards under Kenyan conditions. *Food Chem.* **20**: 225-233.

Owuor, P.O., Obanda, A.M., Tsushida, T., Horita, H., Murai, T. (1987). Geographical variation of theaflavins, thearubigins and caffeine in Kenyan clonal black teas. *Food Chem.* **26**: 223-230.

Pan, X., Niu, G., Liu, H. (2003). Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. *Chem. Eng. Proces.*, **42**: 129-133.

Price, W.E., Spitzer, J.C. (1994). The kinetics of extraction of individual flavanols and caffeine from a Japanese green tea (Sen Cha Uji Tsuyu) as a function of temperature. *Food Chem.*, **50**: 19-23.

Poon, G.K. (1998). Analysis of catechins in tea extracts by liquid chromatography-electrospray ionizaton mass spectrometry. *J. Chromatogr*. **794**: 63-74.

Rapeanu, G., Van Loey, A., Smout, C., Hendrickx, M. (2006). Biochemical characterization and process stability of polyphenoloxidase extracted from Victoria grape (*Vitis vinifera* ssp. *Sativa*). *Food Chem.*, **94**: 253-261.

Reeves, S.G., Gone, F., Owuor, P.O. (1985). Theaflavin analysis of black tea-problems and prospects. *Food Chem.*, **18**: 199-210.

Roberts, E.A.H., Smith, R.F. (1963). The phenolic substances of manufactured tea. IX. – the spectrophotometric evaluation of tea liquors. *J Sci. Food Agric.*, **14**(10): 689-700.

Roberts, E.A.H., Wood, D.J. (1951). The amino-acids and amides of fresh and withered tea-leaf. *Curr. Sci. Assoc.*, **20**: 151-153.

Robertson, A., Hall, M.N. (1989). A critical investigation into the Flavognost method for theaflavin analysis in black tea. *Food Chem.*, **34**: 57-70.

Romani, A., Minnuni, M., Mulinacci, N., Pinelli, P., Vincieri, F.F. (2000). Comparison among differential pulse voltammetry, amperometric biosensor and HPLC/DAD analysis for polyphenol determination. *J. Agric. Food Chem.*, **48**: 1197-1203.

Ruan, J., Wu, X., Härdter, R. (1999). Effects of potassium and magnesium nutrition on the quality components of different types of tea. *J. Sci. Food Agric.*, **79**: 47-52.

Ruan, J., Wu, X., Ye, Y., Härdter, R. (1998). Effects of potassium, magnesium and sulphur applied in different forms of fertilisers on free amino acid content in leaves of tea (*Camellia sinensis* L). *J. Sci. Food Agric.*, **76**: 389-396.

Sadzuka, Y., Inoue, C., Hirooka, S., Sugiyama, T., Umegaki, K., Sonobe, T. (2005). Effects of theanine on alcohol metabolism and hepatic toxicity. *Biol. Pharm. Bull.*, **28** (9): 1702-1706.

Saijo, R. (1982). Isolation and chemical structures of two new catechins from fresh tea leaf. *Agric. Biol. Chem.*, **46**: 1969-1970.

Sakanaka, S. (2003). A novel convenient process to obtain a raw decaffeinated tea polyphenols fraction using a lignocellulose column. *J. Agric. Food Chem.*, **51**: 3140-3143.

Sang, S., Lambert, J.D., Tian, S., Hong, J., Hou, Z., Ryu, J., Stark, R.E., Rosen, R.T., Huang, M., Yang, C.S., Ho, C. (2004). Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. *Bioorgan. Medicin. Chem.*, **12**: 459-467.

Sanderson, G.W. (1963). The chloroform test - A study of its suitability as a means of rapidly evaluating fermenting properties of clones. *Tea Quarterly*, **34**: 193-196.

Sano, M., Suzuki, M., Miyase, T., Yoshino, K., Maeda-Yamamoto, M. (1999). Novel antiallergic catechin derivatives isolated from oolong tea. *J. Agric. Food Chem.*, **47**: 1906-1910.

Sano, M., Tabata, M., Degawa, M., Miyase, T., Maeda-Yamamoto, M. (2001). Simultaneous determination of twelve tea catechins by high-performance liquid chromatography with electrochemical detection. *Analyst*, **126** (6): 816-820.

Scharbert, S., Holzmann, N., Hofmann, T. (2004). Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.*, **52**: 3498-3508.

Sharma, V., Gulati, A., Ravindranath, S.D. (2005). Extractability of tea catechins as a function of manufacture procedure and temperature of infusion. *Food Chem.*, **93**: 141-148.

Shen, D., Wu, Q., Wang, M., Yang, Y., Lavoie, E.J., Simon, J.E. (2006). Determination of the predominant catechins in Acacia catechu by liquid chromatography/electrospray ionization-mass spectrometry. *J. Agric. Food Chem.*, **54**: 3219-3224.

Shulz, H., Engelhardt, U.H., Wegent, A., Drews, H., Lapczynski, S. (1999) Application of near-infrared reflectance spectroscopy to the simultaneous prediction of alkaloids and phenolic substances in green tea leaves. *J. Agric. Food Chem.*, **47**(12): 5064-5067.

Smit, H.J., Blackburn, R.J. (2005). Reinforcing effects of caffeine and theobromine as found in chocolate. *Psychopharmacol.*, **181**: 101-106.

Sood, C., Jaggi, S., Kumar, V., Vipin Kumar, Ravindranath, S. D., Adarsh Shanker. (2004). How manufacturing processes affect the level of pesticide residues in tea. *J. Sci. Food Agric.*, **84**: (15) 2123-2127.

Spiro, M., Price, W.E. (1986). Determination of Theaflavins in tea solution using the flavagnost complecation method. *Analyst*, **111**: 331-333.

Subramanian, N., Venkatesh, P., Ganguli, S., Sinkar, P.S. (1999). Role of polyphenol oxidaze and peroxidase in the generation of black tea theaflavins. *J. Agric. Food Chem.*, **47**: 2571-2578.

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. *J. Agric. Food Chem.*, **48**: 5649-5653.

Takeuchi, A., Matsumoto, S., Hayatsu, M. (1994). Chalcone synthase from Camellia sinensis: isolation of the cDNAs and the organ-specific and sugar-responsive expression of the genes. *Plant Cell Physiol.*, **35**(7): 1011-1018.

Temple, S.J., Temple, C.M., Boxtel, A.J.B., Clifford, M.N. (online 2001). The effect of drying on black tea quality. *J. Sci. Food Agric.*, **81**: 764-772.

Thangapazham, R.L., Singh, A.K., Sharma, A., Warren, J., Gaddipati, J.P., Maheshwari, R.K. (2007). Green tea polyphenols and its constituent epigallocatechin gallate inhibit proliferation of human breast cancer cells in vitro and in vivo. *Cancer Letters*, **245**: 232-241.

Thomas, J., Saravanan, M., Kumar, R.R., Pius, P.K. (2005). Influence of age after pruning on the levels of flavanols and other bioconstituents in tea (Camellia sinensis). *J. Sci. Food Agric.*, **85**: 931-934.

Tian, W., Li, L., Wu, X., Chen, C. (2004). Weight reduction by Chinese medicinal herbs may be related to inhibition of fatty acid synthase. *Life Sci.*, **74**: 2389-2399.

Tsushida, T., Murai, T. (1985). Selective extraction of caffeine with hot water from instant tea leaves. *Nippon Nôgeikagaku Kaishi,* **59** (9): 917-919.

Uefuji, H., Tatsumi, Y., Morimoto, M., Koathien-Nakayama, P., Ogita, S., Sano, H. (2005). Caffeine production in tobacco plants by simultaneous expression of three coffee N-methyltransferases and its potential as a pest repellent. *Plant Mol. Biol.*, **59**: 221-227.

Vuataz, I., Brandenberger, H., Egli, R.H. (1959). Pant phenols I. Separation of the tea leaf polyphenols by cellulose column chromatography. *J. Chromatogr.*, **2**: 173-187.

Wachira, F.N., Tanaka, J., Takeda, Y. (1995). Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome*, **38**: 201-210.

Wang, L., Kim, D., Lee, C. (2000). Effects of heat processing and storage on flavanols and sensory qualities of green tea beverage, *J. Agric. Food Chem.*, **48**: 4227-4232.

Wang, X., Hu, S., Wan, X., Pan, C. (2005). Effect of microbial fermentation on caffeine content of tea leaves. *J. Agric. Food Chem.*, **53**: 7238-7242.

Wright, L.P. (2005). Biochemical analysis for identification of quality in black tea *(Camellia sinensis)*, PhD thesis, University of Pretoria, Pretoria, South Africa: 63.

Wright, L.P., Aucamp, J.P., Apostolides, Z. (2001). Analysis o f black tea theaflavins by non-aqueous capillary electrophoresis. *J. Chromatogr*., **919**: 205-213.

Wright, L.P., Mphangwe, N.I.K., Nyirenda, H.E., Apostolides, Z. (online: 2000). Analysis of caffeine and flavan-3-ol composition in the fresh leaf of *Camellia sinensis* for predicting the quality of the black tea produced in Central and Southern Africa. *J. Sci. Food Agric*., **80**: 1823-1830.

Wright, L.P., Mphangwe, N.I.K., Nyirenda, H.E., Apostolides, Z. (2002). Analysis of the theaflavin composition in black tea (*Camellia sinensis*) for predicting the quality of tea produced in Central and Southern Africa. *J. Agric. Food Chem.*, **82**: 517-525.

Wu, Q., Wang, M., Simon, J.E. (2003). Determination of proanthocyanidins in grape products by liquid chromatography/mass spectrometric detection under low collision energy. *Analyt. Chem.*, **75**: 2440-2444.

Xiaogang, W., Xiaochun, W., Yinghui, H., Xiaoqiu, L. (2005). Study on the improvement upon ultraviolet spectrophotometry method for determination of caffeine in Chinese green teas, International Tea Symposium, p 702-708.

Yanagimoto, K., Ochi, H., Lee, K., Shibamoto, T. (2003). Antioxidative activities of volatile extracts from green tea, oolong tea, and black tea. *J. Agric. Food Chem.*, **51**: 7396-7401.

Yang, Y.C., Lu, F.H., Wu, J.S., Wu, C.H., Chang, C.J. (2004). The protective effect of habitual tea consumption on hypertension. *Arc. Intern. Med*., **164** (14): 1534-1540.

Yao, L., Liu, X., Jiang, Y., Caffin, N., D'Arcy, B., Singanusong, R., Datta, N., Xu, Y. (2006). Compositional analysis of teas from Australian supermarkets. *Food Chem.*, **94**: 115-122.

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

Zellner, D.A., Durlach, P. (2002). What is refreshing? An investigation of the colour and other sensory attributes of refreshing foods and beverages. *Appetite*, **39**: 185-186.

Zhang, Q., Lian, H., Wang, W., Chen, H. (2005). Separation of caffeine and theophylline in poly(dimethylsilozane) microchannel electrophoresis with electrochemical detection. *J. Chrom*., **A1098**:172-176.

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