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Biochemical analysis for identification of quality in black tea *(Camellia sinensis)*

By: Louwrance Peter Wright

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List of Abreviations

2-D PC	Two-dimensional paper chromatography
ACN	Acetonitrile
BSA	Bovine serum albamin
C	(+)-Catechin
cDNA	complementary DNA
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CHS	Chalcone synthase
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CoA	Coenzyme A
CTC	Cut tear and curl maceration machine
CZE	Capillary zone electrophoresis
DMF	*N*,*N*-Dimethylformamide
DNA	Deoxyribonucleic acid
DT	Dried tea
DTL	Dried tea leaf
EC	(-)-Epicatechin
ECg	(-)-Epicatechin gallate
EGC	(-)-Epigallocatechin

EGCg	(-)-Epigallocatechin gallate
EOF	Electroosmotic flow
GC	Gas chromatography
GLC	Gas-liquid chromatography
HPLC	High performance liquid chromatography
IBMK	Isobutyl methyl ketone
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LDL	Low-density lipoproteins
LOD	Limit of detection
MEEKC	Microemulsion electrokinetic capillary chromatography
MEKC	Micellar electrokinetic capillary chromatography
MeOH	Methanol
MPU	Mini processing unit
MT	Made tea
NACE	Non-aqueous capillary electrophoresis
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NMR	Nuclear magnetic resonance spectroscopy
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonium lyase
POD	Peroxidase
PPO	Polyphenol oxidase
RFLP	Restriction fragment length polymorphism

RSD	Relative standard deviation
S/N	Signal to noise
SDS	Dodecyl sulphate
SIF	Sum of individual flavan-3-ols
SIT	Sum of the individual TF
TAL	Tyrosine ammonium lyase
TCU	Tea clarification units
TF	Theaflavin
TF-A	Theaflavin-3-monogallate
TF-B	Theaflavin-3'-monogallate
TF-dg	Theaflavin-3,3'-digallate
TF-f	Free theaflavins
TLC	Thin layer chromatography
TR	Thearubigin
TRF(CA)	Tea Research Foundation of Central Africa
UDP	Uridine 5'-diphosphate

Chapter 1

Introduction

1 Historical importance of tea.

Tea has been cultivated and consumed in China for more than two thousand years (Dodd, 1994). Today it is the most widely consumed caffeine-containing beverage in the world. Its worldwide consumption is second only to water. The importance of tea is however much more than just the consumption of it by millions of people. Tea also had a marked effect on human development in both the East and West.

Tea arrived in London for the first time during 1652. During this time the hygiene in Europe was dubious. Because of the threat of waterborne disease, water was often boiled before drinking. The addition of tea gave it both flavour, and stimulative properties due to the presence of caffeine. Although coffee and chocolate also became available in Europe during the same time, it was unaffordable for the general public until after the Second World War. The only other alternative was alcohol strong enough to kill the waterborne pathogens. Since people do not work, learn or function properly while constantly drunk, this was impractical.

The East India Companies started the tea trade. These groups of European merchants started to sail the seas in search of valuable commodities since the end of the 16th

century. They bought the tea in Canton, however, the Chinese demanded to be paid in silver. This practice led to massive inflation in Europe as silver prices in Europe climbed. The rising popularity of tea only worsened this situation. In 1801 each person in England consumed more than a kilogram of tea. This prompted the East India Company to find some other trading commodity to replace silver. They found this in opium. This highly addictive drug they produced from the opium poppy in India. The Chinese were willing to provide tea in return for opium, which was illegal in China. By 1830 the British exported 1.5 million kg of opium to China annually. This nearly destroyed Chinese society by leaving millions addicted. This also led to the Opium War (1840-1842) between Britain and China. By winning the war the British forcibly opened China to trade and ravaged the country's cultural and intellectual heritage. A society, which was centuries ahead of the rest of the world in inventions and technology, was consequently ruined by opium. Arguably this also prompted the communist uprising of the 20th century.

Tea also influenced the history of other parts of the world. Most of the tea now originates from India, Sri Lanka, Indonesia and Africa, where the Europeans introduced tea. Today India is the biggest producer of tea in the world, and two-thirds of that produced is for home consumption. Before 1840 tea drinking was unknown in India. In the United States of America, tea also played a crucial role in the build-up to the War of Independence in America. In 1773 a group of colonials objected to British taxes on tea. That led to the famous Boston Tea Party when they dumped the cargo of three tea ships into the harbour of Boston, Massachusetts (Fullick 1999).

2 Manufacturing of black tea.

In its simplest form the manufacture of tea entails the collection and drying of tea leaf. The manufacturing practices and factory equipment, however, plays a vital role in the optimum development of flavour and quality and the production of an attractive and clean product. Since the selling price of tea depends on its quality, the manufacturing practices are very important. Basically the manufacturing of tea consists of operations in the following order.

1) Partial removal of moisture (withering).
2) Leaf disruption into small pieces (maceration).
3) Quality development by exposure to air (fermentation).
4) Completion of moisture removal (drying).
5) Sieving into size fractions with fibre removal (sorting).

The withering stage is important for chemical changes taking place and for the reduction of the moisture levels from 75–80% to 55-70% depending on the specific manufacturing process being used. The withering time is usually between 12 and 16 h, during which time air is blown through the leaves in order to carry away the moisture. It is important to keep the leaves near ambient temperature since overheating leads to adverse chemical reactions with an adverse effect on the quality.

The purpose of cutting is to reduce the size of the leaf particles as well as cell disruption. By breaking up the cell compartments, the cytoplasmic polyphenol oxidase can come into

contact with its flavan-3-ol substrates in the vacuole. This is important for the oxidation process taking place during the next fermentation stage.

During the fermentation stage the polyphenols are oxidized to form the characteristic compounds of black tea. The biochemical and chemical reactions taking place during this stage are exothermic. The rise in temperature must be controlled to prevent a drop in tea quality due to unwanted secondary reactions taking place too quickly. Passing air through the macerated leaf provides temperature control as well as the provision of oxygen needed for the oxidation reactions. As a result of the temperature control function, leaves are treated with air quantities in vast excess of that needed for the fermentation process only. The duration of the fermentation stage is around 60 minutes, but can be much shorter or longer depending on the factory machinery used to disrupt the leaf and the ambient temperature.

After the fermentation the moist leaf is dried using heated air. The drying process is important for some of the flavour characteristics of the final product. The increased temperature and reduction of moisture levels in the leaf has also the effect of denaturing and inactivating the enzymes to preserve the black tea. Tea enters the drier with 60-72% moisture and is dried down to moisture content of 2.5-3.5%.

Sorting is used to separate the different sized tea particles into even sized groups and to remove the fibres and stalk in the tea. The stalk and fibre are removed by electrostatically charged rollers, which attract the fibre and stalk preferentially. After sorting, the tea is packed and transported to the market (Hampton 1992).

3 Conditions affecting quality

The quality of the black tea produced in Southern Africa, based on organoleptical evaluation, has always been “medium” by international standards. This prompted the Tea Research Foundation of Central Africa TRF(CA) to adopt a long-term tea plant improvement programme in 1956 with the main emphasis on quality. A wide range of local tea plants with differing characteristics was used as starting material for the plant improvement programme (Ellis and Nyirenda, 1995).

For quality assessment, quantitative biochemical tests were developed to monitor biochemical reactions taking place during fermentation and black tea manufacturing. The most reliable biochemical methods are based on assays of polyphenol oxidase activity and the catechin content of tea plants. These factors are best manifested in the theaflavin (TF) and thearubigin (TR) content in the black tea beverage. Another biochemical indicator of the quality of brewed black tea is the “volatile flavour index”, based on the volatile compounds in black tea (Owuor *et al*, 1988).

Together with the manufacturing practices followed, black tea quality is also influenced by a variety of environmental and horticultural factors. Environmental factors include variations in temperature, rainfall and the amount of sunlight. Horticultural factors include fertilisation, plucking standards and the frequency of plucking. A specific clone is thus evaluated in relation to other clones. Testing includes manufacturing of tea from samples in a small

factory. Tea is then judged organoleptically by an experienced tea taster on the basis of taste, aroma, colour and mouth feel (Ellis and Nyirenda, 1995).

Since much emphasis in this work was in the development of biochemical understanding of black tea quality, the concept of tea quality will be discussed more thoroughly in the remainder of this section.

The quality of the tea is very important since it depicts the price. Any increase in prize will thus result in an increase in the profit and economic viability of a tea estate. Several factors affect the quality of black tea. These can be roughly divided into three categories, namely; (a) environmental influences on the quality, (b) the manufacturing practices used in the factory and (c) the genetical make-up of the clones.

3.1 The environment and quality

Climatic conditions are harsh in Southern Africa for producing high quality tea. A hot, wet summer is usually followed by a cool, dry winter and a hot, dry spring, resulting in regular moisture stresses. The Southern Africa region also experiences changing rainfall patterns at about ten year intervals. One prerequisite is thus for drought and heat tolerant plants.

The environment has several profound influences on the quality of tea. Most noticeably of these in Southern Africa are temperature and soil moisture content. This part of the world experience considerable climatic changes throughout the year. During the hot wet months of

December to April most of the crop is harvested. This is followed by the colder and shorter days during May to July with almost zero growth rates. From August to September the warmer weather initiates some growth. In Central Africa the next months of October to November typically have low soil moisture accompanied by extreme temperatures that slow or halt the growth during these periods (Hilton *et al*, 1973). In South Africa the raining season usually starts earlier (October – November).

Hilton *et al* (1973) showed that in Central Africa the quality of the tea was inversely related to the growth rate of the tea bush. The faster the tea growth, the lower the quality. This was also reflected in the flavan-3-ol content of the tea as well as the theaflavin content of the made black tea. In fast growing tea the flavan-3-ol concentration decrease whereas slow growing tea bushes have a higher concentration of flavan-3-ols and the ratio of ungallated flavan-3-ols to gallated flavan-3-ols increase. The same tendency of higher quality tea produced during slower growing periods of the year was observed by Gulati and Ravindranath (1996) for black tea produced in Himachal Pradesh, India. They, however, only looked at the quality of the various growth flushes during the season. They observed the highest contents of theaflavins, thearubigins and caffeine during early flush with a gradual decrease to a minimum during main flush and then a slight improvement during backend flush. The same results were obtained by Sud and Baru (2000) for Kangra orthodox black tea in Himachal Pradesh, India. They once again observed a decrease in the TF and TR levels as well as a decrease in quality during the fast growing rainy seasons. They, however, concluded that this decrease in black tea quality were partly due to a low degree of withering and high chlorophyll content. When they circulated hot dry air through the

withering troughs to increase the evaporation of leaf moisture, the quality of the tea also increased.

Soil fertility also has a significant effect on the quality of made black tea. Hilton *et al* (1973) found that the addition of nitrogen fertiliser had a deleterious effect on the quality of black tea. This effect was contributed to the increased growth rate of the tea after fertiliser application. Ruan *et al* (1998) observed an increase in the amino acid content of green tea produced after the application of potassium, magnesium and sulphur in different forms of fertiliser. The free amino acid content in green tea is positively correlated with the quality of the green tea. The increased amino acid content of the leaves was probably contributed to an increased activity of nitrate reductase. The effect of these ions on the quality of black tea is not known.

Altitude is also known to have a marked effect on the quality of made black tea. Good proof for this come from the work done by Owuor *et al* (1990). They compared the quality of black tea produced from different plots of land within a 10 km radius. The different plots of tea were treated similarly, the only difference being the altitude of the plots. They observed an increase in the quality of the tea with an increase in altitude. This increase in quality was explained by the decrease in growth rate with an increase in altitude.

Plucking standard also has an important aspect on the quality of the black tea. The plucking standard describes the average size and degree of growth of shoots plucked from a tea garden. This has a large effect on the yield and quality of the tea. Generally the plucking

standard can be explained as fine, medium or coarse. With fine plucking only the first two leaves and the bud are plucked, with medium plucking half the shoots consists of two and a bud and the other half of three and a bud, with coarse plucking the majority of the shoots consists of three and bud or four and a bud. Ellis and Grice (1983) showed a significant influence of the plucking standard on the quality of the made black tea. The finer the plucking standard the higher the quality of the tea. They could, however, find no significant effect of the plucking intervals on the quality of the made black tea. Due to the pattern of shoot growth, taking shoots when they are small leads to a more rapid regeneration of more shoots. However, because of the exponential nature of shoot development a greater weight of leaf will be produced in the form of larger shoots over a longer period of time, despite their lesser number. In practise, longer plucking intervals are accompanied with a coarser plucking standard due to the increase in the number of mature leaves (Owuor and Odhiambo, 1990). It thus seems that the effect of plucking interval on the quality of black tea observed by some researchers are rather due to the influence on the plucking standard. Plucking standard also has a marked effect on the yield. The coarser the plucking standard the higher the yield. On an economical basis a compromise must be reached between the increased quality of fine plucked leaf versus the increased yield of coarser plucked tea. In the field mostly a medium plucking standard is used (Mitini-Nkhoma, 1989). To maintain the tea bushes in a manageable condition, the bushes are pruned every few years. Pruning is accompanied by increased growth rate. Owuor and Langat (1988) observed a decrease in the quality of the tea due to the increased vigour of the bush. With an increase in time from pruning, the quality of the black tea increased.

In conclusion it can be seen that the environment has a marked affect on the quality of black tea. Environmental conditions and plucking practices that promote faster growth or coarser plucked shoots lead to a decrease in the quality of the produced tea. In practice, though, a compromise must be reached between the increased yield of fast growing and coarse plucked tea with the increased quality of slower growing and finer plucked tea.

3.2 Manufacture practices and quality.

Various stages of the manufacturing process are critical for the quality of the final product. The first step in manufacture entails the withering stage. The withering consists of a chemical wither and a physical wither. The chemical wither entails all the biochemical and chemical changes taking place during the withering stage and the physical whither entails the moisture loss of the leaf during the withering stage. Various studies have shown that both the physical and the chemical wither must take place to produce high quality teas. Owour *et al* (1987) and Owour and Orchard (1990) showed that the black tea composition and quality varies when green leaf from which they were manufactured was withered to the same degree but for different periods. Other research (Owuor *et al*, 1989) showed that not just the moisture content after withering (physical wither), but also the duration of withering and the stage during withering when the moisture was lost, play a significant role in the quality of the final product. The leaf temperature during the withering process also has an influence on the black tea quality (Owour and Obanda, 1996), with lower temperatures (below 30ºC) resulting in better quality teas. The plucking standard of the leaf was also shown to affect the influence of the withering process on the quality of the black tea

(Obanda and Owour, 1994). The coarser the plucking standard, the higher the influence of the withering stage on the quality of the black tea manufactured.

The cutting process and its influence on quality are mostly controlled by the machine or combination of machines used to disrupt the tea leaf. In cutting the leaf three factors are important to consider in assuring the quality of the tea. The particle sizes of the cut leaf, the amount of cell disruption important for the ensuing fermentation stage, and heat generation. Heat is produced due to the mechanical process and due to the chemical reactions taking place by the exposure of the freshly cut leaf surfaces to the air. An increase in the temperature is usually accompanied by a decrease in the tea quality. The different leaf cutting machines will differ in their ability to disrupt cells, the particle size of the cut leaf and their heat generation capabilities. The five cutting machines most commonly used in the tea industry are the LTP machine (Lawrie Tea Processor), the CTC machine (crush, tear, curl), the orthodox roller, the rotorvane, the BCR machine (Boruah Continuous Roller) and the Legg cutting machine. The leaf moisture after withering has a significant effect on the particle sizes of the produced tea and must be optimised for each of the cutting procedures used in the factory (Hampton, 1992).

During the fermentation step most of the enzymatic and chemical reactions associated with the making of black tea occur. These reactions are dominated by the oxidation of polyphenols, mainly flavan-3-ols, to form theaflavins and thearubigins. The synthesis of volatile flavour compounds during this stage also play an important role in specifically the more aromatic teas e.g. the Kenyan black teas. Various parameters were monitored to

optimise the fermentation stage in the factory. These include monitoring of the reduction of the flavan-3-ols, measurement of the colour change and the measurement of TF and TR formation (Owour *et al*, 1994). For Central African teas and Southern African teas the analyses of TF levels were shown to be the best method for optimising the fermentation stage (Cloughley, 1977).

Manufacturing practices that have the highest influence on the quality of the tea is temperature and fermentation time. Various studies on Kenyan black teas have shown that the TF contents and tasters' evaluations change in a quadratic manner with fermentation time. These studies also showed that the formation of the individual TFs differed in each clone and also between different clones. Since the astringencies of the individual TFs differ, the astringencies of black teas will differ with the composition and total amount of TFs. Therefore it was concluded that for Kenyan black teas the measurement of total theaflavins may not correctly predict total astringency and hence value of the tea (Owour *et al*, 1994; Owuor and McDowell, 1994; Owuor and Obanda, 1998). Research in Kenya showed that the maximum levels of TFs were reached after approximately 90 min of fermentation (Owuor and Obanda, 1998), whereas Turkish black tea reached their maximum level of TFs after approximately 80 min of fermentation (Tufekci and Guner, 1997). These differences in the optimum fermentation times are most probably due to changes in fermentation temperature as well as polyphenol oxidase (PPO) activity. The temperature of fermentation also has a marked effect on the quality of the produced black teas (Cloughley, 1977). With low temperature fermentation, the rate of TF formation was reduced, however the maximum TF levels were higher than the teas produced with higher fermentation temperatures. Higher

fermentation temperatures resulted in higher levels of TRs, at the expense of TF levels. This reduction of TF levels at higher fermentation temperatures was most probably due to denaturation of PPO (Cloughley, 1977).

3.3 The genetical make-up of the tea plant and quality.

Due to their genetics there are significant differences between the individual clones. For their ability to produce high quality black tea the contents of flavan-3-ols of the different clones are of most importance. Although the absolute amounts of the flavan-3-ols will be influenced by environmental conditions, the relative quantities will remain constant. The effect of the flavan-3-ol contents in the green tea leaf on the quality of the made black tea will be discussed more thoroughly in chapter 2 of this thesis. Most of the flavan-3-ols are, however, oxidised and polymerised during the fermentation stage into the dimeric TFs and the poorly understood polymer like TRs. The importance of the flavan-3-ol profiles in the green leaf is thus not directly responsible for the quality attributes in the final black tea product. The flavan-3-ol contents in the leaves will, however, play a significant role in the amounts and identities of the oxidised polyphenolic compounds in the black tea. For Southern African teas correlations between the TF content of the black tea and quality were shown previously. In chapter 3 an in depth discussion on the importance of the TFs for quality, and on the relationship between the flavan-3-ol contents and the TFs produced, will be given.

Another inherited trait with a large influence on the quality of black tea is PPO activity. Tests for PPO activity showed that this enzyme varies widely between clones. Black teas also possess a spectrum of volatile flavour compounds that are very important for the quality of the final product. This ability to produce flavour is also an inherited trait coming mainly from China hybrid teas. Flavour is highly dependent on the environmental conditions with conditions resulting in slow plant growth contributing to favourable flavour (Ellis and Nyirenda, 1995).

4 Tea breeding

4.1 The taxonomy and genetic background of tea

The genus *Camellia* with its 82 species belongs to the family *Theaceae*. All the tea clones in cultivation are botanically called *C. sinensis* (L) O. Kuntze, irrespective of species-specific differences. Tea is a heterogeneous plant with many overlapping morphological, biochemical and physiological attributes. Most vegetative characteristics of tea show a continuous variation and a high degree of plasticity, and hence, cannot be separated into discrete groups to identify various taxa. Hardly any vegetative feature can be said to have a discontinuous variation.

Based on leaf pose and growth habitat, Kitamura and later Sealy, identified two intra-specific forms of *C. sinensis* (L.), the China variety, *Camellia sinensis* var. *sinensis* (L.) and the Assam variety, *Camellia sinensis* var. *assamica* (Masters) Kitamura. Sealy also

recognised two distinct forms under *C. sinensis* var. *sinensis*, namely f. *parviflora* and f. *macrophylla* (Banerjee, 1992).

The grouping of tea into an erect small-leafed China variety and a horizontal broad-leafed Assam variety was rather subjective. Plants with intermediate leaf characteristics could not always be assigned to either of these two varieties. Hence, distinction on more reliable floral morphology was developed for differentiating various taxa. Relying mostly on characteristics of styles, Wight (1962) divided tea plants into *C. sinensis* (L.), *C. assamica* (Masters) and a third southern form of tea or Cambod race, the sub-species *C. assamica* ssp. *lasiocalyx*. Other authors recognised five distinct types of tea. The latter types were not claimed to be separate taxa, but variations related to either extreme China or extreme Assam plants.

In the Indian sub-continent the classification proposed by Wight (1962) is popular. Otherwise the practice the world over is to put tea plants under the name *C. sinensis* (L.) O. Kuntze, irrespective of taxonomic variation. Reference to varieties is however still common; China, Assam and Cambod varieties are generally referred to as *C. sinensis*, *C. assamica* and *C. assamica* ssp. *lasiocalyx*, respectively (Banerjee, 1992).

Hybrids between the varieties are readily obtained, resulting in highly heterozygous commercial clones of *C. sinensis*. Indeed, because of the extreme homogenisation, it is doubtful whether the archetypal teas still exist. However, many hybrids currently available

are still referred to as China, Assam, or Cambod, depending on their morphological proximity to the main taxa.

There are no absolute crossing barriers within the genus *Camellia*, creating the possible involvement of taxa other than *assamica*, *sinensis* and *assamica* ssp. *lasiocalyx* in the tea genetic pool. Of particular interest is the suspected involvement of *Camellia irrawadiensis* Barua. Other *Camellia* species suspected to have contributed to the tea genetic pool by hybridisation include *C. flava* (Pitcard) Sealy, *C. petelotii* (Merrill) Sealy, and possibly *C. Lutescens* Dyer. This sexual compatibility of *C. sinensis* with other *Camellia* species provides the potential for the introduction of other desirable genes into the *C. sinensis* gene pool. For example, using this breeding strategy, resistance to a wider range of pests can be obtained.

Besides a few natural triploids, the chromosome number for all varieties of *C. sinensis* studied is 2n = 30 (Bezbaruah, 1971; Kondo, 1977).

4.2 The propagation of tea.

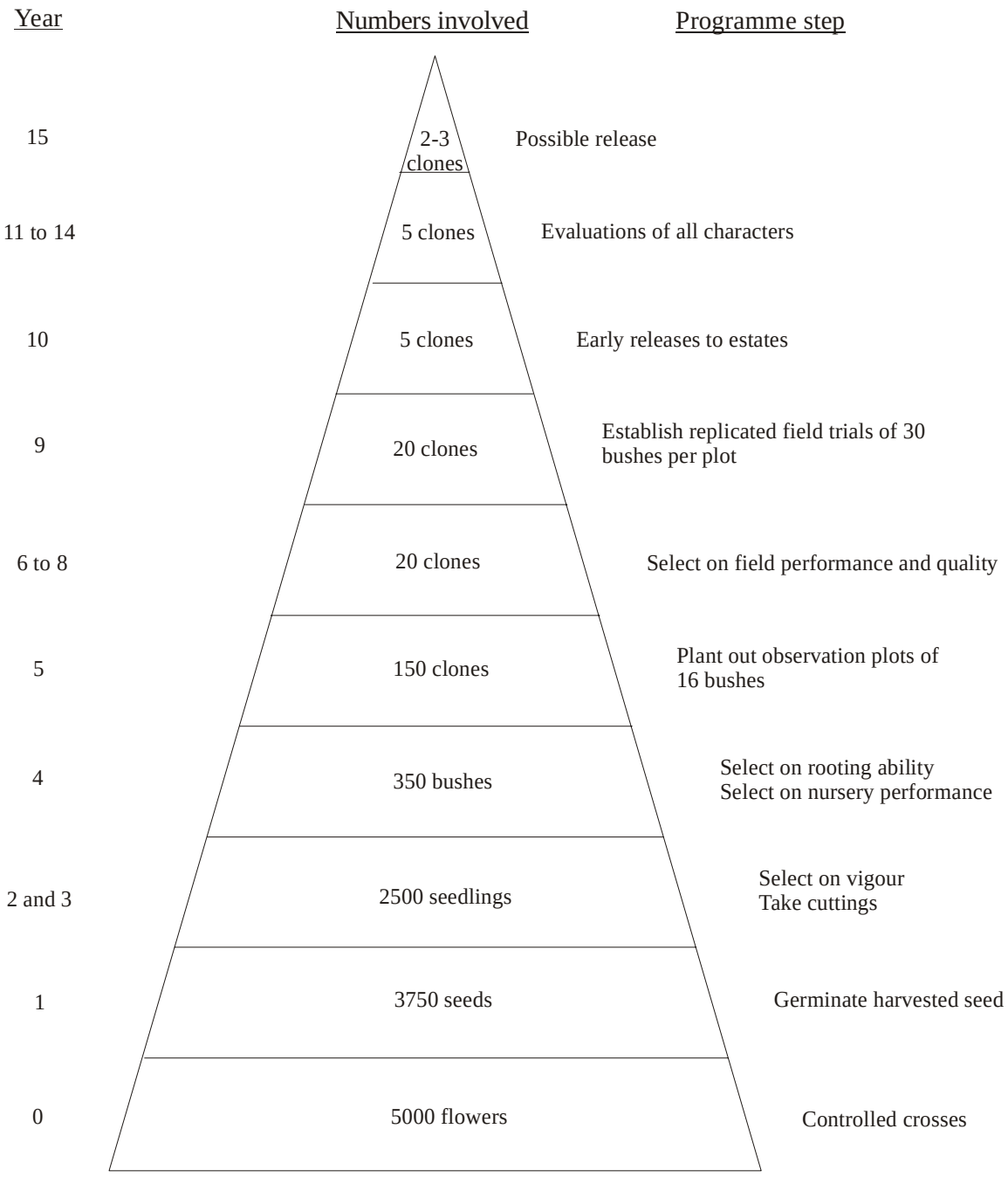
Until the 1950's, tea was mainly propagated from seed obtained from seed gardens. The seed bearers were selected for yield, quality and other desirable characteristics. The majority of tea produced commercially, is seedling tea. Variability in yield and quality is an expected problem within seedling plantations. This led to the preferred use of vegetative propagated plants. Elite plants were selected and multiplied vegetatively by single node cuttings. These

plants were released as clones or used in hybridisation programmes. Many seedling plantations in India, Sri Lanka and Indonesia are older than 50 years, resulting in lowered yields, and in some parts lowered productivity due to disease. This created a demand for clonal planting material of high quality and high yield and disease resistance potential.

The aims of the Plant Improvement Programme at the TRF(CA) are to develop new tea clones with improved quality and yield. To obtain this goal field selections and crosses are undertaken followed by various trails to determine the growth characteristics and quality of each clone. This process takes a minimum of 15 years (Table 1.1).

The bulk of Africa's initial tea populations used for clonal development, are thought to be obtained from a few random pollinated crosses undertaken in Assam (India). These crosses possibly account for only a small portion of the total available tea gene pool in the world. Recent studies, however, demonstrated wide genetic differentiation of Kenyan teas compared to tea populations from other parts of the world (Wachira *et al*, 1995). This tendency was also confirmed by Wright (1996) which showed wide genetic diversity between five randomly selected TRF(CA) clones (SFS150, SFS204, PC1, PC81, MFS87). Using the coefficient of Jaccard, the genetic diversity between the five clones were determined to range from 19% to 45%. Thus, although not significant yet, the increased use of clonal teas might lead to a restriction of the level of genetic diversity within the cultivated tea gene pool.

Table 1.1. Breeding and selection programme at TFR(CA).



5 The flavan-3-ol content of tea.

5.1 The flavonoids

The flavonoids are polyphenolic in nature. Polyphenols are substances that contain an aromatic ring bearing one or more hydroxyl substituents. Phenolic substances usually are water-soluble and are frequently combined with a sugar as glycosides and are usually located in the vacuole. The flavonoids are the largest group of the thousands of polyphenolic structures known, but simple monocyclic phenols, phenylpropanoids and phenolic quinones are also abundant. Several polymers in plants are also polyphenolic in nature, namely the lignins, melanins and tannins. Phenolic units are also encountered in proteins, alkaloids and terpenoids (Harborne, 1998).

The flavonoids are all structurally derived from the parent substance flavone. Flavonoids are mainly water-soluble compounds. The flavonoids can be extracted with 70% ethanol and will remain in the aqueous phase after partitioning of this extract with petroleum ether. Because of their phenolic nature, the flavonoids change in colour when treated with base or with ammonia. The flavonoids also show intense UV and visible absorption due their conjugated aromatic systems (Harborne, 1998). The basic C15 unit of flavonoids consists of two benzene rings (A and B) connected by a three-carbon chain. This chain is closed in most flavonoids to form the heterocyclic ring (C) (Figure 1.1). The natural flavonoids are divided into classes based generally on the oxidation state of their C-ring (Stafford, 1990). The major subgroups of flavonoids are shown in Figure 1.2.

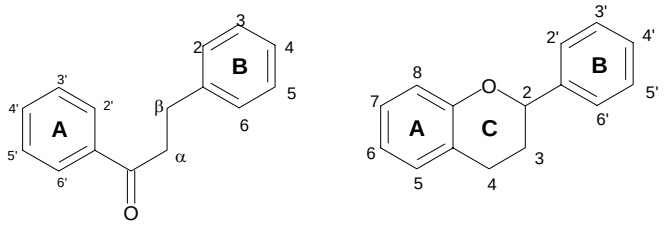


Figure 1.1. The basic ring structure and numbering systems of chalcone and flavonoid are illustrated.

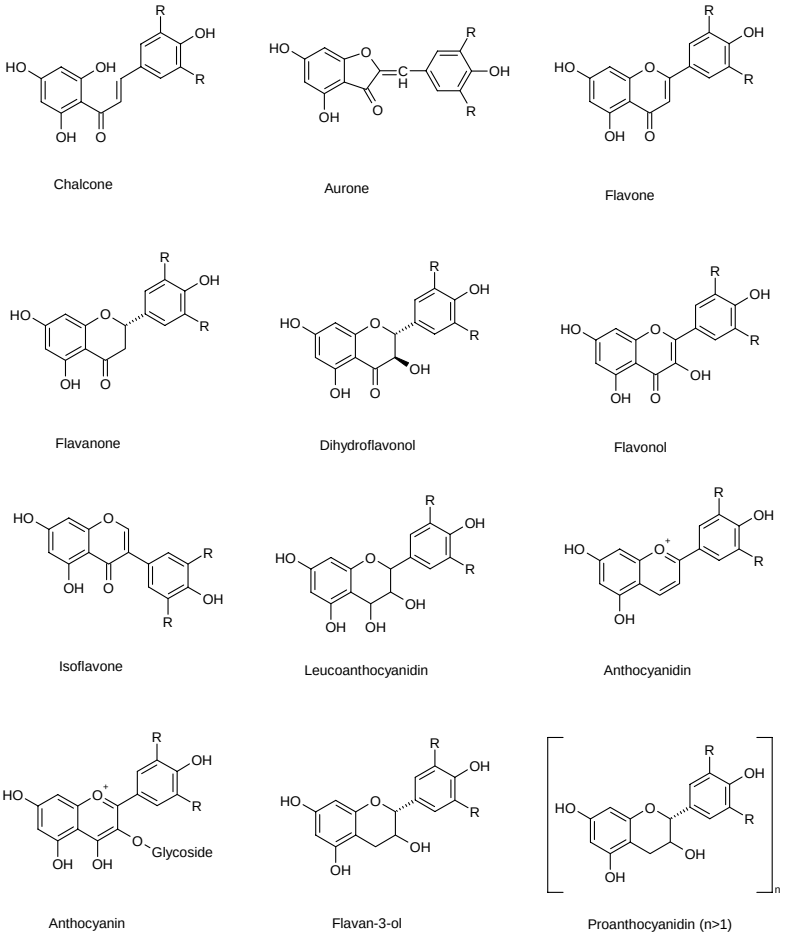


Figure 1.2. The major subgroups of flavonoids with 5,7-hydroxy A-rings (R = H or OH).

5.2 Flavonoid biosynthesis.

Although much work have been done on the flavonoid metabolism in other plants, very little detailed knowledge is available on the metabolism of the flavan-3-ols and other polyphenolics in tea. Most of the understanding of the flavonoid metabolism in tea is based on assumptions made from the metabolism in other plants (Magoma *et al*, 2000). However, no information in the literature was found to suggest major deviations from the proposed pathways. In the first part of the introduction on flavonoid metabolism will be a general description of flavonoid metabolism. The second part will be on the assumed flavonoid metabolism in the tea plant. Lastly the steps responsible for the synthesis of the different flavan-3-ol compounds in the tea leaves will be discussed.

5.3 General flavonoid biosynthesis.

The flavonoids are also known as secondary metabolites. Secondary metabolites are all the compounds that are (apparently) not absolutely essential to the life and growth of the producing organism. In other words, if a secondary metabolic pathway is eliminated (e.g., by a mutation) the organism will continue to grow. Secondary metabolism utilizes a limited number of primary metabolites in their biosynthesis. In the secondary metabolism there are four major pathways; the isoprene pathway, the polyketide pathway, the shikimate pathway and the amino acid pathway (Figure 1.3) (Bentley, 1999). As will come apparent from later discussions, the shikimate and amino acid pathways have a major influence on the metabolism of the tea flavan-3-ols.

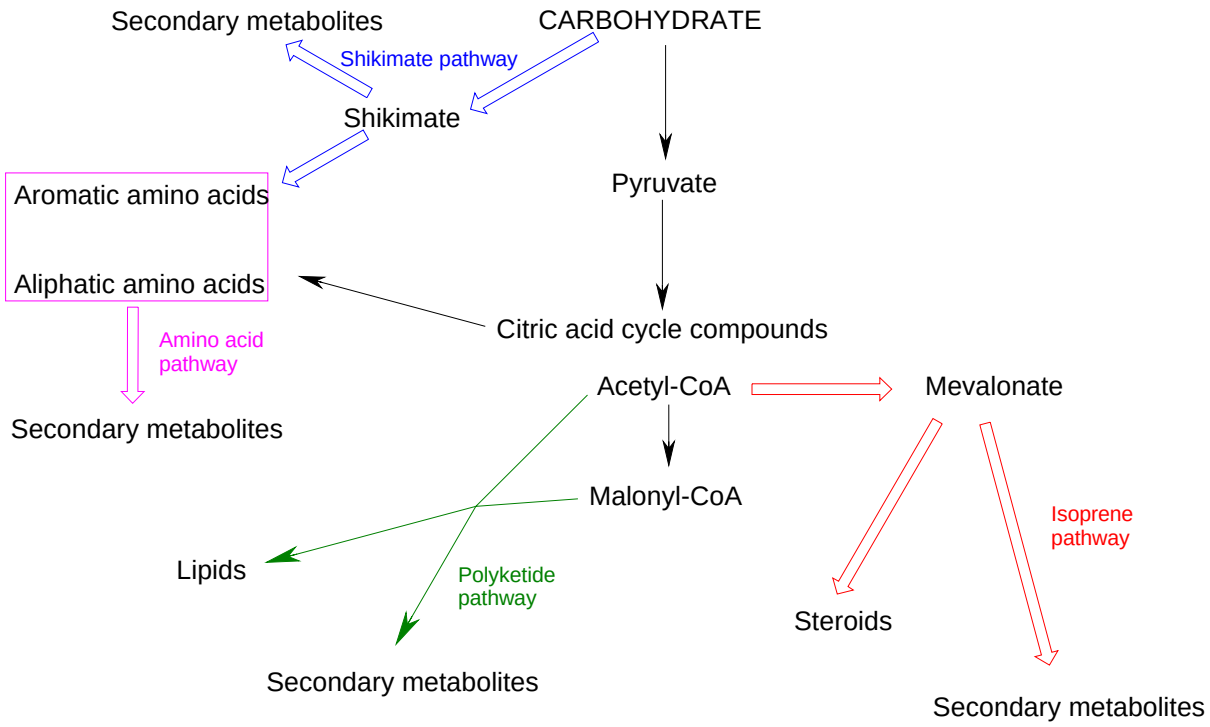


Figure 1.3. The four major pathways for secondary metabolite biosynthesis.

The biosynthesis of the flavonoids initiates from the carbohydrate metabolism. The shikimate pathway is connected to carbohydrate metabolism through the pentose phospate pathway. The shikimate pathway is responsible for the synthesis of phenylalanine that is the precursor of the flavonoid pathway through the phenylpropanoid pathway. The shikimate pathway is also responsible for the formation of gallic acid, which will presumably be attached to the flavan-3-ols with an ester bond in the last stages of the biosynthesis process of the tea flavan-3-ols. However, malonyl-Coenzyme A (-CoA) is the other precursor for flavonoid biosynthesis. This compound is synthesised from acetyl-CoA, which originates from the citric acid cycle (Figure 1.4) (Chu and Juneja, 1997).

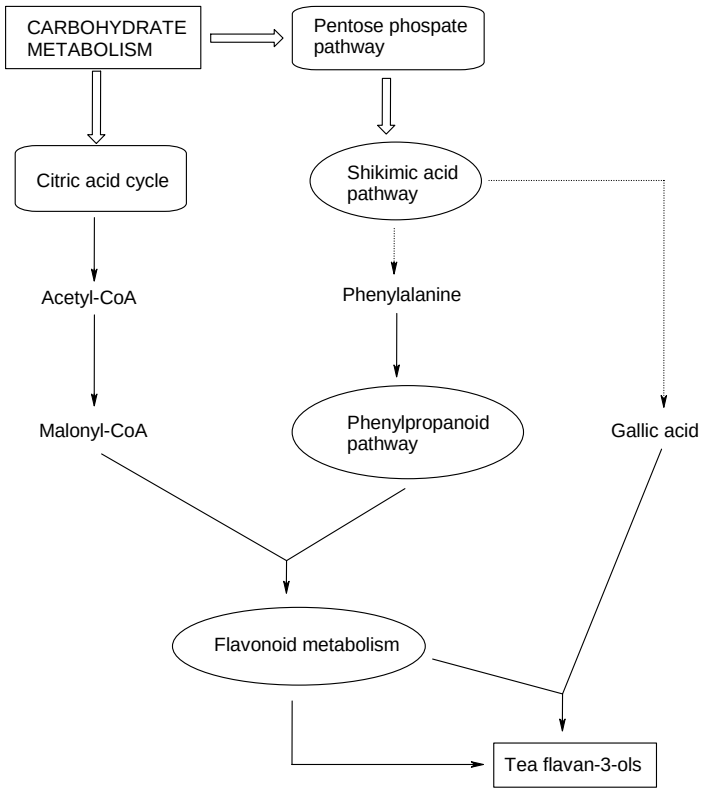


Figure 1.4. The proposed general biosynthetic pathway of flavan-3-ols in the tea plant.

The phenylpropanoid pathway is the first committed stage in the biosynthesis of a large number of phenolic compounds in plants, including the flavonoids. The general phenylpropanoid pathway starts with phenylalanine and ends with 4-coumaryl-CoA (Weisshaar and Jenkins, 1998). The phenylpropanoid pathway, however, produce the precursors for a whole range of compounds. The functions of these compounds are as diverse as its structural variations and include flower pigments, phytoalexins, UV protectants, insect repellents and signal molecules in plant-microbe interactions. These compounds also function as structural polymers such as suberin, lignin and other cell wall components (Figure 1.5) (Hahlbrock and Scheel, 1989).

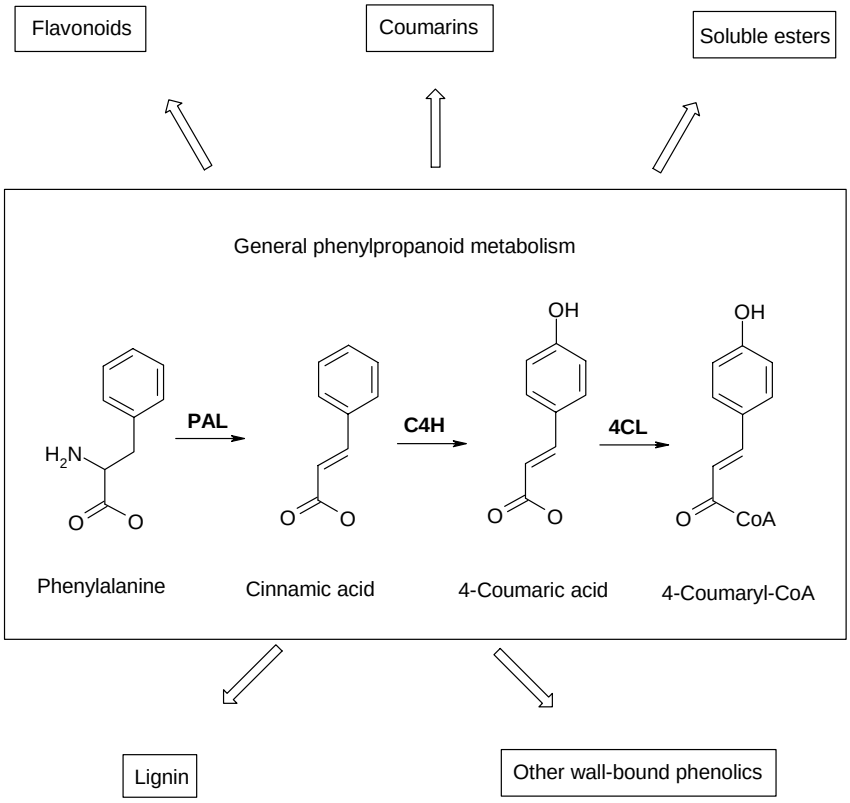


Figure 1.5. The general phenylpropanoid pathway and some of its major branch pathways.

The first enzymatic reaction in the phenylpropanoid pathway is the deaminantion of phenylalanine to produce cinnamic acid by phenylalanine ammonia lyase (PAL). This enzyme is also an important regulatory enzyme for the formation of various pathways downstream. The PAL gene has been shown in various plant species to be encoded by a small multi-gene family (Liang *et al*, 1989; Butland *et al*, 1998). PAL activity is primarily regulated at the gene expression level. However, PAL was also shown to be regulated by feed-back inhibition or activation by products produced from branches of the phenylpropanoid pathway, by enzyme activation, and by phosphorylation. PAL activity is

regulated by factors such as injuries, infections, environmental stimuli (including UV-light exposure) and the developmental stage of the plant (Liang *et al*, 1989). Various isoforms was also shown to occur for many species (Sarma *et al*, 1998).

The PAL enzyme was also shown to be important in the biosynthesis of the tea flavan-3-ols. Sangwan and Ravindranath (1997) showed a positive correlation between the enzyme activities of PAL and tyrosine ammonia lyase (TAL) and the total phenolics in tea. This was determined by monitoring the total phenolic content and PAL activity in various tea clones during different growth seasons of the year. Other research also indicated this correlation by measuring the PAL enzyme activity and catechin content in different parts of the tea plant (Iwasa, 1977). A positive correlation between the PAL activity and catechin content was measured in different tea clones (Iwasa, 1977). Shade treatment of tea clones indicated a decrease in the catechin content of tea leaves, which also coincided with a decrease in the PAL activity in the tea leaves (Iwasa, 1977; Saijo, 1980). Thus there are various proofs for the positive correlation between PAL activity and catechin content in tea leaves.

The PAL gene for tea was cloned and classified by (Matsumoto *et al*, 1994). Using tea PAL cDNA the PAL genes of the tea plant was investigated by restriction fragment length polymorphism (RFLP). The authors showed the PAL genetic variation to be much higher than predicted from various PAL-hybridization bands seen in most Assam hybrids. On the other hand, they could divide the Japanese green tea cultivars belonging to the *C. sinensis* variety into five groups. They concluded that the PAL gene is present as a single gene in the

tea haploid genome. They were also successful in distinguishing between high and low catechin content in Assam hybrids and Japanese green tea cultivars from RFLP analysis.

The first committed step in the flavonoid pathway is the synthesis of the C15 chalcone intermediate (4,2',4',6'-tetrahydroxychalcone) by the chalcone synthase (CHS) enzyme. For the sake of clarity all the flavonoid compounds shown in Figure 1.6 are underlined, however, only the names of the flavonoid classes are shown in Figure 1.6. This reaction takes place by the condensation of three malonyl-CoA molecules with a hydroxycinnamic acid CoA ester (usually 4-coumaroyl-CoA). The next reaction catalyses the direct hydroxylation at position 3 by the dioxygenase, flavanone-3-hydroxylase. The dihydroflavonol (dihydrokaempferol) formed is the precursor for the leucoanthocyanidins by the reduction of the carbonyl group in position 4 by dihydroflavonol 4-reductase. The leucoanthocyanidin formed in Figure 1.6 is leucopelargonidin. The leucoanthocyanidins are the direct precursors for the formation of the flavan-3-ols (also known as catechins) and the proanthocyanidins. The flavan-3-ols (in Figure 1.6 afzelechin) are formed by the enzyme flavan 3,4-*cis*-diol reductase. The proanthocyanidins are formed by a condensation of flavan-3-ols and leucoanthocyanidins. However, the leucoanthocyanidins are also precursors for the important classes of anthocyanidin (pelargonidin) and anthocyanin (pelargonidin 3-glucoside). The reactions from leucoanthocyanidin to the anthocyanidins are still unknown. The anthocyanidins are not very stable and usually a glycosylation transfers a glycoside (usually glucose) to position 3 of the anthocyanidin to form a stable anthocyanin. This reaction is catalysed by UDP glucose flavonoid 3-O-glucosyltransferase. Two other important classes formed during flavonoid metabolism are

the flavones and the flavonols. The flavones (e.g. apigenin) are formed by the introduction of a C-2 to C-3 double bond in a flavanone by the flavone synthase enzyme. The flavonols (kaempferol) are synthesised from dihydroflavonols by the introduction of a double bond between C-2 and C-3 by the enzyme flavonol synthase (Heller and Forkmann, 1988; Stafford, 1990; Dooner *et al*, 1991).

The general biosynthetic pathway for flavan-3-ols with a 2,3-*trans* stereochemistry and with a 5,7-hydroxy A-ring is well known. It starts with chalcone synthase and chalcone iosmerase to form the flavanone naringenin with its 2R stereochemistry due to the stereospecificity of the chalcone isomerase enzyme. Next a hydroxyl group is attached at position 3 to form a 2,3-*trans* dihydroflavonol. This is followed by two NADPH-dependent reductases, dihydroflavonol 4-reductase and flavan 3,4-*cis*-diol reductase, to synthesise firstly a flavan-3,4-diol and then the flavan-3-ol. This pathway from the flavanone level is shown in Figure 1.7. The flavan-3-ols in tea, however, consists of a mixture of six different compounds, namely: (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-epigallocathechin gallate. These flavan-3-ols differ in the number of hydroxyl groups on their B-rings, the stereochemistry of C3 and the attachment of an extra galloyl group at the 3 hydroxyl group.

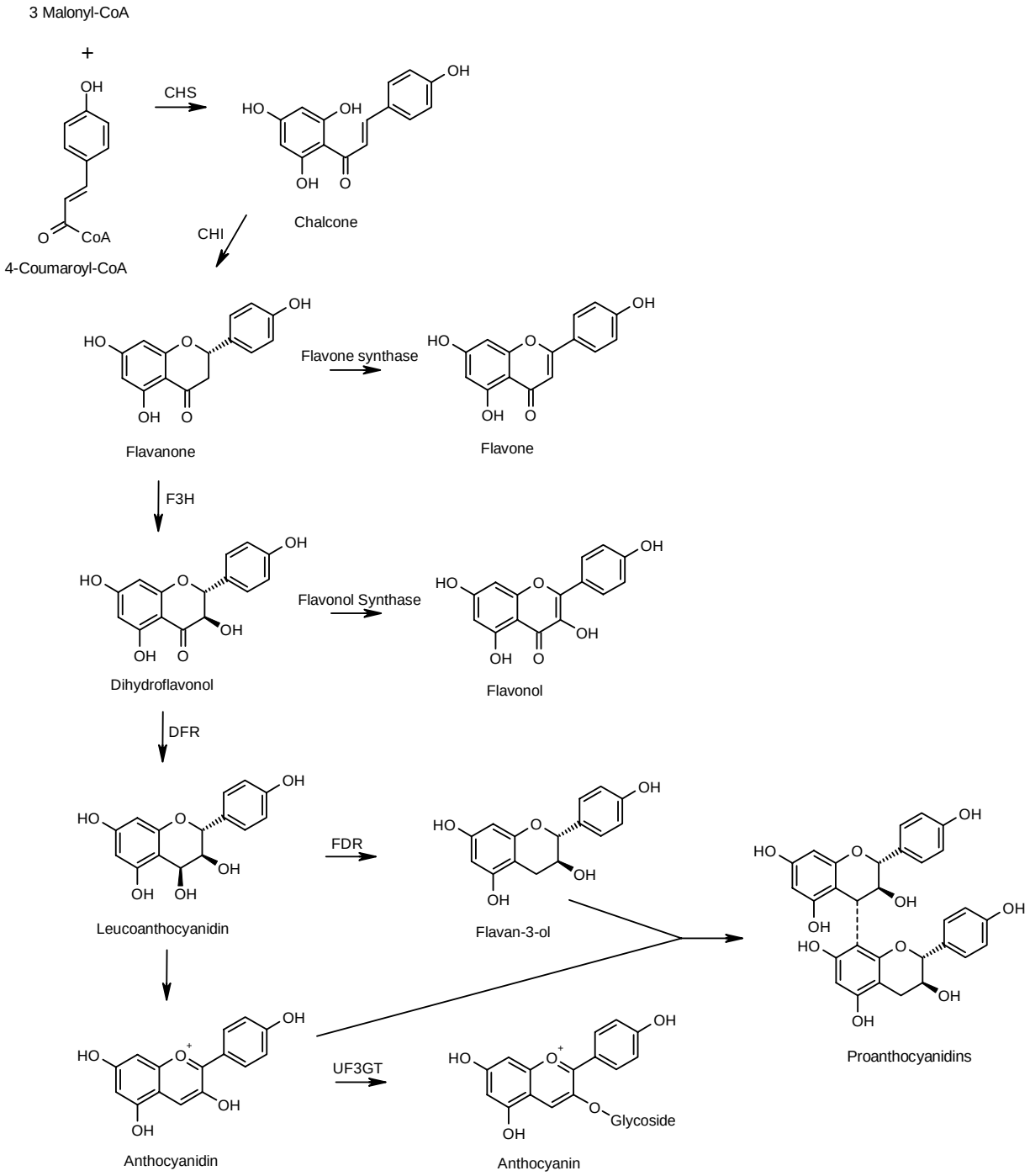


Figure 1.6. The general flavonoid pathway showing the major classes of flavonoids formed. The enzyme abbreviations are: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; DFR, dihydroflavonol 4-reductase; FDR, flavan 3,4-*cis*-diol reductase; UF3GT, UDP glucose flavonoid 3-O-glucosyltransferase.

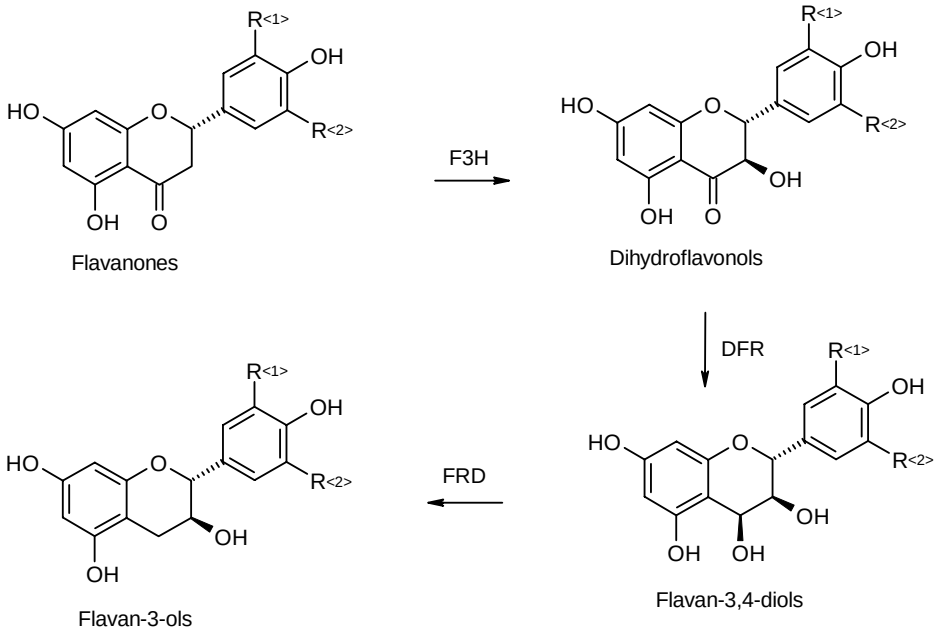


Figure 1.7. The biosynthetic pathway of 2,3-*trans* flavan-3-ols from the flavanone level. R<1> and R<2> can be either H or OH. The enzymes shown are: F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; FDR, flavan 3,4-*cis*-diol reductase.

The method of producing the 2,3-*cis* configuration in the flavan-3-ols is unknown at this point. Stafford (1990) proposes two possibilities for the formation of the 2,3-*cis* flavan-3-ols. Firstly it is possible that a α-3-hydroxylation takes place during the hydroxylation by flavanone 3-hydroxylase. This hypothesis is supported by the occurrence of plants that only produce 2,3-*cis* flavan-3-ols. A second possibility is the occurrence of a 3-hydroxyflavanone epimerase enzyme that will change the 2,3-*trans* stereochemistry to a 2,3-*cis* configuration. The occurrence of an epimerization step at the 3,4-diol level is considered unlikely because of the identification of a 2,3-cis isomer of the 3-glycoside of dihydroquercitin as a natural product. Due to the presence of both the 2,3-*trans* and the

2,3-*cis* isomers in tea, one would suspect the presence of the epimerase enzyme in the biosynthetic pathway of tea flavan-3-ols. Closer inspection of the typical flavan-3-ol profile of tea leaves, however, shows that the majority have the 2,3-*cis* conformation. This might indicate the change to the 2,3-*cis* stereochemistery at the flavanone 3-hydroxylation step.

The tea flavan-3-ols also differ according to the degree of hydroxylation of their B-ring. The tea flavan-3-ols can either have a dihydroxy B-ring (C, EC, ECg) or a trihydroxy B-ring (GC, EGC, EGCg). The 4'-hydroxyl group originates from the 4-coumaroyl-CoA precursor used by chalcone synthase. The 3' and 5'-hydroxyl groups are usually added during the flavonoid pathway. There is also a possibility that the 3'-hydroxyl group is attached during the phenylpropanoid pathway, however, from the flavonoid metabolism in other plants this seems highly unlikely. The 3' and 5'-hydroxyl groups are usually attached at the C_{15} level within the so-called "grid" pathway (Stafford, 1990; Stotz *et al*, 1984; Stich and Forkmann, 1987; Holton *et al*, 1993; Menting *et al*, 1994; Nielsen and Podivinsky, 1997). The hydroxylases specific for the B-ring are quite different from the hydroxylases specific for hydroxylation of the C-ring. The B-ring is aromatic whereas the C-ring is non-aromatic. These enzymes are however similar in their nonspecificity towards the hydroxylation pattern of the other ring (ring B or C) not involved in the specific reaction. That means that the 3'- and 3',5'-hydroxylases are not specific for the absence or presence of a hydroxyl group at position 3, and F3H are not specific for the presence or absence of the 3' or 3',5'-hydroxyl groups. This is responsible for the "grid"

pathway shown in Figure 1.8, whereby dihydroquercetin can be produced by two pathways and dihydromyricetin by three pathways.

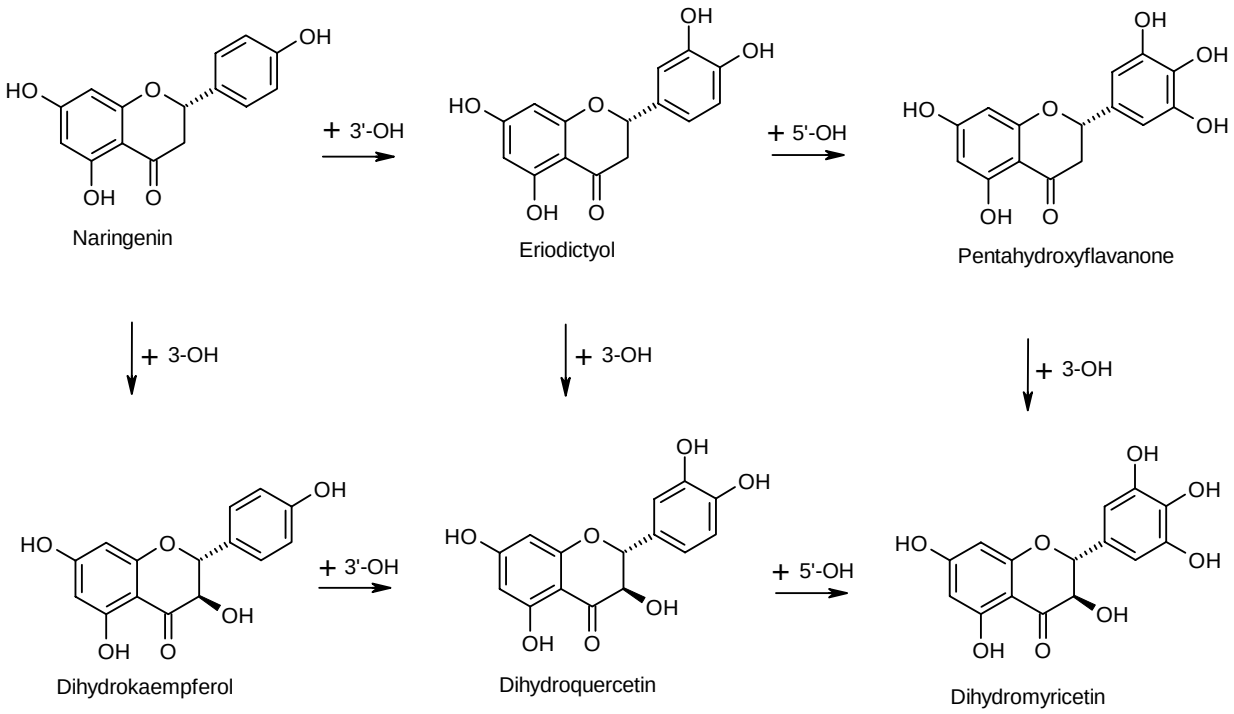


Figure 1.8. The “grid” pathway for the 3’- and 5’-hydroxylation of the B-ring.

6 Analysing tea for their flavan-3-ol and theaflavin content.

Traditionally professional tea tasters assess tea quality organoleptically. The tea industry is interested in obtaining correlations between the quality of tea and their chemical composition. Finding such an analysis method will provide the industry with an objective and reliable alternative for analysing their product. The analysis of tea can be used in four different areas: (i) to identify the constituents which are responsible for tea quality; (ii) to

optimise the manufacturing process; (iii) to identify the constituents which are responsible for the health effects of tea; and (iv) to screen for clones with good catechin profiles early in the clonal development programme.

Because of the abundance of the flavan-3-ols in the green leaf of tea, and the emphasis thereof in the research performed by the author, only the green leaf analysis of the flavan-3-ols will be discussed. This is however not the only abundant constituents in tea. Other compounds which have been analysed in tea leaf include: the flavonols and their glycosides (McDowell *et al*, 1990; Price *et al*, 1998); the flavones and their glycosides; phenolic acids and esters, including free gallic acid and theogallin; carotenoids and chlorophylls (Taylor *et al*, 1992); amino acids (Ruan *et al*, 1998); different bio-available metals (Nagata et al, 1992); and vitamins (Liang *et al*, 1990).

For analysis of the black tea constituents, the discussion will focus on the TFs. Unlike the TRs, the TFs are well characterised and were already shown to have a correlation with the quality of black tea produced in Central Africa (Hilton and Ellis, 1972; Hilton *et al*, 1973).

6.1 Analysis of tea flavan-3-ols.

The flavan-3-ols are the most abundant group of compounds in the green leaf of tea. Up to 30% of the dry matter of young tea leaves are composed of the flavan-3-ols. The major flavan-3-ols occurring in tea are shown in Figure 1.9. The five most abundant flavan-3-

ols in tea are (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCg). Of these five flavan-3-ols the EGC and EGCg flavan-3-ols are usually the most abundant flavan-3-ols occurring in the tea produced in Central and Southern Africa. Other flavan-3-ols also occurring in tea are (-)-gallocatechin, (-)-catechin gallate and (-)-gallocatechin gallate.

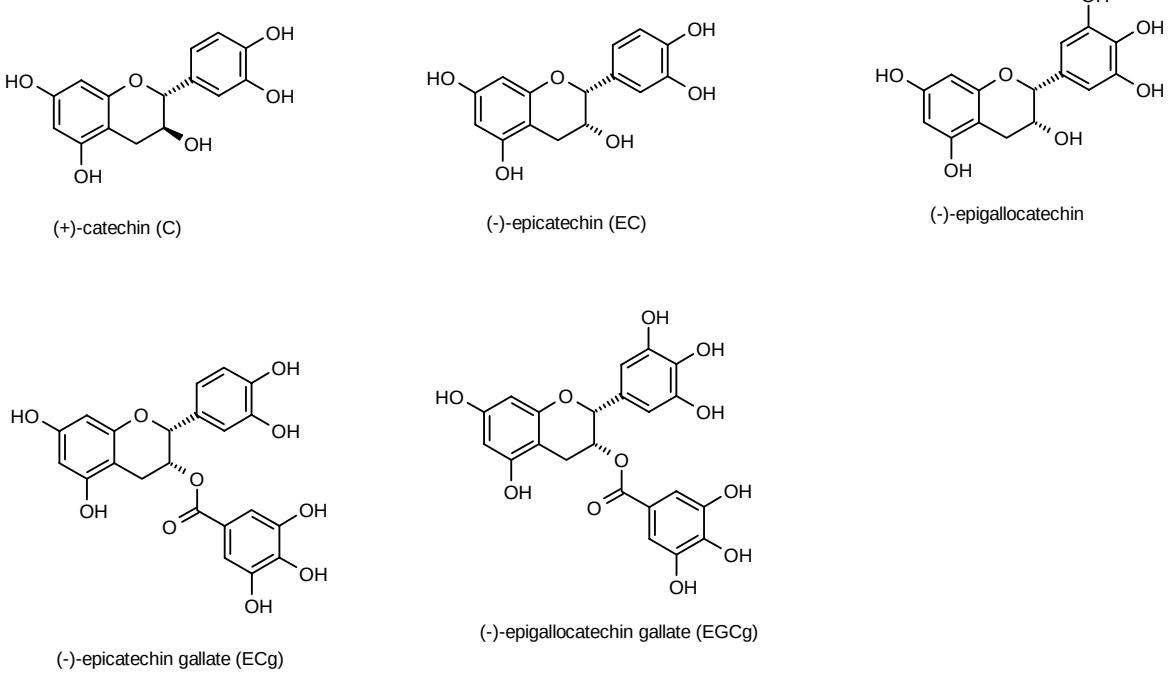


Figure 1.9. The major flavan-3-ols occurring in green tea leaves.

Originally the flavan-3-ols of tea were separated and analysed with two-dimensional paper chromatography (2-D PC). The different flavan-3-ols were quantified by eluting the flavan-3-ol spots with water and measuring the absorbance (Roberts and Wood, 1951; Nakagawa and Torii, 1964). Similarly, the flavan-3-ols were also separated and analysed using thin layer chromatography (TLC). Various stationary phases have been used for

TLC analysis, including cellulose (Forrest and Bendall, 1969), polyamide (Finger *et al*, 1992) and silica gel (Dalluge and Nelson, 2000). Because of long analysing times and quantification problems, these methods are not widely used anymore.

To improve on the methods of PC and TLC, gas-liquid chromatography (GLC) methods were developed. Collier and Mallows (1971) were the first to develop a temperature programmed GLC method to analyses the flavan-3-ols within one run. However, before separation on the GLC capillary columns were possible, the flavan-3-ols had to be derivatised with bistrimethylsilylacetamide.

Various other methods exist for the determination of the flavan-3-ols in tea. They include spectrophotometric methods for the measurement of total catechins by the formation of a coloured complex with either 4-dimethylaminocinnamaldehyde or diazotized sulfanilamide. The measurement of catechins with a biosensor consisting of a source of PPO and an oxygen electrode was also reported. Catechins were also analysed based on their chemiluminescent emmision and were identified and described using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) (Dalluge and Nelson, 2000).

A few normal pressure liquid chromatographic (LC) methods have also been developed, mostly for the preparative isolation of the tea flavan-3-ols. Various stationary phases have been used, including Sephadex LH-20, styrene-divinylbenzene copolymer or methacrylate esters, MCI gel CHP-20P or Bondapak C_{18} Porasil B, and high-porosity polystyrene gel Diaion HP20 (Finger *et al*, 1992).

The first real break-through concerning the fast and reliable analysis of tea flavan-3-ols came with the introduction of reverse phase high performance liquid chromatography (HPLC) by Hoefler and Coggon (1976). The researchers used a µBondapak C_{18} column with a mobile phase of acetic acid-methanol-DMF-water (1:2:40:157) and used a UV detector measuring the absorbance at 280 nm. According to Dalluge and Nelson (2000) the next significant improvement was by Goto *et al* (1996), who developed gradient elution system capable of separating all eight naturally occurring tea flavan-3-ols. Their method also used a C_{18} stationary phase with a mobile phase consisting of water-acetonitrile-phosphoric acid. Recently Dalluge *et al* (1998) undertook a systematic study of the effect of column selection and mobile phase composition on the separation of six prominent tea flavan-3-ols and caffeine. They concluded that the separation and resolution was dependent on the column and the presence of acid in the mobile phase. End-capped, deactivated, monomeric C_{18} columns were preferable over non-deactivated monomeric or polymeric C_{18} columns. The presence of acid was particularly important for the elimination of peak tailing. Liquid chromatography-mass spectrometry (LC-MS) was also used for the identification of flavan-3-ols in tea (Lin *et al*, 1993). Direct MS characterization of flavan-3-ols in tea extracts without the use of LC has also been demonstrated (Poon, 1998).

An interesting new development in the separation and analysis of tea flavan-3-ols is the use of capillary electrophoresis (CE). CE has the advantages of faster analysis times, less running buffers used and cheaper columns. Modern CE instruments also have as standard programmable sample trays, with the advantage of 24-hour analysis. Because of the

importance of CE analysis in the research of this thesis, a general introduction into the principles and applications of CE will be given later in this chapter. A review of the CE methods in the literature, used to analyze tea components (including the flavan-3-ols) will also be given later.

6.2 The analysis of theaflavins

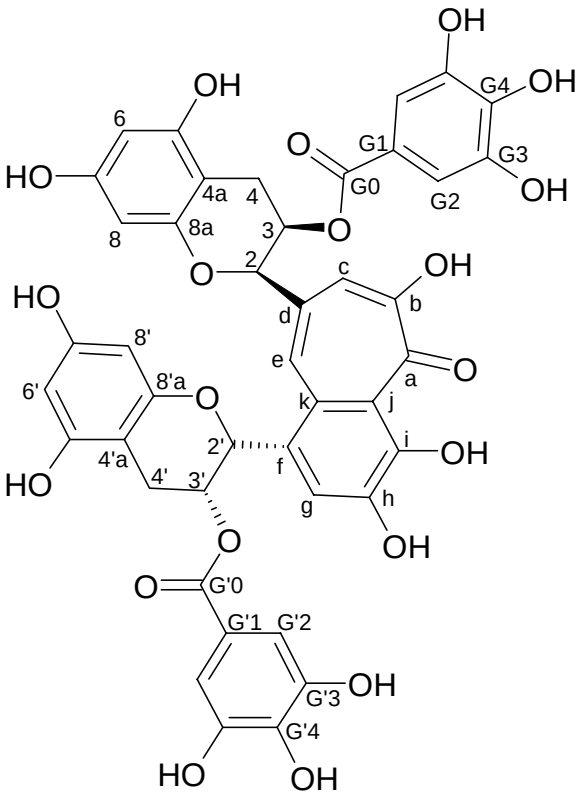


Figure 1.10. The numbering scheme for theaflavin digallate.

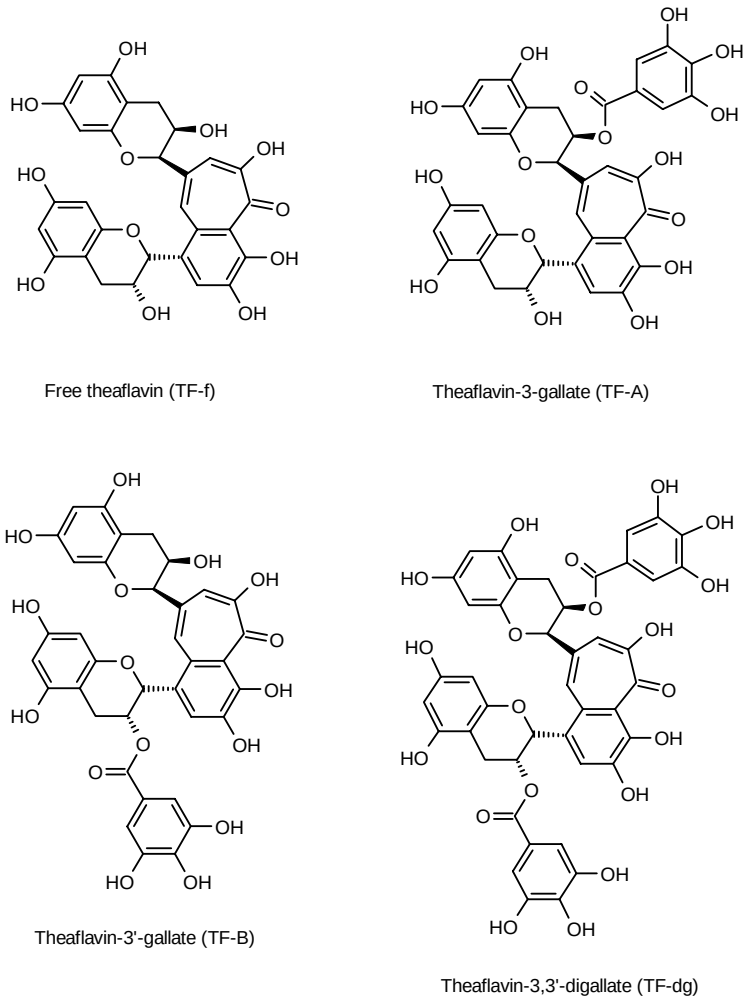


Figure 1.11. The major theaflavins occurring in black tea.

The theaflavins are formed during the fermentation stage of the black tea manufacturing process. These TFs are formed in the enzyme-catalysed oxidation reactions between one dihydroxy flavan-3-ol and one trihydroxy flavan-3-ol. The TFs are orange-red in colour and contain a 7 membered benzotropolone ring. Although the TFs only constitute about two percent of black tea on a dry mass base, they have a very significant influence on the quality. TFs were shown to contribute to the bright colour and briskness or astringency of

black tea and were correlated with the quality of black teas produced in Central Africa (Hilton and Ellis, 1972). The major TFs occurring in black tea are shown in Figure 1.11.

Originally the individual TFs were isolated with the aid of 2-D PC and gel chromatography, and their structures determined with MS and NMR. However, at first only the total TFs were determined with the aid of spectrophotometry. The first method extracted the TFs from hot aqueous tea infusions with either ethyl acetate or isobutyl methyl ketone (IBMK), and measured the absorbance at 380 nm and 460 nm (Roberts and Smith, 1961). This method was discontinued after it was discovered that the values obtained depended on the composition of the TF fraction. Another spectrophotometric method developed involved the flavognost reagent (2-aminoethyl diphenylborate in ethanol). The flavognost reagent forms a green complex with the *cis*-h,i-dihydroxybenzene ring associated with the TFs (Spiro and Price, 1986). Another less expensive method using aluminium chloride instead of flavognost was also developed (LikoIeche-Nkhoma and Whitehead, 1988). The limitation of the spectrophotometric methods is their inability to quantify the individual TFs.

To be able to analyze the individual TFs Collier and Mallows (1971) developed a GLC method to separate and quantify the individual TFs. This method, however, had a few disadvantages. Extensive cleanup is necessary prior to analysis, involving extraction with ethyl acetate and chromatography on Sephadex LH-20. Furthermore, derivatisation with trimethylsilyl are needed and the two monogallates were not resolved. (Lea and Crispin,

1971) developed a method to isolate free theaflavin from digallated theaflavin and the monogallated theaflavins. The monogallated theaflavins were also not resolved.

The first HPLC method was developed by (Hoefler and Coggon, 1976). This reverse phase method used a µBondapak C_{18} column and a mobile phase of acetic acid-acetone-water with detection at 365 nm. A slightly modified version of this method was used by researchers in investigations on model fermentation systems (Robertson, 1983). Wellum and Kirby (1981) developed a new method by systematically looking at factors influencing the TF analysis. Their method used a Partisil ODS column at 80ºC with a gradient elution system with acetone-water-methanol, however, the monogallates remained unresolved. The separation of the two monogallates was achieved when an acidifier was added to the mobile phase (Finger *et al*, 1992). Recently, the use of photodiode-array detection was recommended to avoid misleading results of contamination of TF peaks with flavonol glycosides (McDowell *et al*, 1991). The use of a citrate buffer also led to improvements in resolution and peak shape due to masking of surface metals by the chelating action of citric acid (Bailey *et al*, 1991). Temple and Clifford (1997) could not find any improvement when using citric acid instead of acetic acid when analysing TFs. They did however use decaffeinated tea samples in their analysis.

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7 Capillary electrophoresis

7.1 Introduction

Electrophoresis is the migration of ions in a solution when an electrical potential is applied to the solution. Cations will migrate to the negative electrode (cathode), and anions will migrate to the positive electrode (anode). Capillary electrophoresis is when this migration of ions occurs in a thin capillary tube. The ions will migrate at different velocities according to their mass to charge ratio. During electrophoresis different ions will thus tend to separate according to their size, shape and charge.

In physical terms, the velocity of the ions in an electrical field is dependent on the driving force and the frictional drag on the ion. The driving force depends on the electrical field strength and the effective charge on the ion. The stronger the electric field and the higher the charge on the ion, the higher the mobility of that ion. The frictional drag depends on the viscosity of the running buffer and the solvated radius of the ion. The higher the viscosity and the larger the solvated radius, the higher the frictional drag will be and the slower the ion will move.

A very important factor in CE is the occurrence of electroosmotic flow (EOF). Silica tubing is the capillary generally used for CE. The silica contains surface silanol groups (Si-OH), which are ionized above pH 2 to give the capillary wall a fixed negative charge. The fixed negative charges attract cations in the solution to form the double layer of negative and positive charges at the capillary wall. When an electric field is applied to the capillary, the cations attracted to the capillary wall migrate to

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the cathode. The movement of the cations in the double layer imparts a flow to the liquid in the capillary. This electrically induced movement of the liquid in the capillary is the EOF.

The EOF significantly affects the migration of ions through the capillary. The positively charged ions will migrate faster towards the cathode, the neutral compounds without any net charge will also move towards the cathode at a rate similar to the velocity of the EOF. Even the anions will move to the cathode if the force of the EOF is larger than the force due to the electrical field. The EOF effect is described in Figure 1.12.

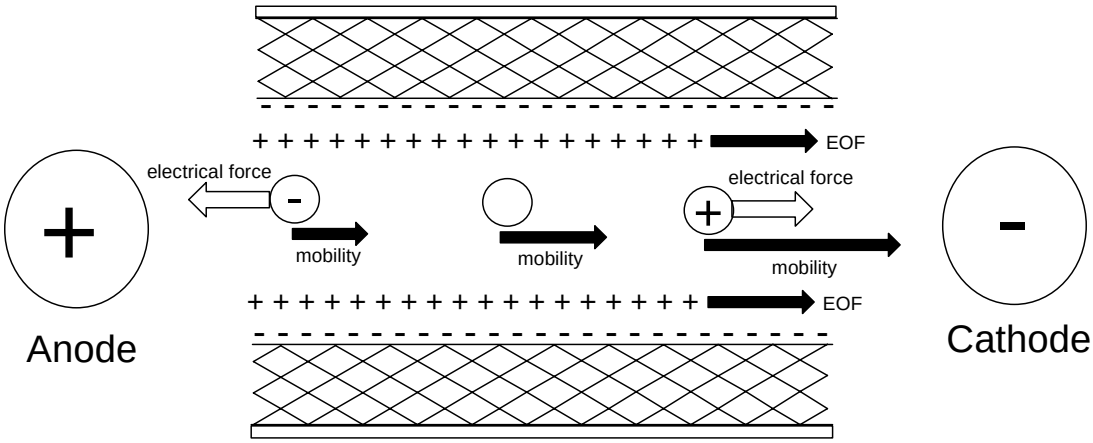

Figure 1.12. A schematic drawing of a capillary showing endoosmotic flow (EOF) and its effect on migration of cations, anions and neutral compounds. The solid arrows represent the direction and velocity of the migration of the different analytes, and the EOF. The open arrows represent the force on the different analytes due to the electrical field.

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The EOF also has a dramatic effect on the flow profile in the capillary. In normal pressure induced chromatography, the flow in the capillary will have a parabolic shape. The flow in the center of the capillary will be faster due to frictional drag at the wall of the column. This results in band spreading of the analytes. With EOF the flow profile will be flat, because the flow originates at the capillary wall. This will significantly reduce the band spreading in CE.

7.2 Band broadening

Even though EOF significantly reduces band spreading, it still occurs. Factors having an influence on band spreading include diffusion, Joule heating, viscosity, wall interactions and buffer mobility. Diffusion is the main source of band spreading in CE. Diffusion proceeds as the band of analyte moves through the capillary. The extent of diffusion depends on the diffusion coefficient of the compound and the time it spends in the capillary.

The electrical field across the capillary filled with buffer induces the flow of current. The flow of current is responsible for heating of the running buffer in a process called Joule heating. Heat is dissipated through the capillary wall resulting in an uneven temperature profile across the capillary, with the highest temperature at the center of the capillary. The effect of this Joule heating is band broadening. Band broadening due to Joule heating can be minimized by using small diameter capillaries, limiting the current flow, thermostatting the exterior capillary wall and using a low buffer concentration.

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The analytes can also have interactions with the capillary wall that can lead to band broadening. Due to the negative charge of the capillary wall, cations tend to be electrostatically attracted to it. Hydrophobic compounds also tend to adsorb to the capillary wall. Coating or deactivating the surface of the capillary minimizes wall interactions.

The buffer mobility also affects band broadening and peak skewing. This happens when the analyte has a different mobility than the buffer. The difference in mobility of the analyte results in a localized difference in the field strength in the analyte zone. For example, if a compound has mobility lower than the buffer mobility, the field strength will be higher within the analyte zone. The analytes will thus move faster in the analyte zone than in the pure buffer. As the solutes moves ahead it enters into the pure buffer and slows down, this tends to sharpen the front of the peak. As analytes diffuses into the pure buffer at the back of the analyte zone, it also slows down, leading to peak tailing. When the analyte has a mobility faster than the buffer, the peaks will tend to form peak fronting (Li, 1992; Weinberger, 1993).

7.3 Modes of capillary electrophoresis

Several different modes of CE exist, each with its own sets of advantages and disadvantages. Capillary zone electrophoresis (CZE) is the most common mode of CE. In CZE the capillary is filled with a dilute buffer solution and the different compounds are separated on the basis of their charge to weight ratio. This mode of analysis is applicable to a wide variety of ionic substances (Dolnik, 1997).

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A mode of capillary electrophoresis, which is very similar to CZE, is non-aqueous capillary electrophoresis (NACE). This method differs from CZE in that only non-aqueous solutions are used. NACE is an alternative for compounds that are difficult to separate using normal aqueous running buffers (Bjornsdottir, 1998; Altria, 1999).

Another popular mode of CE is micellar electrokinetic capillary chromatography (MEKC). This method has the advantage of using both principles of electrophoresis and chromatography in the separation of analytes. The principle of chromatography is added by the introduction of surfactant micelles. These micelles act as a pseudo stationary phase and separation occurs because analytes have different affinities towards the micelles and buffer. Compounds with a more hydrophobic nature will spend more time in the micelles and less in the buffer, whereas more hydrophyllic compounds will spend more time in the buffer and less time in the micelles. MECC can thus be used to separate both ionic and neutral compounds (Altria, 1999; Quirino and Terabe, 1999).

Another mode of CE that is similar to MEKC is microemulsion electrokinetic capillary chromatography (MEEKC). The differences are that where MEKC used surfactants as its pseudo stationary phase, MEEKC uses microemulsions. A microemulsion is a solution containing a dispersion of nanometer droplets of an immiscible liquid. In MEEKC oil droplets are used in an aqueous buffer with added ionic surfactants and a short tail alcohol as stabilizers to reduce the surface tension between the two liquid layers. Solutes penetrate the surface of the droplets in MEEKC much easier than the micelles in MEKC that have a more rigid structure. MEEKC can thus be applied to a wider range of compounds than MEKC (Altria, 1999).

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Capillary gel electrophoresis (CGE) fills the capillary with an aqueous gel such as cross-linked polyacrylamide or an entangled polymer solution. Separation occurs through a molecular sieving process as the analytes migrate through the pores of the gel. The ionic analytes are thus mainly separated on size (Rickwood and Hames, 1990).

For capillary isotachophoresis (CITP) the sample is injected between a high mobility leading buffer and a low mobility terminating buffer. The analytes are then separated on the basis of differing mobilities in a way resembling displacement chromatography. The analytes form adjacent zones whose length depends on the amount of analyte in the zone. This technique can only be used for ionic compounds (Li, 1992).

Capillary isoelectric focusing (CIEF) separate different zwitterionic compounds by creating a pH gradient throughout the capillary. The different analytes will migrate until they reach a pH zone were the net charge of the compound will be zero. CIEF is limited to amphoteric compounds with an isoelectric point (Li, 1992).

Capillary electrochromatography (CEC) is a new technique developed to combine the advantages of HPLC and CE. The principle of CEC is the packing of the capillary with a stationary phase similar to those used in HPLC and where the flow of the mobile phase is maintained by the EOF effect of CE rather than the pressure system used for HPLC. CEC has the advantages over HPLC of a flat flow profile and since the flow rate is maintained by the EOF it is not influenced by the particle size of the

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stationary phase. This results in the use of smaller stationary phase particles and the use of longer columns with the consequent increase in the number of theoretical plates (Bartle and Myers, 2001).

7.4 Capillary electrophoresis of tea flavan-3-ols

CE has only recently been applied for the analysis of tea catechins. Almost all analyses of tea catechins were done by HPLC until as recently as 1997, when the first publication on CE analysis of tea flavan-3-ols appeared (Horie *et al*, 1997). This publication was soon followed by a number of other publications. A summary of the CE methods in the literature used to analyze the flavan-3-ols and theaflavins are shown in Table 1.2.

Of the ten methods in the literature, only three used CZE (Horie *et al*, 1997; Arce *et al*, 1998; Lee and Ong, 2000), while the rest used the MEKC mode. Borate (Horie *et al*, 1997; Arce *et al*, 1998; Horie and Kohata, 1998; Nelson *et al*, 1998), or a combination of borate and phosphate (Larger *et al*, 1998; Watanabe *et al*, 1998; Barroso and van de Werken, 1999; Lee and Ong, 2000) was the buffer of choice, with only two methods using only a phosphate buffer for separation of the flavan-3-ols (Aucamp *et al*, 2000; Worth *et al*, 2000). Both these two methods however added methanol to their running buffers to obtain the necessary resolution. Other researchers also added organic modifiers to their running buffers, including methanol (MeOH) (Nelson *et al*, 1998), and acetonitrile (ACN) (Larger *et al*, 1998; Lee and Ong, 2000). Other buffer additives introduced to the running buffer to enhance selectivity include urea (Nelson *et al*, 1998), and ß-cyclodextrin (CD) (Nelson *et al*, 1998; Lee and Ong,

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2000).

The pH of the running buffers varied widely from a low of pH 2.5 to a high of pH 8.5. Most running buffers, however, had a pH of 7.0 and above. All the MEKC methods used SDS as their surfactant and all the analytical methods used UV detection and open fused-silica capillaries.

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Table 1.2. A summary of the published methods used to analyse the flavan-3-ol and theaflavin content of tea with capillary electrophoresis.

CE mode	Running buffer	Capillary length*	Voltage	Temperature	Detection**	Analytes	Reference
CZE	20 mM borate at pH 8.0	77cm, 70 cm, 50 um	30 kV	23°C	UV, 200 nm	EC, EGC, ECg, EGCg, C, caffeine, thaenine, ascorbic acid	Horie *et al*, 1997
CZE	150 mM borate at pH 8.5	57 cm, 50 cm, 75 um	20 kV	20°C	UV, 210 nm	EC, EGC, ECg, EGCg, C, gallic acid, caffeine, adenine, theophylline, quercetin, caffeic acid	Arce *et al*, 1998
MEKC	80 mM borate, pH 8.4, 50 mM SDS	77 cm, 70 cm, 75 um	25 kV	30°C	UV, 194 nm and 270 nm	EC, EGC, ECg, EGCg, theanine, caffeine, ascorbic acid	Horie and Kohata, 1998
MEKC	25 mM phosphate, 50 mM borate, pH 7.0, 25 mM SDS	36 cm, 32 cm, 50 um	15 kV	20°C	UV, 280 nm	EC, EGC, ECg, EGCg, C, Cg, GCg, Caffeine, ascorbic acid	Watanabe et al, 1998
MEKC	80 mM borate, pH 8.0, 110 mM SDS, 14% MeOH, 1.5 M urea, 1.0 mM beta-CD	67 cm, 60 cm, 50 um	20 kV	20°C	UV, 280 nm	EC, EGC, ECg, EGCg, C, GCg, caffeine, L-tryptophan	Nelson *et al*, 1998
MEKC	50 mM phosphate, 200 mM borate, pH 6.0, 20 mM SDS, 10% ACN	64.5 cm, 56 cm, 50 um	30 kV	25°C	UV, 278 nm	EC, EGC, ECg, EGCg, C, caffeine, theobromine, chlorogenic acid, kaempferol derivative, bisflavanols, theogallin, quercetin-3-glucoside	Larger *et al*, 1998
MEKC	12 mM K_2HPO_4, 12 mM borate, pH 7.0 40 mM SDS	85 cm, 70 cm, 50 um	30 kV	21°C	UV, 200 nm, 266 nm	EC, EGC, ECg, EGCg, C, caffeine, ascorbic acid	Barroso and van de Werken, 1999
MEKC	25 mM phosphate, pH 7.0 100 mM SDS, 6% MeOH	57 cm, 50 cm, 50 um	14 kV	25°C	UV, 200 nm	EC, EGC, ECg, EGCg, C, caffeine, ascorbic acid, theanine, gallic acid	Aucamp *et al*, 2000
MEKC	20 mM phosphate, pH 2.5, 100 mM SDS, 10% MeOH	50 cm, 45.4 cm, 50 um	20kV	25°C	UV, 195 nm	EC, EGC, ECg, EGCg, C, Cg, caffeine	Worth *et al*, 2000
CZE	10 mM KH_2PO_4, 200 mM borate, 27.5% ACN, 4.5 mM beta-CD	40 cm, 32 cm, 50 um	25 kV	30°C	UV, 205 nm	EC, EGC, ECg, EGCg, C, Cg, caffeine, adenine, theophylline, gallic acid, caffeic acid, the four major TFs	Lee and Ong, 2000
NACE	90 mM CH_3COONH_4, 71% ACN, 25% MeOH, 4% CH_3COOH	40 cm, 32 cm, 50 um	27.5 kV	18.5°C	UV, 380 nm	The four major TFs	Wright *et al*, 2001

* First is given the total capillary length, then the separating capillary length, and then the diameter of the capillary used.

** First is given the detection method, and then the wavelength used to measure spectrophotometric absorbance.

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7.5 Capillary electrophoresis of theaflavins

Only two methods have been published describing the analysis of TFs with CE. The first mention of an attempt to analyze the TFs with CE was by (Larger *et al*, 1998) who unsuccessfully tried to detect the TFs with MEKC. They hypothesized that the TFs were either degraded or were strongly retained by the capillary wall. The first researchers to detect the theaflavins with CE were Lee and Ong (2000), but their relative standard deviations for the linear regression of the TFs were 32.2% and the within day repeatability was less than 80%. The method of (Lee and Ong, 2000) was developed for the analysis of both the TFs and the flavan-3-ols. They used high amounts of ACN in their running buffer to counteract the interactions between the TFs and the capillary wall. Wright *et al* (2001) used only organic solvents in their NACE method to analyze the TFs. They obtained acceptable repeatability (< 6%) with their method developed specifically for analyses of only the TFs. The method developed by Wright *et al* (2001) is described in detail in chapter 3 of this thesis.

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8 Aims of study.

i) Determine the correlations between the individual flavan-3-ol content of the fresh tea leaf and the quality in the black tea derived thereof for Central and Southern African black tea clones.

ii) Develop a method to analyze the major theaflavins in black tea with CE.

iii) Determine the correlation between the major theaflavins in black tea and the quality of black tea for Central and Southern African tea clones.

iv) Determine the influence of tannase on the quality of black tea due to its alteration of the amount of gallated flavan-3-ols in tea.

9 Hypotheses

i) There is a correlation between the flavan-3-ol profiles in the fresh leaf and the quality of the black tea derived there from.

ii) The major theaflavins can be analyzed with CE.

iii) There is a correlation between the theaflavin profile and the quality in black tea.

iv) Good quality tea is associated with higher amounts of free theaflavin (TF-f) and mono-gallated theaflavins (TF-A and TF-B), while digallated theaflavin has no effect on the quality of black tea.

v) Tannase can alter the amounts of gallated flavan-3-ols, and thereby alter the quality of black tea.

10 Null hypotheses

i) There is no correlation between the flavan-3-ol profiles in the fresh leaf and the quality of the black tea derived there from.

ii) The major theaflavins can not be analyzed with CE.

iii) There is no correlation between the theaflavin profile and the quality in black tea.

iv) Good quality tea is not associated with higher amounts of free theaflavin (TF-f) and mono-gallated theaflavins (TF-A and TF-B), while digallated theaflavin has an effect on the quality of black tea.

v) Tannase can not alter the amounts of gallated flavan-3-ols, and thereby alter the quality of black tea.

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Chapter 2

Analysis of caffeine and flavan-3-ol composition in the fresh leaf for predicting the quality of the black tea (*Camellia sinensis*) produced in Southern Africa.

1 Introduction

The quality of the manufactured black tea produced in Southern Africa, based on organoleptic evaluation, has been medium by international standards. This prompted the TRF(CA) to adopt a long-term tea plant improvement programme in 1956 with the emphasis on quality (Ellis and Nyirenda, 1995). Evaluation of new clones is a long-term process requiring 15 years before release.

The aim of the work in this chapter was to develop a method for predicting quality early in this programme. This will assist the plant breeders in removing the poor quality potential clones early in the breeding programme.

Flavan-3-ols synthesised in tea leaves are the most important non-volatile constituent substrates of black tea. The flavan-3-ols provide the characteristic taste and visual appeal to the liquor (Robertson, 1992). Six major flavan-3-ols occur in tea; (+)-catechin (C), (+)-gallocatechin (GC), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECg) and (-)-epigallocatechin-3-gallate (EGCg). In black tea manufacture, the leaves are macerated to break down sub cellular compartments. This allows cytoplasmic polyphenol oxidase (PPO) (EC 1.10.3.1) to oxidise the flavan-3-ols in the vacuoles. The main consequence of this enzymatic

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oxidation process, formally known as the fermentation process, is the polymerisation of the flavan-3-ol monomers to form thearubigins (TRs) and theaflavins (TFs). It must be emphasised that this is not an anaerobic fermentation process and no alcohol is produced.

Various biochemical methods exist to assess quality in black tea, the most important being the analysis of thearubigins (TR) and theaflavins (TF) (Wood and Roberts, 1964). The chemical nature of the TRs is still poorly understood, making it difficult to analyse (Whitehead and Temple, 1992). Theaflavins, on the other hand, are well characterised. Total theaflavins were shown to be closely related with quality and market value for Central African seedling black teas (Hilton and Ellis, 1972; Hilton *et al*, 1973). This relationship was less apparent for Kenyan black teas (Owuor *et al*, 1986; Owuor *et al*, 1987). The theaflavins digallate equivalents were more important in predicting quality in Kenyan black teas (Owuor and Obanda, 1995; Owour *et al*, 1994).

A major disadvantage of methods evaluating quality based on TR and TF content is the need for samples of manufactured black tea. Sufficient amounts of tea leaves are necessary before a representative black tea sample of a clone can be prepared. As many as six years can pass before new tea clones in a breeding programme can be assessed for quality (Temple, 1995). Biochemical analysis of green leaf may open the possibility of selecting for quality much earlier in the breeding programme. This will also mean that more clones can be screened for quality since no laborious and time consuming vegetative propagation of poor clones would be necessary (Ellis and Nyirenda, 1995).

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Biochemical tests for predicting black tea quality from green leaf composition include carotenoid and chlorophyll content (Taylor *et al*, 1992), caffeine content (Bhatia, 1963; Millin *et al*, 1969) and flavanol composition (Hilton and Palmer-Jones, 1973; Madanhire, 1995; Obanda *et al*, 1997). The discrimination between tea clones on their carotenoid and chlorophyll level is still in a developing stage and not widely used. Selecting cultivars using chlorophyll florescence also seems promising (Mphangwe and Nyirenda, 1997). Caffeine is important in cream formation and the briskness of tea (Smith, 1968). Flavan-3-ol composition is important for formation of TRs and TFs. Especially in TF formation the flavan-3-ol composition seems to be of importance (Hilton and Palmer-Jones, 1973). Although the sand grinding method, using five fresh shoots, could be used to accurately analyse TF content (Hilton and Palmer-Jones, 1975), the chloroform test was preferred because of its rapidity and cost (Temple, 1995). Ding *et al* (1992) also speculated on the association of unoxidized flavan-3-ols with the astringency of black tea. In comparing flavan-3-ol composition with black tea quality, Obanda *et al* (1997) found ECg, EGCg and caffeine content to be indicators of quality. Similarly, it was suggested that the higher EGCg and ECg content in fresh leaf might lead to the formation of higher amounts of theaflavin-digallates in the black tea (Madanhire, 1995). It was also established that high total TF levels in black tea were associated with high levels of EGC and EC in green leaf (Hilton and Palmer-Jones, 1975; Robertson, 1983), but flavan-3-ol analysis was discontinued in the TRF(CA) breeding and clonal selection programme because the thin layer chromatography then used was laborious and time consuming with the large numbers involved.

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Except for the chloroform test, none of the above methods is being regularly used at present in any breeding and selection programme as far as we could ascertain.

In this work we investigated the flavan-3-ol composition of various tea clones grown in Malawi and South Africa. Correlations between flavan-3-ol contents, theaflavin content and tea taster scores were then determined. The emphasis was put on discriminating between poor quality and good quality clones, rather than between good and excellent clones (Wright *et al*, 2000; see Appendix A).

2 Materials and methods

2.1 Leaf

The TRF(CA) has been breeding and selecting tea clones since 1956. Many of these clones are available in 16 bush plots for production of sufficient fresh leaf for mini-manufacture of black tea samples for tea taster evaluation. Twenty good and twenty poor quality clones were selected from 16 bush plots based on prior tea taster scores determined in Blantyre, Malawi by tea taster A. The bushes were at least 6 years old. The first two leaves and the bud were collected for flavan-3-ol profile analysis. The leaves were collected at Mimosa and Nsuwadzi research stations of the TRF at Mulanje in Malawi during December of 1998. After collection a small sample of the tea leaves (10 g) were steamed for one minute and then dried for two hours in an oven at 95°C. The leaves were then stored frozen in airtight plastic bags at -20°C until analysis, except for the day taken to travel from Mulanje to Pretoria when they were kept at ambient temperature. The leaves that were not prepared for flavan-3-ol

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analysis (1 Kg) were sent to the Mini Processing Unit (MPU) at Mimosa for black tea manufacture.

2.2 Reagents

The flavan-3-ol standards used in running the capillary electrophoresis (CE) for analysing the flavan-3-ol profiles were kind gifts from Dr Y Hara (Mitsui Norin Corporation, Japan). Flavognost reagent (diphenylboric acid 2-aminoethyl ester) was obtained from Sigma (Sigma Aldrich, Jhb, RSA). All other reagents and solvents were of analytical grade.

2.3 Green leaf flavan-3-ol extraction

The dried tea leaves were pulverised to a powder in a mortar and pestle. This powder (50 mg) was then extracted with 5 ml of 40 % ethanol for 30 minutes at room temperature. The extract was then filtered through Schleicher and Schüll no. 595 fluted filter paper. An aliquot (0.45 ml) of this filtrate was then diluted ten times with the internal standard (0.1 mg / ml *para*-nitrophenol) to give a final volume of 4.5 ml. Samples with flavan-3-ol contents higher than the linear range on the CE were diluted twenty times with the internal standard. All extractions were done on the same day of CE analysis.

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2.4 Black tea manufacture

Black tea was manufactured at the MPU of the TRF(CA) at Mimosa. After collection of leaf the previous day, it was withered overnight at ambient temperature. The withered leaf was then passed 3 times through a cut, tear and curl (CTC) maceration machine and then fermented at 25ºC for 70 minutes. The fermented leaf was then dried in a miniature fluid bed drier to achieve 3% moisture content. The fibre was then removed with electrostatic rollers and the tea sieved to obtain particles between 500 and 1500 micrometer.

2.5 Total green leaf polyphenol determination

In determining the total polyphenol content the flavan-3-ol extraction was performed as described for CE flavan-3-ol extraction. No internal standard was, however, added. The filtered extract was diluted five times with deionised water before the polyphenol content was determined with the Folin-Ciocalteu reagent. The Folin-Ciocalteu reaction was performed as described by Julkunen-Tiito (1985).

2.6 Theaflavin determination

The flavognost method was used for determination of total TF content according to the method of Robertson and Hall (1989).

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2.7 Sensory analysis

Tea taster A in Blantyre had scored the teas on at least three different occasions on a ten-point scale for brightness, briskness, colour and strength of liquor. Additional scores were provided for the colour of the liquor with milk and for the colour of the infusion. The six scores were added for a total score out of 60.

Tea taster B in Johannesburg, South Africa scored the freshly made black tea samples from the forty clones. Tea taster B used a twenty-point scale for colour of the infusion, brightness, strength, briskness, quality and value. The six individual parameter scores were added to obtain the total score out of 120 points. Both tea tasters have expert knowledge of Southern African teas.

2.8 Statistical methodology

The flavan-3-ol profiles of the tea clones were separated in two groups according to the total scores of tea taster A. Twenty good clones were then compared with twenty poor quality clones using the unpaired Student's t-test to obtain (P) values. Regression analysis was used to find the Pearson correlation coefficients (r) between the measured parameters and the tea taster results and tea valuation. All the parameters measured in the fresh leaves and the black teas were correlated with the results of both tea tasters and the valuation. The SAS (1996) software package was used for these analyses.

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The LOGISTIC procedure of SAS (1996) was used to predict the quality of a clone according to the content of the individual measured parameters or combinations of them. The LOGISTIC procedure fitted linear logistic regression models for binary response data (good quality, poor quality) by the method of maximum likelihood. The stepwise assay was used with a significance level of entry into the model of 0.1 (90%) and a significance level for staying in the model of 0.15 (85%). This produced different formulas with which to calculate the probability of a clone to be of good quality according to the content of a specific parameter. With these formulas the ability to distinguish the different tea clones as either good or poor quality were determined for each of the different parameters. This gave the percent concordant value, the percentage of the clones that could be classified correctly. The statistical significance between the percent concordant values was determined by investigating the equality of proportions using the following equations to determine the Z statistic.

$$Z = \frac{P_1 - P_2}{\sqrt{P(1-P)\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}} \quad \text{with} \quad P = \frac{X_1 + X_2}{n_1 + n_2}$$

P_1 and P_2 = Percent concordant / 100 of the two parameters compared.

X_1 and X_2 = Amount of clones predicted correctly as either good or poor quality for each of the parameters compared.

n_1 and n_2 = Size of the sample for each of the parameters compared.

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2.9 Capillary electrophoresis analysis

Capillary electrophoresis was performed on a Beckman P/ACE 2100 using a fused silica capillary with a diameter of 50 μm with a total length of 58 cm and a separating length of 51 cm. The detector was set at 200 nm and the sample injected by pressure for 2s. The running buffer was 25 mM phosphate at a pH of 7.0 containing 100 mM sodium dodecyl sulphate (SDS) and 6% methanol (v/v). The applied voltage was 14 kV and the temperature was maintained at 25°C. Each capillary electrophoresis analysis was done in duplicate. For a more detailed account see the method of Aucamp et al (2000).

3 Results

A typical electropherogram of the flavan-3-ols and their structures are shown in Figure 2.1. The flavan-3-ols were also grouped according to the presence or absence of the 5'-hydroxyl group on the B ring (tri-hydroxy flavan-3-ols and di-hydroxy flavan-3-ols respectively) and the presence or absence of gallic acid (gallated flavan-3-ols and non-gallated flavan-3-ols respectively).

The forty tea clones were screened for their flavan-3-ol profiles and the total polyphenol content of their fresh leaves. However, (+)-gallocatechin occurs at trace amounts below the limit of detection of our analysis method. (+)-Gallocatechin is also not a precursor to one of the major TFs occurring in tea, and therefore should not have

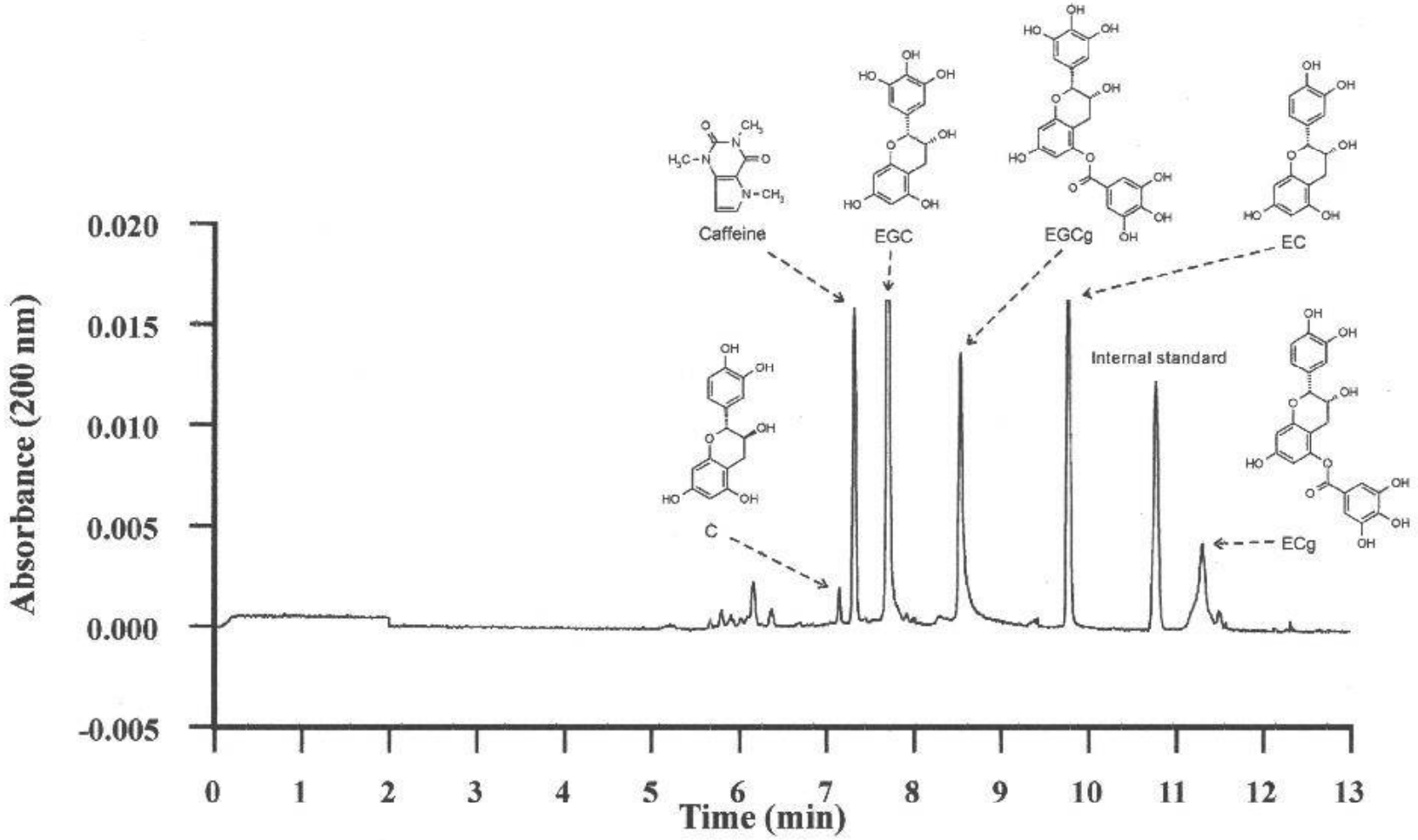

Figure 2.1. Electropherogram of a green leaf extract. Running buffer 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol at pH 7.0. Capillary length 57 cm (50 cm to the detector), 50 µm internal diameter, sample injection 0.5 psi for 2 seconds. Applied voltage 14 kV, capillary temperature 25C and detection at 200 nm. The peak identities are indicated on the electropherogram.

Table 2.1. Total polyphenol, caffeine and individual catechin contents of fresh leaf, TF content of black tea, total tea taster scores and valuation for forty TRF tea clones.

Clone	C (umol/g DL)	EGC (umol/g DL)	EGCg (umol/g DL)	EC (umol/g DL)	ECg (umol/g DL)	SIF (umol/g DL)	Caffeine (umol/g DL)	Di-hydroxy flavan-3-ols (umol/g DL)	Tri-hydroxy flavan-3-ols (umol/g DL)	Gallated flavan-3-ols (umol/g DL)	Non-gallated flavan-3-ols (umol/g DL)	Total polyphenols (mg/g DL)	Total TF (umol/g DT)	Taster A score	Taster B score	Valuation (UK pence/kg)
Good quality																
SFS204	9.44	78.13	235.42	32.97	78.39	434.35	155.88	120.80	313.55	313.82	120.53	208.22	11.79	23.6	70	130
PC1	9.87	250.50	174.94	90.93	58.09	584.33	168.80	158.89	425.44	233.03	351.30	224.99	17.19	22.1	54	117.2
88/79-2	3.91	87.84	257.76	26.79	60.05	436.35	193.06	90.76	345.60	317.81	118.54	166.51	7.72	18.2	58	109.4
PC117	13.39	140.76	244.53	68.05	89.05	555.79	193.20	170.49	385.29	333.59	222.20	219.76	13.04	24	74	147.7
PC213	9.49	161.97	200.54	63.42	64.41	499.83	193.85	137.32	362.52	264.95	234.88	131.27	12.06	21.7	74	140.6
PC190	5.81	108.36	329.29	40.62	99.39	583.46	170.73	145.82	437.64	428.67	154.79	276.08	15.18	20	64	151
PC108	8.56	176.73	251.69	68.87	80.41	586.25	213.16	157.83	428.42	332.10	254.15	194.52	17.73	25.1	72	150.5
15M-58	7.79	140.80	232.12	57.19	71.70	509.60	186.61	136.68	372.92	303.82	205.78	198.21	8.75	22.1	68	128.5
15M-39	4.43	130.47	243.78	36.91	54.73	470.33	165.52	96.07	374.26	298.52	171.81	211.91	9.11	20.6	74	121.5
NKW51	11.71	132.52	225.34	54.83	67.65	492.06	194.72	134.19	357.86	292.99	199.07	205.76	14.96	25.6	64	143.3
88/3-3	6.90	61.97	243.42	24.82	76.68	413.78	201.72	108.39	305.39	320.10	93.68	230.84	6.56	21.2	64	123.3
15M-1	11.24	123.13	258.95	64.94	100.03	558.28	209.36	176.21	382.08	358.98	199.31	180.36	17.28	21.9	64	118.7
88/5-2	8.20	100.35	230.55	39.97	70.20	449.26	194.42	118.36	330.90	300.75	148.51	160.51	12.77	21.7	78	123.8
PC200	5.91	119.25	219.56	48.31	66.71	459.73	217.96	120.93	338.81	286.27	173.46	133.43	9.33	21.9	64	141.3
PC192	8.94	183.64	226.64	81.56	92.46	593.23	267.10	182.96	410.28	319.10	274.13	146.51	15.67	22.1	74	153
PC119	9.01	212.17	227.22	76.49	73.96	598.85	145.25	159.45	439.40	301.18	297.67	238.38	15.63	24.1	64	152.1
PC168	9.95	87.17	241.08	66.39	131.23	535.81	206.68	207.57	328.25	372.30	163.51	234.07	6.65	25	54	140.3
33/10-47	11.78	161.22	182.72	69.86	54.93	480.51	181.07	136.57	343.94	237.65	242.86	219.45	13.80	25.2	76	137.3
PC104	11.57	189.09	272.28	114.67	129.72	717.34	163.59	255.97	461.37	402.01	315.34	162.67	18.44	30.1	64	150.2
PC118	6.65	191.62	221.08	93.50	88.90	601.74	196.62	189.05	412.70	309.98	291.77	253.62	14.02	23.8	74	149.1
Low quality																
88/50-5	6.94	107.65	221.61	45.54	72.20	453.94	161.38	124.68	329.26	293.81	160.14	180.67	8.44	19.8	52	116.2
PC206	11.25	117.82	294.18	35.26	74.34	532.85	153.63	120.85	412.00	368.52	164.33	233.76	12.32	16.2	52	98.8
SFS42	12.32	137.48	233.86	48.17	74.34	506.17	142.92	134.82	371.35	308.20	197.97	203.76	10.76	n.a.	45	n.a.
NKW30	6.60	76.22	232.32	23.55	57.01	395.71	201.71	87.16	308.54	289.33	106.38	152.35	5.22	17.4	45	113.2
88/60-9	5.44	123.22	248.78	39.80	62.71	479.96	181.66	107.95	372.01	311.49	168.47	207.76	8.39	17.7	50	108.8
NKW20	3.49	71.98	246.99	17.28	43.32	383.06	205.08	64.09	318.97	290.31	92.75	155.74	4.82	14.7	40	107.6
NKW44	9.12	49.71	233.10	26.99	50.16	369.07	176.24	86.26	282.81	283.25	85.82	180.21	8.39	14.5	50	106.7
88/79-1	4.11	69.82	220.26	25.43	51.29	370.91	180.58	80.83	290.08	271.54	99.37	154.66	6.12	17	45	114.8
88/54-11	6.36	118.27	223.06	42.46	68.34	458.48	222.07	117.16	341.33	291.40	167.09	157.28	10.45	18.4	52	110
PC169	7.76	38.88	165.90	21.11	72.54	306.18	233.83	101.40	204.78	238.44	67.74	126.96	7.59	19.3	50	113
PC186	4.63	98.40	236.42	34.61	58.90	432.97	160.77	98.14	334.83	295.32	137.65	104.03	12.23	18.3	50	135
CL12	2.38	136.22	253.76	47.51	66.51	506.38	130.52	116.40	389.98	320.27	186.11	290.09	12.19	n.a.	50	n.a.
11M-3	5.13	135.99	228.31	55.75	68.24	493.42	200.76	129.12	364.30	296.55	196.87	168.36	9.02	17.6	45	102.8
PC211	7.26	98.34	289.98	26.76	66.60	488.94	188.99	100.61	388.32	356.58	132.36	188.83	7.46	16.4	45	125.5
88/61-11	6.89	39.18	279.01	21.57	82.09	428.74	243.10	110.55	318.19	361.10	67.64	215.14	6.03	14.4	40	88.6
88/119-1	8.53	122.59	270.72	38.38	67.15	507.36	202.38	114.05	393.31	337.87	169.50	208.53	10.72	18.1	53	112
88/54-14	6.40	115.66	229.29	35.58	51.16	438.09	160.75	93.14	344.95	280.45	157.64	129.12	7.72	17.4	52	108.8
15M-18	7.31	139.88	188.37	51.57	51.70	438.84	187.90	110.59	328.25	240.07	198.76	179.90	6.65	20.1	50	116.1
PC194	5.28	122.35	226.85	40.05	53.90	448.43	187.21	99.23	349.20	280.75	167.69	190.37	10.09	18.8	50	142
88/110-4	4.90	28.22	230.95	16.32	70.13	350.52	226.85	91.34	259.18	301.08	49.44	147.74	6.38	16	45	102.4

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a significant impact on the TF content (Robertson, 1983). The TF content of the black tea manufactured from each clone was determined. These parameters and the total scores for tea taster A and B and the valuation are given in Table 2.1. The tea clones were separated in two groups of twenty good quality tea clones and twenty poor quality tea clones. This grouping was done according to tea taster results done previously on at least two occasions.

All the appropriate data sets were tested for normality using the normal probability plot (Figure 2.2 to 2.4). Since many of the data sets showed divergence from normality, the distribution free Kruskal-Wallis test was also used to test for significant differences between data sets (see Table 2.2). Although the P-values obtained with the Kruskal-Wallis test differ from those obtained with the Student's t-test, both tests showed significant differences between the same data sets. The P-values in the text were calculated with the Kruskal-Wallis test.

EGCg was the most abundant flavan-3-ol for all the clones except for PC1. Levels for EGCg ranged between 165.90 and 329.29 µmol g^{-1} dried tea leaf (DTL). There was no significant difference (P = 0.7868) between the EGCg values of the good and poor quality tea clones, see Table 2.2.

The second most abundant flavan-3-ol was EGC, which ranged between 28.22 and 250.50 µmol g^{-1} DTL. There was a significant difference in the EGC values between the poor and good quality tea clones (P = 0.0049).

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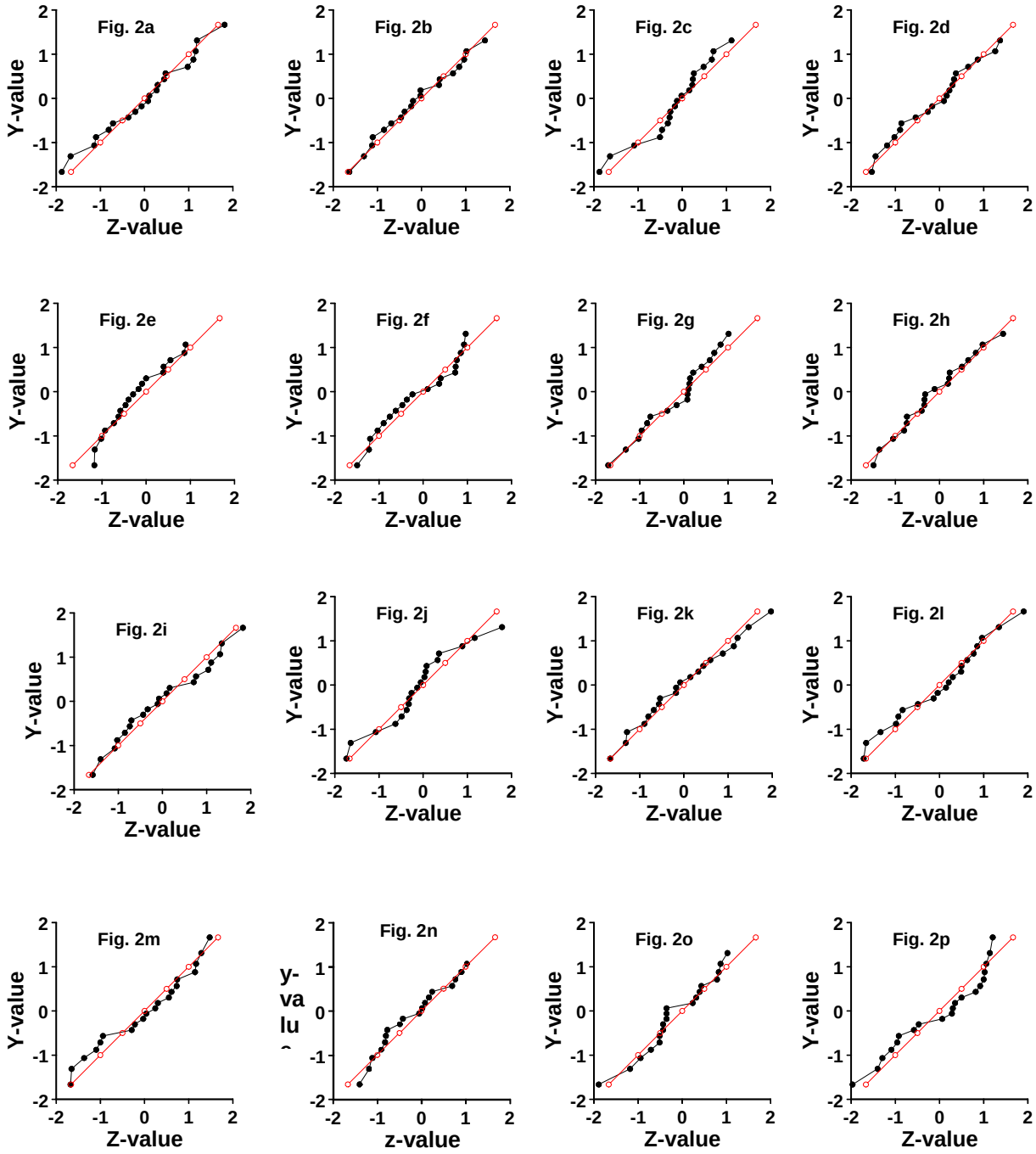

Figure 2.2. Normal probability plot for variables of good quality clones (a-p). The variables tested for normality were: (a), C; (b), EGC; (c), EGCg; (d), EC; (e), ECg; (f), SIF; (g), caffeine; (h), non-gallocatechins; (i), gallocatechins; (j), gallated catechins; (k), non-gallated catechins; (l), total polyphenols measured with Folin-Ciocalteu; (m), total theaflavins measured with flavognost; (n), taster B score; (o), taster A score; (p), value.

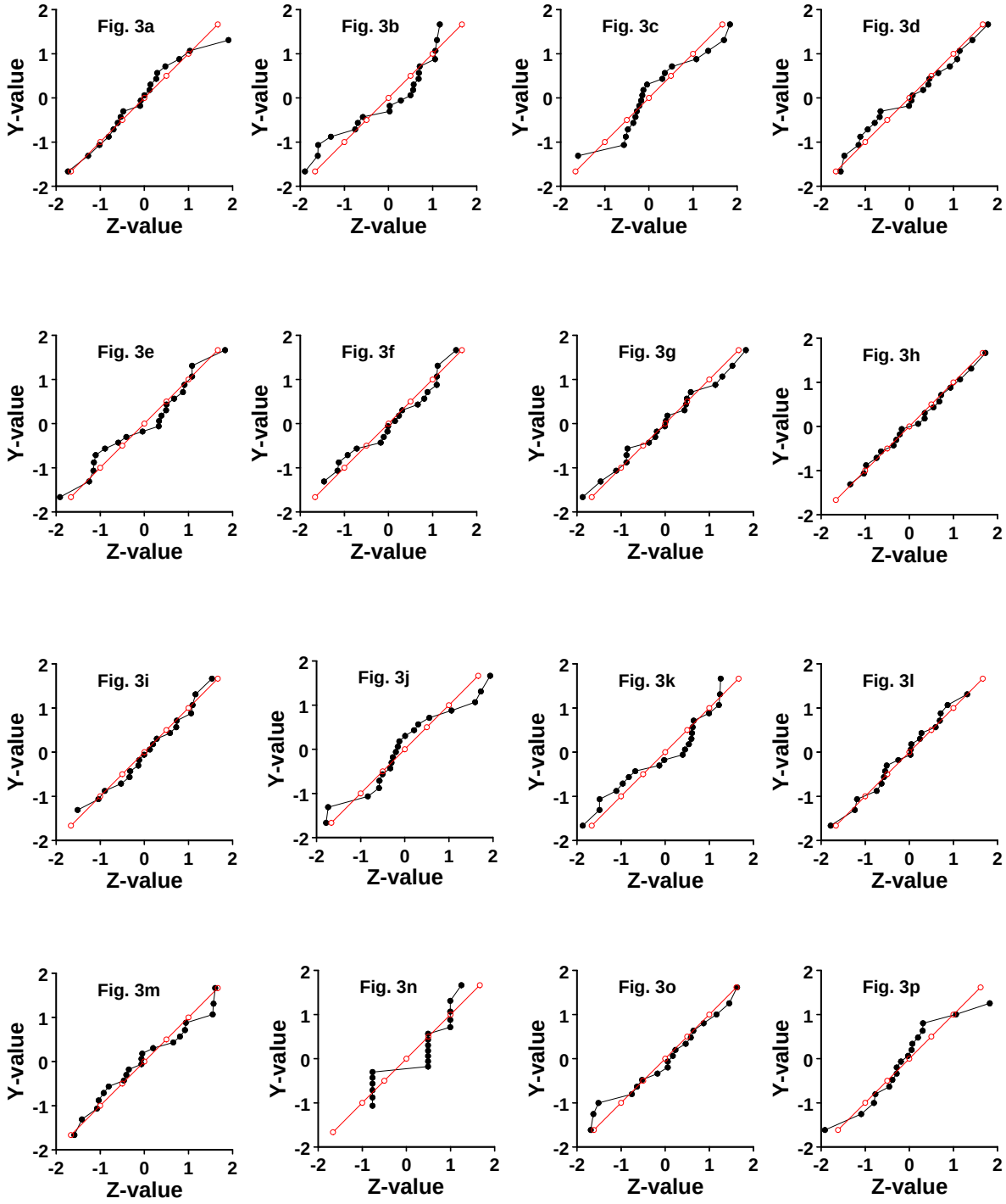

Figure 2.3. Normal probability plot for variables of poor quality clones (a-p). The variables tested for normality were: (a), C; (b), EGC; (c), EGCg; (d), EC; (e), ECg; (f), SIF; (g), caffeine; (h), non-gallocatechins; (i), gallocatechins; (j), gallated catechins; (k), non-gallated catechins; (l), total polyphenols measured with Folin-Ciocalteu; (m), total theaflavins measured with flavognost; (n), taster B score; (o), taster A score; (p), value.

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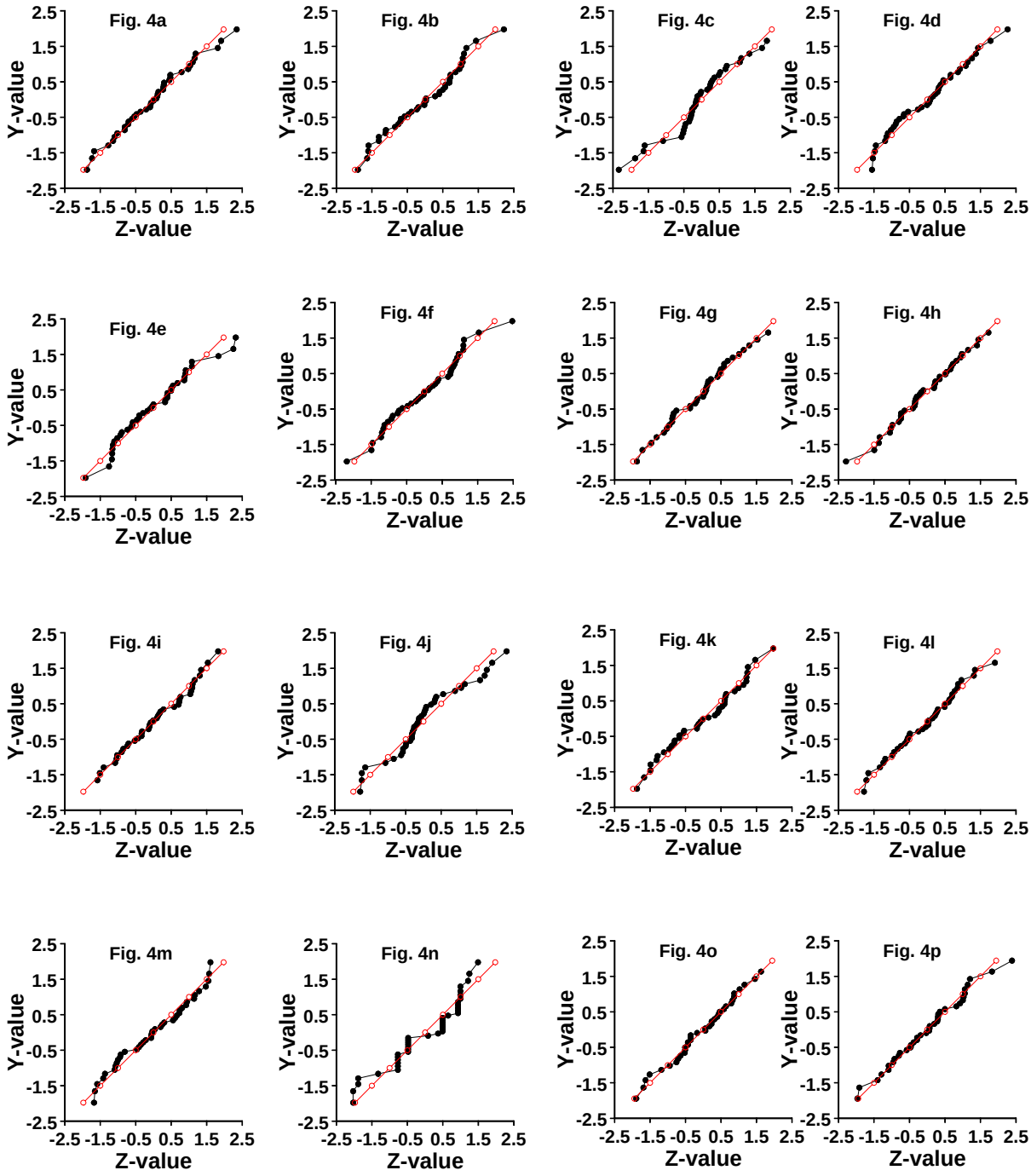

Figure 2.4. Normal probability plot for variables of a combination of good quality and poor quality clones (a-p). The variables tested for normality were: (a), C; (b), EGC; (c), EGCg; (d), EC; (e), ECg; (f), SIF; (g), caffeine; (h), non-gallocatechins; (i), gallocatechins; (j), gallated catechins; (k), non-gallated catechins; (l), total polyphenols measured with Folin-Ciocalteu; (m), total theaflavins measured with flavognost; (n), taster B score; (o), taster A score; (p), value.

Table 2. Student's t-test and Kruskal-Wallis test significance between the good and poor quality tea clones and the regression analysis between the measured parameters and the tea taster results and tea valuation.

	C (umol/g DL)	EGC (umol/g DL)	EGCg (umol/g DL)	EC (umol/g DL)	ECg (umol/g DL)	SIF (umol/g DL)	Caffeine (umol/g DL)	Di-hydroxy flavan-3-ols (umol/g DL)	Tri-hydroxy flavan-3-ols (umol/g DL)	Gallated flavan-3-ols (umol/g DL)	Non-gallated flavan-3-ols (umol/g DL)	Total [c] polyphenols (mg/g DL)	Total TF (umol/g DT)	Taster A score	Taster B score	Valuation (UK pence/kg)
Mean	8.73	141.88	235.95	61.05	80.43	528.05	190.97	150.22	377.83	316.38	211.67	199.85	12.88	23.0	67.40	136.44
SD(n-1)	2.57	48.92	32.54	23.68	21.91	76.45	26.69	39.82	45.85	47.96	70.79	40.00	3.77	2.54	7.11	13.74
(for good quality clones)																
Mean	6.60	97.40	237.69	34.69	63.13	439.50	187.42	104.42	335.08	300.82	138.69	178.76	8.55	17.30	48.05	112.35
SD(n-1)	2.44	36.53	30.73	11.81	10.36	60.91	30.43	17.62	50.19	34.97	47.74	41.93	2.35	1.72	3.97	12.42
(for poor quality clones)																
Mean	7.67	119.64	236.82	47.87	71.78	483.77	189.19	127.32	356.46	308.60	175.17	189.31	10.72	20.32	57.73	125.03
SD(n-1)	2.69	48.20	31.25	22.79	19.05	81.64	28.31	38.23	52.15	42.17	70.13	41.83	3.80	3.59	11.33	17.19
(for all the clones)																
Student's t-test (P value)	0.0108	0.00249	0.863	0.000123	0.00355	0.000258	0.697	0.0000725	0.00776	0.249	0.000549	0.0559	0.000128	2.08E-09	1.2E-11	1.88E-06
Kruskal-Wallis test (P value)	0.0110	0.0049	0.7868	0.0003	0.0053	0.0009	0.6652	< 0.0001	0.0179	0.1677	0.0012	0.0659	0.0004	< 0.0001	< 0.0001	< 0.0001
r (Taster B)	0.4**	0.478**	-0.099	0.541**	0.305*	0.479**	0.028	0.503**	0.382**	0.0644	0.519**	0.174	0.607**			
r (Taster A)	0.608**	0.62**	-0.182	0.802**	0.581**	0.674**	-0.131	0.807**	0.466**	0.128	0.708**	0.265*	0.684**			
r (Valuation)	0.363*	0.562**	-0.019	0.649**	0.446**	0.621**	-0.088	0.633**	0.510**	0.187	0.610**	0.216	0.631**			
m (Valuation)[a]	2.570	0.203	-0.011	0.493	0.406	0.132	-0.060	0.287	0.171	0.077	0.151	0.097	2.880			

[a] The slope of the linear regression obtained with the different variables against value as the dependent variale.
*,** Significant at P < 0.05 and 0.01, respectively.

[c] Total polyphenols were determined as catechin equivalants using the Folin-Ciocalteu reagent.

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All the other flavan-3-ols (C, EC, and ECg) occurred in much lower amounts. This was determined by comparing the overall contents of the different flavan-3-ols with each other, using Student's t-test and the Wilcoxon rank sum test (Table 2.3). There was a significant difference between the amounts of all the flavan-3-ols. The flavan-3-ol occurring at the highest amounts were EGCg, followed by EGC, ECg, EC and then C.

C, EC and ECg showed a significant difference between the good and poor quality tea clones. EC showed the clearest difference between good and poor quality clones of all the flavan-3-ols (P = 0.0003). ECg and C had P values of 0.0053 and 0.0110 respectively. The contents of these flavan-3-ols ranged between 2.38 - 13.39 µmol g-1 DTL, 16.32 - 114.67 µmol g^{-1} DTL and 43.32 - 131.23 µmol g^{-1} DTL for C, EC and ECg respectively.

Table 2.3. Student's t-test and Wilcoxon rank sum test for significant differences between the overall contents of the individual flavan-3-ols.

	ECg	EC	EGCg	EGC
Student's t-test				
C	1.828E-23	8.988E-14	4.715E-36	1.616E-17
EGC	3.677E-07	1.177E-11	8.238E-20	
EGCg	2.880E-38	4.947E-43		
EC	2.525E-06			
Wilcoxon rank sum test				
C	< 0.0001	< 0.0001	< 0.0001	< 0.0001
EGC	< 0.0001	< 0.0001	< 0.0001	
EGCg	< 0.0001	< 0.0001		
EC	< 0.0001			

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The flavan-3-ols were grouped according to the presence or absence of the 5'-hydroxyl group on the B ring (tri-hydroxy flavan-3-ols and di-hydroxy flavan-3-ols respectively) and the presence or absence of gallic acid (gallated flavan-3-ols and non-gallated flavan-3-ols respectively). In Table 2.4 the different groups of flavan-3-ols were also tested for significant differences between the overall contents of the different groups. There were significant differences between all the groups tested. The group containing the most flavan-3-ols was the tri-hydroxy flavan-3-ols, followed by the gallated flavan-3-ols, the non-gallated flavan-3-ols and lastly the di-hydroxy flavan-3-ols. The last two groups mentioned contained much less flavan-3-ols. Comparing the amounts of the flavan-3-ols in the different groups is important for understanding their effect on TF formation.

Table 2.4. Student's t-test and Wilcoxon rank sum test for significant differences between the overall contents of different groups of flavan-3-ols.

	Non-gallated flavan-3-ols	Gallated flavan3-ols	Tri-hydroxy flavan-3-ols
Student's t-test			
Di-hydroxy flavan-3-ols	3.510E-04	1.754E-32	4.576E-34
Tri-hydroxy flavan-3-ols	8.945E-21	2.335E-05	
Gallated flavan-3-ols	3.128E-15		
Wilcoxon rank sum test			
Di-hydroxy flavan-3-ols	0.0006	< 0.0001	< 0.0001
Tri-hydroxy flavan-3-ols	< 0.0001	< 0.0001	
Gallated flavan-3-ols	< 0.0001		

The sum of individual flavanols (SIF), as determined by CE, ranged between 306.18 and 717.34 µmol g^{-1} DTL. Generally the good quality tea clones had a higher SIF content than the poor quality tea clones (P = 0.0009). The tea clone with the highest SIF content was PC104, though this clone was not released because of poor nursery performance and field survival. The total polyphenols (catechin equivalents), measured with the Folin-Ciocalteu method, also showed a difference between the poor and good quality tea clones (P = 0.0659). This difference was however not nearly as good as the SIF contents, and the correlation coefficient between total polyphenols and total taster scores (r = 0.174 and r = 0.265, compared to r = 0.479 and r = 0.674 for SIF) and value (r = 0.216 compared to r = 0.621 for SIF) were also low. There was a significant difference only at the 95% confidence level between the correlation coefficients of SIF and total polyphenols for taster A scores and valuation. For taster B there was no significant difference between the mentioned correlation coefficients (Table 2.5).

To compare the amount of the total polyphenols determined with the Folin-Ciocalteu reagent with the SIF amount, the means and standard deviations of SIF was calculated as mg / g DTL. The SIF mean for all the clones was 193.08 ± 28.30 mg / g DTL, compared to 189.31 ± 41.83 mg / g DTL for the total polyphenol mean, with no statistical significant difference between the two values (P = 0.638). There was also no significant difference (good quality, P = 0.485; poor quality P = 0.995) between the means for SIF and total polyphenols for the good quality (207.46 ± 26.78 and 199.85 ± 40.00 mg / g DTL) and for the poor quality (178.70 ± 22.18 and 178.76 ± 41.93 mg / g DTL) clones. The variations in the SIF amounts are however considerably lower than those for the total polyphenol amounts (28.30, 26.78 and

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22.18 compared to 41.83, 40.00 and 41.93). On the other hand, these two parameters differ significantly at the 90% confidence level in their ability to predict the quality of a tea clone (percent concordant 80.8 and 66.5 respectively, see Table 2.6).

PC104 also had the highest EC, di-hydroxy flavan-3-ol and TF contents. PC190 and PC1 had the highest EGCg and EGC content respectively; they also had the highest amounts of gallated flavan-3-ols and non-gallated flavan-3-ols respectively. PC1 also had the highest tri-hydroxy flavan-3-ols. Both PC1 and PC104 were initially selected on the basis of having high EGC and EC levels.

No significant difference could be detected in the amounts of gallated flavan-3-ols between the good and poor quality tea clones. The tri-hydroxy flavan-3-ols, di-hydroxy flavan-3-ols and non-gallated flavan-3-ols did show differences between the good and poor quality tea clones. The di-hydroxy flavan-3-ols had the most significant difference ($P < 0.0001$), followed by the non-gallated flavan-3-ols ($P = 0.0012$) and tri-hydroxy flavan-3-ols ($P = 0.0179$). These observations supported previous findings on Malawi teas (Hilton and Palmer-Jones, 1975; Robertson, 1983) but do not agree with the work of Obanda *et al* (1997), perhaps because we used different clones with different quality parameters than the Kenyan clones.

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Table 2.5. The z-statistic for comparing different r-values obtained from correlating green leaf parameters and flavognost TF content with taster A and taster B scores and value respectively.

	EGC	EGCg	EC	ECg	SIF	Caffeine	Di-hydroxy flavan-3-ols	Tri-hydroxy flavan-3-ols	Gallated flavan-3-ols	Non-gallated flavan-3-ols	Total polyphenols	Total TF
(Taster A)												
C	0.08	3.72**	1.67	0.17	0.47	3.50**	1.73	0.84	2.41*	0.74	1.82	0.55
EGC		3.80**	1.59	0.26	0.39	3.58**	1.65	0.92	2.49*	0.66	1.90	0.47
EGCg			5.39**	3.55**	4.19**	0.22	5.45**	2.88**	1.31	4.46**	1.91	4.27**
EC				1.84	1.20	5.17**	0.06	2.51*	4.08**	0.92	3.48**	1.12
ECg					0.64	3.33**	1.90	0.67	2.24*	0.92	1.64	0.72
SIF						3.97**	1.26	1.31	2.88**	0.27	2.29*	0.08
Caffeine							5.23**	2.66**	1.09	4.25**	1.69	4.05**
Di-hydroxy flavan-3-ols								2.57*	4.14**	0.98	3.54**	1.18
Tri-hydroxy flavan-3-ols									1.57	1.58	0.98	1.39
Gallated flavan-3-ols										3.16**	0.60	2.96**
Non-gallated flavan-3-ols											2.56*	0.19
Total polyphenols												2.36**
(Taster B)												
C	0.42	2.25*	0.78	0.47	0.42	1.70	0.56	0.09	1.54	0.65	1.07	1.21
EGC		2.67**	0.37	0.88	0.01	2.12*	0.14	0.51	1.96*	0.23	1.48	0.79
EGCg			3.03**	1.78	2.67**	0.55	2.81**	2.16*	0.70	2.90**	1.18	3.46**
EC				1.25	0.36	2.48*	0.22	0.87	2.33*	0.13	1.85	0.42
ECg					0.89	1.23	1.02	0.38	1.08	1.12	0.60	1.67
SIF						2.12*	0.14	0.51	1.97*	0.23	1.49	0.78
Caffeine							2.26*	1.61	0.16	2.35*	0.64	2.91**
Di-hydroxy flavan-3-ols								0.65	2.10*	0.09	1.62	0.65
Tri-hydroxy flavan-3-ols									1.45	0.74	0.97	1.3
Gallated flavan-3-ols										2.20*	0.48	2.75**
Non-gallated flavan-3-ols											1.72	0.56
Total polyphenols												2.27*
(Value)												
C	1.07	1.67	1.65	0.42	1.45	1.96*	1.53	0.76	0.80	1.37	0.67	1.52
EGC		2.74**	0.58	0.65	0.38	3.03**	0.46	0.31	1.87	0.31	1.74	0.45
EGCg			3.32**	2.09*	3.12*	0.29	3.20**	2.43*	0.87	3.05**	1.00	3.19**
EC				1.23	0.20	3.61**	0.11	0.88	2.44*	0.27	2.32*	0.13
ECg					1.03	0.44	1.12	0.35	1.22	0.96	1.09	1.10
SIF						3.41**	0.08	0.69	2.25*	0.07	2.12*	0.07
Caffeine							3.49**	2.72**	1.16	3.33**	1.29	3.48**
Di-hydroxy flavan-3-ols								0.77	2.33*	0.16	2.20*	0.01
Tri-hydroxy flavan-3-ols									1.56	0.61	1.44	0.75
Gallated flavan-3-ols										2.17*	0.13	2.32*
Non-gallated flavan-3-ols											2.05*	0.14
Total polyphenols												2.19*

*,** Significant at P < 0.05 and 0.01, if z > 1.96 and 2.58, respectively.

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The TF values obtained with the flavognost method in the made black tea also showed a significant difference between good and poor quality tea clones (P = 0.0004). This supports earlier work by Hilton and Ellis (1972) and by Ellis and Cloughley (1981).

The correlation coefficients between the fresh leaf and black tea parameters showed similar trends for Taster A and Taster B. Parameters with low P values had high r-values. This indicates that parameters with low P values discriminate between clones with good and poor quality as perceived by both tea tasters. For Taster B, total TF had the highest correlation coefficient (r = 0.607), followed by EC (r = 0.541), non-gallated flavan-3-ols (r = 0.519) and di-hydroxy flavan-3-ols (r = 0.503). There is no significant difference between these r-values at the 95% confidence level. The correlation coefficients obtained for the above parameters with the total score for Taster A were higher. The di-hydroxy flavan-3-ols (r = 0.807) and EC (r = 0.802) had the highest correlation coefficient, followed by non-gallated flavan-3-ols (r = 0.708) and then by total TF (r = 0.684). Once again there was no significant difference between these correlation coefficients.

Correlations between the fresh leaf and black tea parameters and the valuation (UK pence / kg) were also determined. EC content had the highest correlation with value (r = 0.649), followed by di-hydroxy flavan-3-ols (r = 0.633), TF (r = 0.631), non-gallated flavan-3-ols (r = 0.610), tri-hydroxy flavan-3-ols (r = 0.510) and ECg (r = 0.446) content. There was no significant difference between these correlation coefficients at the 95% confidence level. As shown in Figure 3a, b and c, the narrowest 90% confidence limits were obtained for TF, followed by EC and the non-gallated flavan-3-ols. When comparing the slopes of the linear regression between the

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parameters and value, the highest m-value was obtained for TF, and then C (m = 2.880 and 2.570 respectively)(Table 2.2). The next highest m-values were obtained with EC and ECg (m = 0.493 and 0.406 respectively). The higher the m-value obtained with a specific parameter, the higher the increase of value with an increase of that parameter. By comparing both the confidence limits and the slopes of the linear regression of the different parameters and value, TF determined according to the flavognost method has the best ability to predict the value of a Southern African tea clones. The total TF content can however only predict 40% (r^2 = 0.40 for TF vs. value) of the variation in the value of the black tea, indicating that it only has a limited ability of predicting the value of Southern African black tea.

To determine which of the parameters had the best ability to distinguish between good and poor quality tea clones, the LOGISTIC procedure of SAS was used to predict the quality of the clone according to the content of the individual parameters. Using different formulas calculated by the above mentioned procedure, the ability to classify the different tea clones as either good or poor quality were determined according to the content of the individual flavan-3-ols. This gave the percent concordant value, the percentage of the clones that could be classified correctly, as shown in Table 2.6. Using the same methods the combination of different parameters was also tested for their ability to predict the quality of a tea clone. This was done by using the stepwise assay of the LOGISTIC procedure, which only identified the EC and ECg content to predict the likelihood for a clone to produce a good or poor quality black tea at the 0.1 significance level.

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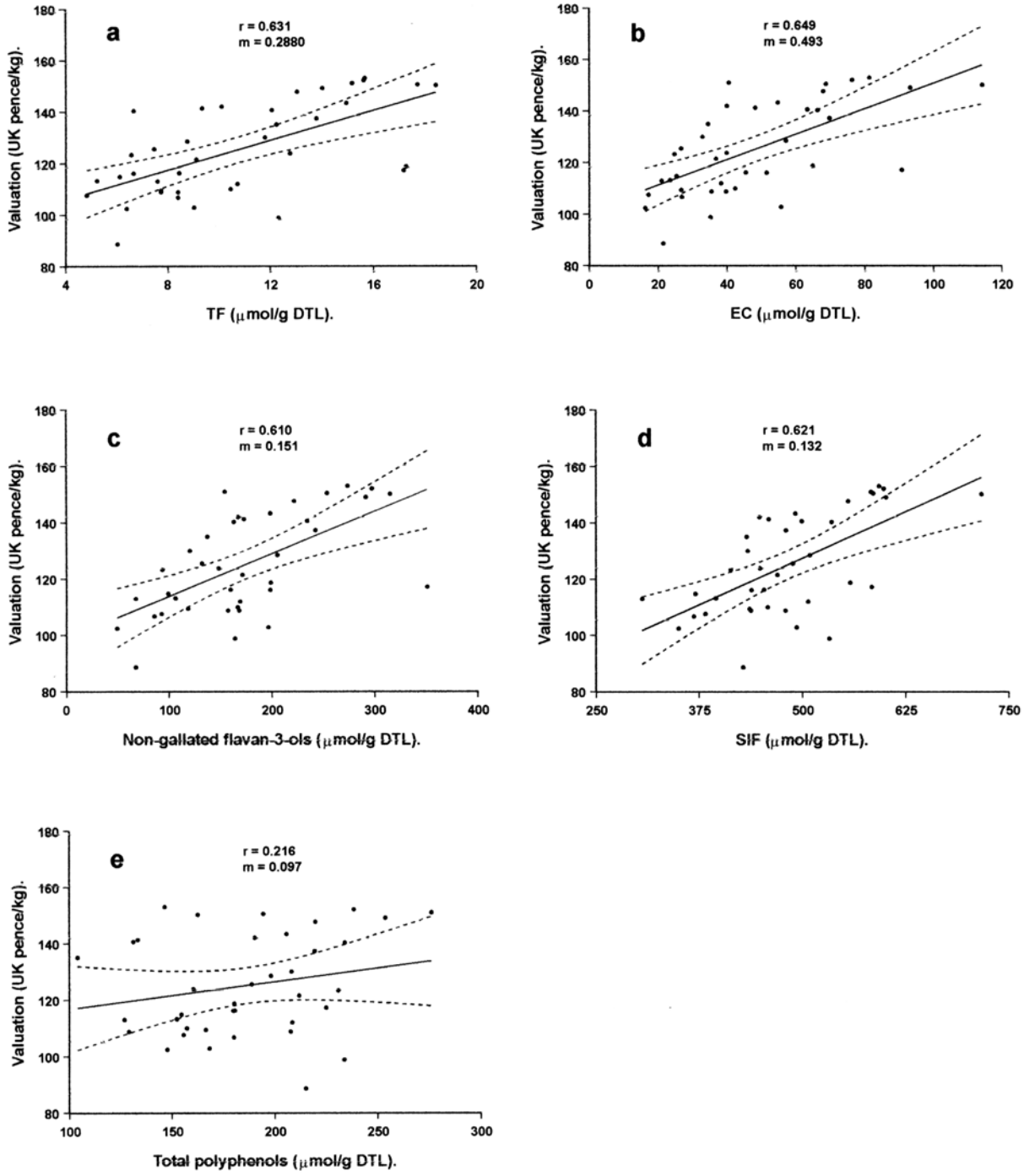

Figure 2.5. The relationship between (a) TF, (b) EC, (c) non-gallated flavan-3-ols, (d) SIF and (e) total polyphenol content and valuation. The dotted lines represent 90% confidence limits.

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Table 2.6. The percent concordant values for predicting tea clones as either good or poor quality as determined using the content of different flavan-3-ol parameters.

Parameters	Percent concordant	Analysis method
EC + ECg	86.8	CE
C	73.3	CE
EGC	76.0	CE
EC	83.5	CE
ECg	75.8	CE
Caffeine	53.3	CE
EGCg	49.5	CE
SIF	80.8	CE
Total polyphenols	66.5	Spectrophotometric
Value	90.8	Organoleptic

As could be expected, the organoleptic evaluation (value) had the best ability to predict the tea quality (90.8%), as this was the parameter used to classify the clones as either good or poor quality. Of the other parameters, a combination of EC and ECg could best predict the quality of a clone (86.8%), followed by EC (83.5%), SIF (80.8%), EGC (76.0%), ECg (75.8%) and C (73.3%). There were, however, no statistical significant differences between these values at the 95% confidence limit (Table 2.7). The parameters having the weakest ability to distinguish between good and poor quality were EGCg (49.5%) and caffeine (53.3%), which were also not significantly different at the 95% confidence limit. The percent concordant values of EGCg and caffeine were significantly lower than the other parameters mentioned. The total polyphenols that were determined spectrophotometrically with the Folin-Ciocalteu reagent had a lower percent concordant (66.5%) than the SIF value (80.8%). Although there was no significant difference between these two values at the 95% confidence limit, there was a significant difference at the 90% confidence limit.

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Table 2.7. The z-statistic for showing the significant differences between the percent concordant values for different flavan-3-ol parameters.

	C	EGC	EC	ECg	Caffeine	EGCg	Total polyphenols	SIF	Value
EC + ECg	1.511	1.241	0.415	1.262	3.271*	3.580*	2.146*	0.728	0.567
C		0.278	1.108	0.257	1.856*	2.186*	0.663	0.798	2.039*
EGC			0.835	0.021	2.124*	2.451*	0.939	0.522	1.779*
EC				0.855	2.905*	3.222*	1.756*	0.315	0.976
ECg					2.103*	2.431*	0.918	0.542	1.799*
Caffeine						0.340	1.204	2.616*	3.737*
EGCg							1.540	2.938*	4.036*
Total polyphenols								1.452	2.652*
SIF									1.281

* Significant at $P < 0.05$, if $z > 1.645$

The relationship between EC and ECg content in green leaf of good and poor quality tea clones is shown in Figure 2.6. All the poor quality tea clones have relatively low EC and ECg contents (EC lower than 60 and ECg lower than 85 µmol g^{-1} DTL, as indicated with the dotted line in Figure 2.6), whereas the good quality tea clones generally had a higher content for both these flavan-3-ols.

Based on the EC content (EC) and ECg content (ECg) in the fresh leaves, the estimated logit of the probability of a clone to be of good quality can be calculated as

$$\text{Logit}(p) = -8.5954 + 0.0781(EC) + 0.0735(ECg)$$

Using this estimate, the probability (p) of a clone to be of good quality can be calculated as follows

$$p = e^{\text{logit}(p)} / (1 + e^{\text{logit}(p)})$$

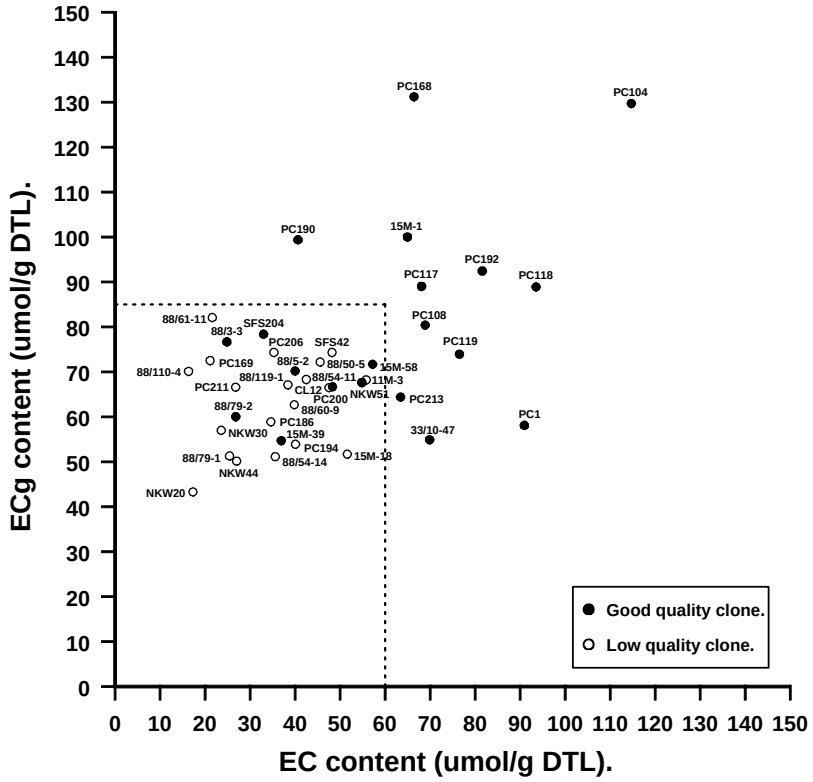

Figure 2.6. Green leaf EC and ECg content of different good and poor quality tea clones. The dotted line was arbitrarily drawn that it would distinguish between good and poor quality tea clones.

4 Discussion

The aim of this study was to investigate whether any correlations existed between the flavan-3-ol profiles in the green leaf of tea clones and the quality of the made black tea produced from these leaves. To assist in identifying flavan-3-ols important for quality, two groups of twenty tea clones in each group were studied. The one group consisted of known good quality TRF clones and the other group of known poor quality TRF clones. The flavan-3-ol contents of these two groups were then compared

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to identify any significant differences between the two groups. Lastly, the flavan-3-ol contents of the forty tea clones were also compared with tea taster scores and valuation to identify any correlations.

These analyses were repeated on a smaller subset of clones obtained at the Grenshoek Tea Estate in the Tzaneen region of South Africa. The significant differences between the good quality tea clones (PC105, PC108, PC113, PC114, PC117 and PC118) and poor quality tea clones (RS193, RS233, 29-44, MFS87 and 5/1/5-14) were confirmed for the same parameters as seen on the tea clones in Malawi. These analyses were done on two separate occasions during January and May 1998. Obanda et al (1997) also observed that the flavan-3-ol composition of their tea clones varied more between clones than with time of the year.

For Central African teas a positive correlation between total TF content and taster evaluations was shown (Hilton and Ellis, 1972). Owuor et al (1986) also showed a positive correlation between TF content and quality for Kenyan tea clones, but found this to be statistically non-significant. The theaflavin digallate equivalents were shown to be of more importance for Kenyan clones (Owuor and Obanda, 1995). The interpretation of our results can best be done when taking into account the formation of TF during black tea fermentation.

Theaflavins are formed from specific pairs of flavan-3-ols during the oxidation occurring during the aerobic fermentation stage. Under the influence of polyphenol oxidase a tri-hydroxy flavan-3-ol and di-hydroxy flavan-3-ol combine to produce a theaflavin (Nakagawa and Torii, 1965; Robertson, 1983). When a gallated di-hydroxy

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flavan-3-ol combines with a gallated tri-hydroxy flavan-3-ol, a TF digallate will form. When only one of the two flavan-3-ol monomers is gallated, a TF monogallate will form.

The tri-hydroxy flavan-3-ols (EGC and EGCg) outnumber the di-hydroxy flavan-3-ols considerably (Brown et al, 1969). TF formation requires both a tri-hydroxy flavan-3-ol and a di-hydroxy flavan-3-ol. Based on availability, di-hydroxy flavan-3-ol contents should be the limiting factor in the amount of TF formed.

This also agrees with the effects of flavan-3-ol contents on the formation of TFs and TRs during *in vitro* oxidation (Robertson, 1983). Di-hydroxy flavan-3-ols are oxidised slower than the tri-hydroxy flavan-3-ols. Highest TF formation should occur when the concentrations of the di-hydroxy and tri-hydroxy flavan-3-ol quinones are equal. Therefore, events that increase di-hydroxy flavan-3-ol oxidation, decrease the tri-hydroxy flavan-3-ol oxidation or increase stability of both quinones, should result in higher TF formation. Based on his *in vitro* oxidation experiments, Robertson (1983) concluded that a high ratio of di-hydroxy flavan-3-ols to tri-hydroxy flavan-3-ols will increase the TF/TR ratio in black tea.

Once flavan-3-ols are oxidised to their quinone form, they can undergo chemical reduction back to their reduced form. Robertson (1992) suggested that the steady state concentration of the di-hydroxy flavan-3-ol quinones will decrease during redox equilibration, whereby the tri-hydroxy flavan-3-ols are oxidised into their quinone forms. This emphasises the importance of higher di-hydroxy flavan-3-ol content to increase TF formation.

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Our results confirm Robertson's (1983) conclusions where the biggest difference between the good and poor quality tea clones is found for the di-hydroxy flavan-3-ols. Of the di-hydroxy flavan-3-ols, catechin shows the least correlation with quality. Probably because catechin is not a precursor of one of the four major TFs (Robertson, 1983). This may explain its low correlation with quality.

Robertson (1983) showed in his *in vitro* oxidation studies of the four major flavan-3-ols, that inhibition of PPO by the gallated flavan-3-ols occurs. When the total gallated flavan-3-ol concentration was increased above 1.2 fold, substrate inhibition of the PPO was observed. Robertson (1983) also showed an increase in the ungallated flavan-3-ols up to two fold the normal levels in his *in vitro* studies, resulted in a linear increase in the TF formation with no apparent PPO inhibition.

The increased inhibition of PPO by the gallated flavan-3-ols can also be explained by the tendencies of polyphenols to complex with proteins (Martin et al, 1987; Spencer et al, 1988; Luck et al, 1994). Recent work suggests that an increase in molecular weight and flexibility of a polyphenol will increase its ability for complexation with proteins (Martin et al, 1987; Spencer et al, 1988). The addition of a gallic acid to gallated flavan-3-ols will increase both the molecular weight and flexibility of the flavan-3-ol. This is confirmed by the observation that TFs with more galloyl groups were precipitated more by gelatine, than ungallated TFs (Spencer et al, 1988). Complexation of gallated flavan-3-ols with caffeine also showed the galloyl ester groups of the flavan-3-ols to be the preferred sites of complexation. Because of the structural similarities between caffeine and peptides, the same interactions between

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polyphenols and caffeine can be expected between polyphenols and proteins (Gaffney et al, 1986).

Of the di-hydroxy flavan-3-ols the EC had higher correlation with quality than ECg probably due to the absence of the gallic acid in the former. This phenomenon can also be seen in the tri-hydroxy flavan-3-ols where the ungallated EGC had a higher correlation with quality than the EGCg. The good and poor quality clones can be distinguished better by the total di-hydroxy flavan-3-ols than by the total tri-hydroxy flavan-3-ols.

Obanda et al (1997) found that the EGCg and ECg occurring in green leaf had the best correlations with tea tasters' scores. In contrast our work shows EC to be of most importance, followed by EGC and then ECg, when comparing twenty good quality tea clones with twenty poor quality tea clones. We also found no difference between the two groups when looking at EGCg contents. These observations may be best explained by taking into account the TF levels in the black tea.

It seems that for TRF(CA) clones the total TF content is important, whereas for Kenyan clones the gallated TF contents are of more importance (Hilton and Ellis, 1972; Owuor et al, 1986; Owuor and Obanda, 1995). This may explain why EGCg and ECg contents of Kenyan clones are correlated with quality. Whether the individual TF contents of Central African clones will also be important is not known at present. The TRF(CA) teas generally sell on the basis of briskness (astringency) and colour whereas the Kenyan clones are sold on good aroma.

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Obanda et al (1997) also showed caffeine content to be of importance when predicting black tea quality. Our results showed no statistically significant difference between caffeine content of good and poor quality tea clones.

Both the CE method for determining SIF and the spectrophotometrical method of Folin-Ciocalteu for determining the total polyphenols gave more or less the same means. These two different methods, however, differed significantly at the 90% confidence level in their ability to predict the quality of a clone. This difference in quality prediction is most probably due to the higher standard deviation of the Folin-Ciocalteu method. This means that the much more cost-effective spectrophotometric method can most probably be utilised for quality prediction if the experimental variation of the test can be improved to the same levels of that of the CE method.

The fresh leaf EC and ECg content together correlated well with the total score obtained from both tea tasters. The probability (p) of a new clone to be of good quality can now be calculated from the fresh leaf EC and ECg content. This p value may become a useful selection criterion in the breeding and selection of TRF(CA) tea clones in the future.

Chapter 3

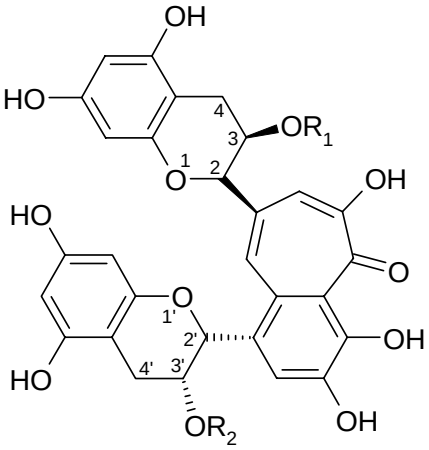
Analysis of black tea theaflavins by non-aqueous capillary electrophoresis (NACE).

1 Introduction

The value of tea is determined by its quality as perceived during organoleptic evaluation by tea tasters. Although the professional tea taster has expert knowledge on tea and a fine tuned sensory ability to identify and describe the various attributes of a tea liquor, the scores between different tea tasters may differ according to personal preferences or the demands of the market. Methods for analysing tea samples for predicting their value are required by the tea industry. Such methods can help with the breeding and selection programme where insufficient material is available for scoring by tea tasters. Analytical methods can also be used during manufacturing for process optimization and quality control.

During black tea manufacture, the fresh leaves are macerated to disrupt the cells and sub-cellular compartments. This allows cytoplasmic polyphenol oxidase (PPO) (EC 1.10.3.1) to oxidize the flavan-3-ols in the vacuoles. During the manufacturing process of black tea, the majority of the monomeric flavan-3-ols are oxidized and polymerized to form thearubigins (TRs) and theaflavins (TFs) (Bailey *et al*, 1992; Bailey *et al*, 1993). The diverse group of TRs is still poorly understood without sufficient analysis methods for their characterization. The TFs, on the other hand, are well characterized and show

significant correlation with quality. A theaflavin is formed from one trihydroxyflavan-3-ol and one dihydroxyflavan-3-ol. If both the trihydroxy- and dihydroxyflavan-3-ols are gallated a theaflavin-digallate (TF-dg) is formed. If neither substrate is gallated, free theaflavin (TF-f) forms. If the trihydroxyflavan-3-ol is gallated the theaflavin-3-monogallate (TF-A) forms, while if only the dihydroxyflavan-3-ol is gallated, the theaflavin-3'-monogallate (TF-B) is formed (Temple, 1999) (Figure 3.1). The flavan-3-ol content of the fresh leaf and the manufacturing procedure determine the TF composition of black tea.



i Free theaflavin (TF-f)
R1 = H
R2 = H

ii Theaflavin-3-gallate (TF-A)
R1 = 3,4,5-trihydroxybenzoyl
R2 = H

iii Theaflavin-3'-gallate (TF-B)
R1 = H
R2 = 3,4,5-trihydroxybenzoyl

iv Theaflavin-3,3'-digallate (TF-dg)
R1 = 3,4,5-trihydroxybenzoyl
R2 = 3,4,5-trihydroxybenzoyl

Figure 3.1. Structures of the four major theaflavins analyzed.

It is important to continuously strive to raise the quality of tea (Ellis and Nyirenda, 1995). For Southern African black tea, there is a significant correlation between total TF content

and value (Hilton and Ellis, 1972). However, research in Kenya, has indicated that the relative amounts of the individual theaflavins might have a considerable effect on the quality of the black tea (Owuor *et al*, 1986; Owuor *et al*, 1987; Owuor, 1995). Analyzing individual TFs can be used to determine their influence on the quality of Southern African black teas. A fast and sensitive method to monitor the formation of the individual TFs during manufacturing will be of great help in optimizing the manufacturing process.

The method most widely used for measuring TFs is the spectrophotometric method involving flavognost reagent (2-aminoethyl diphenylborate in ethanol) (Robertson and Hall, 1989). The limitation of the flavognost method is its inability to quantify the individual TF components. Currently the preferred methods for determining individual TFs are based on isocratic or gradient HPLC methods (Bailey *et al*, 1990; Temple and Clifford, 1997). These methods require large amounts of solvents and long analysis times. A method for analyzing TFs using gas chromatography (GC) has been developed (Collier and Mallows, 1971). This method however has the disadvantage of requiring derivatization before analysis.

A capillary zone electrophoresis (CZE) method was developed to determine both catechins and TFs. Poor sample stability and high relative standard deviation (RSD) values were encountered when analysing TFs with this method (Lee and Ong, 2000). Other researchers also had difficulty in detecting the TFs in their micellar electrokinetic (capillary) chromatography (MEKC) method (Larger *et al*, 1998). Non-aqueous capillary electrophoresis (NACE) is a method that can be used to separate and analyze compounds

that are difficult to analyze in aqueous systems. Various researchers have successfully applied NACE for the analysis of natural compounds (Cherkaoui *et al*, 1999; Matysik, 1999; Song *et al*, 1999), lipophilic compounds (Salimi-Moosavi and Cassidy, 1996), aromatic compounds (Bjornsdottir and Hansen, 1999; Luong *et al*, 1999) as well as pharmaceuticals, food compounds and compounds occurring in biological fluids (Bjornsdottir *et al*, 1998).

We report here a new NACE method developed specifically for determining the individual TF composition in black tea (Wright *et al*, 2001, see Appendix B).

2 Materials and methods

2.1 Instrumentation

Capillary electrophoresis was performed on a HP ^{3D}CE system using a fused silica capillary column from Bio-Rad (Scientific Group, JNB, RSA) with a diameter of 50 μm. For the CZE method a capillary with a total length of 59 cm and an effective separating length of 51 cm was used. Detection was by UV absorbance at 280 nm and wavelength scans were performed form 190 – 500 nm using the diode array detector. For the NACE method the total length of the capillary was 40 cm, giving an effective separating length of 32 cm. The separation was monitored at 380 nm.

The apparent pH values (pH*) of the background electrolyte solutions were measured with a Mettler Toledo MP220 pH Meter with a glass electrode at room temperature. The electrode was calibrated using standard aqueous buffers, pH 4 and 7.

2.2 Reagents

Three different commercial black teas were bought at a local supermarket. The TF standards used in calibrating the capillary electrophoresis (CE) for analyzing the TF profiles were kind gifts from Dr Y Hara (Mitsui Norin Corporation, Japan). Isobutyl methyl ketone (IBMK) complying with ACS standards was purchased from Merck (Merck NT Laboratory Supplies, JNB, RSA). Acetonitrile (ACN) and methanol (MeOH) used for CE analysis were of HPLC grade and purchased from BDH (Merck NT Laboratory Supplies, JNB, RSA). Macherey-Nagel MN 617 filter paper was purchased from Separations, JNB, RSA. All other reagents and solvents were of analytical grade.

2.3 Analytical conditions

The running buffer used for the CZE method consisted of 50 mM K_2SO_4 and 600 mM boric acid titrated to pH 7.0 using 1.0 M NaOH. The applied voltage was 22,5 kV and the temperature was maintained at 25°C. The sample was injected pneumatically for 5 sec (50 mbar). The capillary was rinsed with water for 1 min., 0.1 M NaOH for 2 min. and then again with water for 2 min. after each analysis. At the start of each analysis the capillary was also rinsed with running buffer for 2 min. before injection of sample.

For the non-aqueous capillary electrophoresis (NACE) technique the running solution consisted of 71% ACN (v/v), 25% MeOH (v/v), 4% acetic acid (v/v) and 90 mM ammonium acetate. The analytical conditions for the NACE method was a final applied voltage of 27.5 kV and the temperature was maintained at 18.5°C. Due to current cut-off problems, the voltage was initially increased to 10 kV over the first minute, and then to 27.5 kV over the next two minutes. The sample was injected pneumatically for 5 sec (50 mbar). Before each analysis the capillary was rinsed with 1.0 M NaOH for 2 min., followed by water for 0.5 min., ACN : MeOH (75:25, v:v) for 0.5 min. and finally running solution for 1 min. After each analysis the capillary was rinsed with MeOH for 0.5 min. and water for 1 min. This elaborate rinsing schedule was used to ensure no precipitation of salts when changing between non-aqueous and aqueous solvents. At the end of each day the column was rinsed for 20 min. with 1.0 M NaOH, 5 min. with water, 15 min. with MeOH and 5 min. with water.

2.4 Preparation of samples and standard

For the CZE method dried tea (DT) was extracted by adding 6 g black tea to 250 ml boiling deionized water in a preheated thermos flask and shaking for 10 min. During this time the thermos flask was continuously shaken on a horizontal shaker at approximately 90 rpm. The tea infusion was then filtered through Macherey-Nagel MN 617 fluted filter paper. After cooling to room temperature, the tea infusion was extracted with one volume of chloroform and the chloroform fraction discarded. The aqueous tea extract was then extracted with one volume IBMK and the aqueous phase discarded. The IBMK extract was then once again retained after extraction with 5% sodium bicarbonate (w/v). The IBMK (50 ml) was then evaporated in a Buchi Rotavapor-RE and the remaining residue redissolved in 40% (v/v) ethanol (5 ml). This solution was then stored at -20°C until analysis.

For the NACE method 2 g DT samples were extracted similarly to the CZE method with 100 g boiling water. This tea extract was then filtered through Macherey-Nagel MN 617 fluted filter paper. An aliquot (10 ml) of the filtrate was extracted with one volume (10 ml) of IBMK at room temperature. After phase separation samples of 1 ml of the upper IBMK phase were transferred to separate 4.5 ml amber vials. The IBMK was removed by evaporation on a 40°C heating block under a stream of nitrogen gas. The vials were flushed with nitrogen gas and sealed. The extracts were stored in the dark at room temperature and analyzed within 48 hours. Immediately prior to the analysis on the CE the residue was redissolved in 100 µl loading solution consisting of 74.5% ACN, 25%

MeOH and 0.5% acetic acid (v/v). The TF standards were also dissolved in the loading solution for analysis.

2.5 Method validation

Reproducibility tests were performed to determine both intra-day and inter-day variation in migration times. The statistical evaluation was carried out on TF standards being analyzed seven fold for the intra-day reproducibility test and data from seven days with five repeats per day for the inter-day reproducibility test. Repetitive runs were carried out with commercial black tea samples to determine the reproducibility of both the extraction and analysis of the samples. Each black tea sample was extracted four separate times and each extract was analyzed in four-fold. The RSD values and the means were determined with the Fig.P (version 2.98) software package (1999).

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3 Results

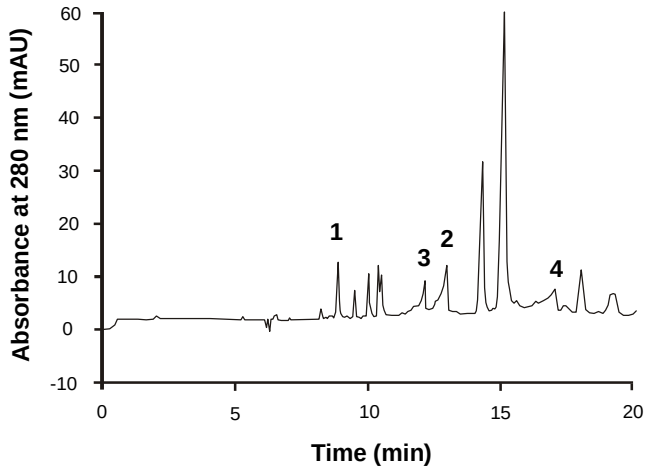
First attempts were made with aqueous buffers using the methods of CZE and MEKC. Of the two aqueous methods CZE was more suitable. We have been unable to analyze TFs by MEKC and others have reported similar problems (Larger *et al*, 1998).

The borate concentration and pH had the most significant influence on the separation of the analytes. Although the four TFs could be separated, peak broadening was a significant disadvantage of this method (Figure 3.2). High buffer concentration, together with potassium sulfate as an additive, decreased peak broadening.

Peak broadening resulted in unacceptable low TF peak heights at 380 nm. This method was discontinued because of the low efficiency and high variation in quantification of complex black tea samples.

Pollutant phenols have been analyzed with a non-aqueous solution consisting of ACN-MeOH-acetic acid, ammonium acetate and potassium hydroxide (Gas *et al*, 1997). Because of the polyphenolic nature of the TFs we decided to develop a non-aqueous method, following a similar strategy.

a



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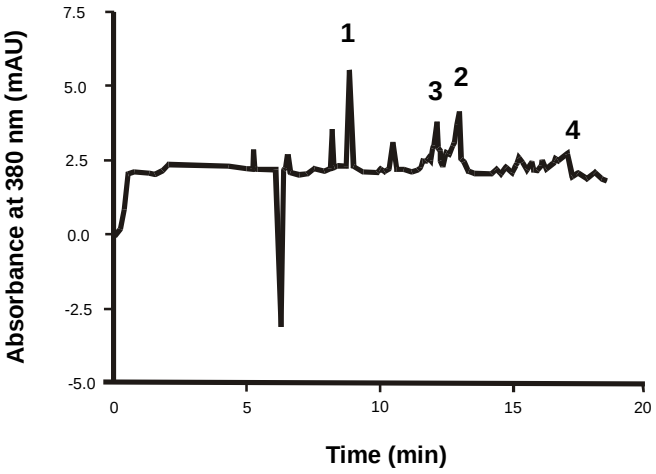


Figure 3.2. Electropherogram obtained with the CZE method of a TF extract prepared from a commercial black tea sample. Running buffer 600 mM boric acid and 50 mM K_2SO_4 titrated to pH 7.0 with 1.0 M NaOH. Capillary length 59 cm (51 cm to the detector), 50 μm internal diameter, sample injection 50 mbar for 5 sec. Applied voltage 22.5 kV, capillary temperature 25°C and detection at (a) 280 nm and (b) 380 nm. Peak 1 = TF-f, peak 2 = TF-A, peak 3 = TF-B and peak 4 = TF-dg.

Acetone was used as the neutral marker in this study when calculating the electrophoretic (actual) mobilities. Because of the gradual increase of the applied voltage over the first minute and then again over the next two minutes at a different rate, the final voltage of 27.5 kV was not used as the applied voltage, but the average voltage as determined at the migration time of each analyte. This slow ramping of the voltage was necessary because of current cut-off problems were experienced with fast ramping. Due to current cut-off the final voltage of 27.5 kV was not used for all the running solutions analyzed. For each non-aqueous solution the highest voltage that did not cause current cut-off problems was used. This was in the range of 22 - 30 kV.

The effect of MeOH content on the actual electrophoretic mobilities of the TFs is shown in Figure 3.3. The TFs showed a decrease in electrophoretic mobility with an increase in MeOH. The migration of the TFs is, however, also dependent on the extent and direction of the EOF (Table 3.1). The apparent mobilities also decreased with an increase in MeOH content. The EOF decreased with an increase in MeOH content.

Table 3.1. The EOF mobilities and apparent pH for different non-aqueous solutions.

MeOH (%)	Acetic acid (%)	ACN (%)	Ammonium acetate (mM)	EOF	Apparent pH (pH*)
10	4	86	90		6.1
25	4	71	90	12.506	6.2
50	4	46	90	11.063	6.1
75	4	21	90	5.731	5.9
90	4	6	90	2.489	5.7
25	4	71	10	22.843	5.4
25	4	71	20	20.321	5.6
25	4	71	30	17.619	5.7
25	4	71	60	13.884	6.0
25	4	71	90	12.506	6.2
25	4	71	120	9.266	6.3
25	1	71	90	11.764	7.2
25	2	71	90	11.748	6.8
25	4	71	90	12.506	6.2
25	6	71	90	11.588	5.8
25	8	71	90	11.671	5.4

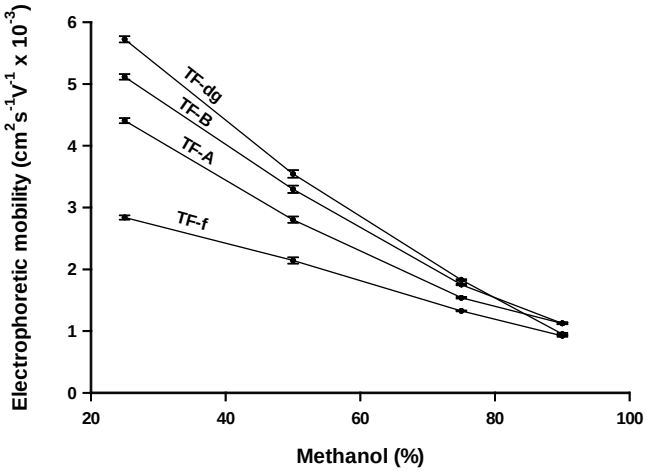


Figure 3.3. Electrophoretic mobilities of the four major TFs in different MeOH/ACN compositions containing 90 mM ammonium acetate and 4% acetid acid (v/v). Capillary length 40 cm (32 cm to the detector), 50 μm internal diameter, sample injection 50 mbar for 5 sec. Capillary temperature 18.5°C and detection at 380 nm.

The effect of added acetic acid on the electrophoretic mobilities is shown in Figure 3.4. An increase of acetic acid resulted in a decrease in actual mobilities for the TFs. Acetic acid was added to act as a hydrogen-bond donor to overcome possible solubility problems of our non-aqueous solution, which consisted of a high percentage of ACN in MeOH. However, the addition of the acetic acid also had a marked effect on the mobilities of the TFs, therefore the amounts of acetic acid added were also optimized. The addition of the acetic acid did not have a considerable influence on the selectivity of the TFs. As seen in Table 3.1, the addition of acetic acid had a large effect on the pH*, compared to changes of pH* with variations in the MeOH or ammonium acetate content. A decrease in the pH* did however not have the expected decreasing effect on the EOF (Table 3.1).

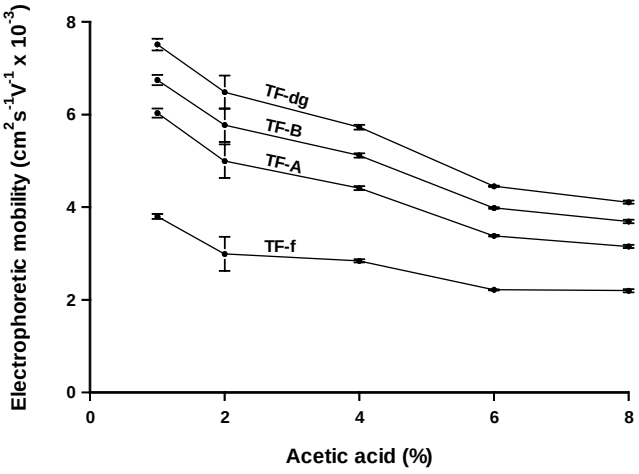


Figure 3.4. Electrophoretic mobilities of four TFs in running solutions containing different acetic acid/ACN compositions and 90 mM ammonium acetate and 25% MeOH (v/v). Other experimental conditions are the same as in Figure 3.3.

Although the EOF decreased with increasing ammonium acetate concentration, the TF mobilities increased (Figure 3.5). The increase in the mobilities of the TFs and the decrease of the EOF coincided with increased resolution.

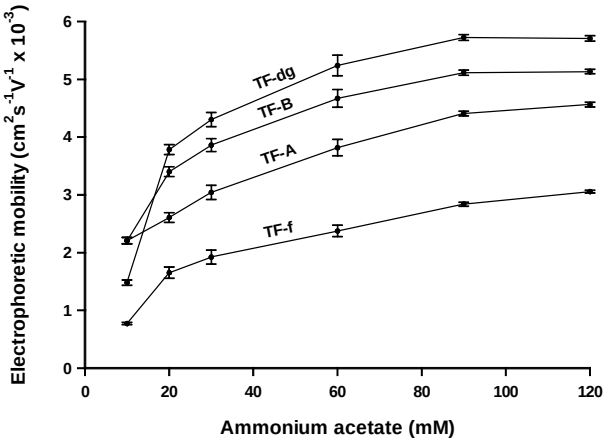


Figure 3.5. Electrophoretic mobilities of four TFs in running solutions containing different amounts of ammonium acetate and 71% ACN, 25% MeOH and 4% acetic acid (v/v). Other experimental conditions are the same as in Figure 3.3.

With to high concentrations of the background electrolyte longer migration times and excessive Joule heating are a disadvantage.

In our method we used the increased separation selectivity observed with an increase in the ammonium acetate concentration, together with the decreased migration times observed with an increase in acetic acid content, to optimize our method. We used a concentration of 90 mM ammonium acetate to achieve the desirable resolution, and a relatively high content of 4% acetic acid to decrease the migration times of the four TFs to well under ten minutes.

Our optimized separation solution thus consisted of 71% ACN, 25% MeOH, 4% acetic acid (v/v) and 90 mM ammonium acetate.

The four major TFs elute within 10 minutes. Column purge and re-equilibration extend the total analysis time by 6 minutes. The analysis time for TFs on HPLC is significantly longer. Using a simple IBMK extraction of a black tea infusion, the individual TFs could be identified and quantified as shown in Figure 3.6 and Figure 3.7. These peaks were identified by their migration times as well as their UV/VIS spectra.

Calibration curves for TF, TF-A, TF-B and TF-dg were linear in the range of interest (100 to 1000 μg/ml), with r-values of 0.9998, 0.9992, 0.9995 and 0.9987 respectively. The repeatability (n = 4) of the method was also evaluated as shown in Table 3.2. The RSD values for the determination of the individual TF concentrations were smaller than 6% for all the different black tea samples. The variation in the migration times of the TF standards is shown in Table 3.3. Intra-day variations (1.7%) and inter-day variations were less than 3.6%. The limit of detection (LOD) values were taken as 3 *S/N* (signal / noise) at 380 nm and were 24, 25, 21 and 23 μg/ml for TF-f, TF-A, TF-B and TF-dg respectively.

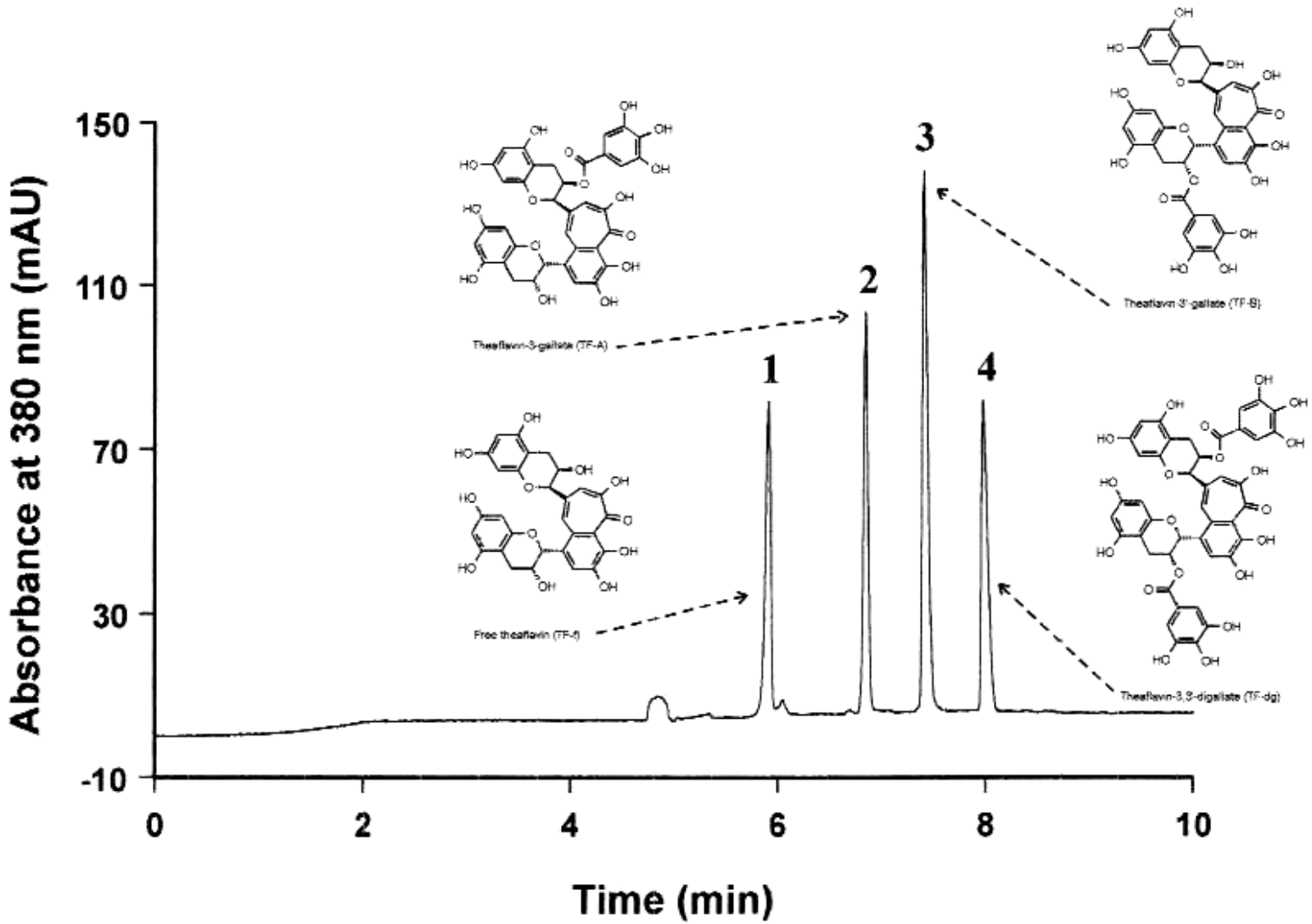
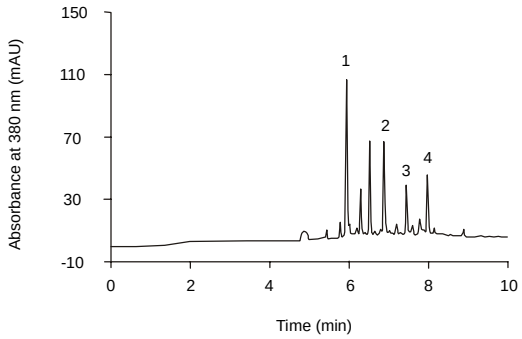
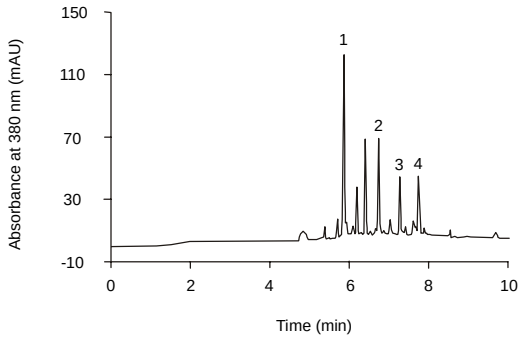


Figure 3.6. Electropherogram of TF standards. Running solution 71% ACN, 25% MeOH, 4% acetic acid (v/v) and 90 mM ammonium acetate. Applied voltage 27.5kV. Other experimental conditions are the same as in Fig. 3.3. Peak identification is the same as in Fig. 3.2.

a



b



c

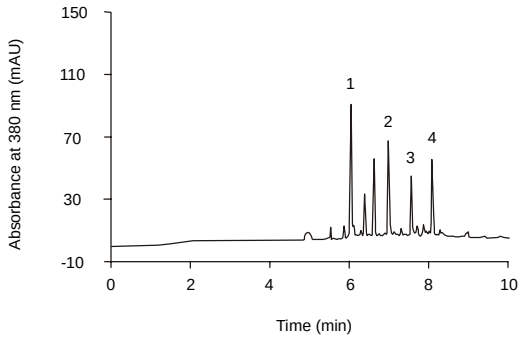


Figure 3.7. Electropherogram of a TF extract prepared from commercial black tea samples, (a) Black tea - A, (b) Black tea - B and (c) Black tea - C. Running solution 71% ACN, 25% MeOH and 4% acetic acid (v/v) 90 mM ammonium acetate. Applied voltage 27.5 kV. Other experimental conditions are the same as in Figure 3.3. Peak identification is the same as in Figure 3.2.

Table 3.2. The variation in individual TF content of three different local black tea samples.

Sample	TF-f Mean (umol/g DT)	RSD (%)	TF-A Mean (umol/g DT)	RSD (%)	TF-B Mean (umol/g DT)	RSD (%)	TF-dg Mean (umol/g DT)	RSD (%)	Total Mean (umol/g DT)	RSD (%)
Higher priced										
Black tea - A	**6.809**	**3.9**	**2.654**	**4.1**	**1.068**	**5.6**	**1.060**	**5.1**	**11.593**	**4.1**
Black tea - B	**6.073**	**3.5**	**2.661**	**3.8**	**0.943**	**3.8**	**1.118**	**3.3**	**10.793**	**3.4**
Lower priced										
Black tea - C	**4.716**	**4.4**	**2.458**	**5.3**	**1.027**	**5.6**	**1.440**	**5.8**	**9.642**	**4.9**

The individual TFs concentrations of three black tea samples were determined (Table 3.2). The samples consisted of two higher priced commercial black teas (Black tea - A and Black tea - B) of above average quality, and one lower priced commercial black tea (Black tea - C). True to findings of previous investigations the total TF concentration determined by the NACE method was higher for the higher priced black teas (11.593 and 10.793 μmol/g DT) than for the lower priced black tea (9.642 μmol/g DT). The relative compositions of the individual TFs also differed between the three tea samples. The higher priced black teas had a higher amount of TF-f and a lower amount of TF-dg.

Table 3.3. The intra- and inter-day variation of the migration times.

Compound	Intra-Day Variation (n=7)		Inter-Day Variation (n=7)	
	Mean (min)	RSD (%)	Mean (min)	RSD (%)
TF-f	**6.05**	**1.4**	**5.90**	**2.6**
TF-A	**7.02**	**1.5**	**6.82**	**3.1**
TF-B	**7.61**	**1.6**	**7.36**	**3.3**
TF-dg	**8.20**	**1.6**	**7.91**	**3.5**

4 Discussion

The aim of this study was to develop a CE method for analyzing the individual TFs in black tea infusions. At first, aqueous based methods were tried unsuccessfully. The non-aqueous NACE method, however, provided more satisfactory results. Factors influencing the NACE method and its analytical procedure were then studied in more detail.

The aqueous based CZE separations of the TFs were based on its complexation with borate (Mortin *et al*, 1993; Pietta *et al*, 1994; Fernandez de Simon *et al*, 1995; Lee and Ong, 2000). Significant peak broadening was however observed. The leading fronts were most probably caused by conductivity differences between the zone of analytes (TFs) and the carrier electrolyte (boric acid), as well as interactions with the capillary wall (Seitz *et al*, 1992).

Another significant drawback of the CZE method was the elaborate extraction procedure of the TFs. The CZE sample preparation procedure had additional chloroform and sodium bicarbonate washing steps, compared to the NACE sample preparation procedure. Without the chloroform washing step capillary blockage occurred, most probably due to the precipitation, or interaction with the capillary wall, of some lipophilic compounds. The sodium bicarbonate washing step was necessary for additional clean up of the sample to prevent interference of the TF peaks.

NACE has emerged as an additional CE method for the analysis of compounds that are difficult to separate in aqueous buffers due to low solubility or lack of selectivity in

aqueous media (Salimi-Moosavi and Cassidy, 1996; Gas *et al*, 1997; Morales and Cela, 1999). Due to the different chemical and physical properties of organic solvents compared with water, selectivity can be improved. For our analytes the main consideration for using NACE was to decrease the band broadening effect observed in aqueous media and to enhance the separation efficiency.

Factors enhancing band broadening include the injection process, electrophoretic dispersion, Joule heating, wall adsorption, local turbulences due to non-uniformly charged capillary walls, and hydrostatic flow. NACE has the most pronounced effects on Joule heating due to the lower conductivities associated with non-aqueous media. Wall adsorption effects might also be significantly influenced by using organic solutions due to their effect on the solubility and the pKa of the analytes (Cherkaoui *et al*, 1999).

The decreased apparent mobilities at a higher MeOH content can most properly be explained by the decrease in the EOF. This change in EOF is the result of the change in ion mobilities as expected for mixtures of solvents that have different dielectric constants and viscosities, as described by the Von Smoluchowski equation (Matysik, 1999). The increased viscosity and decreased dielectric constant of the media containing higher amounts of MeOH should result in a decreased EOF. ACN has a higher dielectric constant and lower viscosity than MeOH. This tendency was also observed by other researchers (Matysik, 1999). Decreased EOF mobilities were also observed for the additions of organic solvents to an aqueous buffer, with the EOF decreasing more with the addition of MeOH than with the addition of ACN (Ward and Khaledi, 1999).

In solvents with low hydrogen acceptor abilities, such as ACN, heteroconjugated anion formation may play an important role (Sarmini and Kenndler, 1997). Anions added to ACN undergo very weak solvation and will thus rather interact with a stronger hydrogen donor Brønsted acid than be solvated. In our application the TFs and other polyphenolic substances in the sample will act as Brønsted acids and undergo ion pairing with the separation electrolyte. At higher ACN content increased ion pairing would decrease the ionic strength, which will lead to an increase in the EOF and the apparent mobilities of the analytes. The actual mobilities of the analytes will also increase due to the ion pairing (Matysik, 1999).

The observed decrease in apparent mobilities (and EOF) at even a lower MeOH content of 25 % (except for TF-f) and lower (results not shown) might be due to an effect of the higher ACN content on the effective charge to solvation radius ratio of the analyte. This tendency was also observed for the separation for pharmaceuticals in non-aqueous media (Okada, 1998). The reduced EOF at high ACN content might also be because of a decrease in the effective charge at the capillary surface due to an increase of the pKa of silanol groups, which would decrease the ζ potential at the silica surface.

The most important effect on the selectivity of organic solvents on the analytes is due to its influence on the pKa values of the analytes. The effect of MeOH (Porras *et al*, 1999) and of ACN (Sarmini and Kenndler, 1998) on the pKa values are described based on the theoretical model of the transfer activity coefficient (medium effect). For the addition of the above-mentioned solvents to water, an increase in the pKa values was observed. An

increase in the pKa values of the TFs might also be responsible for the reduced wall adsorption in the non-aqueous solution system. A more protonated state of the TFs should result in reduced ionic interaction with the negatively charged capillary wall. This also coincides with the ion pairing effect described earlier.

The decreased mobilities might be due to a decrease in the pH* which should reduce the charge of the anionic TFs, this will then increase the apparent mobility. The increased mobility can also be explained by the ion pairing effect. With the addition of acetic acid as hydrogen donor to ACN, the solubility of ions increases and this will result in reduced ion pairing with the consequent decrease in mobility of the solutes.

Studies in aqueous buffers, predicted a decrease in the solute mobilities and the EOF with an increase in the ammonium acetate concentration due to the increased ionic strength (Sarmini and Kenndler, 1999).

This observation can be explained by the ion pairing effect. With higher concentrations of ions in the non-aqueous solution, increased ion pairing will result in increased mobilities of the TFs due to the increased charge of the analytes.

To our knowledge, no prior method for analyzing tea by NACE existed. This is also the first method developed exclusively for TF analysis with CE. This NACE method has the advantages of speed and low capillary and running costs compared to the HPLC methods.

This new CE method could be used for evaluating new tea clones and optimizing quality during black tea manufacturing.

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Chapter 4

Analysis of the theaflavin composition in black tea (*Camellia sinensis)* for predicting the quality of tea produced in Central and Southern Africa

1 Introduction

An increase in tea quality is continuously sought in the tea industry. Since 1956 the Tea Research Foundation (Central Africa) TRF(CA) has invested in a long-term tea plant improvement programme (Ellis and Nyirenda, 1995). The aim of the work described here was to develop a method for predicting the quality of the final black tea product by analysis of the individual theaflavins.

During the manufacturing process of black tea, the flavan-3-ol monomers are oxidized and polymerized to form mainly theaflavins (TF) and thearubigins (TR), the former being dimers bearing a benzotropolone system, while the latter is little understood flavonoid polymer mixtures. This happens when the tea leaves are macerated to expose the vacuolar flavan-3-ols to the plant oxidases. Both polyphenol oxidase (PPO) (EC 1.10.3.1) and peroxidase (POD) are capable of oxidising the flavan-3-ols, although only PPO is believed to take part in tea fermentation, due to the absence of peroxide needed by POD as an electron acceptor (Robertson, 1992; Davies *et al*, 1999). The six major flavan-3-ols occurring in tea are; (+)-catechin (C), (+)-gallocatechin (GC), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECg) and (-)-epigallocatechin-3-gallate (EGCg). The specific contents of these flavan-3-ols differ between different tea clones (Robertson, 1992). In the final

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black tea product the flavan-3-ols and their resulting TF and TR products make up most of the non-volatiles. The character and quality of the black tea is very dependent on this composition (Wood and Roberts, 1964).

The major TFs are dimers formed by PPO from one di-hydroxy flavan-3-ol (EC or ECg) and one tri-hydroxy flavan-3-ol (EGC or EGCg). The four major TFs form from specific pairs of substrates. When the tri-hydroxy flavan-3-ol is gallated (EGCg) theaflavin-3-gallate (TF-A) will form (2), if the di-hydroxy flavan-3-ol (ECg) is gallated theaflavin-3'-gallate (TF-B) will form (3). When both flavan-3-ols are gallated (EGCg and ECg) theaflavin-3,3'-digallate (TF-dg) will form (4). If neither of the flavan-3-ol substrates (EC and EGC) are gallated free theaflavin (TF-f) will form (1). The theaflavin profile of the black tea should thus be determined by the flavan-3-ol composition of the green leaf used in the manufacturing of the black tea (Robertson, 1992). Robertson (1983) confirmed this relationship with *in vitro* studies. Other types of TF and dimers are also formed in a smaller scale. These include theasinensis, theaflavates, oolongtheanin and theacitrins. These compounds can also contribute to the colour of the tea and might be utilized in TR formation (Davies *et al*, 1999).

Di-hydroxy flavan-3-ols		Tri-hydroxy flavan-3-ols		Theaflavins	
EC	+	EGC	→	TF	(1)
EC	+	EGCg	→	TF-A	(2)
ECg	+	EGC	→	TF-B	(3)
ECg	+	EGCg	→	TF-dg	(4)

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The major polyphenol group in black tea is TR, constituting approximately 30-60% of the soluble solids. These compounds are still poorly understood and little is known on their chemical character as well as their influence on quality (Finger *et al*, 1992; Whitehead and Temple, 1992; Temple, 1999; Horie and Kohata, 2000). Three groups of TR are defined: (i) SI, soluble in ethyl acetate; (ii) SIa, soluble in water and diethyl ether; (iii) SII, soluble in water (Davies *et al*, 1999).

The TF, on the other hand, are well characterized. The influence of TF on quality, however, is still controversial. Earlier work done by the TRF(CA) indicates a correlation between total (flavognost) TF content and market value for Central African seedling teas (Hilton and Ellis, 1972; Hilton *et al*, 1973). Work done in Kenya however suggests the composition of the individual TF to be of more importance in predicting the quality of Kenyan black teas (Owuor *et al*, 1986; Owuor *et al*, 1987). Owuor and Obanda (1995) and Owuor *et al* (1994) concluded that theaflavin digallate equivalents are preferred to total (flavognost) TF content for predicting the quality in Kenyan black tea. Whether the TF composition will also be of importance in predicting the black tea quality of Central and Southern African teas was not known prior to this research.

Wright *et al* (2000) predicted that for the TRF(CA) tea clones the sum of the individual TF (SIT) content will be more important than the contents of individual TF. This prediction was based on the significance of the flavan-3-ol contents and the relative proportions of the individual flavan-3-ol in the green leaf, on the quality of the black tea. These results were explained by considering the contribution of the flavan-3-ols to the formation of the total theaflavins in the black tea. A higher total

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content of theaflavins will form by an increase in the di-hydroxy flavan-3-ol content and a decrease in the gallated flavan-3-ol content. The di-hydroxy flavan-3-ol contents were better in predicting the quality than the tri-hydroxy flavan-3-ol contents because the former are less abundant and may therefore be the limiting compound. The non-gallated flavan-3-ols were also shown to be better predictors of quality than the gallated flavan-3-ols, probably due to PPO inhibition by the latter. Wright *et al* (2000) showed that the flavan-3-ols, which best predicted the quality of the made black tea were EC and ECg. From the work done by Wright *et al* (2000) follows the hypothesis that the SIT contents will be of most importance in predicting the quality of TRF(CA) tea clones. Furthermore, the non-gallated TF (TF-f) and mono-gallated TFs (TF-A and TF-B) will be more important for predicting quality than the di-gallated TF (TF-dg), because they are the biggest contributors to the SIT.

In this work the contents of individual TF in the black tea manufactured from various tea clones grown in Malawi were investigated. The ability of the contents of individual TF to distinguish between good quality and poor quality tea clones was subsequently determined by student's t-test analysis. Correlations between the TF composition and the tea taster's scores and value of the resulting black tea were determined by regression analysis.

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2 Experimental

2.1 Black tea clones and manufacture

Tea clones growing at the TRF(CA) in Mulanje, Malawi were used in this study. These clones have already passed the chloroform test (Samaraweera and Ranaweera, 1988) and are available as 16 bush plots. The same clones used in the study of Wright *et al* (2000) were used, with the exception of PC118 and NKW51, which were replaced by PC76 and PC79. As before the twenty good and twenty poor quality clones were selected based on repeated prior tea taster scores done in Blantyre, Malawi by tea taster A. These clones are a good representation of the spectrum of clones produced by the tea plant improvement programme at the TRF(CA). Only good clones are released to the tea industry. Shoots of the age and appearance normally used for black tea manufacture were collected during February of 2000 at the Mimosa and Nsuwadzi research stations. These leaves were sent to the Mini Processing Unit (MPU) at Mimosa for black tea manufacture. Black tea was manufactured according to the description given by Wright *et al* (2000). After manufacturing the black teas were stored in foil-lined paper bags at room temperature until transported to Pretoria, South Africa where the bags were stored in airtight containers at 4°C until analysis. The tea samples were transported to Pretoria during the beginning of March of 2000 (Wright *et al*, 2002, see Appendix C).

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2.2 Reagents

The TF standards used in calibrating the capillary electrophoresis (CE) for analyzing the TF profiles were kind gifts from Dr Y Hara (Mitsui Norin Corporation, Japan). Isobutyl methyl ketone (IBMK) complying with ACS standards was purchased from Merck (Merck NT Laboratory Supplies, JNB, RSA). Acetonitrile (ACN) and methanol (MeOH) used for CE analysis were of HPLC grade and purchased from BDH (Merck NT Laboratory Supplies, JNB, RSA). All other reagents and solvents were of analytical grade.

2.3 Sensory analysis

Two professional tea tasters, with expert knowledge of Central and Southern African teas, scored the black teas on at least two different occasions. The tea taster results used in this study were the same as those used by Wright *et al* (2000). However, the tea taster scores for PC118 and NKW51 were not used and scores for clones PC76 and PC79 were not available. Tea taster A in Blantyre has scored tea made from these clones repeatedly on previous occasions. Tea taster B in Johannesburg scored the teas made for this work in April 2000.

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2.4 Theaflavin sample preparation

The TF samples were prepared by extraction from black tea infusion. The black tea infusion was prepared by extracting 2 g dried tea (DT) with 100 g boiling deionised water in a preheated thermo flask for 10 minutes. During this time the thermo flask was continuously shaken on a horizontal shaker at approximately 90 rpm. This tea extract was then filtered through Macherey-Nagel MN 617 fluted filter paper (Separations, JNB, RSA). An aliquot (10 ml) of the filtrate was extracted with one volume (10 ml) of IBMK at room temperature. After phase separation three samples of 1 ml each of the upper IBMK phase were transferred to separate 4.5 ml amber vials. The IBMK was removed by evaporation on a 40°C heating block under a stream of nitrogen gas in a fume hood. The vials were flushed with nitrogen gas and sealed. The extracts were stored in the dark, at room temperature and analyzed within 48 hours. Immediately prior to the analysis on the CE the residue was redisolved in 100 μl sample solution consisting of 74.5% ACN (v/v), 25% MeOH (v/v) and 0.5% glacial acetic acid (v/v).

2.5 Statistical methodology

The different data sets were analyzed for normality with the Shapiro-Wilk test as well as the normal probability plot using the UNIVARIATE procedure of SAS (1996). The 40 samples were tested for normality for the contents of TF-f, TF-A, TF-B, TF-dg, SIT, and total TFs content according to the flavognost scores. The TF profiles of the tea clones were then separated in two groups according to the total scores of tea taster

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A, namely good quality clones and poor quality clones. Each of the two groups was then tested separately for normality for the different variables.

The twenty good quality clones were then compared with the twenty low quality clones using the unpaired Student's t-test to obtain (P) values. The good quality clones and poor quality clones were then also compared with a distribution free method using the Kruskal-Wallis test. The same tests were also used to compare the individual TF contents with each other (the individual TF values for both good quality and poor quality clones combined). The t-test was performed using the TTEST procedure of SAS and the Kruskal-Wallis test was performed with the NPAR1WAY procedure of SAS (1996).

Regression analysis was used to find the Pearson correlation coefficients (r) between the measured parameters and the tea taster results and tea valuation. All the parameters measured in the black teas were correlated with the results of both tea tasters and the valuation. The REG procedure of the SAS (1996) software package was used for these analyses. The REG procedure was also used to test for any higher order relationships between TF content and taster scores and valuation. To test for significant differences between the different correlation coefficients the Z transformations of each r was determined:

$$Z(r) = \frac{1}{2}\ln\left(\frac{1+r}{1-r}\right)$$

The Z statistic of the different z-values compared were then calculated using the followingformula:

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$$Z = \frac{Z(r_1) - Z(r_2)}{\sqrt{\frac{1}{n_1 - 3} + \frac{1}{n_2 - 3}}}$$

r = One of the two correlation coefficients to be compared.

$Z(r_1)$ and $Z(r_2)$ = The Z transformation for each r compared.

n_1 and n_2 = Size of the sample for determining each r.

All the measured parameters were also analyzed with the LOGISTIC procedure of SAS (1996). The LOGISTIC procedure fitted linear logistic regression models for binary response data (good quality, poor quality) by the method of maximum likelihood. The stepwise assay was used with a significance level of entry into the model of 0.1 (90%) and a significance level for staying in the model of 0.15 (85%).

2.6 Capillary electrophoresis analysis

Capillary electrophoresis was performed on a HP ^{3D}CE system using a fused silica capillary column from Bio-Rad (Scientific Group, JNB, RSA) with a diameter of 50 μm and a total length of 40 cm, giving a separating length of 32.5 cm. The detector was set at 380 nm and the sample injected by pressure for 5s (50 mbar). The non-aqueous capillary electrophoresis (NACE) technique was used for the separation (Wright *et al*, 2001). The running buffer consisted of 71% ACN (v/v), 25% MeOH (v/v) containing 0.1 M KOH, 4% glacial acetic acid (v/v) and 90 mM ammonium acetate. The NACE was run at constant current of 35 μA (approximately 22.5 kV) and the temperature was maintained at 18.5°C. Each clone was analyzed in triplicate.

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3 Results

The tea clones were separated into two groups of 20 good quality clones and 20 poor quality clones according to at least two previous tea taster scores. The content of the individual TFs, SIT, total TFs and total tea taster scores for tasters A and B and their valuations are given in Table 4.1. The structures of the four major TF are shown in Figure 4.1.

Table 4.1. Individual and total TF contents of black tea, total tea taster scores and valuation for forty TRF tea clones (n.a. = not available).

Clone	Flavognost *(umol/g DT)*	Taster A score	Taster B score	Valuation *(UK pence/kg)*	TF-f *(umol/g DT)*	TF-A *(umol/g DT)*	TF-B *(umol/g DT)*	TF-dg *(umol/g DT)*	SIT *(umol/g DT)*
Good quality									
SFS204	11.8	23.6	70	130.0	5.3	3.2	1.6	2.8	13
PC1	17.2	22.1	54	117.2	14	4.4	2.0	1.4	21
88/79-2	7.72	18.2	58	109.4	4.5	3.7	1.5	2.7	12
PC117	13.0	24.0	74	147.7	12	5.6	2.3	2.0	22
PC213	12.1	21.7	74	140.6	15	4.7	1.9	1.5	23
PC190	15.2	20.0	64	151.0	9.2	4.9	2.2	3.5	20
PC108	17.7	25.1	72	150.5	16	5.8	2.7	2.1	27
15M-58	8.75	22.1	68	128.5	12	4.6	1.7	1.3	20
15M-39	9.11	20.6	74	121.5	9.6	3.1	1.3	0.9	15
88/3-3	6.56	21.2	64	123.3	6.3	4.7	1.5	2.0	14
15M-1	17.3	21.9	64	118.7	10	4.4	1.7	1.7	18
88/5-2	12.8	21.7	78	123.8	7.6	3.5	1.7	1.6	14
PC200	9.33	21.9	64	141.3	13	5.5	2.2	1.8	22
PC192	15.7	22.1	74	153.0	13	5.4	2.4	1.9	22
PC119	15.6	24.1	64	152.1	17	4.9	2.9	1.8	26
PC168	6.65	25.0	54	140.3	11	6.3	2.8	3.7	24
33/10-47	13.8	25.2	76	137.3	14	4.5	2.3	1.8	22
PC104	18.4	30.1	64	150.2	13	5.6	2.7	2.8	24
PC76	n.a.	n.a.	n.a.	n.a.	15	6.3	3.5	3.0	28
PC79	n.a.	n.a.	n.a.	n.a.	14	6.7	3.5	4.3	29
Poor quality									
88/50-5	8.44	19.8	52	116.2	7.6	3.6	1.4	1.6	14
PC206	12.3	16.2	52	98.80	6.1	3.5	1.7	2.2	14
SFS42	10.8	n.a.	45	n.a.	6.5	4.1	2.0	2.9	16
NKW30	5.22	17.4	45	113.2	5.0	2.8	1.2	1.7	11
88/60-9	8.39	17.7	50	108.8	12	4.3	1.9	1.6	19
NKW20	4.82	14.7	40	107.6	3.8	2.2	0.8	1.4	8.2
NKW44	8.39	14.5	50	106.7	3.9	2.6	0.9	1.5	8.9
88/79-1	6.12	17.0	45	114.8	6.9	3.6	1.6	1.9	14
88/54-11	10.4	18.4	52	110.0	8.6	3.2	1.5	1.2	15
PC169	7.59	19.3	50	113.0	7.3	5.0	2.0	3.1	17
PC186	12.2	18.3	50	135.0	7.0	3.8	1.8	2.1	15
CL12	12.2	n.a.	50	n.a.	6.0	4.8	2.5	4.3	18
11M-3	9.02	17.6	45	102.8	8.4	3.1	1.0	0.9	13
PC211	7.46	16.4	45	125.5	9.1	3.6	1.5	1.2	15
88/61-11	6.03	14.4	40	88.60	3.0	2.2	0.7	1.4	7.3
88/119-1	10.7	18.1	53	112.0	7.1	4.3	1.7	2.2	15
88/54-14	7.72	17.4	52	108.8	9.8	4.7	2.2	2.2	19
15M-18	6.65	20.1	50	116.1	11	3.6	1.5	1.1	17
PC194	10.1	18.8	50	142.0	7.9	3.8	1.8	2.0	16
88/110-4	6.38	16.0	45	102.4	3.4	2.8	1.0	2.7	10

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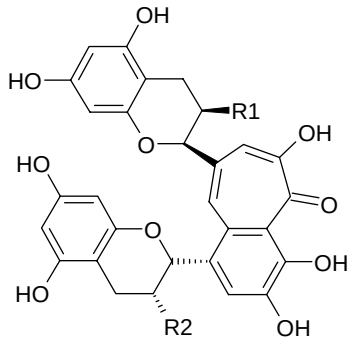

Free theaflavin (TF-f)
R1 = OH
R2 = OH

Theaflavin-3-monogallate (TF-A)
R1 = 3,4,5-trihydroxybenzoyl
R2 = OH

Theaflavin-3'-monogallate (TF-B)
R1 = OH
R2 = 3,4,5-trihydroxybenzoyl

Theaflavin-3,3'-digallate (TF-dg)
R1 = 3,4,5-trihydroxybenzoyl
R2 = 3,4,5-trihydroxybenzoyl

Figure 4.1. The TFs analyzed in black tea.

All the appropriate data sets were tested for normality using the Shapiro-Wilk test as well as the normal probability plot (Table 4.2 and Figure 4.2). Since many of the data sets showed divergence from normality, the distribution free Kruskal-Wallis test was also used to test for significant differences between data sets (see Table 4.3). Although the P-values obtained with the Kruskal-Wallis test differ from those obtained with the Student's t-test, both tests showed significant differences between the same data sets. The P-values in the text were calculated with the Kruskal-Wallis test.

Table 4.2. Testing for normality with Shapiro-Wilk showing the probability of a data set to be normally distributed.

	TF-f	TF-A	TF-B	TF-dg	SIT	Flavognost	Taster B score	Taster A score	Valuation
Normality for variables (p) *(for good quality clones)*	0.2878	0.6849	0.2108	0.1423	0.2415		0.1153	0.0916	0.126
Normality for variables (p) *(for poor quality clones)*	0.8403	0.6303	0.9566	0.0483	0.2171		0.0061	0.5598	0.2064
Normality for variables (p) *(for combination of good and poor quality clones)*	0.3235	0.6974	0.2322	0.0076	0.4078	0.0506			

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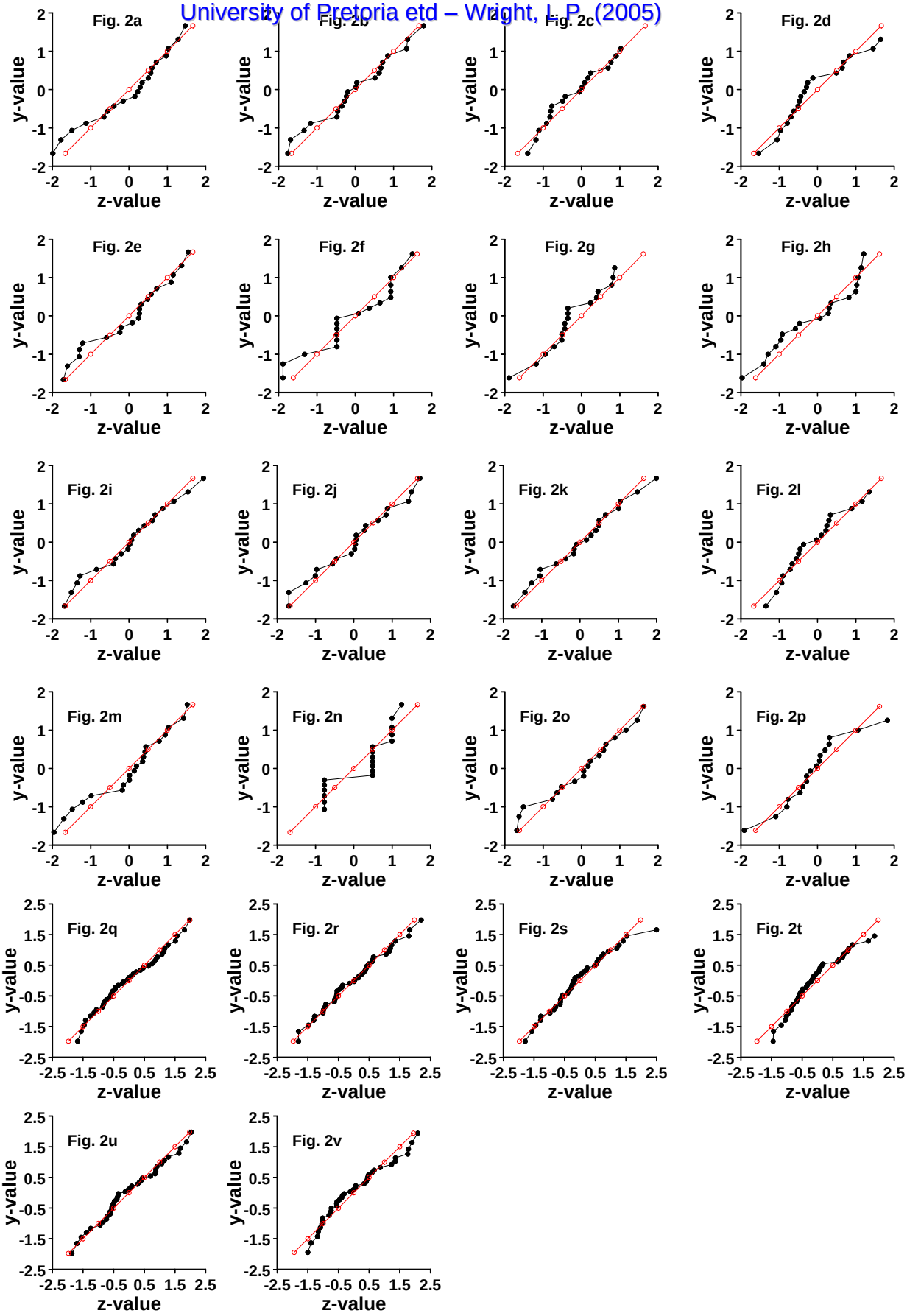

Figure 4.2. Normal probability plot for variables of good quality clones (a-h), poor quality clones (i-p) and a combination of good and poor quality clones (q-v). The variables tested for normality were: (a, i, q), TF-f; (b, j, r), TF-A; (c, k, s), TF-B; (d, l, t), TF-dg; (e, m, u), SIT; (f, n), Taster-B; (g, o), Taster-A; (h, p), value; (v), flavognost.

Table 4.2. Student's t-test and Kruskal-Wallis test significance between the good quality and poor quality tea clones and the regression analysis between the measured parameters and the tea taster results and tea valuation.

	TF-f *(umol/g DT)*	TF-A *(umol/g DT)*	TF-B *(umol/g DT)*	TF-dg *(umol/g DT)*	SIT *(umol/g DT)*	Flavognost *(umol/g DT)*	Taster B score	Taster A score	Valuation *(US cents/kg)*
Mean	**11.54**	**4.90**	**2.22**	**2.22**	**20.88**	**12.88**	**67.40**	**23.00**	**136.44**
SD(n-1)	**3.54**	**1.01**	**0.65**	**0.88**	**4.98**	**3.77**	**7.11**	**2.54**	**13.74**
(for good quality clones)									
Mean	**6.99**	**3.58**	**1.53**	**1.96**	**14.07**	**8.55**	**48.05**	**17.30**	**112.35**
SD(n-1)	**2.39**	**0.80**	**0.48**	**0.82**	**3.46**	**2.35**	**3.97**	**1.72**	**12.42**
(for poor quality clones)									
Student's t-test (P value)	**3.62E-05**	**6.00E-05**	**5.09E-04**	**0.340**	**1.60E-05**	**1.28E-04**	**1.18E-11**	**2.08E-09**	**2.00E-06**
Kruskal-Wallis test (P value)	**0.0002**	**0.0004**	**0.0016**	**0.358**	**0.0002**	**0.0012**	**< 0.0001**	**< 0.0001**	**< 0.0001**
r (Taster B)	**0.601****	**0.513****	**0.485****	**-0.026**	**0.594****	**0.591****			
r (Taster A)	**0.732****	**0.750****	**0.779****	**0.285***	**0.802****	**0.662****			
r (Valuation)	**0.705****	**0.741****	**0.788****	**0.316***	**0.785****	**0.607****			
m (Valuation)[a]	**3.34**	**12.48**	**24.90**	**8.20**	**2.68**	**2.73**			

[a] The slope of the linear regression obtained with the different variables against value as the dependent variable.

*, ** Significant at P < 0.05 and 0.01, respectively.

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All the clones showed a relative standard deviation (RSD) of less than 6% for the content of the four TF analyzed.

The TF occurring in the highest abundance in all 40 tea clones analyzed was TF-f. The content for TF-f ranged from 3.0 to 17 μmol g^{-1} dried tea (DT). There was a significant difference between the TF-f content of the good and poor quality tea clones (P = 0.0002), see Table 4.3. The other three TF occurred at significantly lower levels than that for TF-f, see Table 4.4.

The TF occurring at the second highest abundance was TF-A. The content of TF-A in the tea ranged from 2.2 to 6.7 μmol g^{-1} DT. There was also a significant difference between the TF-A values for the good quality and poor quality clones (P = 0.0004). Although the overall content of TF-A was significantly lower than the TF-f content (P < 0.0001), it was significantly higher than the content of TF-B (P < 0.0001) and TF-dg (P < 0.0001) (Table 4.4).

Table 4.4. Student's t-test and Kruskal-Wallis test for significant differences between the overall contents of the inividual TFs.

	TF-dg	TF-B	TF-A
Student's t-test			
TF-f	**2.56E-15**	**1.27E-15**	**1.11E-10**
TF-A	**5.67E-15**	**2.07E-17**	
TF-B	**0.110**		
Kruskal-Wallis test			
TF-f	**< 0.0001**	**< 0.0001**	**< 0.0001**
TF-A	**< 0.0001**	**< 0.0001**	
TF-B	**0.4025**		

As seen from Table 4.4, the overall contents of TF-B and TF-dg occurred significantly lower compared with TF-f ($P < 0.0001$; < 0.0001) and TF-A ($P < 0.0001$; < 0.0001). There is no significant difference between the content of TF-B and TF-dg ($P = 0.403$). The content for these TF ranged between 0.70 and 3.5 μmol g^{-1} DT for TF-B and between 0.85 and 4.3 μmol g^{-1} DT for TF-dg. There was a significant difference between the content of TF-B in the good quality clones and poor quality clones ($P = 0.0016$). No significant difference could be found in the TF-dg content between good and poor clones ($P = 0.358$).

The SIT content was calculated by the sum of the individual TF measured on the CE (SIT = TF-f + TF-A + TF-B + TF-dg). This differed from the total (flavognost) TF content ($P < 0.0001$). The flavognost spectrophotometric method uses the flavognost reagent (2-aminoethyl diphenylborate in ethanol), which forms a green complex with the cis-1,2-di-hydroxy-benzene ring associated with the TF. The total (flavognost) TF contents are significantly lower than the SIT values determined with our CE method. Our CE results suggest that the formula used by the flavognost method to calculate the TF content (Robertson and Hall, 1989) may have to be revised, since it under estimates the TF content by about 40%.

The clone with the lowest levels for TF-f, TF-A, TF-B and SIT, was 88/61-11. Clone 11M-3 had the lowest levels of TF-dg. The clone with the highest levels of TF-f was PC119, Clone PC79 had the highest levels for both TF-A and SIT, whereas the highest levels for TF-B and TF-dg occurred in clones PC76 and CL12, respectively. It is interesting to note that the clone with the highest levels for TF-dg was classified as a poor quality clone. The SIT values ranged from 7.3 to 29 μmol g^{-1} DT with a

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significant difference between the values for the good and poor quality clones (P = 0.0002).

Correlation coefficients between the combined content (good quality and poor quality scores) of individual TF and tea taster scores are shown in Table 4.3. All the parameters looked at showed a low probability of having any higher order relationships with the taster scores (results not shown). The correlation coefficients obtained with the total score for taster A are higher than those for taster B. The highest correlation coefficient for taster A was obtained for SIT (r = 0.802), followed by TF-B (r = 0.779), TF-A (r = 0.750) and TF-f (r = 0.732). There was no significant difference between these correlation coefficients at the 95% confidence level (Table 4.5). TF-dg levels showed significantly poorer correlation with taster A (r = 0.285). For taster B the highest correlation coefficient with total taster scores was for TF-f (r = 0.601). The TF with the second best correlation coefficient for taster B scores was SIT (r = 0.594), followed by flavognost TF score (r = 0.591), TF-A (r = 0.513) and TF-B (r = 0.485). At the 95% confidence level there was no significant difference between these correlation coefficients (Table 4.5). Once again TF-dg levels showed the poorest and significantly lower correlation coefficient with taster B (r = -0.026).

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Table 4.5. The z-statistic for comparing different r-values obtained from correlating individual TF contents and SIT with taster B and taster A scores and value respectively.

	TF-A	TF-B	TF-dg	SIT
(Taster B)				
TF-f	0.54	0.69	3.02**	0.05
TF-A		0.16	2.48*	0.49
TF-B			2.32*	0.65
TF-dg				2.97**
(Taster A)				
TF-f	0.16	0.45	2.60**	0.70
TF-A		0.28	2.76**	0.53
TF-B			3.05**	0.25
TF-dg				3.29**
(Value)				
TF-f	0.31	0.77	2.23*	0.74
TF-A		0.46	2.54*	0.429
TF-B			3.00**	0.03
TF-dg				2.97**

*, z > 1.96 have 95% probability of differing significantly.
**, z > 2.58 have 99% probability of differing significantly.

The correlation coefficients between the content of individual TF with the value of the tea showed the same general trend as the tea taster scores. The valuation was as perceived by taster A. The TF-B had the highest correlation coefficient (r = 0.788), followed by SIT (r = 0.785), TF-A (r = 0.741), TF-f (r = 0.705) and total TFs (r = 0.607). There was no significant difference between these correlation coefficients at the 95% confidence level (Table 4.5). TF-dg had a significant lower correlation coefficient for value (r = 0.316). The r^2-values show that 62% of the variation in value can be predicted with TF-B and SIT content. For TF-A and TF-f, 55% and 50% of the variation in value can respectively be explained by their content. For Central and Southern African teas this indicates that SIT, TF-B, TF-A or TF-f values have a limited ability for predicting the value of the tea. As seen in Figure 4.3, the narrowest

90% confidence limits for the correlation with value were obtained for SIT contents, followed by TF-B, TF-A and TF-f. The width of the 90% confidence limit between these four parameters are, however, very similar. The TF-dg values, on the other hand, show no ability to predict the value of the above-mentioned teas.

The slopes (m-value) of the linear regressions obtained with the content of the individual TF and value are shown in Table 4.3. The content of TF-B had the highest m-value (24.90), followed by TF-A (m = 12.48) and TF-dg (m = 8.20). Because of the low r-value of TF-dg with value, the high m-value obtained with TF-dg is not considered to be of much importance. The SIT and total TFs had low m-values of 2.68 and 2.73 respectively.

Using the stepwise assay of the LOGISTIC procedure, only the TF-f and total TF content according to the flavognost method met the 0.1 significance level for predicting the likelihood for a clone to produce a good or poor quality black tea. Based on the TF-f content (TF-f) and TF (flavognost) content, the estimated logit of the probability of a clone to be of good quality can be calculated as

$$\text{logit}(p) = -6.4403 + 0.2981(\text{flavognost}) + 0.3686(\text{TF} - \text{f})$$

The TF-f content and the TF (flavognost) content must be given as μmol g^{-1} DT. Using this estimate, the probability (p) of a clone to be of good quality can be calculated as follows

$$p = \frac{e^{\text{logit}(p)}}{1 + e^{\text{logit}(p)}}$$

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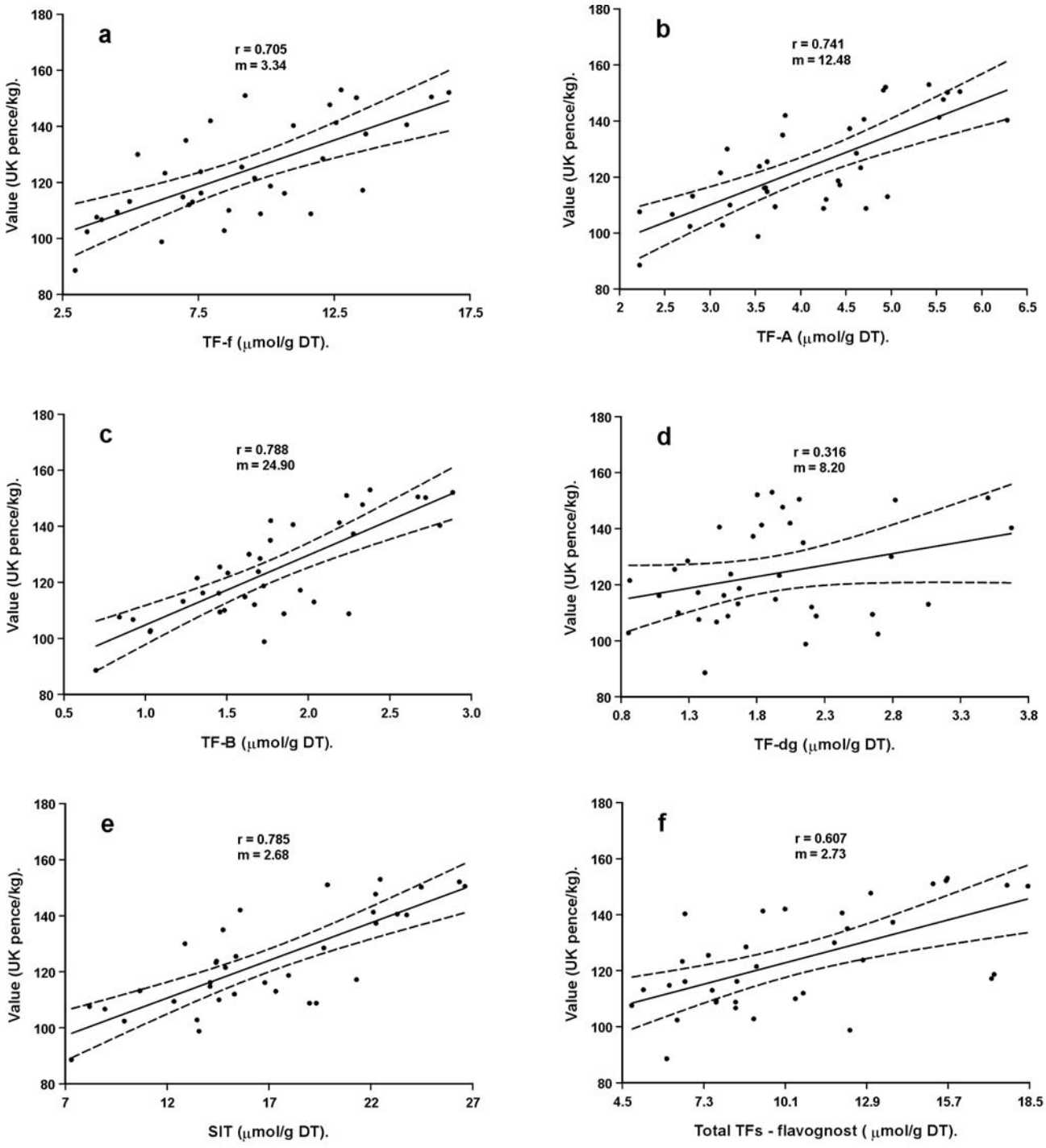

Figure 4.3. The relationship between (a) TF-f, (b) TF-A, (c) TF-B, (d) TF-dg, (e) SIT and (f) total TF content and manufactured black tea valuation. The dotted lines represent 90% confidence limits.

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The LOGISTIC procedure of SAS was also used without the stepwise assay to determine the equations for all the different parameters to predict the probability of a clone to be a good quality. The different equations for the different parameters, or combinations of them were then tested on the data. This gave the percent concordant values, the percentage of the clones that could be classified correctly, as shown in Table 4.6.

Table 4.6. The percent concordant values for predicting tea clones as either good or poor quality as determined using the content of different theaflavin parameters.

Parameters	Percent concordant	Analysis method
TF-f	84.8	CE
TF-A	82.8	CE
TF-B	79.0	CE
TF-dg	58.3	CE
SIT	84.3	CE
Total TFs + SIT	85.6	CE + Spectrophotometric
Total TFs + TF-f	86.1	CE + Spectrophotometric
Total TFs	80.6	Spectrophotometric
Value	90.8	Organoleptic

Due to the fact that the value was one of the parameters used to separate the clones in good and poor quality groups, it also had the highest percent concordant values. Of the other parameters a combination of total TFs and TF-f content (86.1%) had the highest percent concordant, followed closely by total TFs and SIT (85.6%). The next best parameter was TF-f (84.8%), followed by SIT (84.3%), TF-A (82.8%), total TFs (80.6%) and TF-B (79.0%). There were no significant differences between these parameters at the 95% confidence level (Table 4.7). TF-dg, on the other hand, had a significantly lower percent concordant value of 58.3%.

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Table 4.7. The z-statistic for showing the significant differences between the percent concordant values for different theaflavin parameters.

	TF-f	TF-A	TF-B	TF-dg	SIT	Total TFs	Total TFs + SIT	Total TFs + TF-f
TF-f		0.243	0.674	2.627*	0.062	0.497	0.101	0.165
TF-A			0.432	2.404*	0.181	0.254	0.343	0.407
TF-B				1.995*	0.612	0.178	0.773	0.837
TF-dg					2.570*	2.165*	2.718*	2.775*
SIT						0.435	0.163	0.227
Total TFs							0.597	0.660
Total TFs + SIT								0.064

* Significant at $P < 0.05$, for $z > 1.645$.

4 Discussion

The aim of this research was to investigate the relevance of the contents of individual TF in black tea from Central and Southern Africa to their quality. Twenty good quality tea clones and twenty poor quality tea clones were compared for identification of significant differences in the TF content and profile between these two groups. Furthermore, the correlations between the TF content and profile and the total tea taster scores of two tea tasters as well as the valuation of the chosen tea clones were investigated.

Earlier research already indicated a correlation between total (flavognost) TF content and the quality of the black teas of Central African origin (Hilton and Ellis, 1972; Hilton *et al*, 1973). Furthermore, Owuor *et al* (1987) and Owuor and Obanda (1995) have shown the importance of the TF composition on the quality of the black tea manufactured from Kenyan clones. They found the theaflavin digallate equivalents to be correlated with the quality of the clones they investigated, but could not find a significant correlation between the total (flavognost) TF or the TF-A and TF-B content and quality.

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Madanhire emphasized the need for investigating the importance of the profiles of individual TF on the quality of black tea manufactured from TRF(CA) tea clones, and the flavan-3-ol composition responsible for it (Madanhire, 1995). Wright *et al* (2000) showed in their work that there indeed exists a significant correlation between the flavan-3-ol contents and quality of the TRF(CA) black tea clones. This raised the question whether the correlation between flavan-3-ol composition and TRF(CA) tea quality are due to a correlation between the flavan-3-ol composition and TF composition. Because of the complexity of the interactions between the flavan-3-ols and the PPO enzyme responsible for TF formation, simple statistical analysis could not satisfactorily explain the correlation between the flavan-3-ol composition and the TF composition (results not shown). Other factors, which might be involved, include peroxidase activity, thearubigin formation, the effect of redox equilibration, enzyme inhibition by gallated flavan-3-ols and a substrate limiting effect of di-hydroxy flavan-3-ols. A more thorough kinetic analysis also accounting for PPO activity should provide a better model for explaining the relationship between the flavan-3-ol and TF composition. Clearly there is no simple explanation for the correlation between the flavan-3-ol content in the leaves and the TF content in the manufactured black tea.

Our results confirm the importance of SIT content in predicting the quality of TRF(CA) tea clones. However, the non-gallated TF (TF-f) and mono-gallated TFs also showed a significant ability to distinguish between good and poor quality tea clones. In contrast to the Kenyan tea clones, the TF-dg contents showed no significant difference between the good and poor quality tea clones.

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These findings can be explained if the quality of the Central and Southern African teas is more dependent on the total amount of TFs, than the composition of the individual TFs. The significant effect of the non-gallated TFs and mono-gallated TFs might be explained by the effect of their flavan-3-ol precursors on TF enzymatic synthesis.

TFs are formed by the enzymatic oxidation and condensation catalyzed by PPO, whereby one di-hydroxy flavan-3-ol and one tri-hydroxy flavan-3-ol are combined. The major TF has its specific flavan-3-ol precursors as shown in equation 1 to 4. According to the *in vitro* oxidation studies done by Robertson (Robertson, 1983), the affinities of the different flavan-3-ols for PPO differ. Robertson (Robertson, 1983) showed a linear increase in the amounts of TFs formed with up to a two fold increase in the non-gallated flavan-3-ols. An increase of more than 1.2 fold of gallated flavan-3-ols, however, resulted in substrate inhibition and a decrease in the amounts of TFs formed. This might explain why the non-gallated and mono-gallated TFs are associated with quality, while the digallated TF is not. Since gallated flavan-3-ols may inhibit PPO it is not surprising that TF-f has the highest correlation with valuation. The TF-f levels had the best ability of the individual TFs to distinguish between good and poor quality tea clones. TF-f also occurs in the highest amounts of the four TFs. Of the two mono-gallated TFs, TF-A contents were better in predicting the quality of the tea clone. This can also be explained by the hypothesis that the total amount of TFs are of most importance when predicting the quality of TRF(CA) tea clones. The TF-A content is significantly higher than the content of TF-B or TF-dg.

The increased inhibitory effect of the gallated TFs can also be explained by the protein complexation tendencies of different polyphenols (Martin *et al,* 1987; Spencer *et al,* 1988; Luck *et al,* 1994). Generally, the higher the molecular weight and the higher the flexibilities of a polyphenol, the higher its ability for protein complexation. The gallated flavan-3-ols have higher molecular weights and flexibilities than the non-gallated flavan-3-ols. Similarly, the mono-gallated TFs and di-gallated TF have higher molecular weights and flexibilities that should also increase their abilities for product inhibition of PPO due to protein complexation. This is confirmed by the observation of Spencer *et al* (1988) that TFs with more galloyl groups were precipitated more efficiently by gelatine than non-gallated TFs.

With the exception of TF-dg, all the individual TF and SIT showed a significant correlation with tea taster scores and value. These correlations, however, don't differ significantly from each other. When looking at the slopes of the linear regressions obtained with value, TF-B and TF-A had much higher m-values. A higher m-value will be desirable since it implies that a small change in the independent variable (TF content) will result in a large change in the dependent variable (value). When considering both the correlation coefficients and the slopes of the linear regressions obtained with value (Figure 4.3), TF-B and then TF-A are best suited to predict the value of Southern African black tea. Although TF-B and TF-A could only respectively predict 62% and 55% of the variation in value, their content might be used as chemical indication of the value of Central and Southern African black tea in the absence of better indicators. The di-hydroxy flavan-3-ol precursor of TF-A is EC, whereas ECg is the di-hydroxy flavan-3-ol precursor of TF-B. This correlates well with the research of Wright *et al* (2000), showing that EC and ECg have the best

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ability of the flavan-3-ols to predict the quality of Southern African tea clones. A combination of TF-f content and the TF (flavognost) content have the best ability to predict whether a Southern African tea clone will be of good or poor quality.

In conclusion, the results presented in this work are in agreement with the hypothesis that the SIT content in the black tea is associated with the quality of Central and Southern African clones. Furthermore, in contrast to the Kenyan tea clones, TF-dg is not associated with quality. Using TF-f content and flavognost TF content the quality of a clone can be predicted. The content of TF-B and TF-A can also be used as indicator of the value of Central and Southern African black tea. The analysis of the individual TF content may also be used in the optimization of the manufacturing process for black tea produced in Central and Southern Africa.

Chapter 5

The effect of tannase on the quality of black tea.

1 Introduction

Black tea are sold on the world market and prized according to their quality. The quality and thus prize of the black tea are determined organoleptically by professional tea tasters in relation to market conditions (McDowell *et al*, 1991). The manufacturing of high quality black tea is very important to increase the profit and maintain the economic feasibility of an estate. This is particularly important in regions producing medium quality teas and with relative high labour costs, as is the conditions in South Africa. Any cost productive measure to increase the quality will be welcomed by the tea industry.

Black tea is manufactured by macerating green leaf with subsequent oxidation of the flavan-3-ol monomers to form the theaflavin dimers and thearubigin polymers characteristic of black tea. Various researchers investigated the correlation between the flavan-3-ol profile in the green leaf and the quality in the manufactured black tea. According to (Obanda and Owuor, 1997) the catechins occurring in green leaf with the best correlations with tea taster scores are EGCg and ECg. This is in contrast with the work by (Wright *et al*, 2000) showing EC to be of most importance, followed by EGC and then ECg, when comparing twenty good quality tea clones with twenty low quality tea clones (see chapter 2). We also found no difference between the two groups when

looking at EGCg contents. In summary it seems that a high amount of ungallated flavan-3-ols and di-hydroxy-flavan-3-ols are associated with high quality for Southern and Central African black tea.

TF content is already well established as a marker of quality for Central and Southern African black tea (Wood and Roberts, 1964; Hilton and Ellis, 1972; Ellis and Cloughley, 1981; Whitehead and Ndalama, 1991). Four major theaflavins occur which can be ungallated (TF-f), have one gallic acid attached (TF-A and TF-B) or have two gallic acids attached (TF-dg). Recently work done on the TF profiles of different black tea clones showed a correlation with quality. Once again differences were observed between the black teas produced in Kenya and Southern and Central Africa. In Kenya the amount of TF-dg showed the highest correlation with quality, whereas the total amount of TFs did not show a significant correlation with quality (Owuor *et al*, 1986; Owuor and Obanda, 1995). In Southern Africa the sum of the individual TFs (SIT), TF-f and TF-A showed the highest correlation with quality, whereas the TF-dg amounts showed no correlation with quality (Wright *et al*, In press)(See chapter 4). This research indicates that for Southern African black teas the quality might be improved if the amount of TF-f can be increased whereas a decrease in the amount of TF-dg should not have a detrimental effect on the tea quality.

Tannase (tannin acyl hydrolase; EC 3.1.1.20) is an inducible enzyme produced by fungi, bacteria and yeast (Bajpai and Patil, 1996). Tannase hydrolyzes the ester bonds of tannic acid to produce gallic acid and glucose. Tannase also has the ability to hydrolyze the ester

bonds of other tannins, for example the catechin tannins in tea (the gallated flavan-3-ols) and the condensed tannins consisting of polymerized proanthocyanidins (Hatamoto *et al*, 1996; Bhat *et al*, 1998). Tannase is widely used in industry as a crude extract from *Aspergillus niger* or *Trichoderma* species (Barbe and Dubourdieu, 1998). It is used as an inhibitor of creaming down in tea, and as a clarifier in the manufacturing of beer and fruit juices. Tannase is also used in the manufacture of gallic acid (Hatamoto *et al*, 1996).

Tannase was isolated and characterized from *Aspergillus niger* LCF 8 by (Barthomeuf *et al*, 1994). They found it to be a glycoprotein of about 186,000 Dal containing 43% carbohydrates. The enzyme had a pI of 4.3 and optimal activity at a temperature of 35ºC. The enzyme showed optimum esterase activity at pH 5.0 and an optimum tannase activity at pH 6.0, while the enzyme stayed stable between pH 3.5 and 8.0. Tannase enzyme was isolated from *Aspergillus oryzae*, cloned and studied (Hatamoto *et al*, 1996). They estimated the molecular weight of the enzyme to be between 290,000 and 310,000 Dal with a carbohydrate content of 22.7%. With cloning they characterized the enzyme to contain 588 amino acids with a molecular weight of about 64,000 Dal. They further discovered the tannase enzyme to consist of two subunits of 30,000 and 33,000 Dal respectively, linked with a disulfide bridge. They concluded that the native tannase consisted of four pairs of two subunits, forming a hetero-octamer.

In this work we investigated the effect of a commercial tannase extract on the TF profile of black tea produced from tea clones developed by the Tea Research Foundation of Central Africa (TRF(CA)). We also investigated the effect of the added tannase on the

characteristics of the manufactured black tea, including quality and the effect of creaming down.

2 Materials and methods

2.1 Tea samples used

Tea samples from Mukubani tea estate in the Venda region of South Africa were collected to investigate the effect of tannase on creaming down and the flavan-3-ol profile. The tea samples were collected from the factory directly after maceration, but before fermentation took place. The dhool was stored in airtight plastic bags and then put in a cool box on dry ice until it was transferred to a freezer at -20ºC approximately two hours later. In this frozen state it was transported to Pretoria where it was stored at -20ºC. The dhool consisted of a mixture of clones SFS150, PC81 and BB35.

Tea samples from the Grenshoek tea estate in the Tzaneen region of South Africa were used for the studies involving the effect of tannase on the TF profile and sensory evaluation of black tea. The tea was transported as fresh tea leaf of seedling origin to Pretoria. The leaf was plucked in the early morning and losely packed in open boxes after which it was transported to Pretoria. Once it arrived in Pretoria, approximately five hours later, it was immediately processed.

2.2 Reagents

The reagents used for flavan-3-ol extraction and analyses were the same used by (Wright *et al*, 2000). The reagents used for TF extraction and analysis were the same as described by (Wright *et al*, 2001). Two tannase enzyme extracts were used. Kikkoman tannase was obtained in powder form from Kikkoman, Japan (Lot # KTFH6181, 5000 Kikkoman units per gram). Novo tannase was obtained in a concentrated liquid form from Novo, Switzerland, and was labeled as batch 120997.

2.3 SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) gel was prepared according to the method of (Laemmli, 1970). A separating gel of 12% was prepared and a stacking gel of 6%. The proteins were visualized with Coomassie blue stain. The Bradford method was used to determine the protein concentrations in the Kikkoman and Novo enzyme samples (Bradford, 1976). Bovine serum albumin (BSA) was used for preparing the standard curve.

2.4 Black tea manufacturing

The tea leaves obtained from Grenshoek were immediately spread out on the tile floor in the laboratory in a swallow layer just covering the surface. The leaves were then left overnight at 22ºC in the air-conditioned room, to wither. The leaf was then macerated by

passing through a CTC machine three times, and then stored in airtight plastic bags at -20ºC until used. The rest of the manufacturing process was the same for both Mukubani and Grenshoek samples.

Prior to fermentation the dhool was defrosted in an 800-Watt domestic microwave oven. The Mukubani dhool was defrosted as 400 g sub-samples at 50% power for seven minutes, and the Grenshoek dhool was defrosted as 200 g sub-samples for five minutes. The samples were then fermented at 22ºC and approximately 95% humidity for 60 minutes. The fermented dhool was then dried in a mini fluid bed drier, the fiber removed with electrostatic rollers and the tea sieved to obtain particles between 500 and 1500 micrometer. After manufacture the black tea samples were stored in foil-lined bags in a closed container at 4ºC until needed. The freeze-dried samples of the Mukubani dhool were stored in airtight polytops at 4ºC in a cold room.

2.5 Tannase method

The Mukubani samples were treated with different amounts of Kikkoman tannase or Novo tannase that was dissolved in 20 ml of deionized water. The Grenshoek samples were treated with Kikkoman tannase that was dissolved in 5 ml of water. The amounts of protein and tannase in each sample were first determined with the Bradford method and SDS PAGE respectively, to determine the doses of tannase enzyme to add. The tannase was mixed into the dhool just after it was thawed and placed in airtight plastic bags and kept at anaerobic conditions at 22ºC for 30 minutes. At the end of the anaerobic

incubation samples of the Mukubani dhool was taken and frozen in liquid nitrogen and then freeze-dried.

2.6 Green leaf flavan-3-ol extraction and analysis

The flavan-3-ols were extracted with 40% methanol with the method as described by (Wright *et al*, 2000). The extracted flavan-3-ols were analyzed with capillary electrophoresis using the micellar electrokinetic capillary chromatography (MECC) as described by Aucamp *et al* (2000).

2.7 Theaflavin sample preparation and analysis

The theaflavins were extracted with IBMK from a 2% black tea infusion as described by (Wright *et al*, 2001). The TFs were also analyzed with CE using the non-aqueous capillary electrophoresis (NACE) method described by (Wright *et al*, 2001).

2.8 Tea cream analysis

Tea liquors (0.8% w/v) were prepared by adding 100 ml boiling deionized water to 800 mg tea in a thermo flask. The infusion was then incubated in the thermo flasks for ten minutes while shaken on a horizontal shaker at approximately 90 rpm. The tea infusion was then filtered through Macherey-Nagel MN 617 fluted filter paper. The tea was then kept in test tubes at 4ºC for 24 hours and the absorbance measured at 540 nm.

2.9 Sensory analysis

The Grenshoek black tea samples were evaluated by tea taster A in London, United Kingdom, and tea taster B in Johannesburg, South Africa. Both tasters evaluated the samples on a twenty-point scale for colour, brightness, strength, briskness and quality. The individual scores were added to get a total score out of 100. The tea tasters were also not given any indication of how the samples were prepared.

2.10 Statistical methodology

The TF amounts of the Grenshoek samples were analyzed with ANOVA to indicate any significant variations between the controls and tannase treated samples. The amounts of TF-f, TF-A, TF-B, TF-dg and SIT were analyzed in this manner. The individual TFs that showed significant variations after tannase treatment were then compared with each other using the Student's t-test. The same analyses were done for the flavan-3-ol profiles of the different Mukubani samples.

3. Results

The Mukubani tea samples were treated with Kikkoman and Novo tannase. Dhool with only water added and then incubated under anaerobic conditions before manufactured according to standard practice was used as the control (0 tannase added). The other samples were treated with different amounts of either Kikkoman tannase or Novo tannase as shown in Table 1. After manufacture of the different black tea samples, the creaming down effect of the different samples was determined (Table 5.1). The ability of the tannase to inhibit tea cream formation was measured in tea clarification units (TCU). TCU is defined as that amount of enzyme activity that produce a decrease of 0.01 absorbance units at 540 nm relative to the control tea liquor after 24 hours at 4ºC. The protein content of the Kikkoman and Novo tannase preparations was determined, and used to calculate the specific activities at the different amounts used (Table 5.1).

The tannase had a significant effect on the creaming down of the enzyme treated tea samples. Tea with absorbance values (540 nm) of 1.5 or higher show heavy cream, tea with an absorbance between 0.7 and 1.5 will have light creaming, while teas with an absorbance below 0.7 will appear clear. The teas with TCU values of more than 40 will still be clear, while all the teas with TCU values less than 40 will show light creaming. As can be seen in Table 5.1, all the tea samples treated with 1 g or more Kikkoman enzyme powder per Kg made tea (MT) produced a clear tea infusion after 24 hours at 4ºC. All the other tea samples, including all the samples treated with the Novo enzyme

Table 5.1. Comparison of the Kikkoman and Novo tannase preparations.

	Kikkoman tannase							Novo tannase				
Kikkoman tannase (g/Kg MT)	0.0	0.2	0.4	0.6	1.0	1.5	2.0	*	*	*	*	*
Novo tannase (ml/Kg MT)	*	*	*	*	*	*	*	0	12	25	50	100
Total protein (mg/Kg MT)	0	2.4	4.8	7.2	12.2	18.3	24.4	0	15	30	60	120
Absorbance of 0.8% (w/v) tea infusion after 24 hours at 4ºC	1.10	0.70	0.88	0.82	0.48	0.62	0.50	1.10	1.04	1.01	1.02	0.80
TCU	0	40	22	28	62	48	60	0	6	9	8	30
Specific activity (TCU/mg protein)	0.00	16.70	4.60	3.90	5.10	2.60	2.50	0.00	0.40	0.30	0.13	0.25

Table 5.2. The profile of the major flavan-3-ols and gallic acid after treatment with Kikkoman tannase.

	Control	Tannase (0 g/Kg MT)	Tannase (0.1 g/Kg MT)	Tannase (2 g/Kg MT)
EGC				
Mean	21.14	1.16	3.77	5.96
SD(n-1)	0.45	0.33	0.10	0.12
EGCg				
Mean	40.05	7.80	15.16	10.78
SD(n-1)	0.45	0.16	0.12	0.19
EC				
Mean	0.77	0.21	0.82	4.01
SD(n-1)	0.03	0.03	0.03	0.00
ECg				
Mean	16.81	7.73	10.05	5.81
SD(n-1)	0.14	0.09	0.09	0.06
g				
Mean	50.77	4.07	22.09	33.47
SD(n-1)	0.07	0.75	0.02	0.14

extract, showed light creaming. Table 5.1 shows a general trend of decreasing specific activity with an increase in the amounts of enzymes used.

The flavan-3-ol profiles of the Mukubani tea samples treated with different amounts of Kikkoman tannase are shown in Table 5.2. All the samples were analyzed in triplicate. The control was taken before any fermentation took place and also before the 30 minutes anaerobic incubation of the tannase treated samples. For the tannase treated samples (0, 0.1 and 2.0 g/Kg MT) the flavan-3-ol profiles are shown after the 30 minutes anaerobic incubation. A very significant decline in all the flavan-3-ols after the 30 minutes anaerobic incubation period is seen by comparing the flavan-3-ols in the control and zero tannase samples.

Table 5.3. ANOVA table of the different Kikkoman tannase treatments for the contents of the major flavan-3-ols and gallic acid.

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-statistic	F-probability distribution
EGC					
Treatment	368.75	2	184.38	377.56	4.90E-07
Error	2.93	6	0.49		
Total	371.68	8			
EGCg					
Treatment	390.93	2	195.47	1584.85	6.74E-09
Error	0.74	6	0.12		
Total	391.67	8			
EC					
Treatment	296.72	2	148.36	22254.00	2.45E-12
Error	0.04	6	0.01		
Total	296.76	8			
ECg					
Treatment	138.43	2	69.22	1887.68	3.99E-09
Error	0.22	6	0.04		
Total	138.65	8			
g					
Treatment	37267.52	2	18633.76	2982.99	1.01E-09
Error	37.48	6	6.25		
Total	37305.00	8			

The ANOVA analysis identified all parameters to have more than a 99% probability (F-probability distribution < 0.01) to have a significant difference between the samples due to tannase treatment (Table 5.3). Because of the known external variable of the limited oxidation of the flavan-3-ols during the anaerobic incubation, only the samples taken after the anaerobic incubation were compared. All the different parameters looked at showed significant variance between all three tannase treatments compared (Table 5.4), namely 0, 0.1 and 2.0 g / Kg MT tannase added.

Table 5.4. The student's t-test for the different tannase treatments for the major flavan-3-ols and gallic acid.

	Tannase (0.1 g/Kg MT)	Tannase (2 g/Kg MT)
EGC		
0 g/Kg	3.01E-03	5.11E-04
0.1 g/Kg		2.44E-05
EGC		
0 g/Kg	8.42E-07	3.98E-05
0.1 g/Kg		1.95E-05
EC		
0 g/Kg	1.48E-05	2.08E-05
0.1 g/Kg		2.72E-05
g		
0 g/Kg	5.77E-04	2.98E-05
0.1 g/Kg		2.18E-04
ECg		
0 g/Kg	7.29E-06	3.10E-05
0.1 g/Kg		2.17E-06

As could be expected, an increase in the amounts of ungallated flavan-3-ols (EGC and EC) and gallic acid with an increase of tannase (0, 0.1 and 2.0 g/Kg MT) is seen (Table 5.2). The increase in the gallated flavan-3-ols with an increase of tannase, is however, unexpected. The samples treated with 0.1 and 2.0 g tannase per Kg MT showed higher amounts of EGCg (15.16 and 10.78 μmol/g MT, respectively) compared with the sample treated with no tannase (7.80 μmol/g MT). The 2.0 g per Kg MT sample (10.78 μmol/g MT), however, had less EGCg than the 0.1 g per Kg MT sample (15.16 μmol/g MT). This tendency was also observed for the amounts of ECg in the 2.0 and 0.1 g tannase per Kg MT samples (5.81 and 10.05 μmol/g MT, respectively). For ECg contents, the

amount in the 2.0 g tannase per Kg MT sample was however lower than the sample treated with zero tannase (5.81 and 7.73 μmol/g MT, respectively).

Table 5.5. The profile of the major TFs after treatment with Kikkoman tannase.

	Control	Tannase (0 g/Kg MT)	Tannase (0.1 g/Kg MT)	Tannase (2 g/Kg MT)
TF-f				
Mean	5.899	5.950	6.672	14.095
SD(n-1)	0.093	0.369	0.310	1.591
TF-A				
Mean	3.074	3.390	3.569	3.369
SD(n-1)	0.071	0.155	0.191	0.131
TF-B				
Mean	1.102	1.060	1.058	0.913
SD(n-1)	0.030	0.117	0.030	0.032
TF-dg				
Mean	2.023	2.309	2.057	1.063
SD(n-1)	0.169	0.079	0.056	0.109
SIT				
Mean	12.098	12.709	13.356	19.440
SD(n-1)	0.167	0.699	0.510	1.570

The TF profiles of the different Grenshoek tea samples treated with different amounts of Kikkoman tannase are shown in Table 5.5. These samples were treated similar to the samples used for analyzing the flavan-3-ol profiles. The different tannase treatments were done in triplicate and each of the manufactured tea samples were also analyzed in

triplicate. The values in Table 5.5 are the averages and standard deviations (n-1) of the TF contents in three tea samples. The average of the three analyzes done on each sample was used as the value of the individual TFs of each manufactured tea sample. These results were then analyzed with ANOVA as shown in Table 5.6.

Table 5.6. ANOVA table of the different Kikkoman tannase treatments for the contents of the major TFs and SIT.

Source	Sum of squares	Degrees of freedom	Mean square	F-statistic	F-probability distribution
TF-f					
Treatment	142.303	3	47.434	68.447	4.792E-06
Error	5.544	8	0.693		
Total	147.847	11			
TF-A					
Treatment	0.126	3	0.042	2.030	0.188
Error	0.166	8	0.021		
Total	0.292	11			
TF-B					
Treatment	0.062	3	0.021	5.004	0.0305
Error	0.033	8	0.004		
Total	0.095	11			
TF-dg					
Treatment	2.707	3	0.902	72.301	3.88E-06
Error	0.100	8	0.012		
Total	2.807	11			
SIT					
Treatment	103.943	3	34.648	42.774	2.85E-05
Error	6.480	8	0.810		
Total	110.423	11			

The ANOVA analysis identified three parameters to have more than a 99% probability (F-probability distribution < 0.01) to have a significant difference between the samples due to tannase treatment. These were TF-f, TF-dg and the sum of the individual TFs (SIT), which were then analyzed with Student's t-test to see the significance of the differences between the different treatments (Table 5.7).

Table 5.7. The student's t-test for the different treatments for TF-f, TF-dg and SIT.

	Tannase (0 g/Kg MT)	Tannase (0.1 g/Kg MT)	Tannase (2 g/Kg MT)
TF-f			
C	4.19e-1	2.01e-2	6.05e-3
0		3.10e-2	4.77e-3
500			6.26e-3
TF-dg			
C	4.10e-2	3.86e-1	1.14e-3
0		6.86e-3	7.89e-5
500			3.89e-4
SIT			
C	1.34e-1	1.99e-2	7.05e-3
0		1.35e-1	4.22e-3
500			7.23e-3

No significant differences (P < 0.01) were found between the control (normal black tea manufacturing) and the zero amount of tannase used (only anaerobic incubation with water with no added enzyme), for all three parameters looked at. For the treatment with

0.1 g/Kg MT tannase only TF-dg showed a significant difference from the control and 0 g/Kg MT tannase, with the 0.1 g/Kg MT tannase treatment significantly lower than the 0 g/Kg MT tannase treatment (P = 0.00686). However, for the treatment with 2 g/Kg MT tannase al three parameters showed a significant difference compared to their respective controls, 0 g/Kg MT tannase and 0.1 g/Kg MT tannase treatments. TF-dg contents were shown to differ most with treatment of 2 g/Kg MT tannase, with a decrease in TF-dg contents. As could be expected, the TF-f levels increased significantly with treatment with 2 g/Kg MT tannase. SIT contents also showed an unexpected increase after the treatment with 2 g/Kg MT tannase.

Table 5.8. Tea taster scores and value of black tea after treatment with Kikkoman tannase.

	Control	Tannase (0 U)	Tannase (500 U)	Tannase (10000 U)
Taster A (total score)				
Mean	78.0	75.0	71.0	66.0
SD(n-1)	4.5	7.6	4.5	1.0
Taster A (value)				
Mean	118.0	116.0	117.0	111.0
SD(n-1)	2.6	4.4	2.6	1.0
Taster B (total score)				
Mean	53.3	59.3	63.3	47.3
SD(n-1)	1.2	1.2	1.2	1.2

The tea taster scores are shown after different treatments with tannase (Table 5.8.). The tea scores for both tea tasters showed lower values for the highest amount of tannase used (2 g/Kg MT) compared with the control and 0 g/Kg MT tannase treatments. The same tendency was seen for the value of the tea according to tea taster A. Where only 0.1 g/Kg MT tannase was added, a slight increase in the total score of taster B and the value according to taster A were detected, compared to 0 g/Kg MT tannase used. The total taster score for taster A, however, was lower for 0.1 g/Kg MT tannase, compared to 0 g/Kg MT tannase.

Table 5.9. The student's t-test for the different tannase treatment for tea taster scores and value.

	Tannase (0 g/Kg MT)	Tannase (0.1 g/Kg MT)	Tannase (2 g/Kg MT)
Taster A (total score)			
Control	0.675	0.161	0.041
0 g/Kg MT		0.485	0.164
0.1 g/Kg MT			0.172
Taster A (value)			
Control	0.542	0.667	0.032
0 g/Kg MT		0.755	0.180
0.1 g/Kg MT			0.045
Taster B (total score)			
Control	0.0031	0.00045	0.0031
0 g/Kg MT		0.013	0.00022
0.1 g/Kg MT			0.000071

Table 5.9 shows the Student's t-test for the different tannase treatments for taster scores and value. There is no significant difference between the total taster score for tea taster A and value between 0.1 g/Kg MT tannase and the 0 g/Kg MT tannase treatments (P = 0.485, P = 0.755). There is also not a significant difference between the 2.0 g/Kg MT and 0 g/Kg MT tannase treatments for taster A score and value (P = 0.164, P = 0.180). The 2.0 g/Kg MT tannase treatment is, however, significantly lower than the control for taster A score and value (P = 0.041, P = 0.032), and the 0.1 g/Kg MT tannase treatment for value (P = 0.045). For taster B total tea score, the 0.1 g/Kg MT tannase treatment show a significant higher score compared to the control and the 0 g/Kg MT tannase treatment (P = 0.00045, P = 0.013). The 2.0 g/Kg MT tannase treatment is significantly lower than the control, 0 g/Kg MT and the 0.1 g/Kg MT treatments for taster B tea scores (P = 0.0031, P = 0.00022, P = 0.000071).

The Kikkoman and Novo tannase preparations were also analyzed on a SDS PAGE gel as shown in Figure 5.1. Protein determination indicated that the Kikkoman tannase preparation contained 12.2 mg protein per gram powder, while the Novo enzyme preparation contained 1.2 mg protein per ml. Figure 5.1 clearly shows that the enzyme preparations of both Kikkoman and Novo contain a range of proteins of different molecular weights. This is in agreement with other researchers who found many secondary reactions in crude industrial tannase preparations (Barbe and Dubourdieu, 1998).

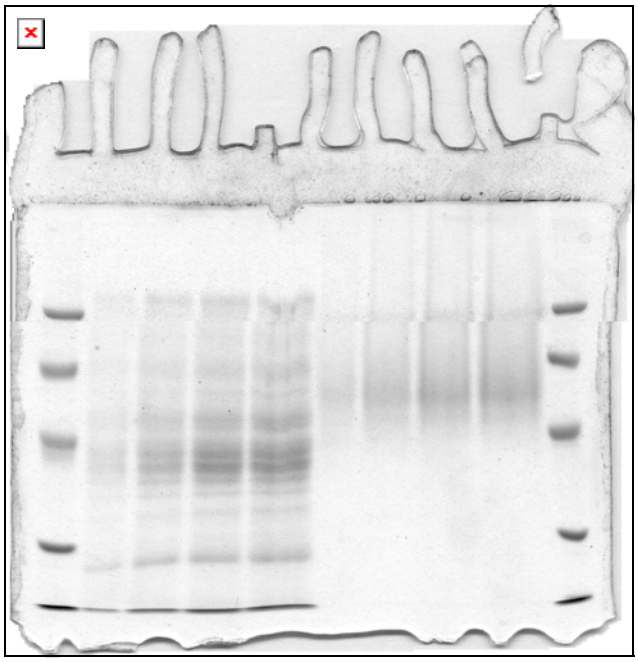

Figure 5.1. The SDS PAGE gel for the tannase preparations from Kikkoman and Novo. Molecular weight markers in the range of 14.5 to 94 kD are shown in lanes 1 and 10. Lanes 2, 3, 4 and 5 contain 12.2, 9.2, 6.1 and 3.1 µg protein respectively from the Kikkoman enzyme preparation, while lanes 6, 7, 8 and 9 contain 12, 9, 6 and 3 µg protein respectively of the Novo enzyme preparation.

4 Discussion

The aim of this study was to determine the effect of tannase treatment during the manufacturing process of black tea on the TF profile and the quality of the end product. Macerated tea leaves were incubated anaerobic for 30 minutes after the addition of tannase prior to the aerobic fermentation stage. Samples were taken before the anaerobic incubation period (control) and after the anaerobic incubation period with and without the addition of tannase. These samples were then analyzed for their individual flavan-3-ol

contents. Samples were again taken after completion of the manufacturing of the black tea and analyzed for their individual TF contents. The black tea samples were also tested for their ability to inhibit tea cream formation with and without the treatment of two different industrial tannase preparations.

Similar to other investigations a decrease in tea cream formation was observed with the addition of tannase (Hatamoto *et al*, 1996; Iacazio *et al*, 2000). A decrease in the specific activity was also observed with an increase in the amount of tannase added. This is in agreement with end-product inhibition by gallic acid as shown by Barthomeuf *et al* (1998). The oligomeric nature of the tannase enzyme (Hatamoto *et al*, 1996) also lends itself to alosteric feed-back inhibition (Palmer, 1987). Another possible explanation for the decreased specific activity with an increase in the amount of tannase added might be the different activity towards different tannins (Barthomeuf *et al*, 1994). All the easy degradable gallated polyphenols will first be hydrolyzed, after which the remainder of the polyphenols will be degraded slower, resulting in a decrease in the specific activity with an increase in ezyme concentration.

The flavan-3ol analysis in the macerated tea leaf before and after anaerobic fermentation shows that a very significant amount of fermentation took place during this time, indicating that enough oxygen is present in the leaf to allow some degree of aerobic fermentation to take place. This makes it impossible to accurately monitor the hydrolysis of the flavan-3-ols by tannase in the macerated tea leaf. While the flavan-3-ols are hydrolyzed by the tannase, they are simultaneously oxidized and polymerized by PPO.

These fermentation products can themselves be substrates, or even inhibitors of the tannase enzyme. Once flavan-3-ols are oxidized to their quinone form, they can undergo chemical reduction back to their reduced state, which can also influence the apparent flavan-3-ol profile observed in the dhool. The production of gallic acid by tannase will also lower the pH (results not shown), which will also affect the fermentation rate of the different flavan-3-ol compounds. One can thus reason that the contents of the flavan-3-ol compounds in the dhool after the tannase treatments are only apparent amounts, and not the actual amounts because of fermentation already taking place.

The flavan-3-ol profiles, however, did show changes due to the tannase treatments. When comparing the samples taking after treatment of 0 g, 0.1 g and 2.0 g / MT tannase, the amounts of EC, ECG and gallic acid increased with an increase in tannase, as was expected. When comparing the amounts of ECg and EGCg, an unexpected increase was observed with an increase in tannase. This apparent increase in the gallated flavan-3-ols is most possible due to some reactions taking place during the fermentation process. This observation of a change in the flavan-3-ol contents with the addition of tannase implies an influence of the tannase on the flavan-3-ol profiles.

In future, the tannase activity on the tea flavan-3-ols can be monitored after heat denaturation of the enzymes in the dhool. The disadvantage of this method will be that fermentation to produce the black tea product will be impossible. Another possible procedure to monitor the tannase activity in tea leaves will be to replace all the oxygen in

the dhool with nitrogen for the incubation period with tannase, followed by the standard fermentation period.

Analyses of the four major TF in the black tea before and after tannase treatments show a significant effect on the TF profile. TFs are dimers synthesized from specific flavan-3-ol precursors. The addition of tannase will decrease the amount of gallated flavan-3-ols, leading to a decrease in the gallated TF and an increase in the ungallated TF. As expected, the digallated TF-dg decreased and the ungallated TF-f increased after the addition of tannase.

The mono-gallated TFs are synthesized from one gallated and one ungallated flavan-3-ol. A decrease in the monogallated TFs is thus also expected because of the decrease in the gallated flavan-3-ols. The amounts of the two monogallated TFs, however, remain the same. The reason for this can be explained by the enzyme activity of the PPO responsible for the formation of the TF. The TFs form only a small percentage of the oxidized end products in black tea with most of the flavan-3-ols ending as precursors for TR. The TF themselves are also precursors for the TR. The individual amounts of the flavan-3-ol substrates for TF synthesis are thus not the only attributes determining the TF formation. Robertson (1983) showed in his *in vitro* oxidation studies of the four major flavan-3-ols, that inhibition of PPO by the gallated flavan-3-ols occur. An increase in the ungallated flavan-3-ols up to twice the normal levels, however, resulted in a linear increase in the TF formation with no apparent PPO inhibition. The decrease in gallated flavan-3-ols will thus lower the inhibition of the PPO catalyzed TF formation, while the increased

ungallated flavan-3-ols can increase the TF formation. This can explain why the amounts of the ungallated TF stay the same, in spite of the decrease of the gallated flavan-3-ols.

The SIT content in the black tea show an unexpected increase with an increase in the amount of tannase used. As with the monogallated TF, this tendency can best be explained by looking at the enzyme kinetics responsible for TF formation. The decrease in the gallated flavan-3-ols also decreased the inhibitory effect of the gallated flavan-3-ols while the increase of ungallated flavan-3-ols increased the formation of TF by PPO.

The analyses of the two tannase preparations with SDS-PAGE show various different proteins. This is in agreement with other researchers showing industrial tannase to be crude preparations with many other activities (Barbe and Dubourdieu, 1998; Bhat *et al*, 1998). Other active enzymes that have been identified in tannase preparations include hemicellulase, cellulase, pectinase and glucosidase. Secondary esterases identified in tannase preparations comprise three classes. Cinnamate esterase specific to ester linkages involving the carboxylic group of cinnamic acids, depsidase specific to the ester link between two phenolic rings, and phenyls esterase specific to the ester link between a phenolic acid and a straight-chain alcohol. These secondary enzyme activities were shown to be involved in an increase in undesired phenolic aromas in white wine, following the tannase treatment of white musts (Barbe and Dubourdieu, 1998).

The addition of a high amount of tannase (2.0 g/Kg MT) resulted in a decrease in the quality of the black tea as perceived by two professional tea tasters. The addition of a

small amount of tannase (0.1 g/Kg MT) on the other hand resulted in a slight, but significant increase in quality according to one of the two tea tasters. The other tea taster could not detect any significant difference after the addition of 0.1 g/Kg MT. According to the results presented in chapter 4, a higher amount of ungallated TF-f is associated with quality. The addition of 0.1 g/Kg MT tannase resulted in a significant increase in the amount TF-f in the black tea. This might explain the increased quality after the addition of the small amount of tannase.

The decrease in quality after the addition of a large amount of tannase is contradictory to findings in chapter 4. Considering the impurities in our tannase extracts, one explanation for the decreased quality might be the secondary enzyme activities associated with tannase extracts (Barbe and Dubourdieu, 1998). These secondary reactions are for example responsible for a decrease in quality of white wine due to undesirable aroma produced due to tannase enzyme treatment of white musts. The addition of 2.0 g/Kg MT tannase also resulted in the appearance and relative increased size of other unknown peaks in the NACE electropherogram of the TF extracts prepared from the appropriate black tea samples (results not shown). The secondary enzymes might form these unknown peaks. These reactions might be more significant due to the higher amount of enzyme added, especially considering the decrease in specific activity with increased tannase concentrations. The relative increase of these unknown compounds might also be responsible for the decrease in black tea quality. In future the uncertainty of the effect of the tannase extract on the quality of the black tea, due to the presence of the secondary enzymes, might be removed by using a highly purified tannase enzyme extract. Another

possible explanation for the decreased quality might be the existence of certain optimum levels for the different individual TFs. If the individual TF amounts fall outside that level, either more or less, the quality of the black tea might decrease. In this case the TF-f levels might be to high, and the TF-dg levels might even be to low.

In conclusion it seems that a high amount of tannase added during black tea manufacturing lower the quality of the tea. However, when only a small amount of tannase is added, the black tea quality might increase.

Chapter 6

Concluding discussion

1 Discussion

Mankind has known tea for almost 5000 years. According to legend tea was discovered by the mythical emperor Shennong, and used as a medicinal drink. By the end of the sixth century the Chinese began to consume it as a beverage. Tea was introduced to the West at the beginning of the seventeenth century. In the West tea quickly became popular for the flavour and stimulative properties it gave to boiling water. Due to poor hygiene during that time the boiling of water was commonplace. In the following centuries wars have been fought over tea, and nations were irreversibly changed by it (e.g. the opium war of 1840–1842). The positive effects tea had on humanity, however far outweighs its drawbacks. Today tea provides employment to hundreds of thousands of people and brings in foreign currency to many third world countries. Recent research on the health aspects of tea also shows that the first assumptions of the Chinese that tea was a medicinal drink might have been true all along.

About 40% of all human cancers are diet related. Food-borne carcinogens are produced during food preservation and preparation. Research on some of these pro-carcinogenic compounds showed tea polyphenols to inhibit their activation (Apostolides, 1995). Tea was shown to inhibit various other carcinogenic and mutagenic activities. Numerous cell

culture and animal models indicate anti-carcinogenic activity mediated through a range of mechanisms including antioxidant activity, enzyme modulation, gene expression, apoptosis, up regulation of gap junction communication and P-glycoprotein activation. Tea flavonoids also have protective properties against heart diseases due to its ability to prevent the oxidation of low-density lipoproteins (LDL) and thus help prevent the formation of atherosclerotic plaques (Duthie *et al*, 2000).

The main aim of this research was to assist the tea industry in improving the quality of the tea clones developed for propagation in Central and Southern Africa. The quality of teas is determined on the basis of its flavour and appearance. These parameters are used to determine the prize of tea in relation to market conditions. Professional tea tasters usually determine the quality organoleptically. Any measure to increase the quality and thus the prize of black tea is highly desired.

The three major factors responsible for the quality of Central and Southern African black teas are the environment, manufacturing practices and the characteristics of the plant material used to manufacture the black tea. Teas produced in Central and Southern Africa are not cultivated under optimum environmental conditions. The typical cold winters and frequent droughts of this region are obstacles in producing high quality teas. The TRF(CA) is trying to compensate for this by using optimized manufacturing procedures and by developing high quality tea clones best suited for the above-mentioned environmental conditions.

In this study a method was developed to quantify the major TFs, which can assist in optimizing the manufacturing process of black tea production. This study, however, focuses on the development of methods that can help to improve the quality characteristics of TRF tea clones. Two different approaches to improve tea clone quality were investigated. The first approach uses improved breeding techniques, whereas the second approach tried to improve tea quality after harvesting.

To improve efficiency in breeding new tea clones, it is important to identify the specific characteristic important for the quality of the tea. An identified characteristic correlated with tea quality will streamline the breeding programme by providing a strategy by which to improve the genetic material. Such a quality character might also speed-up the breeding programme by providing early identification of quality in newly developed clones.

To identify a quality compound in tea material, the technique of capillary electrophoresis (CE) was utilized. At the start of this study capillary electrophoresis was used due to financial and apparatus constrains. Prior to this research HPLC was the technique of choice in analyzing tea material. CE, however, has a range of advantages compared to HPLC. The amount of sample required is in the nanoliters range and the solvent wastage is very little. CE is also faster and has higher resolving power that is necessary for complex tea samples. The major disadvantage of CE compared to HPLC is its lower sensitivity.

To analyze the flavan-3-ols in green tea leaf, the method of MECC was developed in our laboratory by Jean Aucamp (2000). This method has the advantage of using both principles of electrophoresis and reverse phase liquid chromatography in the separation of analytes. The principle of chromatography is added by the introduction of surfactant micelles. These micelles act as a pseudo stationary phase and separation occurs because analytes have different affinities towards the organic micelles and aqueous buffer. MECC can thus be used to separate both ionic and neutral compounds, an important aspect to analyze the caffeine in tea leaves (Altria, 1999; Quirino and Terabe, 1999).

For the analysis of the TF in black tea, the NACE method was used. Prior to this research no method existed to analyze TF with CE. Other researchers found difficulty in developing a method due to interactions of the TF with the capillary wall (Larger *et al*, 1998). NACE is very similar to CZE, differing from CZE in that only non-aqueous solutions are used. NACE is a good alternative for compounds that are difficult to separate using normal aqueous running buffers due to low solubility or lack of selectivity in aqueous media (Altria, 1999). For our analytes the main consideration for using NACE was to decrease the band broadening effect observed in aqueous media and to enhance the separation efficiency.

The effects of the organic solvent composition and background electrolyte concentration on the separation selectivity and electrophoretic mobilities were investigated. The TFs showed a decrease in electrophoretic mobility with an increase in MeOH and a decrease in ACN content. This phenomenon can be explained by the change in the EOF due to the

change of ion mobilities as expected for mixtures of solvents that have different dielectric constants and viscosities. ACN has a higher dielectric constant and lower viscosity than MeOH. Other aspects of ACN and MeOH content that may play a role is (i) heteroconjugated anion formation in solvents with low hydrogen acceptor abilities, such as ACN, (ii) the effect of ACN on the effective charge to solvation ratio of the analyte, (iii) the influence of the organic solvents on the pKa values of the analyte and the silanol groups at the capillary silica surface. An increase in acetic acid resulted in a decrease in mobilities of the TFs due to a decrease in the apparent pH*. With an increase in the ammonium acetate concentration the EOF decrease and the TF mobilities increased. This coincided with increased resolution. This observation can be explained by the ion pairing effect.

In our optimized method we used the increased separation selectivity observed with an increase in the ammonium acetate concentration, together with the decreased migration times observed with an increase in acetic acid content. Our optimized separation solution consisted of 71% acetonitrile, 25% methanol, 4% acetic acid (v/v) and 90 mM ammonium acetate.

The developed NACE method achieved base-line separation of the TFs within ten minutes. This method was used to analyze three black tea samples. Calibration curves for TF, TF-A, TF-B and TF-dg were linear in the range of interest (100 to 1000 μg/ml), with r-values of 0.9998, 0.9992, 0.9995 and 0.9987 respectively. The repeatability (n = 4) showed RSD values of the individual TF concentrations smaller than 6% for all the different black

tea samples. The intra-day variations (1.7%) and inter-day variations were less than 3.6% for the migration times. The limit of detection (LOD) values were taken as 3 *S/N* at 380 nm and were 24, 25, 21 and 23 μg/ml for TF-f, TF-A, TF-B and TF-dg respectively.

As can be seen from the above paragraphs, the compounds investigated for identifying quality markers were the major green leaf flavan-3-ols, caffeine and the major TFs. These compounds were selected because of previous work done on Kenyan clones showing a correlation between flavan-3-ol content and quality (Obanda and Owuor, 1997) and between TF content and quality (Owuor and Obanda, 1995). Earlier research also showed a correlation between total TF content and quality for Central African black teas (Hilton and Ellis, 1972).

A significant advantage of a flavan-3-ol as indicator of quality will be early detection of quality during the breeding programme. For their ability to produce high quality black tea the contents of flavan-3-ols of the different clones are of utmost importance. Most of the flavan-3-ols are oxidised and polymerised during the fermentation stage into the dimeric TFs and the poorly understood polymer like TRs. The flavan-3-ol contents in the leaves will thus play a significant role in the amounts and identities of the oxidised polyphenolic compounds in the black tea. Certain hypothesis concerning the correlation between flavan-3-ol content and quality for Kenyan clones were made. These hypotheses were however not yet tested on TRF clones.

To identify flavan-3-ols related to tea quality, twenty good and twenty poor quality tea clones were selected from the breeding programme at the Tea Research Foundation (Central Africa) (TRF(CA)). The flavan-3-ol profile of fresh tea leaves was analyzed by capillary electrophoresis while total theaflavin (TF) content was determined in the black tea manufactured from the same leaves for each clone. The above parameters were correlated with total scores and valuation from two tea tasters with regression analysis. The significance of the differences between the twenty good and twenty poor quality tea clones was determined with the Student's t-test and the distribution free Kruskal-Wallis test.

The total TF content of the black tea correlated well ($P = 0.0004$, $r = 0.631$) with the value of the tea. Off all the parameters determined in the fresh leaves, the highest correlation was obtained with EC ($P = 0.0003$, $r = 0.649$). The SIF content ($P = 0.0009$, $r = 0.621$), amount of di-hydroxy flavan-3-ols ($P < 0.0001$, $r = 0.633$) and the amount of the nongallated flavan-3-ols ($P = 0.0012$, $r = 0.610$) also showed a correlation with the value of the tea. Unlike the Kenyan tea clones, our results showed no correlation with quality for EGCg, caffeine or the gallated flavan-3-ols. By comparing both the confidence limits and the slopes of the linear regression of the different parameters and value, TF determined according to the flavognost method had the best ability to predict the value of Southern African tea clones. Using combinations of the different fresh leaf parameters tested, only EC and ECg content could successfully predict the quality of a tea clone. This may facilitate early selection of good quality TRF clones in the future.

The interpretation of these results can best be done when taking into account the formation of TF during black tea fermentation. Under the influence of polyphenol oxidase a tri-hydroxy flavan-3-ol and di-hydroxy flavan-3-ol combine to produce a theaflavin. When a gallated di-hydroxy flavan-3-ol combines with a gallated tri-hydroxy flavan-3-ol, a TF digallate (TF-dg) will form. When only one of the two flavan-3-ol monomers is gallated, a TF monogallate will form (TF-A or TF-B depending whether the di-hydroxy or the tri-hydroxy flavan-3-ol is gallated). When neither the di-hydroxy nor the tri-hydroxy flavan-3-ol is gallated, a free TF (TF-f) will form.

Based on availability, di-hydroxy flavan-3-ol contents should be the limiting factor in the amount of TF formed. The importance for TF formation is also emphasized by the reduction of oxidized di-hydroxy flavan-3-ols during redox equilibration. On the other hand, PPO was shown to be inhibited by gallated flavan-3-ols. Increased amounts of gallated flavan-3-ols should therefore result in a decrease in the amount of TFs formed. It thus seems that for TRF(CA) clones the total TF content are important, whereas for Kenyan clones the gallated TF contents are of more importance. Analyzing individual TF contents in the black teas tested this hypothesis.

Prior research already showed the total amount of TF to be important for quality of TRF clones. By analyzing the individual TFs in black teas, the importance of the TFs on quality can be tested, as well as the hypothesis that the total amount of TFs are more important for TRF tea quality. Concerning this hypothesis and the explanation for the correlations detected between the individual flavan-3-ols and black tea quality, a high

correlation between TF-f content and quality was expected. On the other hand, TF-dg content should not show any correlation with quality. Any correlations between the individual TF content and tea quality can also be very useful for optimizing the tea manufacturing process.

To identify the importance of the individual TF for quality, twenty good and twenty poor quality tea clones were once again selected from the breeding programme at the Tea Research Foundation (Central Africa) (TRF(CA)). The theaflavin (TF) composition of the black tea manufactured from each clone was analyzed by capillary electrophoresis and correlated with total scores and valuation from two tea tasters with regression analysis. The significance of the differences between the twenty good and twenty poor quality tea clones was determined with the Student's t-test and the distribution free Kruskal-Wallis test.

The results confirm the importance of SIT content in predicting the quality and value ($P = 0.0002$, $r = 0.785$) of TRF(CA) tea clones. However, the non-gallated TF (TF-f) ($P = 0.0002$, $r = 0.705$) and mono-gallated TFs ($P = 0.0004$, $r = 0.741$ and $P = 0.00016$, $r = 0.788$ for TF-A and TF-B respectively) also showed a significant ability to distinguish between good and poor quality tea clones. In contrast to the Kenyan tea clones, the TF-dg contents showed no significant difference between the good and poor quality tea clones.

These findings can be explained if the quality of the Central and Southern African teas is more dependent on the total amount of TFs, than the composition of the individual TFs.

The significant effect of the non-gallated TFs and mono-gallated TFs might be explained by the effect of their flavan-3-ol precursors on TF enzymatic synthesis. The affinities of the different flavan-3-ols for PPO differ. Since gallated flavan-3-ols may inhibit PPO it is not surprising that the non-gallated TF-f had the best ability of the individual TFs to distinguish between good and poor quality tea clones. TF-f also occurs in the highest amounts of the four TFs. Of the two mono-gallated TFs, TF-A content is significantly higher than the content of TF-B or TF-dg, and is also better suited than TF-B to predict the quality of a tea clone. With the exception of TF-dg, all the individual TF and SIT showed a significant correlation with tea taster scores and value. These correlations, however, don't differ significantly from each other. When considering both the correlation coefficients and the slopes of the linear regressions obtained with value, TF-B and then TF-A are best suited to predict the value of Southern African black tea. This is in agreement with the flavan-3-ol study that showed EC and ECg to be best suited to predict black tea quality. EC is the di-hydroxy flavan-3-ol precursor of TF-A, whereas ECg is the di-hydroxy flavan-3-ol precursor of TF-B. However, when predicting the quality from TF levels, a combination of TF-f content and the total TF (flavognost) content was best suited.

Both the flavan-3-ol and TF analyses show an ability to distinguish between good quality and poor quality black tea. To determine which of these parameters had the best ability to distinguish between good and poor quality teas, the LOGISTIC procedure of SAS was used to predict the quality of the tea according to the content of the individual parameters or combinations of them. Using either the flavan-3-ol or the TF formulas to predict the

quality according to the content of the individual parameters, their ability to classify the different tea clones as either good or poor quality were determined. This gave the percent concordant value, the percentage of the clones that could be classified correctly.

The parameter which could best predict the quality of the tea clones was a combination of EC and ECg (86.8%) followed closely by a combination of total TF (flavognost) and TF-f content (86.1%) and by a combination of total TF (flavognost) and SIT content (85.6%). The next best predictor for quality was TF-f (84.8%), followed by SIT (84.3%), EC (83.5%), TF-A (82.8%), SIF (80.8%) and then total TF determined with the flavognost method (80.6%). There were however no significant differences between these parameters at the 95% confidence limit. This shows that there is no significant difference between analyzing the flavan-3-ols or the TFs to predict the quality of a tea clone. It should thus be possible to predict the quality of a new tea clone during the first year of the breeding programme by analyzing the flavan-3-ol content in the fresh leaf. This prediction should be just as accurate as the one made after six to seven years in the breeding programme by analyzing TF content in black tea manufactured from a new tea clone.

CE analysis costs about 25.00 USA $ per sample, while spectrophotometric analysis costs about 2.50 USA $ per sample. A spectrophotometric method that is selective for di-hydroxy flavan-3-ols would be very useful.

In conclusion, the knowledge of these findings concerning the correlation between flavan-3-ol profile and quality, allows the plant breeder to design techniques that will evaluate the genetic potential of clones. By looking at the proposed flavonoid metabolism in tea plants, the selection of clones with high flavonoid-3'-hydroxylase (di-hydroxy flavan-3-ol producer) and low flavonoid-3',5'-hydroxylase activity (tri-hydroxy flavan-3-ol producer) are desired. At this stage it is however not known whether tea will only use a flavonoid-3',5'-hydroxylase or both a flavonoid-3'-hydroxylase and a flavonoid-3',5'-hydroxylase enzyme to synthesize its di-hydroxy and tri-hydroxy flavan-3-ols. It is also not known whether different genes responsible for different isoforms of flavonoid-hydroxylases regulate the amounts of tri-hydroxy relative to di-hydroxy flavan-3-ols, or whether the regulation is at the transcription level of the specified gene. Once the enzymatic and genetic character of the tea flavanoid-3'-hydroxylase and flavonoid-3',5'-hydroxylase are better understood, the plant breeder can search for sources of the desired genes for quality to utilize in his breeding programme.

Lastly, the suitability of quality improvement after harvesting of the tea leaves was investigated. The analysis of black tea TFs showed that a higher content of ungallated TF-f was associated with good quality clones. The content of the di-gallated TF-dg did not have any correlation with the quality. Tannase has the ability to hydrolyze the ester bonds of tannins, for example the catechin tannins in tea (the gallated flavan-3-ols) and the condensed tannins consisting of polymerized proanthocyanidins. It is used in the ice-tea industry to prevent creaming down and is also used in the manufacture of gallic acid. By adding tannase to the freshly macerated tea leaves during the fermentation stage of

black tea manufacturing, the amounts of gallated flavan-3-ols should be decreased, with the resultant increase in the amount of TF-f produced, and a decrease in the amount of TF-dg and the mono-gallated TFs (TF-A and TF-B).

Analysis shows a large decrease in the flavan-3-ol content after the 30 minutes anaerobic incubation period. This indicates that tea fermentation occurs during the incubation period, whereby the flavan-3-ols are oxidized and polymerized. Due to this it is not possible to monitor the activity of the tannase on the gallated flavan-3-ols during this period. Variations in the flavan-3-ol profiles after the different treatments with tannase do, however, indicate an influence of the tannase on the flavan-3-ol contents.

Significant differences between the tannase treated and the untreated samples for the TF contents do occur. The TF-f content increased while the TF-dg content decreased, as was expected. The two mono-gallated TFs, however, remained unchanged. This can best be explained by the loss of inhibitory activity on the PPO due the decreased gallated flavan-3-ol contents in the dhool. This results in similar TF-A and TF-B levels, even though the levels of their gallated flavan-3-ol precursors decreased.

The quality and value of the tea did not increase with increased tannase used as was expected. One of the two professional tea tasters detected a small but significant increase in quality after treatment with a small amount of tannase. The other tea taster did not detect any significant change in quality. Both tea tasters, however, detected a significant decrease in the black tea quality after treatment with a large amount of tannase. This can

most probably be explained by the presence of secondary enzyme activities as is apparent from SDS-PAGE analysis of the tannase extracts, as well as the appearance of unidentified peaks on the CE chromatogram after treatment of large amounts of tannase.

2 Future work

1) Revise flavognost test molar absorption coefficients with new commercially available TF.
2) Search for new reagent to react specifically with the di-hydroxy flavan-3-ols for spectrophotometric analysis.
3) Search for any other high throughput method for di-hydroxy flavan-3-ols.
4) A method to wither, macerate, ferment and measure TF with flavognost on a single shoot.
5) Can good and poor quality clones be identified on mRNA probes of any genes.
6) Chloroform test and “L a b” colour-test of brown leaf.
7) Improve reproducibility of Folin-Ciocalteu method with an optimization study.

Summary

The main aim of this research was to assist the tea industry in improving the quality of the new tea clones developed for propagation in Central and Southern Africa. An increase in the quality correlates with increased value of the black tea, and is thus highly desired. The three major factors responsible for the quality of Central and Southern African black teas are the environment, manufacturing practices and the characteristics of the plant material used to manufacture the black tea. The Tea Research Foundation (Central Africa) (TRF(CA)) is trying to improve their tea clones by optimising the manufacturing procedures and by developing high quality tea clones best suited for the environmental conditions of the region.

In this study we investigated methods to increase tea quality by developing quality markers for the breeder, as well as methods to improve quality during the manufacturing process. The compounds investigated for identifying quality markers were the major green leaf flavan-3-ols, caffeine and the major TFs. To be able to analyse the TFs in the manufactured black tea, we developed the NACE method. This NACE method can also be used to improve tea quality by optimising the black tea manufacturing procedure. We also investigated the ability of improving the tea quality by the addition of tannase to alter the contents of the individual TFs in the black tea.

A significant advantage of a flavan-3-ol as indicator of quality will be early detection of quality during the breeding programme. To identify flavan-3-ols related to tea quality,

twenty good and twenty poor quality tea clones were selected from the breeding programme at the TRF(CA). The contents of the individual flavan-3-ols of fresh tea leaves were analysed by MECC. Total TF content was determined with the flavognost method in the black tea manufactured from the same leaves for each clone. The above parameters were correlated with total scores and valuation from two tea tasters with regression analysis. The significance of the differences between the good and poor quality clones was determined with the Student's t-test and the distribution free Kruskal-Wallis test.

Unlike the Kenyan tea clones, our results showed no correlation with quality for EGCg, caffeine or the gallated flavan-3-ols. Total TF content had the best ability to predict the value of TRF(CA) tea clones. A combination of EC and ECg content could best predict the quality of a TRF(CA) tea clone.

To identify any correlations between the individual TF contents and tea quality, the same experimental set-up was used as that for the flavan-3-ol experiment. The TF composition of the black tea manufactured from each clone was analysed with the NACE method. The TF results confirmed the importance of SIT content in predicting the quality and value of TRF(CA) tea clones. However, the nongallated TF (TF-f) and mono-gallated TFs (TF-A and TF-B) also showed a significant ability to distinguish between good and poor quality tea clones. In contrast to the Kenyan tea clones, the TF-dg contents showed no significant difference between the good and poor quality tea clones.

The correlations between the individual flavan-3-ols or individual TFs and tea quality were explained by the hypothesis that the total amount of TFs is more important than the composition of the individual TFs. The correlations of the flavan-3-ols with quality were explained by their participation in TF synthesis.

To determine which parameter had the best ability to distinguish between good and poor quality teas, the LOGISTIC procedure of SAS was used to determine the ability of the individual parameters or combinations of them to predict the quality of tea clones. The parameter, which could best predict the quality of the tea clones, was a combination of EC and ECg. Other parameters that could distinguish between poor and good quality clones were total TF, TF-f, SIT, EC, TF-A and SIF. There were however no significant differences between these parameters at the 95% confidence limit. This shows that there is no significant difference between analysing the flavan-3-ols during the first year of the breeding programme, or the TFs after six to seven years, to predict the quality of a tea clone.

Lastly, the suitability of quality improvement during the manufacturing stage was investigated by the addition of tannase. The added tannase resulted in increased TF-f and decreased TF-dg levels, while the mono-gallated TFs remained unchanged. The quality and value of the tea, however, did not increase with increased tannase used. This can most probably be explained by the presence of secondary enzyme activities in the tannase extract.

In conclusion, we developed an analytic technique for early detection of quality during the first year of the TRF(CA) breeding programme. This can be done by analysing the EC and ECg contents of the different clones to predict the quality of the black tea manufactured from these clones.

Abstract

In this study we investigated methods to increase tea quality by developing quality markers for the breeder, as well as methods to improve quality during the manufacturing process. The compounds investigated for identifying quality markers were the major green leaf flavan-3-ols, caffeine and the major theaflavins. To be able to analyze the theaflavins in the manufactured black tea, we developed a non-aqueous capillary electrophoresis method. This non-aqueous capillary electrophoresis method can also be used to improve tea quality by optimizing the black tea manufacturing procedure. We also investigated the ability of improving the tea quality by the addition of tannase to alter the contents of the individual theaflavins in the black tea.

To identify flavan-3-ols and theaflavins related to tea quality, twenty good and twenty poor quality tea clones were selected from the breeding programme at the Tea Research Foundation of Central Africa. The contents of the individual flavan-3-ols of fresh tea leaves and the contents of the individual theaflavins were analyzed with capillary electrophoresis. Total theaflavin and individual theaflavin content was determined in the black tea manufactured from the same leaves for each clone. The above parameters were correlated with total scores and valuation from two tea tasters with regression analysis. The significance of the differences between the good and poor quality clones was determined with the Student's t-test and the distribution free Kruskal-Wallis test. Our results suggest that (-)-epicatechin and (-)-epicatechin gallate contents of the fresh leaf

can be used to predict the quality potential of new clones early in the breeding programme.

The suitability of quality improvement during the manufacturing stage was also investigated by the addition of tannase. The added tannase resulted in statistical significant increase in free theaflavins. With the use of a low dose of tannase, a significant increase in the tea quality was observed by one tea taster.

Samevatting

In hierdie studie is metodes ondersoek om tee se kwaliteit te verhoog deur die ontwikkeling van kwalitietmerkers vir die teler. Verder is ook gekyk na metodes om die tee kwaliteit te verbeter tydens die vervaardigings proses. Die verbindings wat ondersoek is as moontlike kwaliteitsmerkers was die belangrikste groen blaar flavan-3-ole, kaffeïenne, en die belangrikste teeflavienne. Om die teeflavienne te analiseer, het ons n' nie-waterige kapillêre elektroforese metode ontwikkel. Hierdie nie-waterige kapillêre elektroforese metode kan ook dien om die teekwalitiet te verbeter deur dit te gebruik vir optimisering van die vervaardigings proses. Daar is ook gekyk na die moontlikheid om die teekwaliteit te verbeter deur die byvoeging van tannase om sodoende die inhoud van die teeflavienne in die swart tee te verander.

Om flavan-3-ole en teeflavienne te identifiseer wat in verband gebring kan word met teekwaliteit, is twintig goeie en twintig swak kwaliteit teeklone geselekteer vanuit die telingsprogram van die "Tea Research Foundation of Central Africa". Die inhoud van die individuele flavan-3-ole in die vars teeblare en die inhoud van die individuele teeflavienne in die swart tee is geanaliseer met kapillêre elektroforese. Die inhoud van die totale teeflavienne en die individuele teeflavienne is bepaal in die swart tee wat vervaardig is van dieselfde blare van elke kloon. Die bogenoemde parameters is toe deur regressie analise gekorreleer met die totale tellings en waardering gedoen deur twee teeproërs. Die betekenisvolheid van die verskille tussen die goeie en swak kwaliteit klone is bepaal deur Student se t-toets en die distribusievrye Kruskal-Wallis toets. Ons resultate

suggereer dat die inhoud van (-)-epikatechien en (-)-epikatechien gallaat, in die vars blare, gebruik kan word om die potensiële kwalitiet van nuwe klone vroeg in die telingsprogram te voorspel.

Die toepaslikheid van kwaliteits-verbetering gedurende die vervaardigingsproses deur die byvoeging van tannase is ook ondersoek. Die bygevoegde tannase het gelei tot n' statisties beduidende toename in vry teeflavienne. Die byvoeging van n' laë dosis tannase het ook gelei tot n' beduidende toename in die teekwaliteit bepaal deur een van die teeproërs.

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