PROFILING FOR ANTIHYPERTENSIVE AMINO ACID (THEANINE) IN SELECTED KENYAN TEA CLONES

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A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements for the Award of the Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This research thesis is my original work and has not been submitted wholly or in part for the award of degree in any other institution of learning.

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SM11/2553/09

Recommendation

We wish to confirm that this research thesis was done under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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Tea Research Institute

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DEDICATION

I dedicate this work to my loving parents, siblings and my son, Ryan.

ACKNOWLEDGEMENT

I acknowledge the Tea Research Institute (TRI), formerly the Tea Research Foundation of Kenya (TRFK) for funding this project. I am deeply indebted to my supervisors for the time they have sacrificed to guide me during this study. Many thanks to Dr. J. K. Wanyoko and Prof. F. N. Wachira for their diverse knowledge on tea, day to day guide on what to do and for moral support all the way. I am also greatly indebted to the Department of Chemistry staff in Tea Research for their assistance during sampling, processing and analysis. I owe a lot of gratitude to the TRI Library staff for providing me with materials on tea which helped me in writing up this thesis. I am greatly indebted to Mr. K. O. Moseti for his guide, suggestions and reviews in the write up.

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ABSTRACT

Tea is a beverage and also a stimulant that is consumed by many people all over the world. It contains various bioactive compounds like alkaloids, polyphenols, and amino acids among others. Amino acids play the role of moderating taste of the tea infusion. Theanine is a unique amino acid found in the *Camellia sinensis* species and has been reported to reduce high blood pressure, improve learning and concentration and reduce premenstrual symptoms. This study was conducted to determine the content of theanine in different partitions of the tea shoot (four leaves and a bud), green and black tea processed from different selected Kenyan tea clones including one purple coloured tea clone and made tea commercially available in the Kenyan market. High performance liquid chromatography (HPLC) was used to quantify the levels of theanine. The results were analyzed statistically by carrying out ANOVA and Least Significant Difference test using MSTAT statistical package to determine if the levels of theanine were significantly different based on the different clones that were analyzed. The theanine content in the different partitions of the tea shoot varied, with the internodes having the highest amounts in the two clones that were analyzed (3.26 % dw) and the first leaf contained the lowest amounts (0.22 % dw). Among the leaves and the buds, theanine was more concentrated in the younger tissues. Theanine levels in the black and green tea processed from the selected tea clones varied with clones, seasons and also processing with green tea having higher levels than black tea in most clones. Green tea on average contained 0.80 % dw of theanine with TRFK 31/8 having the highest (1.20 % dw) and TRFK 303/216 having the lowest (0.50 % dw) theanine content. Black tea on average contained 0.69 % dw theanine with TRFK 56/89 having the highest (0.97 % dw) and TRFK 7/14 having the lowest (0.43 % dw) theanine content. Based on the current findings, theanine can be considered a tea marker since all the tea clones analyzed in this study contained some amount of theanine. Further, significant differences in the theanine content in the clones and during the three seasons considered (July- September, October - December and January - March) were observed. Theanine levels in the made tea in the Kenyan market were significantly different from each other and some teas did not contain theanine and these were majorly the flavoured teas. Kenyan black tea contained 1.02 % dw of theanine and this was comparable to teas from Rwanda, Tanzania and Uganda thus adding value to the Kenyan tea that can be marketed as a health drink. Theanine is a tea marker since all the test clones in the study contained it and would recommend people with hypertension to drink tea.

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LIST OF ABBREVIATIONS /ACRONYNMS

ANOVA	Analysis of Variance
BP1	Broken Pekoe 1
BOP	Broken Orange Pekoe
BOPF	Broken Orange Pekoe Fannings
C	Catechins
CTC	Crush, Tear and Curl
D1	Dust 1
DM	Dry Matter
dw	dry weight
EEG	Electroencephalograph
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FBOP	Flowery Broken Orange Pekoe
GABA	Gamma amino-butyric acid
HPLC	High Performance Liquid Chromatography
ISO	International Standards Organization
ITC	International Tea Committee
LTP	Lawrie Tea Processor
m. a. s. l	mean above sea level
PD1	Pekoe Dust 1
PF1	Pekoe Fannings 1
TBK	Tea Board of Kenya
TRFK	Tea Research Foundation of Kenya
TRI	Tea Research Institute
UV	Ultra violet

CHAPTER ONE
INTRODUCTION

1.1Background Information

Tea is one of the most popular and widely consumed beverages in the world because of its refreshing taste, attractive aroma, and potential health benefits (Dufresne & Farnworth, 2001; Kuo, Weng, Chiang, Tsai, Lin-Shiau & Lin, 2005; Alan, Jaganath & Clifford, 2009). It is made from the tender leaves of the plant *Camellia sinensis* (L.). This plant is native to East and South-eastern Asia (Yamamoto, Juneja, Chu & Kim, 1997). Although this is the only type of plant that produces tea, there are other beverages that are sometimes called "tea". Infusions made from herbs or flowers are sometimes called "herb teas" or "tisanes" but since they are not from the *Camellia sinensis* plant they are not really tea. The tea plant is an evergreen shrub or small tree (Cabrera, Gimenez & Carmen, 2003)and is usually trimmed to waist height so that the leaves can be easily harvested, but wild tree plants can grow into huge trees up to 30 meters in height (Forest, 1985).

Generally, tea can be broadly classified according to the processing method as un-aerated tea (green tea), semi-aerated tea (Oolong tea), fully aerated tea (black tea) or post-aerated tea (pu-erh tea) (Zhao, Chen & Huang, 2006). Black tea is consumed worldwide, while green and Oolong teas are consumed mainly in Asia and North Africa. The consumption of green tea particularly as a drink has become increasingly popular in western cultures because of its reported positive health effects (Yamamoto et al.,1997; Dulloo et al., 1999; Venables, Hulston, Cox & Jeukendrup, 2008). Indeed, a lot of epidemiological and preclinical studies have demonstrated that drinking tea may reduce the risk of cancer and cardiovascular disease (Yang, Maliakal & Meng, 2002; Khan & Mukhtar, 2007). Moreover, other biological functions of tea have also been reported, such as anti-inflammation, anti-allergy, and anti-obesity (Fujimura, Tachibana & Yamada, 2004; Khan & Mukhtar, 2007). These beneficial effects have been attributed to the presence of compounds such as polyphenols, amino acids, vitamins, carbohydrates, and purine alkaloids (Bolling & Chen, 2009). Catechins which are polyphenols are known for their antioxidant-related effects (Morley et al., 2005; Gradisar, Pristovsek, Plaper & Jerala, 2007). Theanine is one of catechins biosynthetic precursors and this takes place when tea leaves are exposed to sunlight (Kito, Kokura, Izaki & Sasaoka, 1968).

Theanine (2-amino-4-(N-ethylcarbamoyl)butanoic acid, $C_7H_{14}N_2O_3$, molecularmass of 174.198) is a non-protein amino acid that was first discovered in tea leaves (Sakato, 1949) and has also been found in bay bolete mushroom, *Xerocomus badius* (Syu, Lin, Huang & Lin, 2008; Wei-Wei, Shinjiro & Hiroshi, 2010). It is the main free amino acid in teas representing as much as 50% of the total amino acids in black tea and 1 – 2% of the dry weight of green tea (Hara, Luo, Wickremashinghe & Yamanishi, 1995; Palva & Palva, 2007).

Besides the four traditional tastes that is sweet, salty, acid, and bitter; theanine is the predominant amino acid in green tea leaves giving tea its characteristic umami (pleasant savoury taste; brothy) (Le Gall, Colquhuon & Defernez, 2004; Kaneko, Kumazawa & Masuda, 2006; Thippeswamy et al., 2006). It not only plays an important role in the characteristic flavour and delicate taste of tea (Lee et al., 2010), but also shows many biological effects. It is involved in many biological activities such as promoting relaxation, inhibiting caffeine's negative effects, reducing blood pressure, and enhancing anti-tumor activity (Kamath et al., 2003; Sugiyama & Sadzuka, 2003; Kimura, Ozeki, Juneja & Ohira, 2007; Yamada & Terashima, 2009; Zhang, Zhang, Lu, Zhang & Preedy, 2013). Moreover, it has been reported to have physiological activities including neuroprotection and anti-obesity (Egashira et al., 2004; Zheng et al., 2005; Cho et al., 2008).

Consequently, there is an increasing interest in theanine as an important component of tea, as an ingredient for novel functional foods and as a dietary supplement [Juneja, Chu & Okubo, 1999; Wan, Zhang & Li, 2009; Vuong, Bowyer & Roach, 2011].

1.2 Statement of the Problem

Kenya is among the leading tea producers in the world and tea has been shown to be of medicinal importance but it is only marketed as a beverage and thus lacks diversification. Tea contains a rare non-protein amino acid, theanine which can help to lower the blood pressure and premenstrual symptoms, enhance learning and concentration. Even though it has been shown to be of medicinal importance, research on the levels of this amino acid has not been done in most of the released Kenyan tea clones and thus clones with high levels of this amino acid have not been identified with respect to the various seasons of the year. Also, with the standard plucking of two leaves and a bud, no much work has been done on the partition with higher theanine

levels and teas in the Kenyan market have also not been analyzed to determine their theanine content.

1.3 Objectives

1.3.1 Main Objective

To determine the levels of theanine in selected Kenyan tea clones and processed teas in the Kenyan market.

1.3.2 Specific Objectives

i. To determine theanine levels in the selected Kenyan released tea clones in tea shoots (two leaves and a bud) during three different seasons of the year.
ii. To determine theanine levels in the different parts of the shoot in one selected clone with the effect of sunlight and withering durations.
iii. To determine theanine levels in processed tea commercially available in the Kenyan market.

1.4 Hypotheses (H_0)

i. There is no significant difference in the theanine levels in the selected Kenyan tea clones in tea shoot (two leaves and a bud) during the different seasons of the year.
ii. There is no significant difference in the levels of theanine in the different parts of the tea shoot in the selected clone with the effect of sunlight and withering durations.
iii. There is no significant difference in theanine levels contained in the various processed teas in the Kenyan market.

1.5 Justification

Hypertension is a major risk factor for heart attack and stroke which are the leading cause of death in the world. Theanine which has been discovered in tea is of medicinal importance especially to those who are hypertensive because it has been shown to reduce high blood pressure in hypertensive rats (Juneja, Chu & Okubo, 1999). Since tea is a natural source of this amino acid, then, it can be used by those with hypertension because it is cheap and available. Profiling of this amino acid in most of the Kenyan released tea clones has not been done and thus there is need for screening various tea clones and quantifying their amounts. Extracts from tea

clones with high theanine levels can be used by the pharmaceutical companies to prepare anti-hypertensive drugs thus diversifying and adding value to our tea. It has also been reported to be added to sweets and other food stuffs in Japan and other tea growing nations and theanine can be extracted for this.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction of Tea to Kenya

Tea was reportedly introduced in Kenya by the Caine brothers who imported the dark-leafed "Manipuri" hybrid seed from Assam in 1904 and 1905 to establish a plantation at Limuru, Central Kenya. In 1912, the Assamica seeds were imported from Sri-Lanka to establish a plantation of tea with high quality and yield (Matheson, 1950). According to Matheson (1950), little interest existed over the next 12 years except for several small plantations that were established at Limuru in the East of the Great Rift Valley and at Kericho, Kaimosi in the West of the Great Rift Valley. However, advice given by Howland brothers in 1924 on the use of quality seed from the light coloured leaf Assam or Manipuri types for drought resistance stimulated serious planting by several companies. Tea planting expanded rapidly and by 1963 the acreage had increased to 21,448 ha with the latest report by (ITC, 2014) that in 2013the acreage stood at 198,657 ha.

2.2 Varieties of *Camellia sinensis*

There are three common varieties of *Camellia sinensis*: Assam, China and Cambod varieties. Assam variety is characterized by large, horizontal, broad, mostly non serrated and light green leaves. It is the predominant variety grown in Kenya due to its high yield potential. It produces tea which has a malty or earthy flavor. China variety is characterized by small, narrow, serrated, erect, and dark green leaves. It is dwarf and shrub like and originated from China. Cambod variety is it is a hybrid of China and Assam varieties with semi erect leaves. Not common in Africa though Tea Research Institute introduced six clones for use in breeding and clonal selection (TRFK, 2002).

2.3 Tea Growing Areas in Kenya

The tea-growing areas in Kenya are divided into two regions defined by the Great Rift Valley. This is a natural geographical phenomenon that divides the country almost asymmetrically; the Aberdare highlands, Mt. Kenya region and Nyambene hills forming the East of Rift block, whereas the West of the Rift block comprises the highlands of Mau escarpment around Kericho, Nandi Hills, Mt. Elgon and the Kisii highlands. Kericho region, in particular, is

home to many large-scale tea plantations (TBK, 2009). The tea growing regions in Kenya are endowed with the ideal climate for tea with altitudes of between 1,500 and 2,700 metres mean above sea level (m. a. s. l); tropical, volcanic red soils and well distributed rainfall ranging between 1,200 to 1,400mm per annum (p.a) that alternates with long sunny days. These attributes to favorable conditions for production that goes on all round the year with two main peak seasons of high crop between March and June and October and December which coincide with the rain seasons (TBK, 2013).

2.4 Quality and Economic Importance of Tea in Kenya

Kenyan black tea has won international acclaim for its consistent high quality and pleasant aroma. The quality of black tea depends on the number of top young leaves harvested, the mode of harvesting, and the care with which the green leaves are handled. Only the upper two young leaves and a bud are handpicked and skillfully processed. Kenyan tea has a distinct bright color and aromatic flavor (TRFK, 2002). Tea is a major foreign exchange earner and a source of livelihood for millions of people in the tea growing world (TBK, 2013). Currently, tea is the leading cash crop in Kenya and makes a very significant contribution to the economy. For instance, in the year 2013, the country produced 432.2 million kgs of made tea (fig 2.1), of which 494,347 metric tons were exported making it one of the largest foreign exchange earner in 2013 at Kshs 133 billion. 26.5 million kgs were consumed locally. The major importers of Kenyan tea in 2013 were Pakistan (95,056 metric tons), Egypt (95,537 metric tons), United Kingdom (63,374 metric tons) and Afghanistan (61,976 metric tons) (TBK, 2014). It has contributed enormously towards poverty eradication and infrastructural development in the rural areas while also enhancing environmental conservation through enhanced water infiltration, reduced soil erosion, and mitigation of global warming through carbon sequestration.

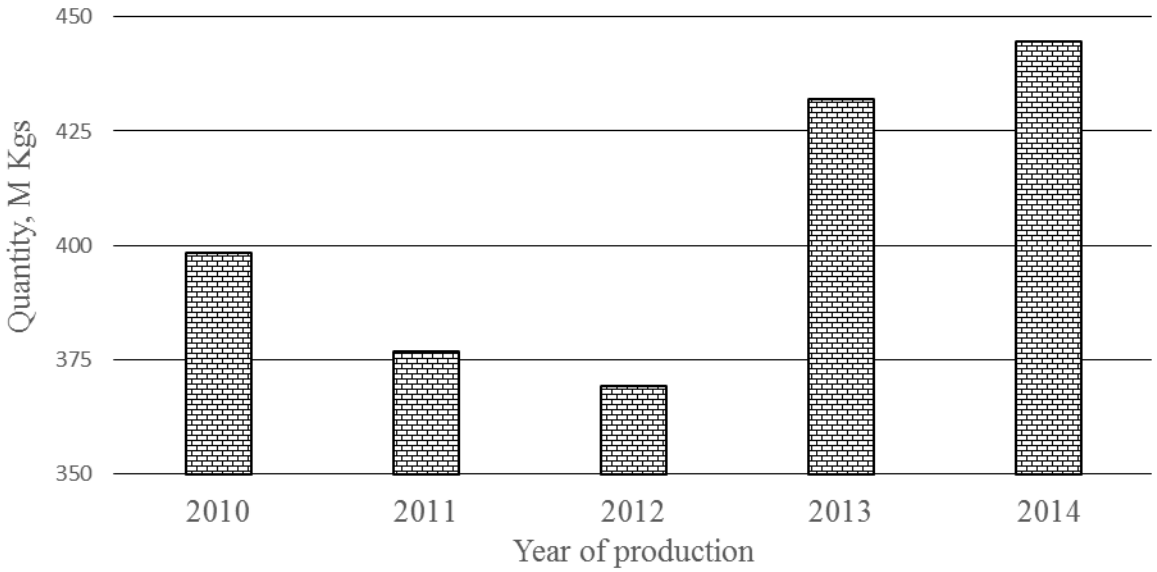


Figure 2.1: Tea production in Kenya (Adapted from Tea Board of Kenya)

2.5 Tea Processing

The different kinds of tea are as a result of how the leaves are processed after harvesting. The biggest factor that determines the kind of tea is the aeration (sometimes called fermentation) of the leaves. Aeration is the natural chemical process that all green matter undergoes after being picked, and the level of aeration of tea leaves produces the various kinds of tea (Lin, Lin, Liang, Lin-Shiau & Juan, 1998; Fernandez, Martin, Gonzalez & Pablos, 2000; Alcazar et al., 2007). There are three major types of tea: green (un-aerated), half-green or Oolong (semi-aerated), and black (fully aerated), (Graham, 1992; Ekborg-Ott, Taylor & Armstrong, 1997; Moderno, Carvalho & Silva, 2009; Sharangi, 2009). Of the main kinds of tea, green tea undergoes the shortest aeration period. The leaves are steamed or fried as soon as possible after they are picked to stop the aeration process. The most popular of all the kinds of tea is black tea and is processed from young, downy leaves, which are then fully aerated before being rolled and dried.

2.5.1 Withering

Withering is the first step in black tea manufacturing and is known as a pre-requisite for tea processing (Borah et al., 2012). The uniformity of oxidation during aeration is ensured by this

stage (Omiadze et al., 2014). During this stage, the moisture content of plucked tea leaves decreases. In addition to physical changes during withering, tea leaves undergo biochemical interactions that play an important role in black tea quality; its aroma in particular (Tomlins & Mashingaidze, 1997; Singh et al., 2012). Freshly plucked leaves spread out on fine meshed screen or troughs and air flow is blown to the leaves so as to draw moisture from the leaves. Withering can be divided to physical and chemical wither. The physical change associated with withering is a loss of moisture from the shoot which leads to changes in cell membrane permeability. Chemical wither involves breakdown of proteins into amino acids and a short chemical wither period favours the formation of theaflavins (Obanda & Owuor, 1992). The rate of moisture loss is related to surface moisture, humidity of the air, air flow, packing density and whether heat is applied during withering (Hampton, 1992).

During the withering and aeration processes for Oolong tea and black tea, the composition of polyphenolic compounds changes significantly (Mukhtar & Ahmad, 2000; Wheeler & Wheeler, 2004). Colorless epigallocatechin (EGC) derivatives are oxidised, polymerised and transferred into theasinensins and reddish theaflavins in Oolong tea, and finally red-brown thearubigins in black tea (Haslam, 2003; Nagao et al., 2009; Schneider & Segre, 2009). Tannin and amino acid levels in tea vary during each manufacturing stage. Amino acids also increase during black tea fermentation, a result of protein hydrolysis (Yao et al., 2006). This is accomplished through the cut, tear and curl (CTC) process, which yields stronger, thicker and brighter teas, ensuring a higher number of cups of tea per unit measure (TRFK, 2002).

2.5.2 Rolling

The leaves are macerated during this step and the cell structures are disrupted bringing various enzymes into intimate contact with their substrates (polyphenols). This may be accomplished by orthodox rollers, Lawrie Tea Processor (LTP) or cut-tear-curl (CTC) machines. Orthodox rolling is widely used in Sri-Lanka which is the world's major producer of orthodox tea. India and Kenya are major producers of CTC teas. In CTC manufacture, the leaves are fed between a pair of stainless steel rollers with etched surfaces, one rotating clockwise the other anticlockwise at different speeds. Polyphenol oxidase enzymes are activated during rolling and their activities are enhanced with the presence of suitable conditions like temperature, oxygen supply and moisture during rolling process. The chemical and biochemical reactions initiated in

the leaves proceed at an accelerated rate during and after the rolling before the leaves progress to the next stage of aeration (Hara, Luo, Wickremanshinghe & Yamanishi, 1995).

2.5.3 Aeration

This step used to be referred to as fermentation, but since no micro-organisms are used in this kind of enzymatic-oxidative condensation process, it is now referred to as aeration (Mo, Zhu & Chen, 2008). The ex-CTC dhool (macerated leaves) is what is aerated immediately after rolling. The high temperature of up to 32°C is progressively reduced by subsequent supply of oxygen where the temperature is reduced to about 22°C and maintained there by controlling air supply. The principal reaction in aeration is the oxidation of catechins and catechin gallates by various enzymes especially polyphenol oxidase (PPO). Other enzymes like peroxidase are also involved and some non-enzymatic reactions take place to form the unique character of black tea (Hara *et al.*, 1995). In this step, there is development of colour, strength and quality of tea brews from the production of non-volatile compounds through the enzymatic oxidation of catechins and their gallates to theaflavins and thearubigins (Haslam, 2003). There is also production of volatile compounds responsible for the characteristic aroma of black tea. High temperatures usually increase the rate of aeration.

2.5.4 Drying

Drying of aerated dhool is aimed at arresting the aeration through cessation of enzymatic activity and reducing moisture content to about 3% of the dry mass. The changes that take place include significant loss of volatile compounds, increase in levels of amino acids, binding of polyphenols to other tea components and increase in carboxylic acids. This step, also referred to as firing is done at 120°C and is necessary for the development of the taste, colour and aroma of black tea (Hara et al., 1995).

2.5.5 Sorting and Grading

This is done to remove excess fibre so as to have clean teas. Grading follows and it is an important stage for marketing tea by ensuring correct particle size, shape and cleanliness. The major primary grades in Kenya are Broken Pekoe 1 (BP1), Pekoe Fannings 1 (PF1), Pekoe Dust 1 (PD1) and Dust 1 (D1). In the tea trade, commonly found grades include Broken Orange Pekoe

(BOP), Flowery Broken Orange Pekoe (FBOP) and Broken Orange Pekoe Fannings (BOPF) (Hara et al., 1995).

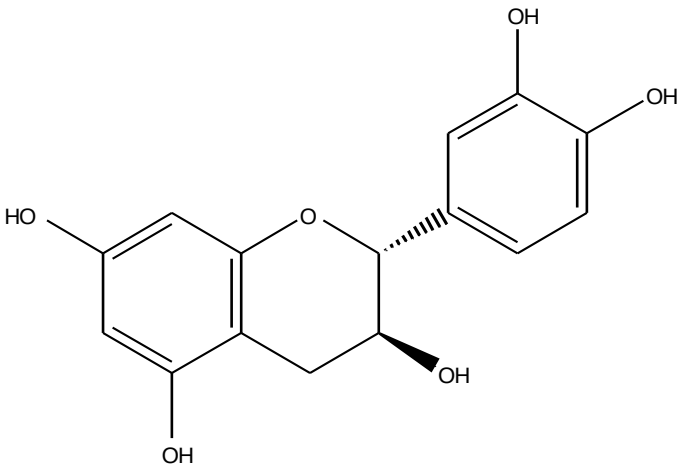
2.6 Tea and Health

Health effects of tea are particularly related to the flavan-3-ols (Modder & Amarakoon, 2002; Higdon & Frei, 2003). Catechins are best known for their antioxidant properties (Cheng a& Breen, 2000; Mira et al., 2002), which has led to their evaluation in a number of diseases associated with reactive oxygen species (ROS) (Heijnen, Haenen, van Acker, van der Vijgh & Bast, 2001; Chun, Kim & Lee, 2003), such as cancer, cardiovascular (Geleijnse, Launer, Hofman, Pols & Witteman, 1999; Sesso, Gaziano, Buring & Hennekens, 1999; Davies et al., 2003) and neurodegenerative diseases (Ramassamy, 2006). Several epidemiological studies as well as studies in animal models have shown that green tea can afford protection against various cancers such as those of the skin, breast, prostate and lung (Mukhtar & Ahmad, 2000; Yang et al., 2002). In addition to the cancer chemopreventive properties, green tea and EGCG have been shown to be anti-angiogenic (prevention of tumor blood vessel growth) (Fotsis, Pepper & Aktas, 1997; Pfeffer et al., 2003) and anti-mutagenic (Kuroda & Hara, 1999). Tea is a good source of essential minerals such as those that are essential for bone growth and development, nerve and muscle function by regulating fluid levels within the cells and prevention of tooth decay. Tea also contains vitamins which are potential antioxidants and help in metabolizing proteins.

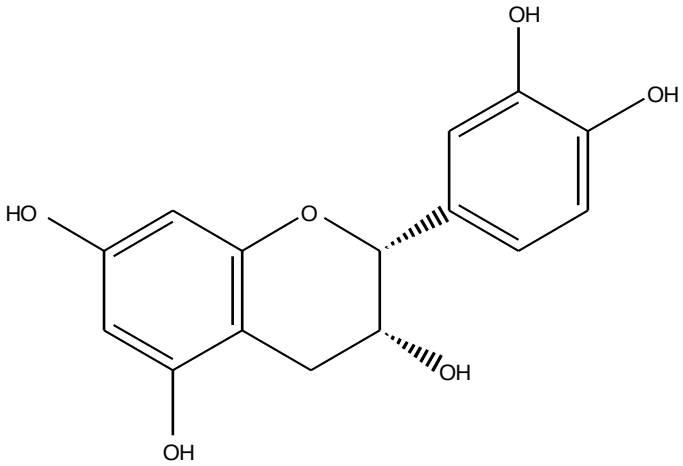
2.7 Tea Chemistry

Tea contains various bioactive compounds like polyphenols, alkaloids, carbohydrates, proteins, chlorophyll, volatile compounds, vitamins, minerals and amino acids (Hilton, 1973; Mondal, Bhattacharya, Laxmikumarran & Ahuja, 2004). The main bioactive constituents of tea leaves belong to the polyphenol group, accounting for 25 to 35% on a dry weight basis (Balentine, 1997; Hara et al., 1995d). They mainly include the following six groups of compounds: flavanols, hydroxyl-4-flavanols, anthocyanins, flavones, flavonols and phenolic acids (Bursill, Abbey & Roach, 2007). The most important and characteristic tea polyphenols are the flavanols, mainly the catechins (flavan-3-ols). Catechins are phytochemical compounds found in high concentrations in a variety of plant-based foods and beverages and the most pre-dominant ones in tea as given in figure 2.2 are: (-)-epicatechin (EC), (-)-epicatechin gallate

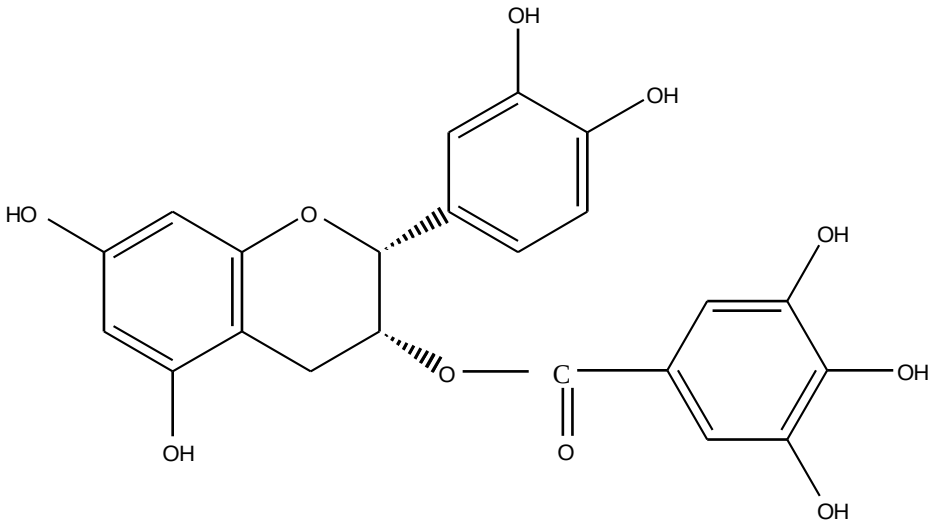
(ECG), (-)-epigallocatechin(EGC), (-)-epigallocatechin gallate (EGCG), (+)-catechin (C), and (+)-gallocatechin (GC) (Hara et al., 1995a; Liang, Lu, Zhang, Wu & Wu, 2003). These compounds contribute to the bitterness, astringency and sweet aftertaste of tea beverages (Hara et al., 1995b, Hirasawa, Takada, Makimura & Otake, 2002, Scharbert & Hoffman, 2005). In black tea, the oxidation of polyphenols during processing leads to the formation of catechins and gallic acid complexes such as theaflavins, theaflavinic acids, thearubigins or theasinensis, and proanthocyanidin polymers (Hara et al., 1995c; Balentine, 1997; Lee, Chen, Lio, Tzen & Chang, 2008).



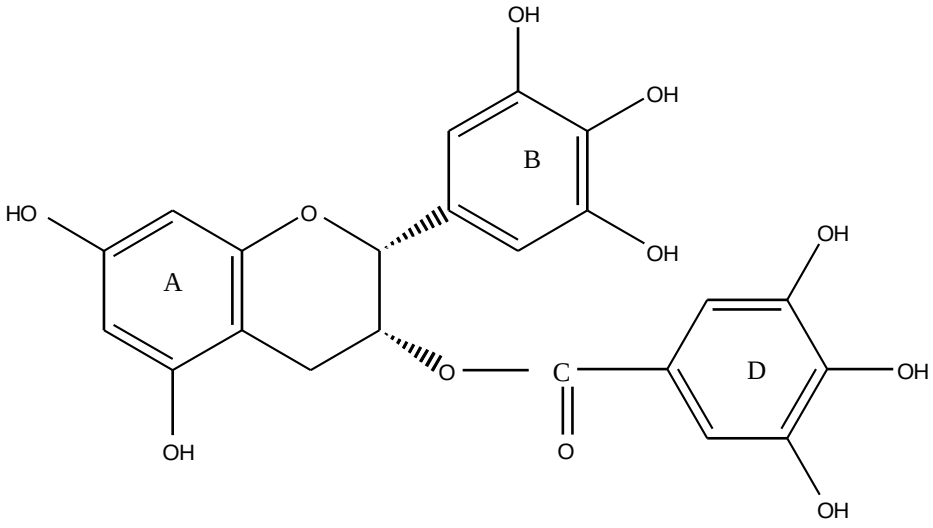
(+) -catechin (C)



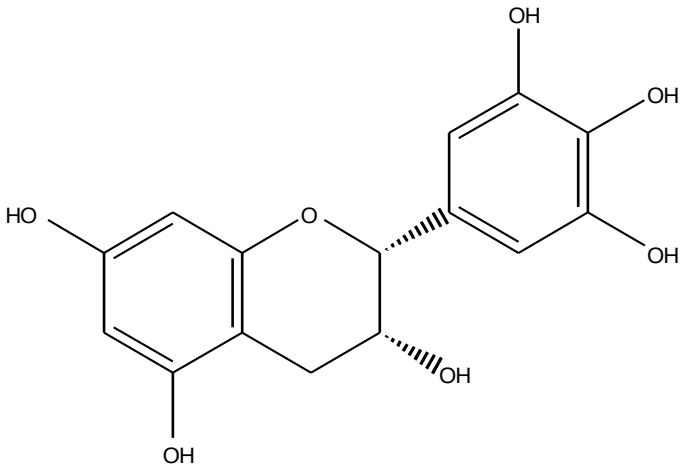
(-)-epicatechin (EC)



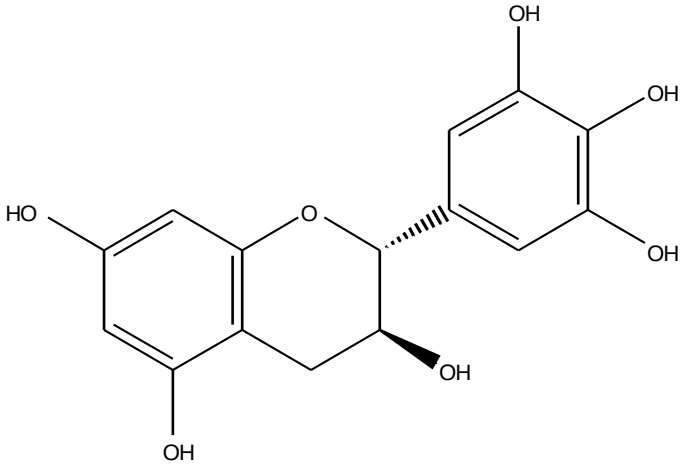
(-) -epicatechin gallate (ECG)



(-) -epigallocatechin gallate (EGCG)



(-) -epigallocatechin (EGC)



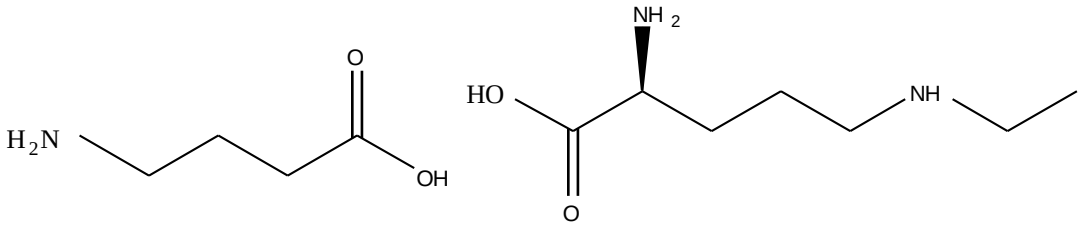
(+) -gallocatechin (GC)

Figure 2.2: Major catechins in tea

Major flavonols in the fresh leaf are kaempferol, quercetin and myricetin (McDowell & Taylor, 1993). Tea also contains sugars constituting 3-5% of the dry weight of the tea flush (2 leaves + bud). These are glucose, fructose, sucrose, raffinose and stachyose. Monosaccharides and disaccharides contribute to the sweet taste of tea infusion (Ou, Huang, Hampsch-Woodil, Flanagan & Deemer, 2002; Baoru, Teemu, Olli, Keith & Heikki, 2009). Methylxanthines are

present in tea with 2 – 4% as caffeine and a small amount of theophylline and of theobromine (Graham, 1992; Hara et al., 1995c). Caffeine is viewed as an important constituent of tea, bestowing mood and cognitive-enhancing properties (Bokuchava & Skobeleva, 1980). Minerals constitute about 4 to 9% of the inorganic matter of tea and include fluorine, potassium, aluminum, iodine, selenium, nickel, and manganese (Hara et al., 1995d). Tea is also a good source of vitamins A, B_1, B_6, C, and folic acid (Liang, Liu, Xu & Hu, 1990).

Tea contains the amino acids common in other biological systems: isoleucine, leucine, methionine, threonine, phenylalanine, glutamine, asparagine, alanine, serine, proline, histidine, glutamic acid, aspartic acid, except theanine which is unique to the tea plant, the most abundant, and accounting for 50% of the total amino acids (Chen et al., 2003). In addition, tea also contains gamma-aminobutyric acid (GABA) in low amounts (Scholz & Bertram, 1995) whose structures are as shown below in figure 2.3. Amino acids in tea play the role in the biosynthesis of polyphenols. Amino acid degradation is involved in the biogenesis of the tea aroma (Balentine, 1997) thus augments the fragrance of tea and improves its flavor (Thippeswamy et al., 2006, Yao et al., 2006). Theanine is also involved in the formation of the inhibitory neurotransmitter, gamma-aminobutyric acid (Juneja et al., 1999).



Gamma amino-butyric acid (GABA)

Theanine

Figure 2.3: Structures of GABA and theanine

2.8 Biosynthesis of Theanine in the TeaPlant

Theanine is synthesized from glutamic acid and ethylamine by theanine synthetase (Deng, Ogita & Ashihara, 2008) in the roots of the tea plant, and is transported to the leaves and accumulated there (Konishi & Takahashi, 1969; Wickremasinghe, 1972). Figure 2.4 is a summary of the biosynthetic pathway of theanine. Theanine is a water-soluble compound and when ingested orally and it is absorbed in the small intestine (Unno, Suzuki & Kakuda, 1999). It

crosses the blood-brain barrier via the large neutral amino acid (leucine-preferring) transport system and upon reaching the brain it has been shown in rats to increase both serotonin and dopamine production (Yokogoshi, Kobayashi, Mochizuki & Terashima, 1998). Serotonin and dopamine are chemicals in the brain associated with positive emotions and relaxation (Le Gall et al., 2004). Theanine is hydrolyzed in the kidney to glutamic acid and ethylamine by the enzyme glutaminase (Terashima, Takido & Yokogoshi, 1999; Unno et al., 1999; Tsuge, Sano, Hayakawa, Kakuda & Unno, 2003).

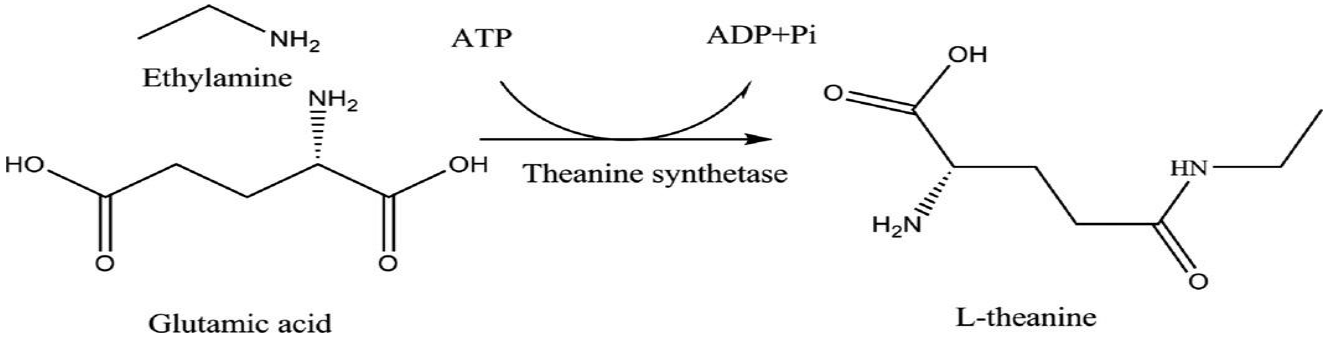


Figure 2.4: Biosynthetic pathway of theanine in the tea plant (Source: Verma, Kumar, KurbaVijaya & Sudhakar, 2013).

2.9 Functions of Theanine

L-theanine may modulate aspects of brain function in humans. Evidence from human electroencephalograph (EEG) studies has shown that it has a direct effect on the brain (Juneja et al., 1999). Brain waves are classified into four categories (delta, theta, alpha, and beta) each with an associated mental state (Figure 2.5). Delta is seen only in the deepest stages of sleep. Theta is seen in light sleep and drowsiness. Alpha is present in wakefulness where there is a relaxed and effortless alertness and Beta is seen in highly stressful situations and where there is difficulty in mental concentration and focus. Theanine directly stimulates the production of alpha brain waves, creating a state of deep relaxation and mental alertness (Nobre, Rao & Owen, 2008). It is well known that alpha brain waves are generated during a relaxed state and therefore alpha waves are used as an index of relaxation (Ito, Nagato & Aoi, 1998). In one study of these mental responses to L-theanine, brain wave topography showed that alpha waves were observed from the back to the top of a person's head (occipital and parietal regions of the brain) within

approximately 40 minutes after the subjects had taken either 50 or 200 mg of L-theanine (Juneja et al., 1999; Unno et al., 1999; Sugiyama & Sadzuka, 2003). In a separate study, the intensity of alpha waves were determined to be dose dependent (with a 200 mg dose showing a significant increase over controls) and detectable after 30 minutes (Juneja et al., 1999; Mason, 2001).

In addition, theanine is also thought to counteract the effects of caffeine possibly by inducing alpha waves and/or directly reducing caffeine levels at the receptor sites (Bryan, 2008; Owen, 2008; Giesbrecht, Rycroft, Rowson & De Bruin, 2010). Since caffeine is naturally present in tea, most people do not find that green tea induces the typical effects expected of a caffeine containing beverage (Kakuda, Nozawa, Unno, Okamura & Okai, 2000).

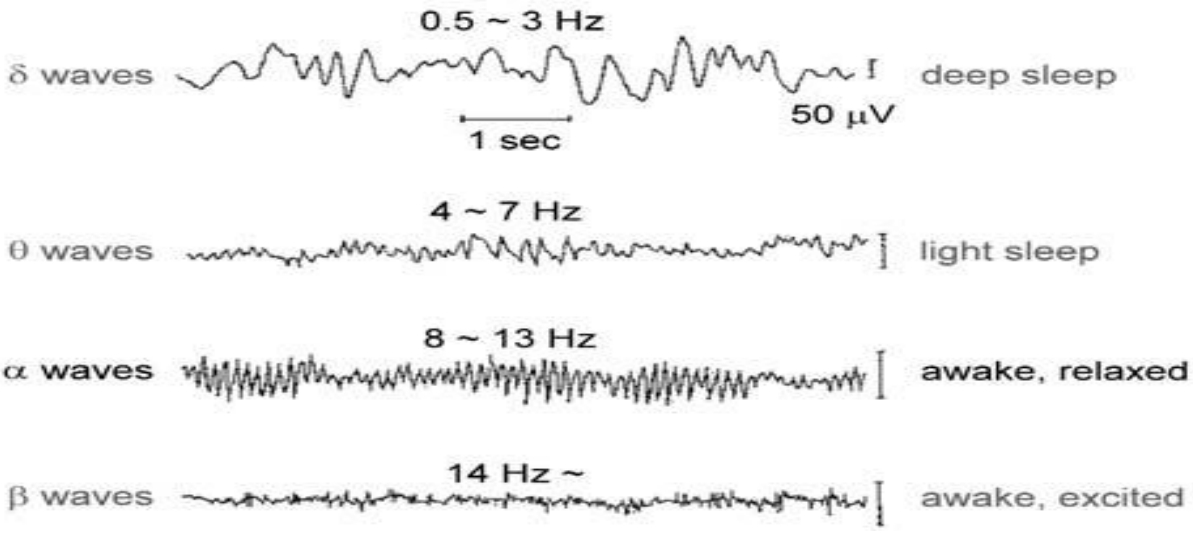


Figure 2.5: Classification of brain waves, (Source: Juneja et al., 1999)

It has been found that theanine has considerable biochemical impact including decreasing the level of norepinephrine and serotonin in brain, and intake of theanine by hypertensive rats resulted in decreased blood pressure (Juneja et al., 1999). The regulation of blood pressure is partly dependent upon catecholaminergic and serotonergic neurons in both the brain and the peripheral nervous system. The lowered blood pressure effect was dose-dependent with the highest test dose creating the most significant drop (glutamine was used as one of the controls). Although glutamine is similar in chemical structure to theanine, it did not exhibit any anti-hypertensive effect (Yokogoshi, Kato & Sagesaka, 1995). Similarly, a recent human study showed that 200 mg of L-theanine with a standardized green tea formulation significantly

reduced blood pressure (Nantz, Cheryl, Bukowski & Percival, 2009). The mechanism of action is thought to be due to the reduction of various neurotransmitters, particularly serotonin, both in the central nervous system and at the peripheral tissue (blood vessel) level (Kimura et al., 2007; Yamada & Terashima, 2009; Deka & Vita, 2011).

Theanine is also involved in the formation of the inhibitory neurotransmitter, gamma aminobutyric acid (GABA) levels in the brain (Yamada et al., 2007) and resulted in enhanced neuroprotective effects (Kakuda, 2002; Nathan, Lu, Gray & Oliver, 2006).GABA influences the levels of two other neurotransmitters, dopamine and serotonin, producing the key relaxation effect (Mason, 2001). GABA serves a sedative function that brings balance to excitability that can lead to restlessness, insomnia, and other disruptive conditions. Theanine also appears to increase levels of dopamine, another brain chemical with mood-enhancing effects, which can reduce blood pressure (Yokogoshi et al., 1998).

Theanine has also been shown to promote a mild, restful, relaxed state without diminishing daytime alertness (Kobayashi, Nagato, Aoi, Juneja, & Kim, 1998). Stress impairs the immune system, making people vulnerable to opportunistic infections, and can cause depression. People under stress can mitigate many of the harmful effects of stress using theanine. Theanine reduces stress and anxiety without the tranquilizing effects found in many other calming supplements (Kimura & Murata, 1971; Kakuda, Matsuura, Sagesaka & Kawasaki, 1996).

Animal studies examining the effect of theanine on memory and learning have consistently shown that the amino acid has a positive effect on these factors. For example, theanine improves the animal's ability to learn its way back through a maze, or to learn to avoid a negative stimulus, like a shock (Yamada, 2008). Neurotransmitters like dopamine and serotonin are both linked to memory and learning. It is thought that theanine's effect on learning and memory is achieved by increasing dopamine levels and reducing serotonin levels (Owen, 2008; Yamada, 2008). For this reason, theanine supplements in foods and beverages are quite popular with children in Japan that wish to improve their memory and learning skills. Theanine exerts protective effects on the brain by antagonizing glutamate toxicity (Borzelleca, Peters & Hall, 2006). Glutamate plays an important role in memory and learning. It is released by nerve cells into the extracellular space, where it normally elicits a desired response and is subsequently taken up by neurons for recycling. Interruptions in blood supply, as in stroke, interfere with this

ability to recycle glutamate. Excess glutamate is released and builds up in the extracellular spaces, where it sets off a chemical chain reaction that result in neuronal death. Theanine is believed to compete with glutamate to bind with glutamate receptors thus reducing glutamate toxicity (Kakuda, Nozawa, Sugimoto & Niino, 2002; Kakuda, 2002; Nagasawa et al. 2004).

Theanine has been found to be increasingly associated with therapeutic benefits in cancer treatment. The cooperative effects of anti-tumour agents and theanine on cancer have been reported (Sugiyama & Sadzuka, 2003). Theanine was used as a modulator with doxorubicin; it enhanced the concentration of doxorubicin in tumors by inhibiting the efflux of doxorubicin, leading to significant antitumor activity. It also reversed the drug's resistance by enhancing the efficacy of its transport into tumor cells. It decreases doxorubicin -induced adverse reactions and decreases doxorubicin concentrations in normal tissues such as the heart and liver. (Sugiyama & Sadzuka, 2003; Sugiyama & Sadzuka, 2004; Wan, Zhang & Li, 2009b; Zhang, Liu & Duan, 2009).

A study of the function of theanine in mitigating the effects of premenstrual syndrome (PMS) was conducted in cooperation with Taiyo Kagaku Co., The University of Shizuoka and The Family Planning Institute of Japan showed that women taking 200 mg theanine daily have lower incidence of Pre-menstrual symptoms (PMS). These symptoms include: physical, mental, and social (Doyle, Ewald & Ewald, 2007) whereby 20 women aged 22 - 49 (mean 30.0) years were administered 2 tablets with 200 mg theanine / day or placebo 2 times a day through three menstruation cycles. Subjects completed a Menstrual Distress Questionnaire with 47 questions addressing the physical symptoms of pain, concentration, behavior change, autonomic reactions, water retention, negative effect, and the mental symptoms of arousal and control. Theanine significantly reduced scores on both physical and mental symptoms of PMS (Ueda et al., 2001).

Several analytical methods have been developed for identification and quantification of theanine, which can be employed for determining theanine concentration, production yield or theanine purity in final products. Analysis of theanine has been mainly facilitated by chromatographic techniques such as high-performance liquid chromatography (HPLC), capillary electrophoresis and micellar electrokinetic chromatography (Ekborg-Ott, Taylor & Armstrong, 1997; Chen et al., 2003; Hsiao, Chen & Cheng, 2010). Even though theanine has been shown to be of health benefit, less studies have been conducted to determine its levels in the various Kenyan released tea clones and thus the need for this study.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental Design

This study was carried out at the Tea Research Institute (TRI), formerly the Tea Research Foundation of Kenya (TRFK) based in Kericho, Kenya. The site is located 12 km from Kericho town (0º 22' South, 35º21' East, elevation 2180 M (m.a.s.l.).The experimental design was a completely randomized design and replicated three times. The experimental plots where the tea clones considered in this study were established in 1983 and receive NPKS fertilizer once a year uniformly at the rate of 150 kg per ha. The study was subdivided into three and carried out as follows; (a) the determination of the theanine levels in selected commercially released Kenyan tea clones, (b) the determination of the theanine levels in different partitions of the tea shoot and (c) the determination of the theanine levels in made teas from different origins commercially available in the Kenyan market. The determination of the theanine levels in the selected commercially released clones was done for three seasons, where the first season (July to September, 2011) was done when weather conditions were cold and wet, the second season (October to December, 2011)was cold and humid (less rainfall) and the third season (January to March, 2012) was hot and dry.

3.2 Sample Collection

Sample collection was done based on the experimental treatments as described below;

3.2.1 Collection and Pre-treatment of Tea Shoots for Clonal Variations Studies

Twenty three (23) clones were selected for this experiment, *viz.*,TRFK 31/8, TRFK 12/12, TRFK 31/11, TRFK 301/4, TRFK 301/5, TRFK K – Purple, TRFK 6/8, TRFK 303/577, TRFK 303/216, TRFK 303/178, TRFK 303/259, TRFK 7/9, TRFK 7/3, TRFK 7/14, TRFK 54/40, TRFK 56/89, TRFK 11/26, TRFK 100/5, TRFK 108/82, TRFCA SFS 150, TRFK 11/52, TRFK 12/19, TRFK 11/4.These clones were selected on the basis that they were of the same age and on the same plantation at the Tea Research Institute Museum, Kericho, whose model is shown in Appendix I. Further, all the agronomic practices on this plantation are always done at the same time and thus, this would not be a variable on the levels of theanine in the teas. Each plot was divided into three for replication purposes and labeled a, b and c. Approximately 4 Kg of tea shoots (Fig 3.1) were hand plucked from the respective plots, placed in well labelled

brown paper bags and taken to a miniature factory within the institute for processing black and green teas.Each replicate sample for each clone was then split into two (2 kg each) and processed as black and green tea respectively.

Figure 3.1: Images of Tea shoot (2 Leaves and a bud)

Green tea was processed immediately by steaming one 2 kg portion on arrival at the miniature factory, rolled using Cut, Tear and Curl (CTC) machine then microwave-dried while the other batch for black tea processing was uniformly spread on withering troughs and left to wither for 18 – 24 hours. The withered leaves were then rolled using a Cut, Tear and Curl (CTC) machine and the resulting dhool (macerated leaves)was placed in aeration cabinets (M501,Tea Craft, UK) for 90 minutes. The dhool was then dried using a fluid bed drier (Tea Craft, UK) at 120 ± 1 ^{0}C for 10 – 15 minutes to reduce the moisture content to below 3^{0}C. This was repeated for the second and third seasons so that the determination of the seasonal variations, if any, in the theanine levels in the selected clones of tea could be determined. The processed green and black tea test samples were then finely ground using an electric coffee grinder (AR 40, Moulinex, China) and stored in well labelled aluminium-lined sachets awaiting analysis.

3.2.2 Collection and Pre-treatment of Tea Shoots for Partition Studies

To determine the best time to sample in order to obtain optimum levels of theanine in the different partitions of the tea shoot and if withering affects these levels, clone TRFK 301/1 was selected. This choice was informed based on findings from a previous study by Kilel, Faraj, Wanyoko, Wachira & Mwingirwa (2013),which had indicated that this clone had high levels of

theanine, 2.03 %, on a dry weight (dw) basis. Tea shoots comprising three leaves and a bud were randomly hand plucked from the respective plot at the Tea Research Institute's Botany/Genetics experimental plots at Kericho. Triplicate tea samples, approximately 2 kg were put in brown paper bags and taken to the miniature factory in the institute for processing. The sampling was done at an interval of three hours with the last sample being collected after twelve hours. The first sample was collected at 6.00 A.M and referred to as 0 hrs in the study, 3hrs (9.00 A.M), 6 hrs (Noon), 9 hrs (3.00 P.M) and 12 hrs (6.00 P.M). All the test tea samples were split into two and one batch was steamed immediately whereas the other batch was left to wither for 3, 6, 9, 12 and 15 hours respectively depending on the time the sampling was done. The first sample collected withered for fifteen hours whereas that which was sampled last withered for three hours. All samples were processed as green tea including those that had withered, that is, both batches were steamed using an electric steamer (HD9120, Philips, China). The three leaves and a bud were then partitioned into seven: a bud, first leaf, first leaf internode, second leaf, second leaf internode, third leaf and third leaf internode. Each partition was microwave-dried (GEO103MB, Samsung, Malaysia) and then finely milled using an electric coffee grinder (AR 40, Moulinex, China) for particle size reduction and test sample homogenization then stored in well labeled aluminium lined sachets awaiting analysis.

Based on the optimum sampling time determined in the above step, two tea clones (TRFK 91/1 and TRFK 301/5) were then sampled for partition studies with respect to leaf colour. Purple leaf coloured clones are rich in anthocyanins while the ordinary green leaf coloured do not contain anthocyanins and anthocyanins are of health benefit. TRFK 91/1 is a purple leaf colored clone while TRFK 301/5 is a green leaf colored clone. TRFK 301/1, which was used in the first step was left out in this study because it was not in the experimental plot where these two clones were and if it could have been collected from another field/plot several variables would affect the concentration of the amino acid including agronomic practices and age of the plant. Further, TRFK 301/1 and TRFK 301/5 clones share some genetic traits, including drought resistance, total polyphenols, catechin content among others. Young tea shoots constituting of four leaves and a bud were randomly hand plucked from the respective plots with each plot partitioned into three for replication purposes and labelled a, b and c as shown in table 3.1.

Table 3.1: Partitioning of the plots for replication

Clone	Partitions / replication		
TRFK 91/1	b	a	c
TRFK 301/5	b	a	c

The samples were put in brown paper bags, sealed, labeled appropriately and transported to the miniature factory for processing. The samples were steamed and partitioned into six: buds, first leaf, second leaf, third leaf, fourth leaf and internodes. They were then microwave-dried (GEO103MB, Samsung, Malaysia), milled into a fine powder using an electric coffee grinder (AR 40, Moulinex, China) and stored in aluminium-lined sachets awaiting analysis.

3.2.3Collection and Pre-treatment of Processed Tea in the Kenyan Market

Processed teas in the Kenyan market were randomly obtained from the Mombasa tea auction, the second largest in the world after Colombo, where teas from various tea growing areas and countries are sold. The teas obtained for this study were from Kenya, Uganda, Tanzania, Rwanda, Cameroon and Sri-Lanka and triplicate samples of both black and green teas were obtained depending on availability. The test samples were finely milled using an electric coffee grinder (AR 40, Moulinex, China) and put in well labeled aluminium lined sachets awaiting analysis. Further, flavoured teas available in the Kenyan market including strawberry, vanilla, peach and pure peppermint flavours were also sampled, milled and stored appropriately awaiting analysis.

3.3 Sample Preparation

Approximately 1.00 ±0.01g of a finely ground sample was weighed into a 200 mL beaker and 100 mL of boiling double distilled water was added. The sample was then allowed to brew for 5 minutes on a hot plate (MHK-4, Mrc, Germany) while stirring. The sample was allowed to cool, made up to volume by double distilled water and filtered using a 0.45µm membrane into sample vials.

3.4 Preparation of Working Solutions

To obtain a standard stock solution of 1000 µg/mL in concentration, 50 ± 0.01 mg of pure L-theanine was accurately weighed into a 50 mL volumetric flask and dissolved with double distilled water by the aid of sonication using Ultra Sonic Bath (XB14, Grant, England) and made up to the volume with the double distilled water. Standard working solutions were prepared by serial dilution of the standard stock solution as shown in Table 3.2 in the concentration range of 20 -80µg/mL.

Table 3.2: Preparation of the working solutions

Standard solution	Working solution (µg/mL)	Volume taken from the stock solution (mL)	Final volume (mL)
A	20	1.0	50
B	40	2.0	50
C	60	3.0	50
D	80	4.0	50

3.5 Determination of Dry Matter Content

Approximately 2.0 ± 0.01 g of the sample was accurately weighed into clean and dry aluminium dishes and heated in an oven at 103 ± 2ºC for 16 hours to constant weight. The differences in weights were the used to compute the dry matter (DM) content of each test sample.

3.6 Chromatographic Determination of Theanine

The theanine content in the test samples was done by High Performance Liquid Chromatography (HPLC). The chromatograph used was a Shimadzu LC 20 fitted with an SIL 20A auto sampler and an SPD-20 UV-Visible detector (set at 210 nm) with a class LC10 chromatograph workstation, manufactured in Kyoto, Japan. The column used was RP-18 (Phenomenex Aqua 250 × 4.6 i.d) and oven was set at 35°C. The mobile phases used were 99.9 % HPLC grade acetonitrile (B) and double distilled water (A). Analysis time was 10 minutes

with 100 % mobile phase A, then the next 8 minutes was washing the column with 20 % mobile phase A and 80 % mobile phase B. The remaining time was conditioning with 100 % mobile phase A before the next injection. The flow rate was 1.0 mL/min and an injection volume of 20µL. Each sample was run for 41 minutes and theanine was eluted at 5.6 minutes.

The values were tabulated and a calibration curve was drawn by using concentration of theanine in the working solutions against peak area. A linear calibration curve (appendix XIII) was obtained with slope and intercept values (m and b as in y = mx + b). The theanine content, $W_{theanine}$, was expressed as a percentage by mass on a dry matter sample basis, as given by the formula:

$W_{theanine}$ (% dw) = $[(A_{sample} - b_{intercept}) \times V_{sample} \times d \times 100] / [m_{std} \times M_{sample} \times 10000 \times W_{DM,sample}]$ (Engelhardt & Simonides, 2007)

Where;

A_{sample} is the peak area obtained for the sample test solution

$b_{intercept}$ is the y intercept

V_{sample} is the injection volume

m_{std} is the slope obtained from the best fit linear calibration

M_{sample} is the mass, in grams, of the sample portion

d is the dilution factor

$W_{DM,sample}$ is the dry matter content, expressed as a mass fraction in percent, of the test sample.

3.7 Statistical Analysis of Data

The results obtained for the various treatments were subjected to Analysis of Variance (ANOVA) using MSTAT statistical software for windows version 2.10 at $p < 0.05$. The Least Significant Difference (LSD) test was used for mean separation where statistically significant differences were observed ($p < 0.05$). Data were presented as a mean of triplicate determinations ± standard deviation (SD).

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Clonal and Seasonal Variations in Theanine Levels in Selected Kenyan Tea Clones

The theanine levels determined in the 23 selected commercially released Kenyan tea clones harvested and processed as black and green teas for three seasons are herein presented. Chromatograms were also obtained after analyzing the samples using HPLC and were as shown below. Theanine peak was identified by comparing the tea sample (Fig. 4.2) chromatogram with that of theanine standard (Fig. 4.1). Figure 4.2 shows the chromatogram of a tea sample and after performing manual integration whereby the peaks were cleaned, the chromatogram appeared as in figure 4.3.

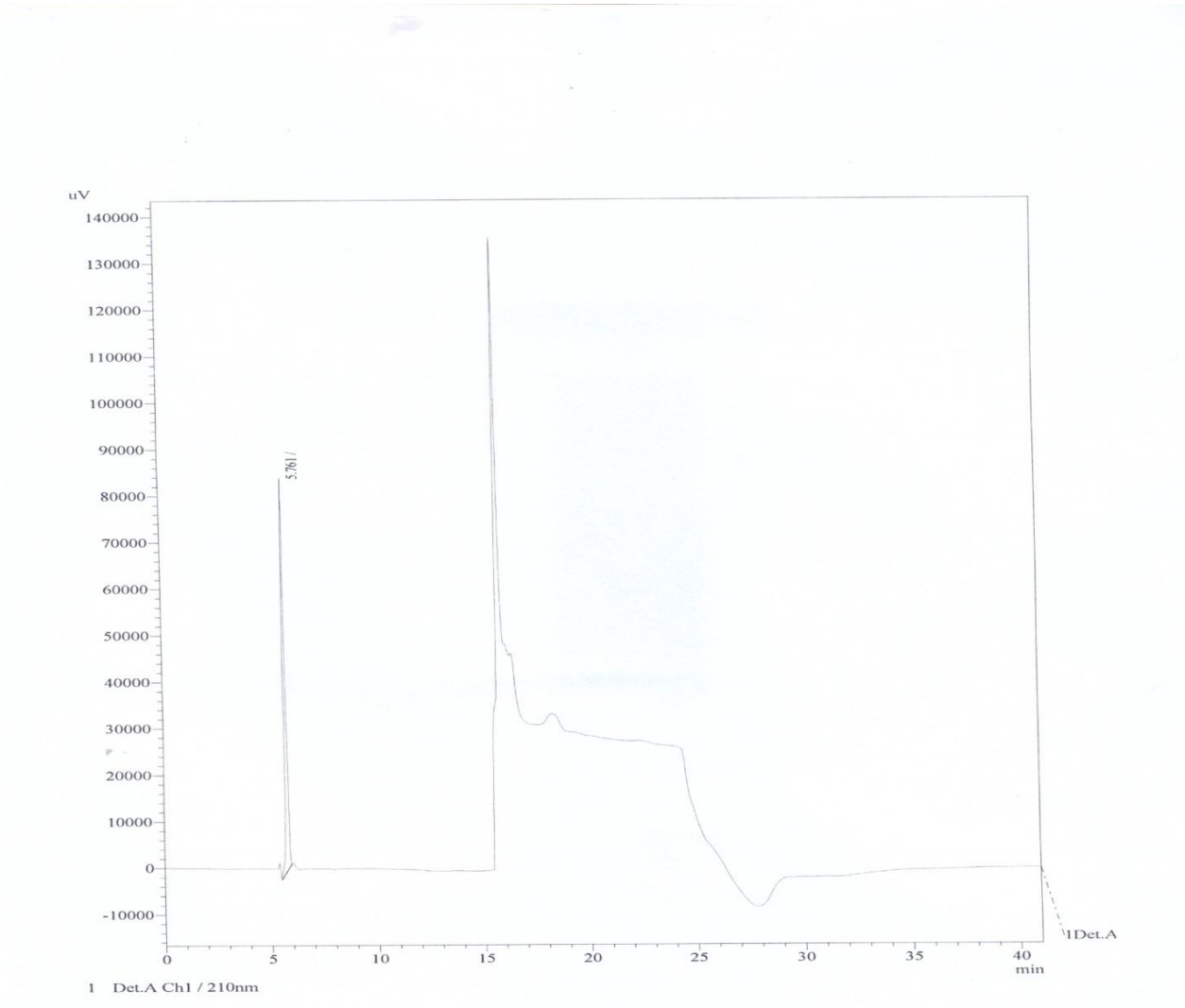


Figure 4.1: Chromatogram of theanine standard

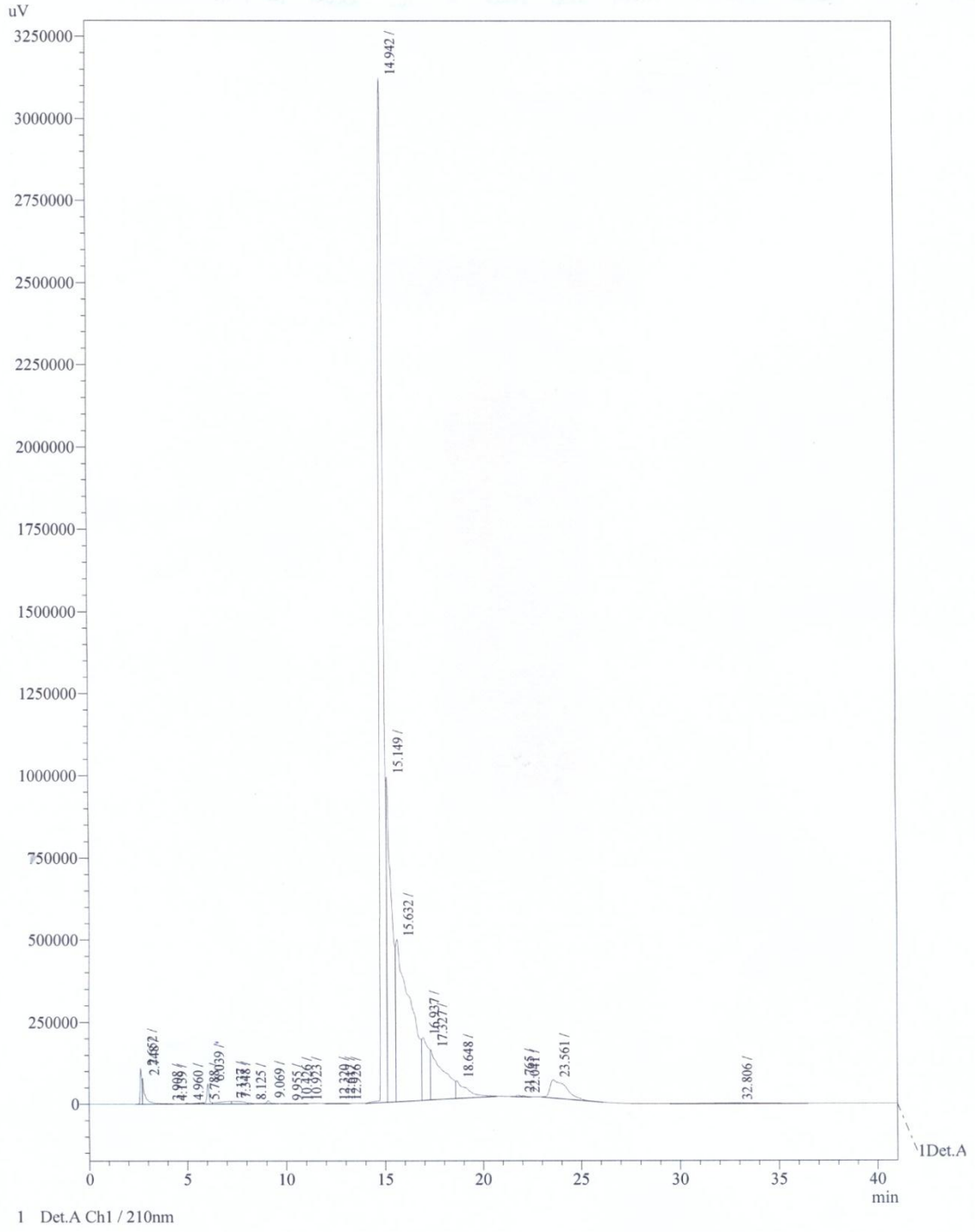


Figure 4.2: Chromatogram of a tea sample

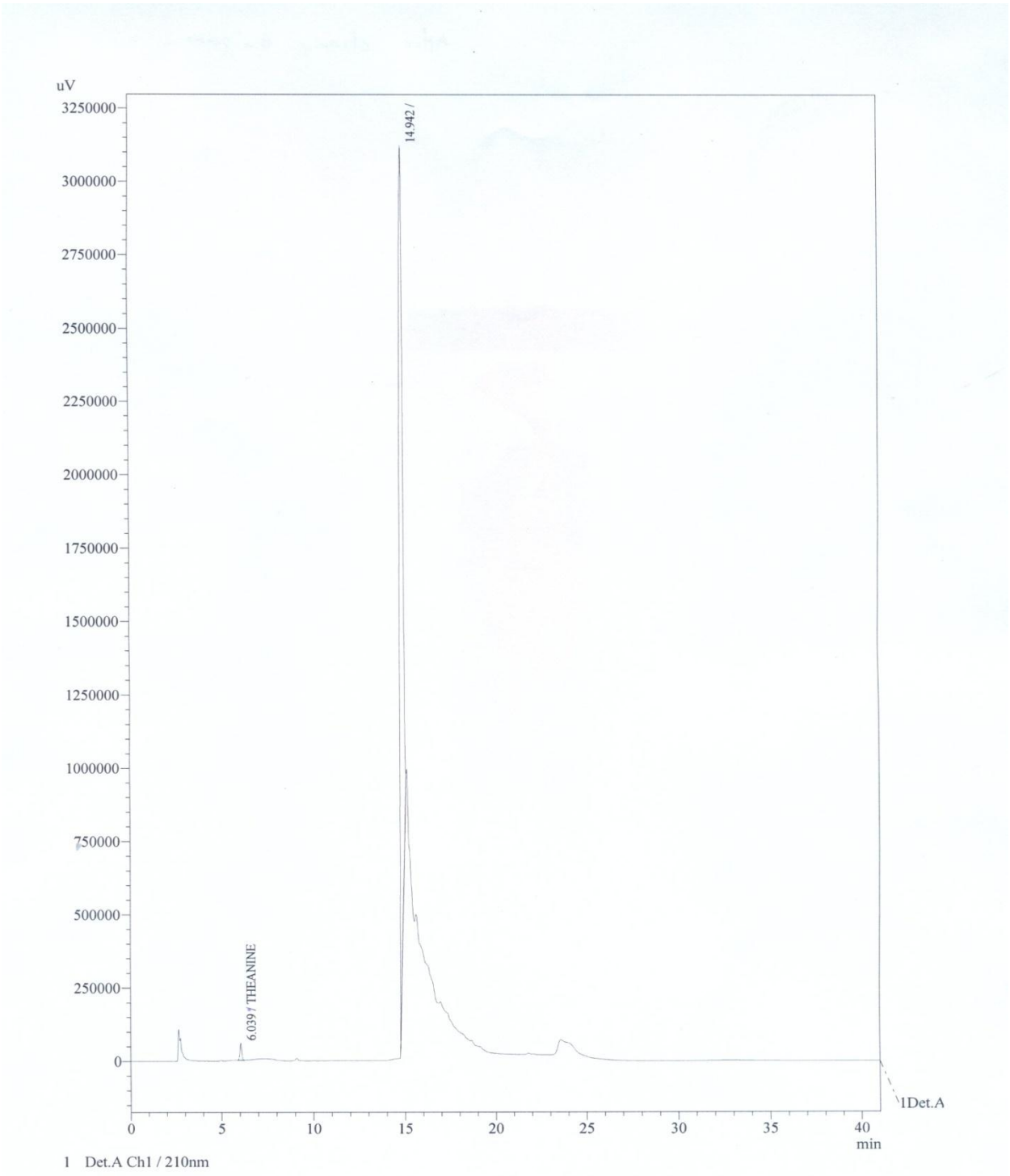


Figure 4.3: Chromatogram of tea sample after cleaning peaks

4.1.1 Green Tea

The levels of theanine in the 23 clones of green tea processed during the three seasons were determined and the results were as shown in the appendix. Analysis of Variance was carried out for every season and a sample of its output has been shown in Appendix II. During the first season (July – September, 2011), the linear calibration curve obtained ($y = 8707.4x + 6075.4$, $r^2 = 0.9996$), demonstrated adequate linearity and was used to calculate theanine levels during this season for both green and black tea. Theanine levels in the twenty three clones ranged between 0.54 – 1.50 % dw (Fig. 4.4, Appendix V). TRFCA SFS 150 (1.50 % dw) contained the highest amount of theanine while TRFK 6/8 (0.54 % dw) contained the lowest amount during this season. TRFK 31/8 (1.35 % dw) and TRFK 7/9 (1.32 % dw) theanine levels were not significantly different from each other ($p > 0.05$). Most of the tea clones analyzed contained 0.6% dw of theanine. TRFK K – Purple (1.04 % dw) was the only purple tea clone in this study and it was among tea clones with high theanine levels. TRFK K - Purple contained theanine in comparable amounts to TRFK 7/3 (1.00 % dw) and TRFK 11/26 (1.04 % dw). Therefore it is double rich as it contains both anthocyanins (potent antioxidants) and theanine.

During the second season (October – December, 2011), the same number of tea clones were analyzed and a linear calibration equation, $y = 10284x + 2520.9$ ($r^2 = 0.9999$) was obtained and used to calculate theanine levels for both green and black tea. The amount of theanine in green tea ranged between 0.59 - 1.34 % dw (Fig. 4.5, Appendix VI). The tea clone that contained the highest theanine level in green tea was TRFCA SFS 150 with 1.34 % dw and the lowest was TRFK 7/14 with 0.59 % dw. On average most of the green tea contained 0.70 % dw of theanine. Though TRFCA SFS 150 contained highest theanine levels (1.34 % dw), its levels were not significantly different ($p > 0.05$) from that contained in TRFK 56/89 (1.28 % dw) and TRFK 7/9 (1.28 % dw). TRFK 7/14 theanine levels of 0.59 % dw were not significantly different ($p > 0.05$) from the levels in TRFK 301/4 (0.63 % dw) and TRFK 303/216 (0.64 % dw) even though it contained the least amount of theanine after ranking all the clones upon subjecting the results to Least Significant Difference (LSD) test. TRFK K – Purple contained 0.73% dw of theanine and itscontent was not significantly different ($p > 0.05$) from some of the green tea clones including TRFK 303/577 (0.74 % dw) and TRFK 31/11 (0.69 % dw).

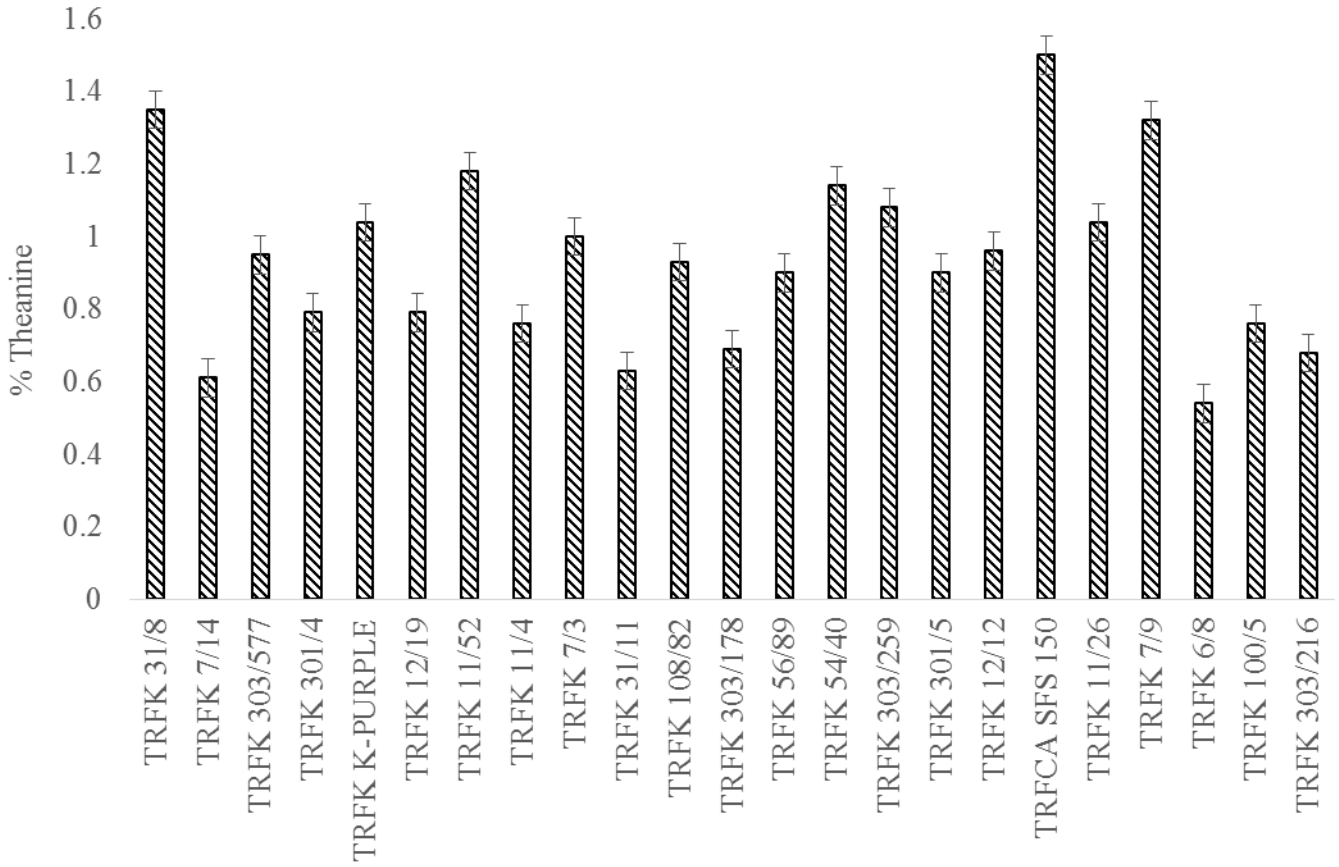


Figure 4.4: Clonal variation in theanine levels in green tea during the first season

In the third season (January – March, 2011), the same number of clones were analyzed and a linear calibration equation, $y = 10100x - 1209.1$, ($r^2 = 0.9961$) was obtained which was then used to calculate theanine levels for both green and black teas. The theanine levels in green tea ranged between 0.14 – 1.09 % dw (Fig. 4.6, Appendix VII). The clone that contained the highest amount of theanine was TRFK 31/8 with 1.09 % dw and the lowest was TRFK 303/178 which had 0.14 % dw. The theanine level in TRFK 31/8 was not significantly different ($p > 0.05$) from the content in TRFK 7/3(1.06 % dw) though when the means were ranked TRFK 7/3 was the second highest. TRFK K - Purple contained 0.48 % dw of theanine and this was not significantly different ($p > 0.05$) from the theanine levels in TRFK 12/19 (0.48 % dw), TRFK 54/40 (0.52 % dw) and TRFK 7/9 (0.52 % dw) among others which are green colored tea clones.

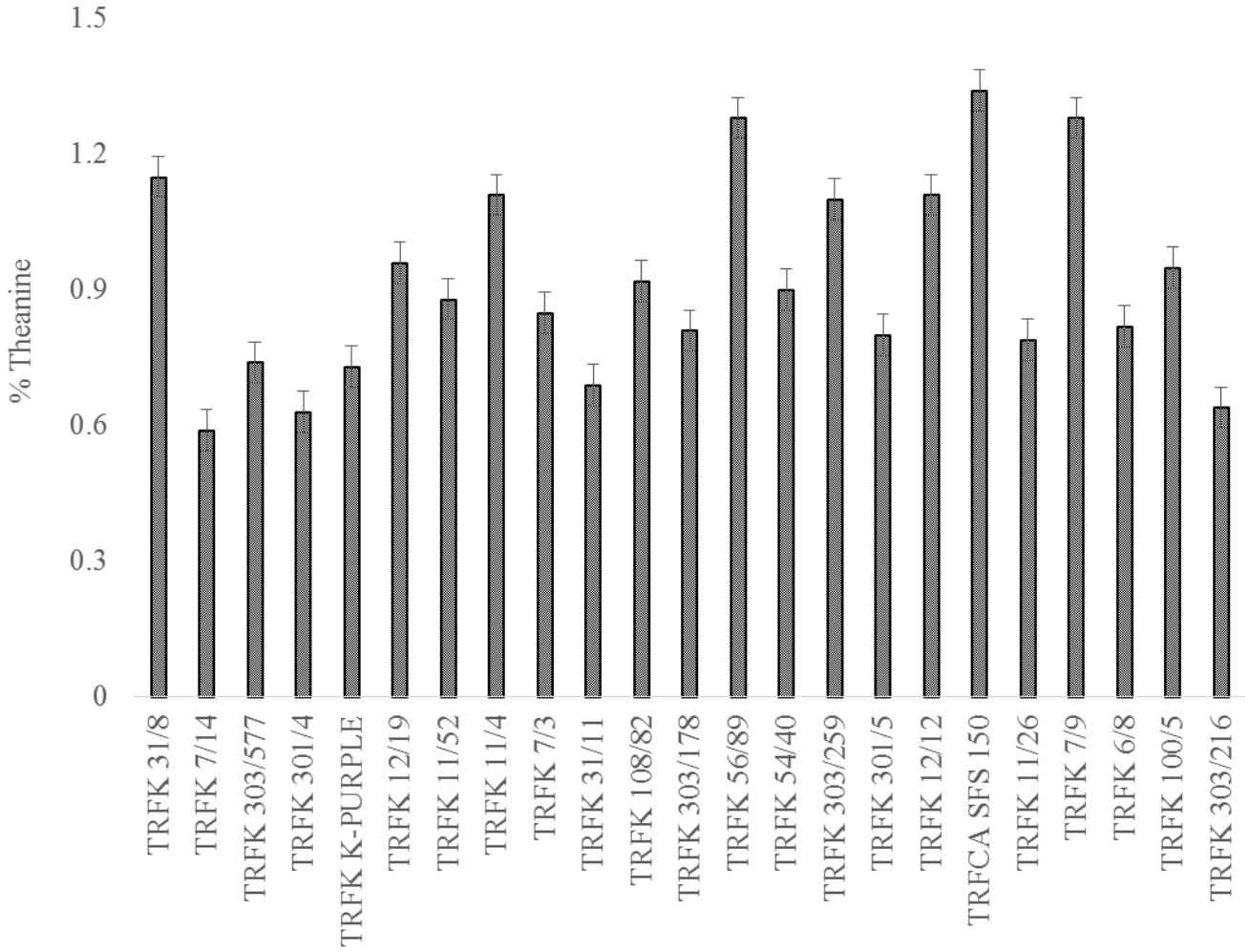


Figure 4.5: Clonal variation in theanine levels in green tea during the second season

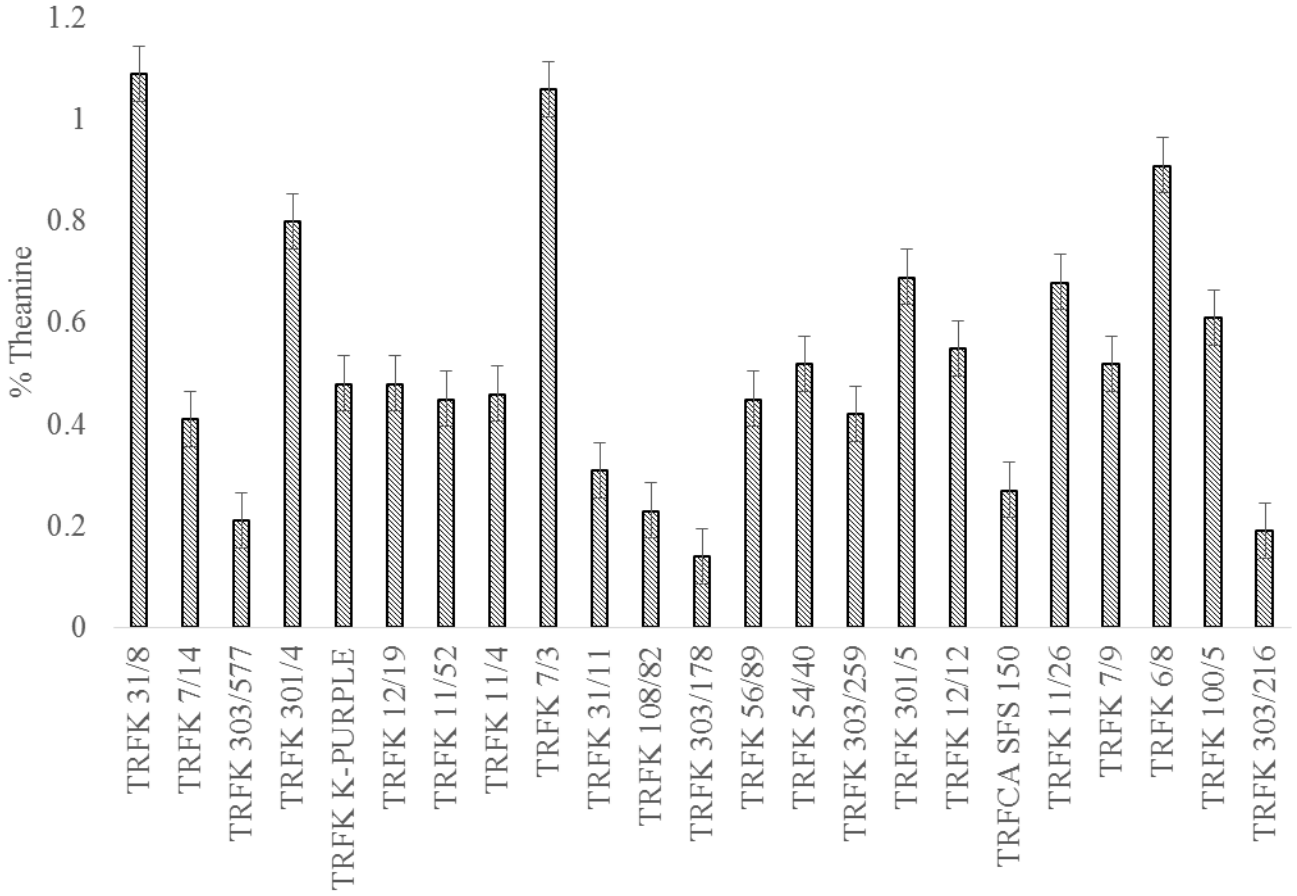


Figure 4.6: Clonal variation in theanine levels in green tea during the third season

Theanine levels varied during the three seasons (Fig. 4.7) with the first and second season having high levels of theanine. The third season showed a decrease in theanine levels in most of the clones except only TRFK 7/3, TRFK 301/4 and TRFK 6/8 whose theanine levels were highest during this season. TRFK 301/4 is a drought resistant clone while TRFK 7/3 is slightly resistant to drought (Wachira, Kamunya, Chalo, Maritim & Kinyangi, 2012) and this could be the reason why theanine levels in these clones were high. TRFK 6/8 is not drought resistant but contained high levels of theanine. There could be genetic traits responsible for this results which need to be further studied. There was a variation in theanine levels among the analyzed clones in all the seasons. Every clone has different genetic composition and some traits might be responsible for this variation and research has to be done on this.

A study was conducted by Yemane *et al.* (2008) on the levels of micronutrients in the leaves of the tea plant and found out that different clones were shown to have different abilities to absorb these nutrients under similar agronomic practices. This could apply also with clones analyzed in this study implying that different clones absorb nitrogen from the soil at different rates thus clonal variation in theanine levels. The best season to obtain optimum theanine levels was the first and second season when the climatic conditions were favourable with average temperatures of 15.93 and 16.75 ^{0}C; rainfall of 198.8 and 270.6 mm respectively.

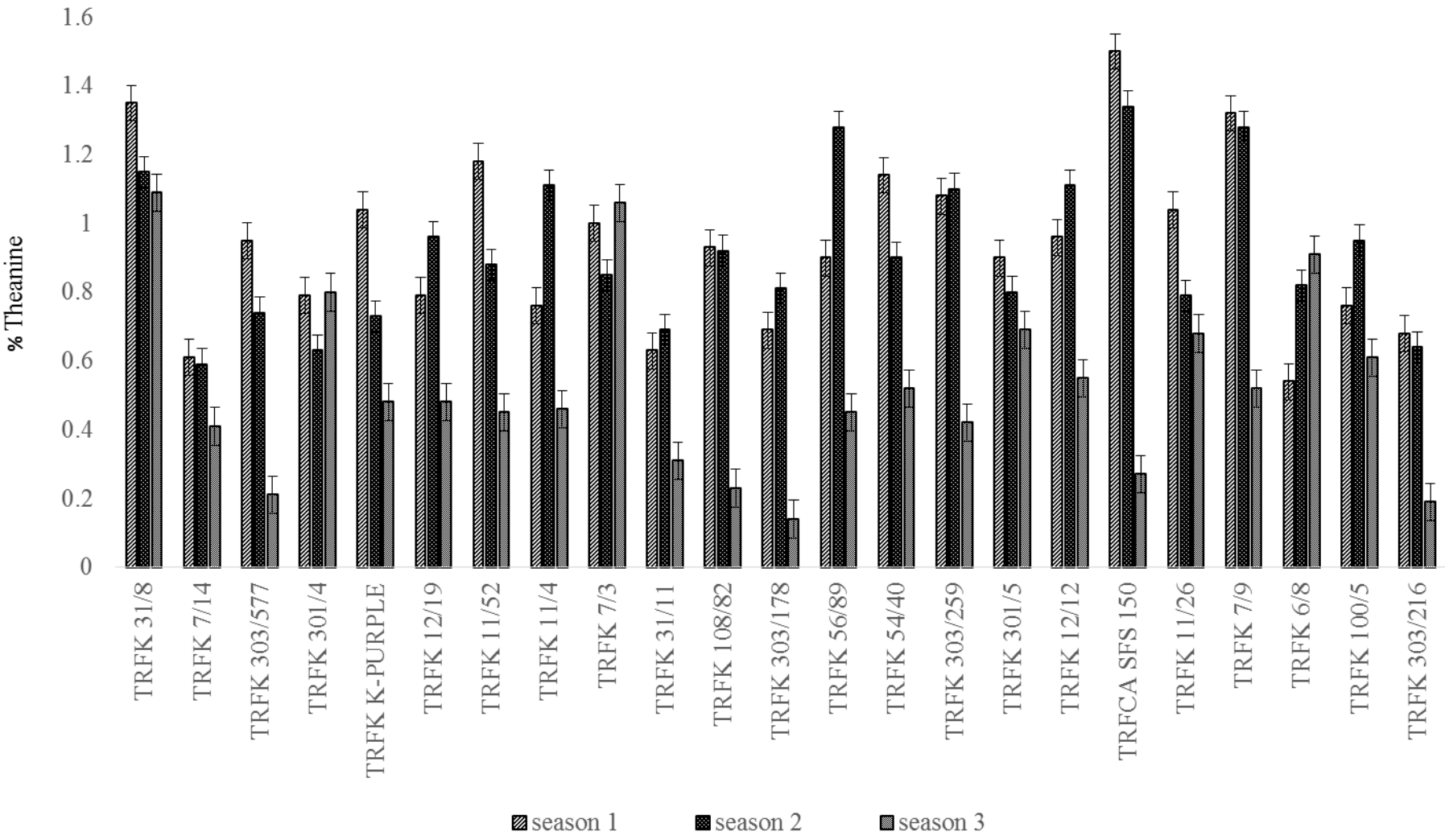


Figure 4.7: Theanine levels in green tea during three seasons

4.1.2 Black Tea

The results for black tea processed from the 23 clones of tea for the three seasons were as shown below. Black tea theanine levels range was between 0.42 – 0.98 % dw (Fig. 4.8, Appendix VIII) during first season. The clone that contained the highest level of theanine was TRFK 108/82 with 0.98 % dw and the lowest was TRFCA SFS 150 with 0.42 % dw. Most of the black teas contained on average 0.50 % dw of theanine. TRFK K- Purple contained 0.80 % dw and was among clones with high levels of theanine with its levels being comparable to ordinary green tea clones: TRFK 31/8 (0.83 % dw), TRFK 301/5 (0.85 % dw) and TRFK 100/5 (0.76 % dw). Nearly half of the clones analyzed contained theanine below that of TRFK K- Purple. The best quality black tea clone TRFK 6/8 contained 0.47 % dw and this was not significantly different ($p > 0.05$) from its progeny TRFK 303/577 (0.49 % dw) but significantly different ($p < 0.05$) from TRFK 303/178 (0.62 % dw) and TRFK 303/216 (0.63 % dw) which are also its progenies.

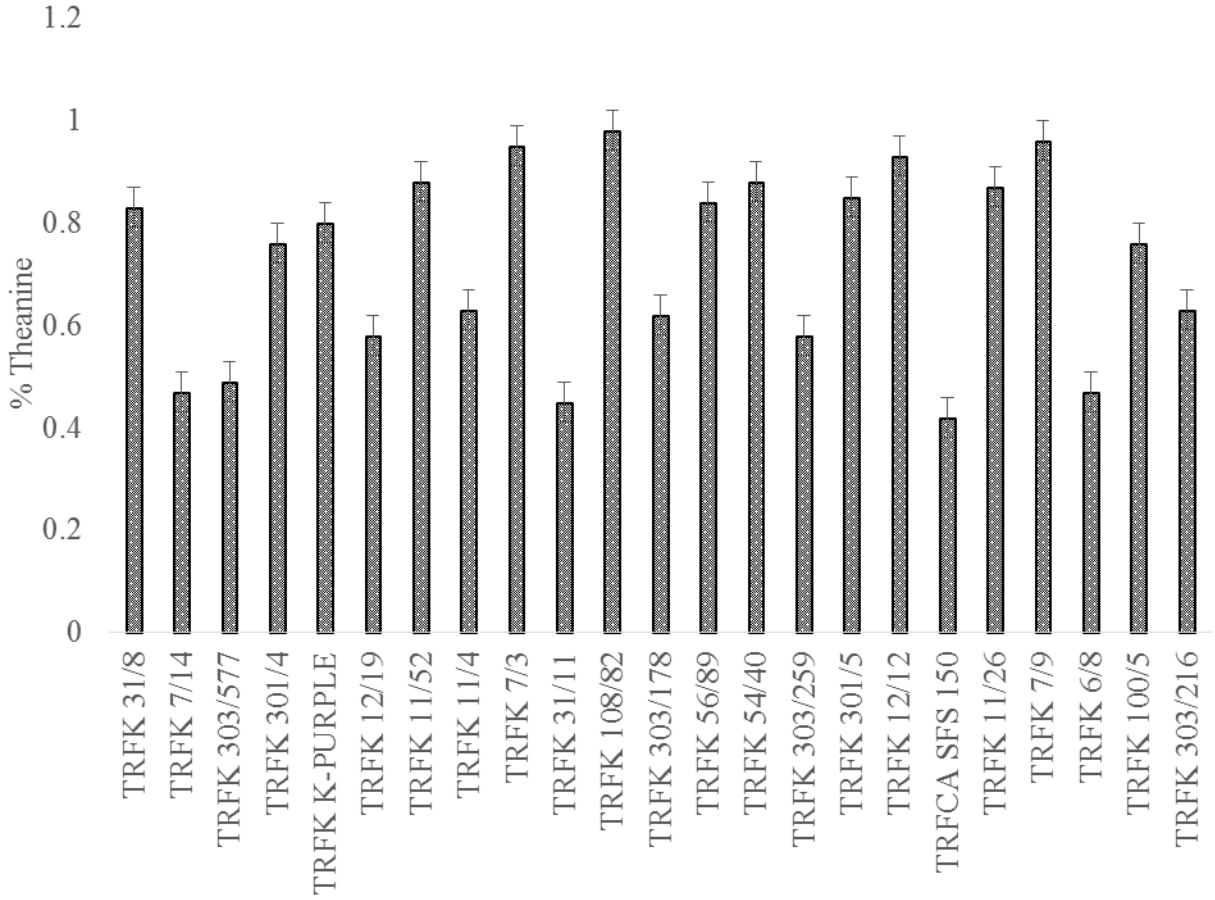


Figure 4.8: Clonal variation in theanine levels in black tea during the first season

The levels of theanine ranged between 0.35 – 1.11 % dw (Fig. 4.9, Appendix IX) during the second season. The clone that contained the highest amount of theanine was TRFCA SFS 150 and the clone that had the lowest was TRFK K – Purple with 0.35 % dw. The theanine levels in TRFCA SFS 150 was not significantly different ($p > 0.05$) from the levels in TRFK 56/89 (1.09 % dw) and TRFK 31/8 (1.08 % dw). Most of the clones contained 0.6 % dw of theanine on average during this season. The best quality black tea clone TRFK 6/8 contained 0.65 % dw and was not significantly different ($p > 0.05$) from TRFK 7/3 (0.63 % dw), TRFK 31/11 (0.64 % dw) TRFK 11/52 (0.67 % dw) and TRFK 303/216 (0.59 % dw) among others. TRFK 303/259 being a progeny of TRFK 6/8 contained 1.02 % dw of theanine level which was higher and significantly different ($p < 0.05$) from its parent (mother bush). TRFK 303/178 contained 0.51 % dw of theanine which was lower and significantly different ($p < 0.05$) from TRFK 6/8 and yet it is its progeny. The differences in theanine levels between the TRFK 6/8 with its progenies could be due to inherited genes from the mother bush. Some genetic traits could be more in one progeny and less in another. This is yet to be established by plant breeders.

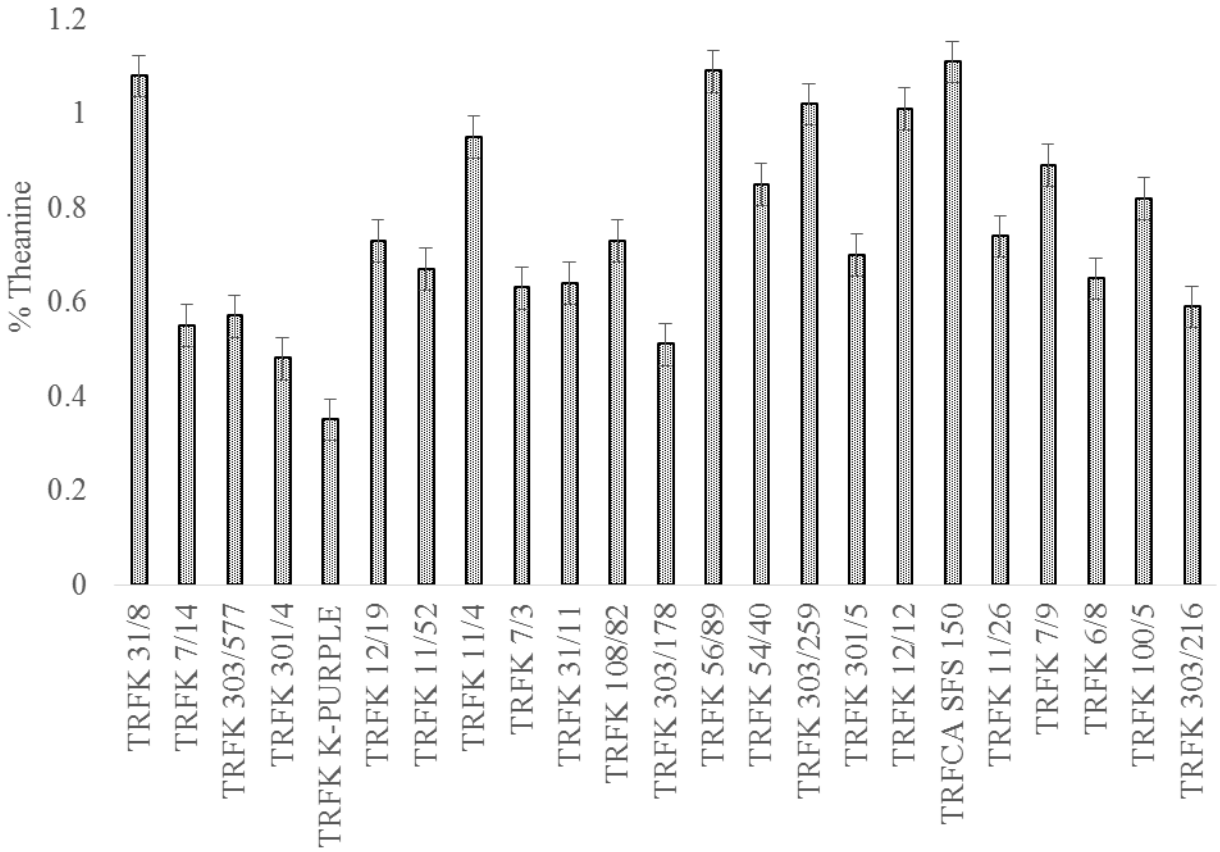


Figure 4.9: Clonal variation in theanine levels in black tea during the second season

The theanine levels ranged between 0.21 – 1.00 % dw (Fig. 4.10, Appendix X) during the third season. The tea clone that had the highest level was TRFK 54/40 with 1.0 % dw and the lowest was TRFK 31/11 with 0.21% dw. TRFK 31/11 cultivar is affected severely by drought and this could be the reason its theanine levels were low. Theanine levels in TRFK 54/40 were not significantly different ($p > 0.05$) from the levels in TRFK 56/89 (0.98 % dw). TRFK K-purple clone contained 0.46 % dw and its theanine levels was also in the range of some ordinary green tea clones. TRFK K – Purple theanine levels were not significantly different ($p > 0.05$) from the levels inTRFK 301/4 (0.45 % dw) and TRFK 6/8 (0.47 % dw) which is well known for the production of high quality black tea. Theanine levels in TRFK 6/8 (0.47 % dw) was also not significantly different ($p > 0.05$) from two of its progenies: TRFK 303/178 (0.58 % dw) and TRFK 303/259 (0.55 % dw) but significantly different ($p < 0.05$) from TRFK 303/216 (0.66 % dw) which is also its progeny. This could also be due to genetic traits inherited from the mother bush.

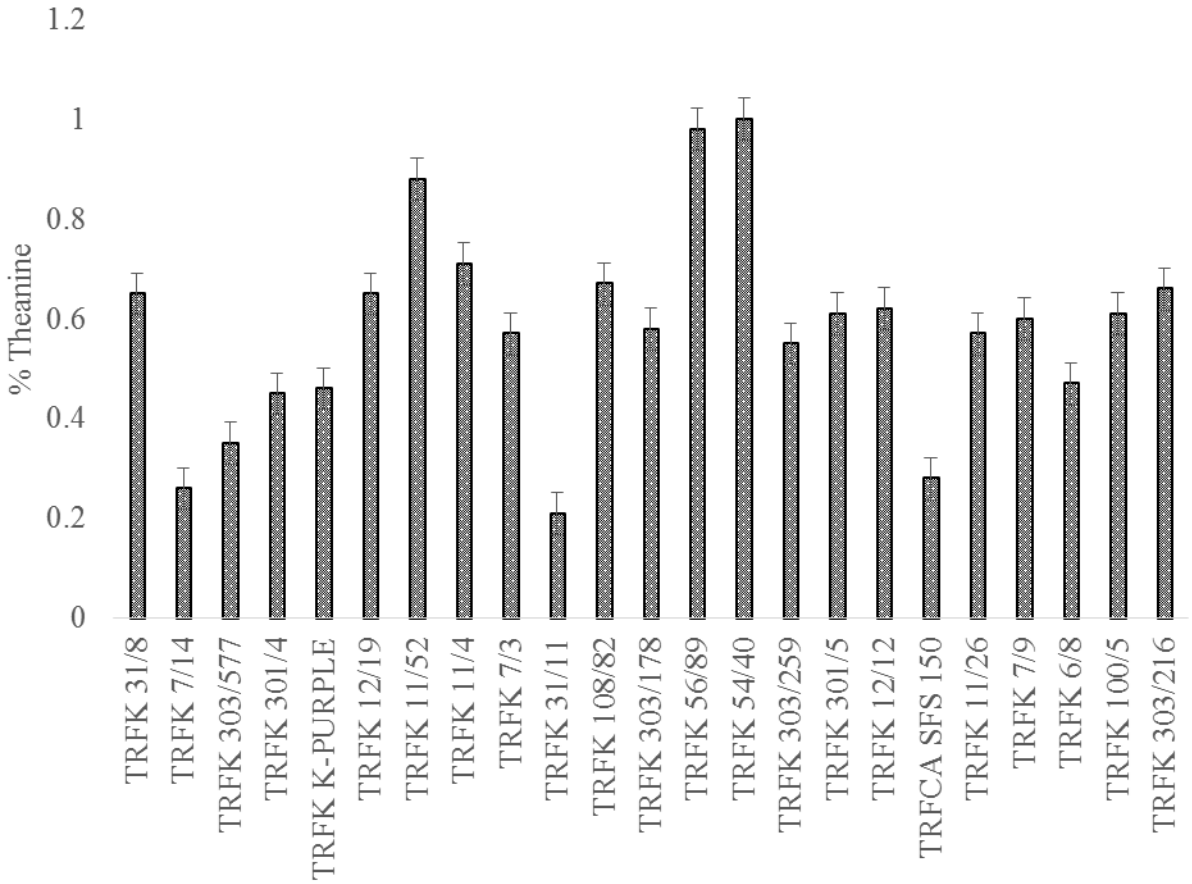


Figure 4.10: Clonal variation in theanine levels in black tea during the third season

Theanine levels varied in the three different seasons for the different clones (Fig. 4.11). Some clones contained high theanine levels during season one then decreased or increased during season two. In the third season, theanine levels decreased in most of the clones except a few which increased. This could have been due to the climatic conditions (Chen, Lin-Shiau & Lin, 2009; Chen et al., 2010) during this season which was hot and dry. There is no much documented work that has been done on theanine levels based on seasonal aspect and especially on these Kenyan tea clones. Theanine level variation in the selected tea clones could also be due to enzymes responsible for theanine synthesis: glutamine synthetase, glutamine synthase, glutamate dehydrogenase, alanine transaminase, alanine decarboxylase and theanine hydrolase which could be expressed differently in the different clones (Li et al., 2011).

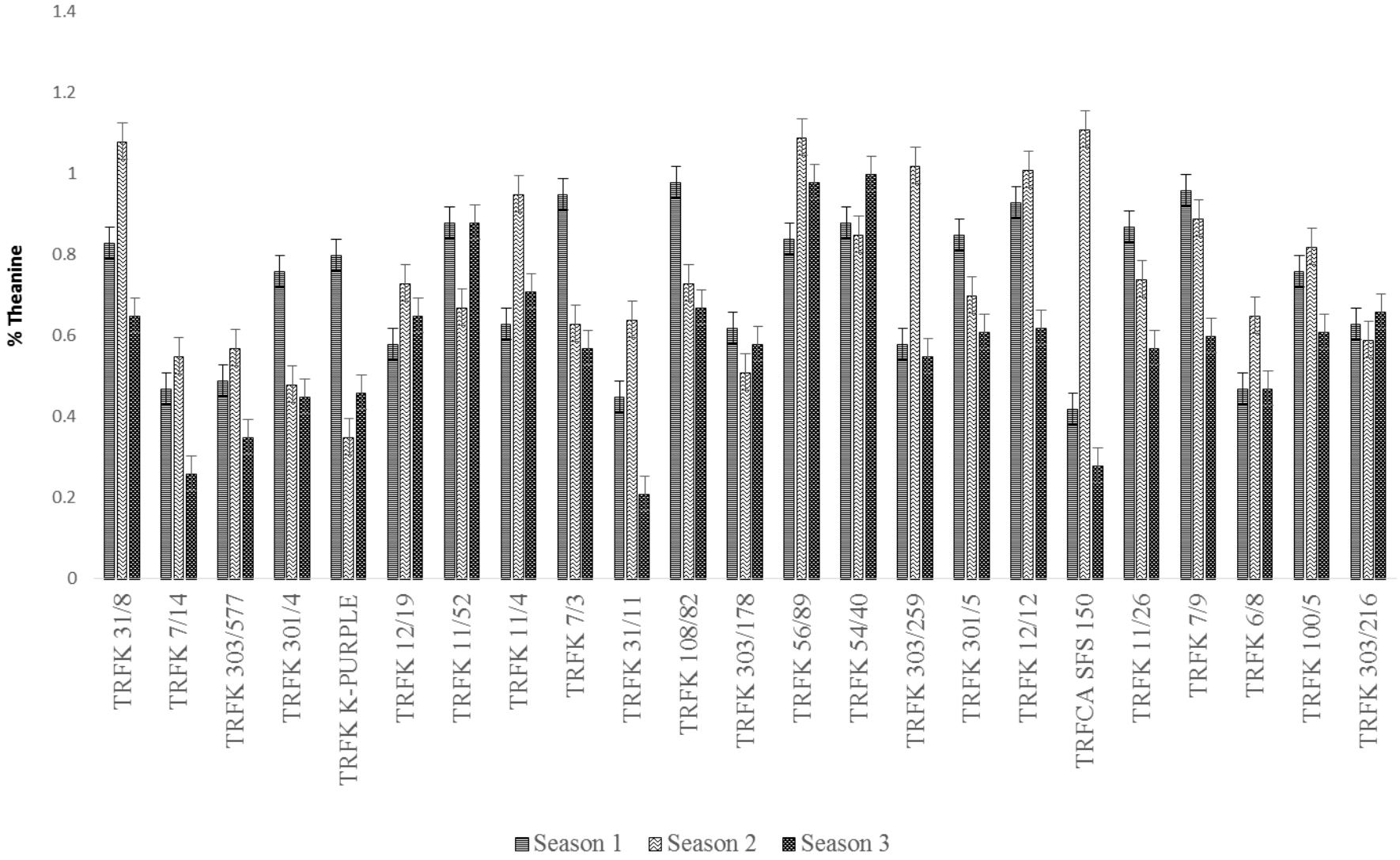


Figure 4.11: Theanine levels in black tea during three seasons

4.1.3 Theanine Levels Seasonal Variation in Green and Black Tea

The results from the seasons were also averaged and graphically represented as in figure 4.12. The results from the three seasons showed that green tea contained more theanine than black tea in most of the clones analyzed. TRFK 31/8 contained the highest theanine level in green tea with 1.20 % dw and the lowest being TRFK 303/216 with 0.50 % dw while in black tea TRFK 56/89 contained the highest theanine with 0.97 % dw and TRFK 7/14, TRFK 31/11 contained the lowest with 0.43 % dw each. TRFK K-Purple contained theanine comparable to the ordinary green tea clones such as TRFK 301/4, TRFK 301/5, TRFK 12/19, TRFK 11/4, TRFK 6/8, and TRFK 100/5 in the green tea and even higher than some other green tea clones in this study. TRFK 6/8 contained 0.53 % dw and has all along been known to be best for black tea manufacture. TRFK 6/8 levels were lower than those of the progenies.

Most of the green tea processed from the selected tea clones and analyzed in this study contained more theanine than black tea processed from the same clones. Also, several challenges were encountered including machine breakdown which could result in green tea processing going on till late at night or until the following day whereby green tea underwent withering and yet they were not supposed to wither. The delay in processing might have also resulted in other clones having no significant difference ($p > 0.05$) in theanine levels for both black and green tea like TRFK K–Purple and TRFK 7/3 on average during the three seasons. Theanine levels variations in clones could have been due to difference in genetic traits since every clone has different gene traits. TRFK 6/8 being well known for black tea also contained theanine in comparable amounts to other clones sampled in this study and especially K- Purple which is also anthocyanin rich. Kilel et al. (2013) analyzed some clones that were done in this study though from a different location (Kangaita) whereby they processed green tea and there was a significant difference in these clones except TRFK 303/577 whose levels were 1.0 and 0.95 % dw in this study. TRFK 31/8, TRFK 303/216, TRFK 6/8 and K- Purple contained 1.01, 1.26, 0.92, 1.10 % dw in Kilel et al. (2013) study and 1.35, 0.68, 0.54, 1.04 % dw, respectively in this study. Further, this comparison suggests regional variations, an aspect not investigated in the current study. Different regions have different climatic conditions and this affects theanine levels. Indeed, climatic and/or regional variations and agronomic practices have been reported to influence the accumulation of theanine in tea leaves (Chen et al., 2009; Chen et al., 2010).

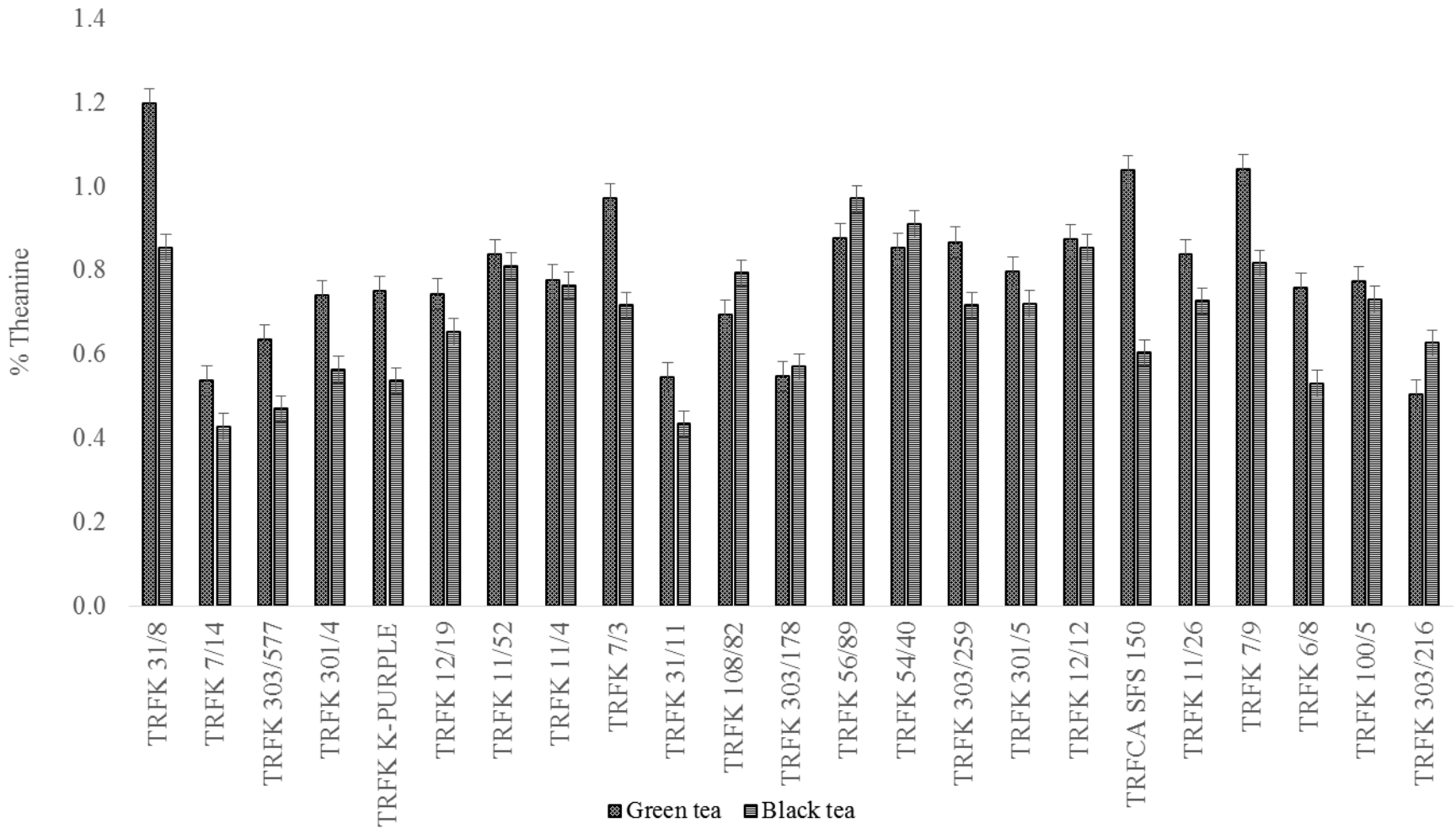


Figure 4.12: Average levels of theanine for green and black teasfrom 23 Kenyan tea clones

Theanine has been reported to be antihypertensive and this was shown to be dose dependent. A dose of 50 - 200 mg per day was shown to be of health benefits (Song, Jung, Oh & Kim, 2003; Nobre et al., 2008). In this study, green and black teas processed from their respective clones were shown to be of health benefit since their theanine levels on average were high. For every cup of tea, 2 g of tea leaves (tea bags) are used and upon conversion into the amount contained in a cup, a clone like TRFK 31/8 (1.20 % dw) contained 24 mg of theanine in a cup of tea assuming a tea bag is infusion (tea bags weighs approximately 2 grams). After drinking 3 cups (72 mg) of tea infusion from this clone (TRFK 31/8) will be of health benefit, antihypertensive. The lowest theanine levels on average obtained in this study was 0.43 % dw in TRFK 31/11, TRFK 7/14 and both were black tea. One would take 6 cups (51.6 mg) of tea to achieve the health benefits of theanine. This could seem impractical but in order to achieve the health benefit, one has to take more cups of tea.

4.2 Theanine Levels in the Different Partitions of the Tea Shoot

This study was further subdivided into two where one clone (TRFK 301/1) was studied to determine effect of sunlight exposure and different withering durations on theanine levels and a further two (TRFK 91/1 and TRFK 301/5) were studied to determine theanine levels in the different partitions of the tea shoot.

4.2.1 Effect of Sunlight Exposure on Theanine Levels

Theanine levels were determined in various partitions of the tea shoot and in this study three leaves and a bud were sampled for partitioning. TRFK 301/1 cultivar was used in this study and it was selected based on earlier findings by Kilel et al., (2013) study who had determined theanine levels in some commercial green clones. This clone contained high levels of theanine (2.03 % dw). Young two-leaf flushes are typically chosen for manufacturing the finest tea because theanine accumulates in growing shoots (Chu et al., 1997). The tea leaves (3 leaves + bud) were partitioned into: buds, first leafs, first leaf internodes, second leafs, second leaf internodes, third leafs and third leaf internodes. The results obtained were as shown in figure 4.13.

The buds contained 1.47 % dw of theanine at the start of the experiment and after three hours the levels decreased to 0.85 % dw. After six hours theanine levels increased to 1.56, 1.67 % dw respectively after nine hours and decreased to 0.63 % dw after twelve hours of sunlight exposure. There was a significant difference ($p < 0.05$) in theanine levels at the

start and after three and twelve hours but there was no significant difference ($p > 0.05$) after six and nine hours.

The first leaf contained 2.02 % dw of theanine at the start and after three hours the levels decreased to 0.95 % dw. The levels increased to 1.12, 1.22 and 1.68 % dw after six, nine and twelve hours respectively. Theanine levels were high at the start and were significantly different ($p < 0.05$) from the rest of the durations. There was no significant difference ($p > 0.05$) in theanine levels in the results obtained after three, six and nine hours. There was a significant difference ($p < 0.05$) in the theanine levels obtained after twelve hours from all the other durations.

The first leaf internode contained 3.88 % dw of theanine at the start and the levels increased to 4.87, 5.20, 5.49 and 5.78 % dw after three, six, nine, twelve hours respectively. There was a significant difference ($p < 0.05$) in theanine levels at the start and during the other durations but there was no significant difference ($p > 0.05$) in theanine levels after three, six, nine and twelve hours.

The second leaf contained 0.98 % dw of theanine at the start and increased to 1.41 % dw after three hours. Theanine levels decreased to 1.27 % dw after six hours and increased to 1.46 % dw and 1.84 % dw after nine and twelve hours respectively. There was a significant difference ($p < 0.05$) in theanine levels at the start of the study and after three, nine and twelve hours but there was no significant difference in theanine levels at the start and after six hours.

The second leaf internode contained 5.62 % dw of theanine at the start and decreased to 5.43 and 2.25 % dw after three and six hours respectively. Theanine levels increased to 6.15 % dw after nine hours and decreased to 4.64 % dw after twelve hours. There was a significant difference ($p < 0.05$) in theanine levels at the start and after six, nine and twelve hours but there was no significant difference ($p > 0.05$) in theanine levels at the start and after three hours.

The third leaf contained 1.20 % dw of theanine at the start and the levels decreased to 0.51 % dw after three hours. Theanine levels increased to 2.00 % dw after six hours, decreased slightly to 1.90 % dw after nine hours and increased to 2.01 % dw after twelve hours. There was a significant difference ($p < 0.05$) in theanine levels at the start and after three, six, nine and twelve hours but there was no significant difference ($p > 0.05$) in the levels obtained after six, nine and twelve hours.

The third leaf internode contained 4.90 % dw of theanine at the start and decreased to 1.71 % dw after three hours. The levels increased to 5.94, 6.11 % dw after six and nine hours

respectively then decreased to 5.54 % dw after twelve hours. There was a significant difference ($p < 0.05$) in theanine levels at the start and after all the durations in the study but there was no significant difference ($p > 0.05$) after six and nine hours.

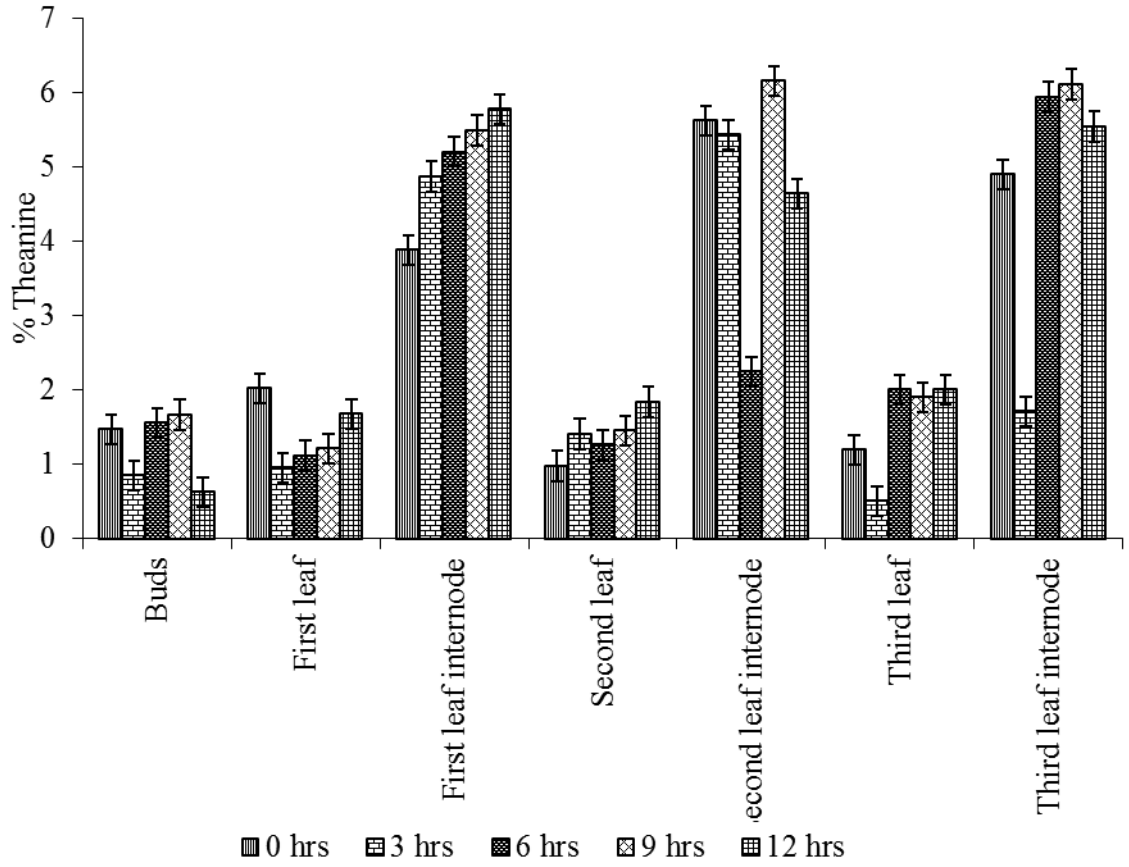


Figure 4.13: Effect of sunlight exposure on theanine levels in tea shoots

Theanine levels have been reported to be affected by sunlight exposure (Kyle et al., 2007). In this study, theanine levels decreased after being exposed to sunlight, this was after 3 hours since this was the only duration where there was maximum sunlight and theanine levels decreased in four of the partitions. There was an increase in theanine levels in the first leaf internode and second leaf, and no a slight decrease though not significant ($p > 0.05$) in the second leaf internode. The internodes were shown to contain high levels of theanine compared to the leaves. There was no linear pattern to show where theanine can be found to be optimum with respect to the different durations. With the recommended plucking standards of two leaves and a bud, theanine levels ranged between 2.23 – 2.98 % dw while for three leaves and a bud the range was 2.25 - 3.43 % dw. Three leaves and a bud were shown to contain higher theanine levels than two leaves and a bud. Chen et al., (2003) conducted a study to determine concentrations of theanine in fresh tea leaves and found out

that there was no linear pattern of increasing or decreasing concentrations with respect to age of the leaf and this concurs with the results obtained in this study. There is no documented work done on the internodes as a separate partition. Baptista, Lima, Paiva, Andrade & Alves (2012), Chen et al.,(2010) and Song, Kelman, Johns & Wright (2012) conducted studies on theanine levels of fresh tea leaves and concluded that theanine levels decrease with age of the leaf and this was not so in this study. The third leaf theanine levels were higher than that of the second leaf and therefore more study has to be done with more leaves.

4.2.2 Effect of Withering Duration on Theanine Levels

Theanine levels were also determined in the different partitions of the tea shoot as was done in the above study but now considering the effect of withering time varying from three to fifteen hours and the results were as shown in figure 4.14. This was meant to determine if theanine levels increase or decrease upon withering in green tea.

The buds contained 0.73 % dw of theanine after three hours and increased to 1.41 % dw after six hours. Theanine levels decreased to 0.66 % dw after nine hours, increased to 1.52 % dw after twelve hours then decreased slightly to 1.49 % dw after fifteen hours. There was no significant difference ($p > 0.05$) in theanine levels after three and nine hours but were significantly different ($p < 0.05$) from the levels obtained after six, twelve and fifteen hours. There was also no significant difference ($p > 0.05$) in theanine levels after six, twelve and fifteen hours of withering.

The first leaf contained 0.61% dw of theanine after three hours and increased to 0.79 % dw after six hours. Theanine levels decreased to 0.54 % dw after nine hours and increased to 0.87, 1.18 % dw after twelve and fifteen hours respectively. There was no significant difference ($p > 0.05$) in theanine levels obtained after three, six, nine and twelve hours. There was also no significant difference ($p > 0.05$) in theanine levels after six, twelve and fifteen hours. There was a significant difference ($p < 0.05$) in theanine levels obtained after three and nine hours of withering with the levels after fifteen hours.

The first leaf internode contained 1.86 % dw of theanine after three hours. Theanine levels increased to 3.94, 5.34 % dw after six and nine hours respectively. The levels then decreased to 1.57 % dw after twelve hours and increased to 5.54 % dw after fifteen hours. There was no significant difference ($p > 0.05$) in theanine levels obtained after three and twelve hours of withering and also after nine and fifteen hours of withering. There was a significant difference ($p < 0.05$) in theanine levels obtained after six hours from the other durations.

The second leaf contained 0.52 % dw of theanine after three hours and the levels increased to 0.95 % dw after six hours. Theanine levels decreased to 0.76 %dw after nine hours, increased to 1.12 % dw after twelve hours and decreased to 0.72 % dw after fifteen hours. There was no significant difference ($p > 0.05$) in theanine levels obtained after three, nine and fifteen hours but a significant difference ($p < 0.05$) in the levels obtained after three hours with levels after six and twelve hours. There was also no significant difference ($p > 0.05$) in theanine levels obtained after six, nine, twelve and fifteen hours of withering.

The second leaf internode contained 2.66 % dw of theanine after three hours and increased to 5.97 % dw after six hours. The levels decreased to 5.24 % dw after nine hours, increased to 6.11 % dw after twelve hours and slightly decreased to 6.02 % dw after fifteen hours. There was a significant difference ($p < 0.05$) in theanine levels obtained after three and nine hours from the other durations but no significant difference ($p > 0.05$) in the levels obtained after six, twelve and fifteen hours of withering.

The third leaf contained 1.14 % dw of theanine after three hours and increased to 2.01 % dw after six hours. The levels decreased to 1.79, 1.64 % dw after nine and twelve hours respectively. After fifteen hours, the levels increased to 1.71 % dw. There was a significant difference ($p < 0.05$) in theanine levels obtained after three hours of withering from the other durations. There was no significant difference ($p > 0.05$) in theanine levels obtained after six, nine, fifteen hours and also after nine, twelve, fifteen hours of withering.

The third leaf internode contained 2.32 % dw of theanine after three hours. The levels increased to 5.01, 5.18 and 5.20 % dw after six, nine and twelve hours respectively. The levels decreased to 5.13 % dw after fifteen hours. There was a significant difference ($p < 0.05$) in theanine levels after three hours of withering from the other durations. There was no significant difference ($p > 0.05$) in theanine levels after six, nine, twelve and fifteen hours of withering.

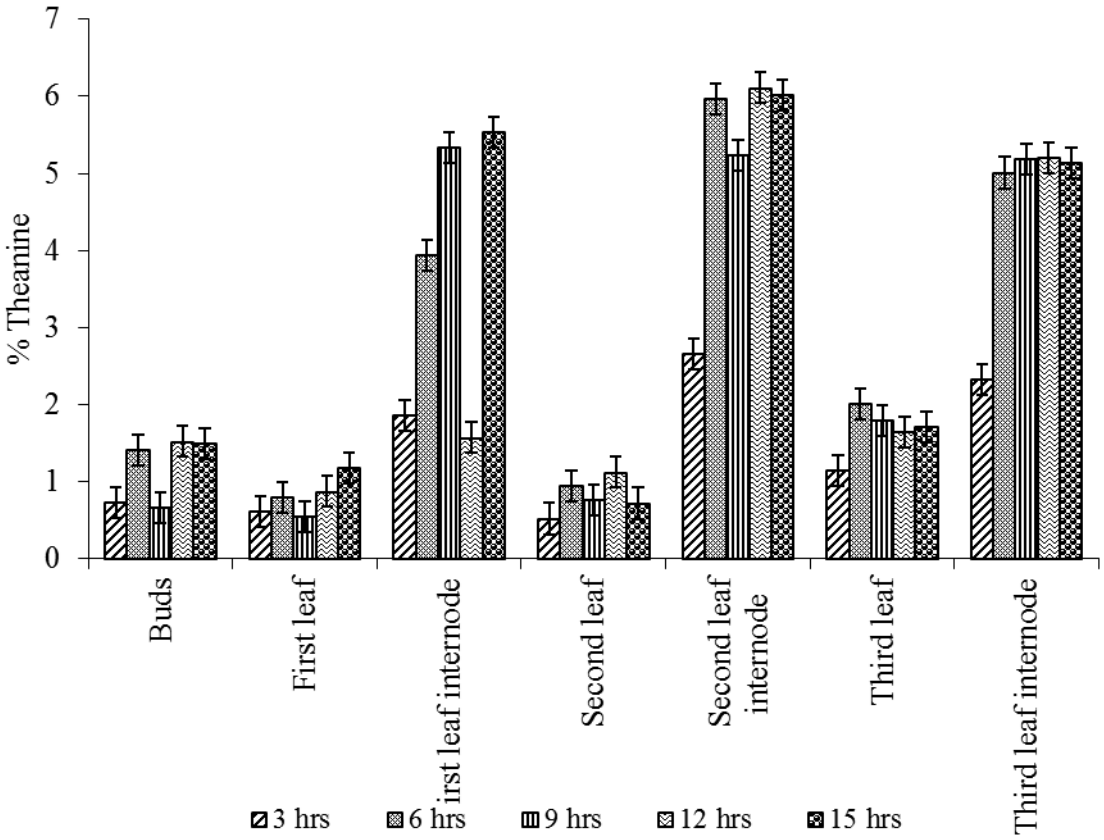


Figure 4.14: Effect of withering duration on theanine levels in tea shoot

Theanine levels varied in all the partitions in this study except in the third leaf internode. The internodes were shown to contain high theanine levels compared to the other partitions and especially the second leaf internode. The first two leaves contained the lowest. Two leaves and a bud are plucked to obtain quality tea though three leaves and bud can be plucked but in small amounts. In appendix IV, theanine levels were shown to increase with age of the leaf; high levels in 3 leaves and a bud than 2 leaves and a bud. Withering duration of 3 hours resulted in a decrease in theanine levels from 3.16 to 1.41 % dw (Fig. 4.15) of the fresh tea leaves.6 hours of withering also resulted in a decrease in theanine levels while there was a slight increase after 9 hours of withering from 2.76 to 2.79 % dw. After 12 hours and 15 hours of withering, theanine levels increased with the highest level shown after 15 hours increasing from 2.87 to 3.11 % dw.

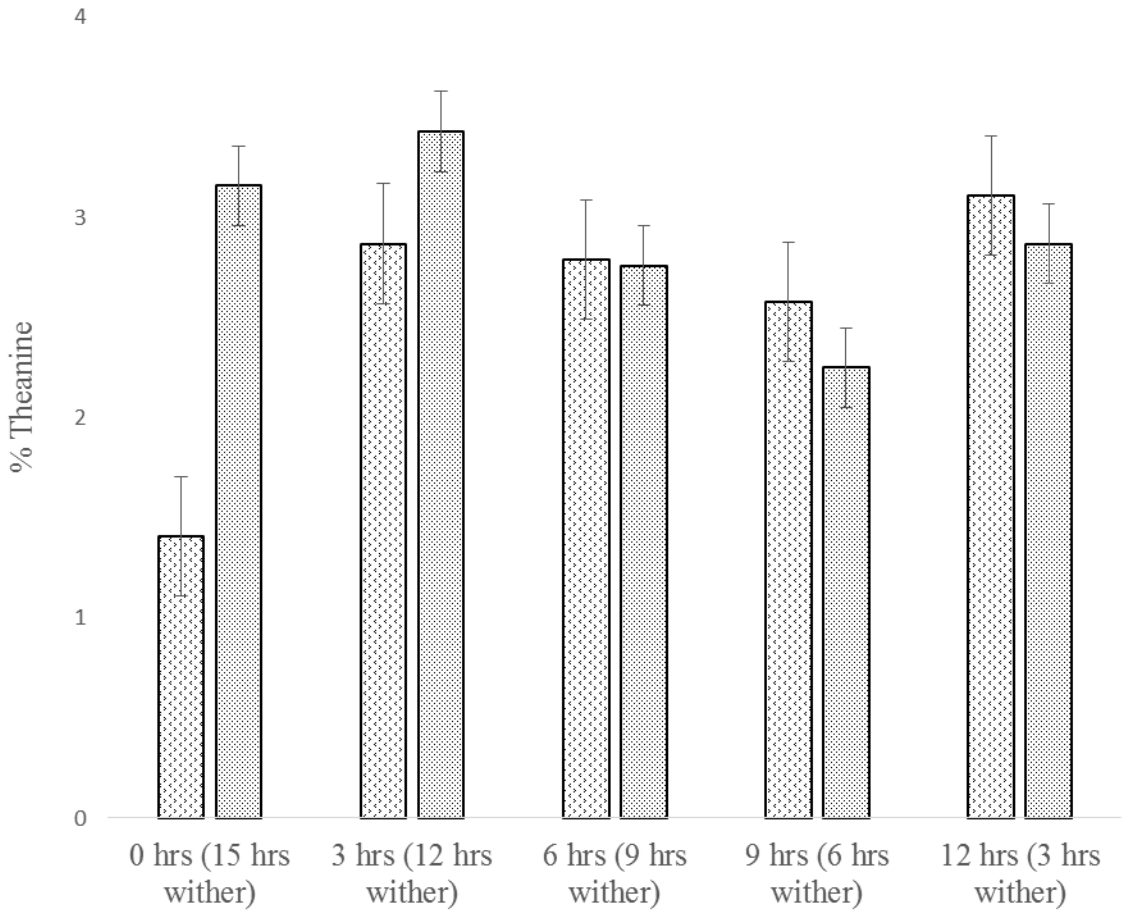


Figure 4.15: Theanine levels on the tea shoot when fresh and upon withering

Jabeen et al. (2015) determined the effect of different withering timings on theanine levels and found out that the levels continually increased until 23 hours of wither then begin to decrease afterwards. This was in agreement with this study even though withering was only done for 15 hours and the withering duration when theanine levels will decline was not established.

4.2.3 Theanine Levels in Two Different Leaf Coloured Clones

Two clones were also analyzed for theanine levels in different partitions: bud, first leaf, second leaf, third leaf and internode. The samples were collected early in the morning and this was done to determine if there could be a variation in theanine content in a purple coloured and green coloured cultivar. Unlike in the previous study where each leaf's internode was partitioned, here the internode was in whole. TRFK 91/1 and TRFK 301/5

were the clones used in this study and the results were as shown in figure 4.16 (Appendix III). A calibration line was obtained and the equation $y = 9482.7x + 9162.8$ (r^2=0.9991).

TRFK 91/1 contained 0.50 % dw while TRFK 301/5 contained 0.79 % dw of theanine in the bud. Theanine levels in this partition for these clones were significantly different ($p < 0.05$). TRFK 91/1 contained 0.22 % dw while TRFK 301/5 contained 0.40 % dw of theanine in the first leaf. Theanine levels in this partition were significantly different ($p < 0.05$) in this partition. TRFK 91/1 contained 0.33 % dw while TRFK 301/5 contained 0.70 % dw of theanine in the second leaf. Theanine levels in this partition were significantly different ($p < 0.05$). TRFK 91/1 contained 0.34 % dw while TRFK 301/5 contained 0.74 % dw of theanine in the third leaf. Theanine levels in this partition were also significantly different ($p < 0.05$). TRFK 91/1 contained 0.42 % dw while TRFK 301/5 contained 0.59 % dw of theanine in the fourth leaf. There was a significant difference ($p < 0.05$) in theanine levels in this partition for the two clones. TRFK 91/1 contained 0.94 % dw while TRFK 301/5 contained 3.26 % dw of theanine in the internode. Theanine levels in this partition for the two clones were significantly different ($p < 0.05$).

TRFK 91/1 was a purple clone while TRFK 301/5 was an ordinary green tea clone. TRFK 301/5 contained the highest levels of theanine in all the partitions and had an average of 1.08 % dw while TRFK 91/1 had 0.46 % dw. The internode was the only partition that contained the highest amount of theanine. The results in this study showed that green tea clones contained more theanine than purple coloured tea clones. A study conducted by Kilel et al., (2013), showed that purple clones (1.44 % dw) contained more theanine than green tea clones (1.12 % dw) and this was not in agreement with this study. This could have been due to difference in the climatic conditions of the locations where these two studies were conducted: Kericho and Kangaita. Theanine levels could be affected by difference in climatic conditions (Lin et al., 1998; Jeng, Chen, Fang, Hou & Chen, 2007; Chen et al., 2009; Chen et al., 2010).

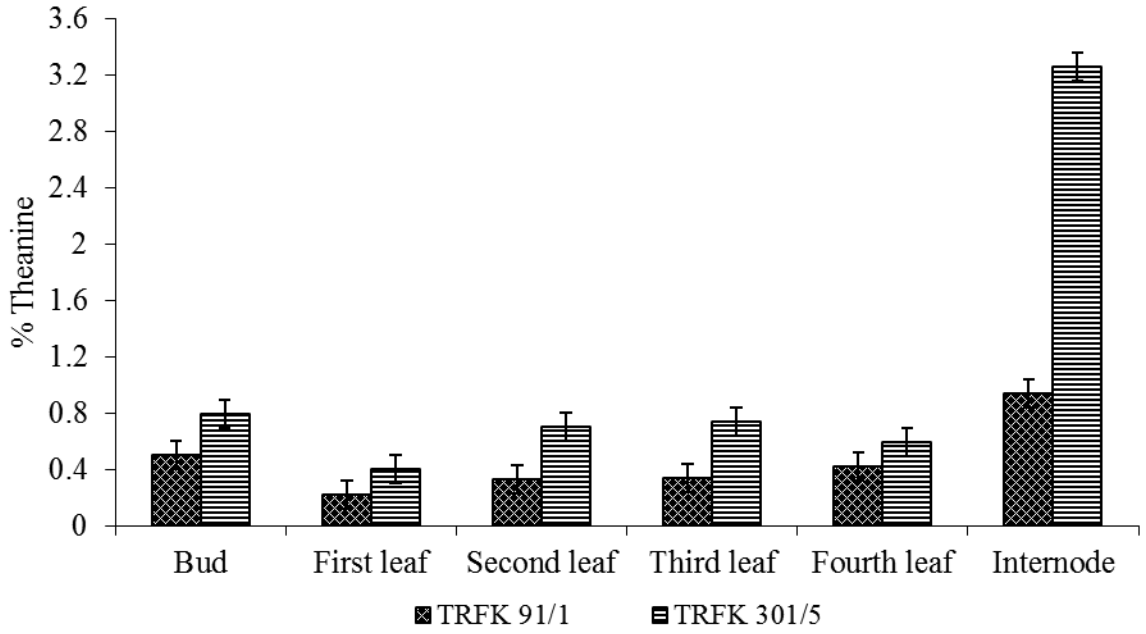


Figure 4.16: Theanine levels in tea shoot oftwo clones

4.3 Theanine Levels in Some Selected Teas Available in the Kenyan Market

This final study was done to determine theanine levels in processed (made) tea from different origins that were available in the Kenyan market. These samples were obtained from Mombasa tea auction and were from Rwanda, Tanzania, Uganda, Sri Lanka, Cameroon and Kenya. The teas were packaged in two forms: loose leaf and tea bags. The results obtained were as shown in figure 4.17 (Appendix XI). The theanine level in Kenyan black tea was 1.02 % dw which was not significantly different ($p > 0.05$) from the Rwandan and Tanzanian black teas whose levels were 0.98 and 1.09 % dw respectively. The Cameroon and Sri Lanka black teas theanine levels were 0.48 and 0.52 % dw respectively and significantly different ($p < 0.05$) and lower than that of the Kenyan black tea. Uganda's black tea theanine level could not be determined in this study since it was below the limit of detection (LOD < 0.01 %). The Rwandan green tea contained the highest level of theanine with 1.6 and 1.16% dw while the Kenyan green tea (flavoured with peach) contained 0.12 % dw of theanine. Theanine can be used as a tea marker and all teas must contain it (Wachira, Kamunya, Karori, Chalo & Maritim, 2013). In this study, there were flavoured teas analyzed and their theanine levels could not be detected since they were below the limit of detection (LOD < 0.01 %). The teas only contained the flavours with no tea in it yet they are marketed as tea. Upon consumption of these teas, they will be of no health benefit.

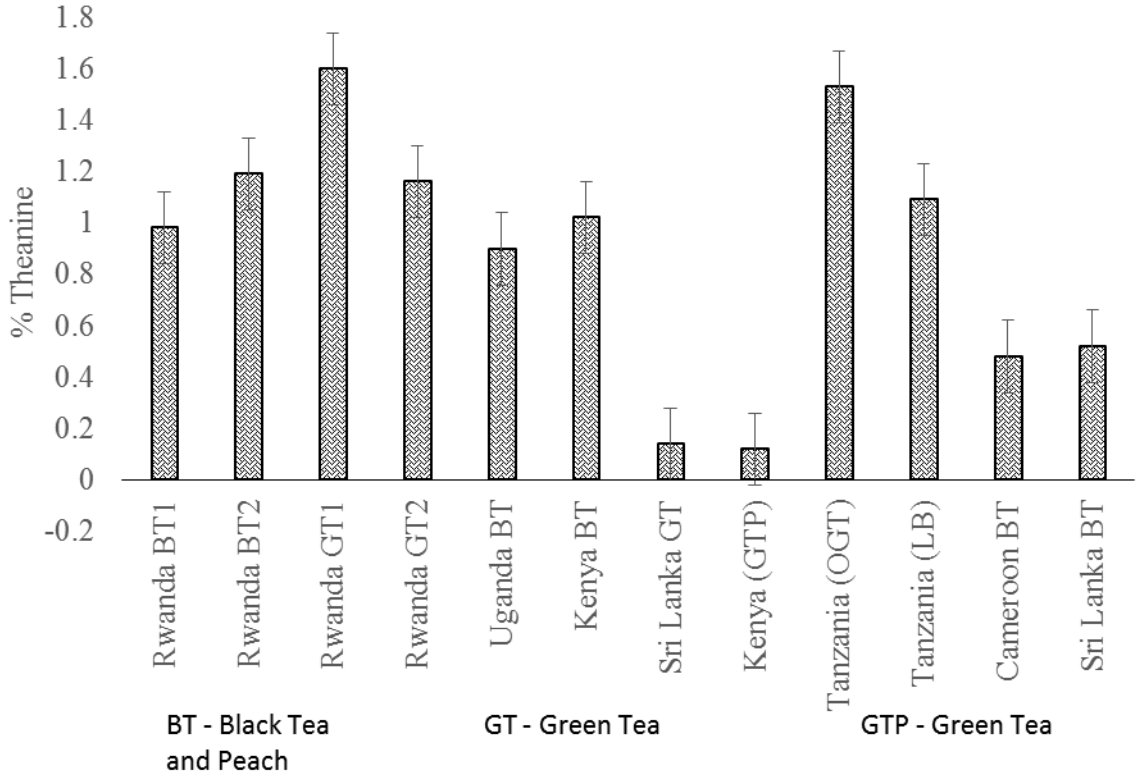


Figure 4.17: Theanine levels in made teain the Kenyan market

Kenyan flavoured green tea (GTP) in the market with theanine levels of 0.12 % dw was comparable to green tea processed from TRFK 303/178 and TRFK 303/216 during the third season (January – March, 2012) which contained 0.14 and 0.19 % dw respectively. This could mean that even flavoured teas are comparable to non flavoured teas because the Kenyan green tea in the market was flavoured with peach. Also the green tea in the market may have been plucked during a season when theanine levels were low (season 3 in this study). Kenyan green tea in the market (GTP) theanine level was 1.2 mg/g (0.12 % dw) in this study and was in agreement with Wang et al., (2010), Alcazar et al., (2007) and Thippeswamy et al., (2006) studies whose levels was 1.13 mg/g. Keenan et al.,(2011), Gong et al., (2012), Karem, Mario & Mario(2013) obtained theanine levels higher than Kenyan green tea in this study: 2.58 – 4.23 mg/g, 14.5mg/g and 4.82 mg/g respectively but Gong et al.,(2012) levels were comparable to the Rwandan green teas. This could have been due to difference in climatic conditions for the different locations.

Kenyan black tea (BT) in the market contained theanine levels of 1.02 % dw which was comparable to black teas processed from different clones in the three seasons conducted

in this study. It was comparable to TRFK 7/3 (0.95 % dw), TRFK 7/9 (0.96 % dw), TRFK 108/82 (0.98 % dw) during the first season; TRFK 12/12 (1.01 % dw), TRFK 303/259 (1.02 % dw) during the second season and TRFK 54/40 (1.00 % dw), TRFK 56/89 (0.98 % dw) during the third season. TRFK 6/8 a well-known cultivar for black tea contained lower theanine levels than the Kenyan black tea analyzed in this study. This could have been because made teas in the Kenyan market are not processed from specific clones but from a variety of clones while others are blended with teas from different origins. Black tea theanine level was 10.2 mg/g in this study and was in agreement with Keenan et al. (2011) theanine levels of 9.1 mg/g. This study also showed that Kenyan black tea (10.2 mg/g) contains high levels of theanine compared to results obtained by Thippeswamy et al. (2006) whose range was 1.31 – 4.16 mg/g. These different results for teas could also have been probably due to the effects of origin, cultivar variety, leaf age, growing area, horticultural practices and plucking season, and the microorganism used for fermentation on metabolites of green tea products (Lin et al., 1998; Jeng et al., 2007; Chen et al., 2009; Chen et al., 2010).

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

i. Clones with high levels of theanine were identified. Upon processing green and black tea from the respective clones, green tea contained higher levels of theanine than black tea. TRFK 31/8, TRFK 7/9, TRFK 303/259, TRFCA SFS 150 were among clones with high theanine levels. There was a variation in theanine levels in the different clones and seasons. Cold and wet season were the best seasons for obtaining high theanine levels

ii. Theanine levels varied in the different partitions of the tea shoot. The internode contained the highest levels of theanine. Three leaves and a bud contained higher theanine levels than two leaves and a bud. It was also shown that withering for long durations resulted in an increase in theanine levels in green tea. The ordinary green tea cultivar (TRFK 301/5) contained high levels of theanine than purple coloured tea clone (TRFK 91/1).

iii. Theanine levels varied with origin of tea. Rwandan green and black tea were shown to contain high levels. Kenyan black tea in the market was comparable to teas from other origins especially black tea from Tanzania (LB) and one of the Rwanda's. Specialty teas did not contain theanine and this could have been flavours only.

5.2 Recommendations

i. Research to be done on the remaining released clones that were not analyzed in this study and clones from other stake holders: Unilever, African Highlands Produce and George Williamson. Plant breeder to determine the trait in clones with high theanine levels and come up with a clone with high theanine levels.

ii. Research to be done on the roots of the tea plant since this is where it is biosynthesized and most of it may be accumulated there. More studies to be carried out to determine a withering duration when theanine levels increases to the maximum. Since the internode was shown to contain high theanine levels, pharmaceutical companies can extract theanine from this partition thus diversifying our tea.

iii. More teas in the Kenyan market need to be analyzed and especially the flavoured teas. Theanine is a tea marker and since flavoured teas analyzed in this study the levels were below the limit of detection (0.01 mg/g) then more research has to be done on this teas to determine whether they are processed from *Camellia sinensis*.

iv. Research to be carried out to determine regional variation on theanine levels: Kangaita, Kericho and Sotik highlands.

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APPENDICES

Appendix I: A Model of the TRI museum

1b	1a	1c		2b	2a	2c		3b	3a	3c		4b	4a	4c
5b	5a	5c		6b	6a	6c		7b	7a	7c		8b	8a	8c
9b	9a	9c		10b	10a	10c		11b	11a	11c		12b	12a	12c
13b	13a	13c		14b	14a	14c		15b	15a	15c		16b	16a	16c
17b	17a	17c		18b	18a	18c		19b	19a	19c		20b	20a	20c
21b	21a	21c		22b	22a	22c		23b	23a	23c				

Appendix II: Sample output of Analysis of variance (ANOVA) for theanine levels in black tea for the three seasons

Function: FACTOR

Experiment Model Number 2: Completely Randomized Design for Factor A, Factor B is a Split Plot

Data case no. 1 to 207.

Factorial ANOVA for the factors:

Replication (reps) with values from 1 to 3

Factor A (seasons) with values from 1 to 3

Factor B (clones) with values from 1 to 23

Variable 4: % theanine

Grand Mean = 0.687 Grand Sum = 142.220 Total Count = 207

T A B L E O F M E A N S

3	2	1	4	Total
*	1	*	0.723	49.910
*	2	*	0.755	52.110
*	3	*	0.583	40.200
*	*	1	0.852	7.670
*	*	2	0.653	5.880
*	*	3	0.854	7.690
*	*	4	0.811	7.300
*	*	5	0.724	6.520
*	*	6	0.762	6.860
*	*	7	0.816	7.340
*	*	8	0.719	6.470
*	*	9	0.530	4.770
*	*	10	0.434	3.910

*	*	11	0.729	6.560
*	*	12	0.794	7.150
*	*	13	0.971	8.740
*	*	14	0.602	5.420
*	*	15	0.427	3.840
*	*	16	0.909	8.180
*	*	17	0.472	4.250
*	*	18	0.719	6.470
*	*	19	0.572	5.150
*	*	20	0.628	5.650
*	*	21	0.566	5.090
*	*	22	0.719	6.470
*	*	23	0.538	4.840

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*	1	1	0.827	2.480
*	1	2	0.580	1.740
*	1	3	0.933	2.800
*	1	4	0.877	2.630
*	1	5	0.867	2.600
*	1	6	0.627	1.880
*	1	7	0.960	2.880
*	1	8	0.953	2.860
*	1	9	0.470	1.410
*	1	10	0.450	1.350
*	1	11	0.757	2.270
*	1	12	0.980	2.940
*	1	13	0.837	2.510
*	1	14	0.420	1.260
*	1	15	0.473	1.420
*	1	16	0.880	2.640
*	1	17	0.497	1.490
*	1	18	0.583	1.750
*	1	19	0.623	1.870
*	1	20	0.633	1.900

*	1	21	0.760	2.280
*	1	22	0.847	2.540
*	1	23	0.803	2.410
*	2	1	1.080	3.240
*	2	2	0.727	2.180
*	2	3	1.013	3.040
*	2	4	0.673	2.020
*	2	5	0.740	2.220
*	2	6	0.953	2.860
*	2	7	0.890	2.670
*	2	8	0.633	1.900
*	2	9	0.653	1.960
*	2	10	0.640	1.920
*	2	11	0.823	2.470
*	2	12	0.730	2.190
*	2	13	1.093	3.280
*	2	14	1.110	3.330
*	2	15	0.550	1.650
*	2	16	0.847	2.540
*	2	17	0.570	1.710
*	2	18	1.020	3.060
*	2	19	0.510	1.530
*	2	20	0.593	1.780
*	2	21	0.477	1.430
*	2	22	0.697	2.090
*	2	23	0.347	1.040
*	3	1	0.650	1.950
*	3	2	0.653	1.960
*	3	3	0.617	1.850
*	3	4	0.883	2.650
*	3	5	0.567	1.700
*	3	6	0.707	2.120
*	3	7	0.597	1.790
*	3	8	0.570	1.710

*	3	9	0.467	1.400
*	3	10	0.213	0.640
*	3	11	0.607	1.820
*	3	12	0.673	2.020
*	3	13	0.983	2.950
*	3	14	0.277	0.830
*	3	15	0.257	0.770
*	3	16	1.000	3.000
*	3	17	0.350	1.050
*	3	18	0.553	1.660
*	3	19	0.583	1.750
*	3	20	0.657	1.970
*	3	21	0.460	1.380
*	3	22	0.613	1.840
*	3	23	0.463	1.390

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
2	Factor A	2	1.164	0.582	50.0878	0.0002
-3	Error	6	0.070	0.012		
4	Factor B	22	4.545	0.207	40.3067	0.0000
6	AB	44	3.432	0.078	15.2179	0.0000
-7	Error	132	0.677	0.005		
	Total	206	9.887			

Coefficient of Variation: 10.42%

s_ for means group 2: 0.0130 Number of Observations: 69

y

s_ for means group 4: 0.0239 Number of Observations: 9

y

s_ for means group 6: 0.0413 Number of Observations: 3

y

Appendix III: Percentage theanine in different partitions

Percentage theanine in for the selected two clones (n=3, M ± SD)

TRFK 91/1		TRFK 301/5	
Bud	$0.50^{ef} \pm 0.02$	Bud	$0.79^{bc} \pm 0.02$
1st leaf	$0.22^{g} \pm 0.01$	1st leaf	$0.40^{f} \pm 0.01$
2nd leaf	$0.33^{fg} \pm 0.02$	2nd leaf	$0.70^{cd} \pm 0.02$
3rd leaf	$0.34^{fg} \pm 0.02$	3rd leaf	$0.74^{cd} \pm 0.02$
4th leaf	$0.42^{ef} \pm 0.01$	4th leaf	$0.59^{de} \pm 0.02$
Internode	$0.94^{b} \pm 0.02$	Internode	$3.26^{a} \pm 0.35$

Appendix IV: Theanine levels in the pluckable tea shoots

Effect of sunlight exposure (hrs)	3 leaves + bud (% theanine)	2 leaves + bud (% theanine)	Withering time (hrs)	3 leaves + bud (% theanine)	2 leaves + bud (% theanine)
0	2.87	2.76	15	3.11	2.78
3	2.25	2.98	12	2.58	2.14
6	2.76	2.23	9	2.79	2.39
9	3.43	2.34	6	2.87	2.51
12	3.16	2.53	3	1.41	1.25

Appendix V: Theanine levels in green tea during season one

Percentage theanine during season 1 (n=3, M ± SD)

TRFK 31/8	$1.35^{ab} \pm 0.04$	TRFK 56/89	$0.90^{efgh} \pm 0.02$
TRFK 7/14	$0.61^{ij} \pm 0.01$	TRFK 54/40	$1.14^{bcd} \pm 0.11$
TRFK 303/577	$0.95^{defg} \pm 0.08$	TRFK 303/259	$1.08^{cde} \pm 0.05$
TRFK 301/4	$0.79^{fghi} \pm 0.02$	TRFK 301/5	$0.90^{efgh} \pm 0.05$
TRFK K-PURPLE	$1.04^{cde} \pm 0.01$	TRFCA SFS 150	$1.50^{a} \pm 0.25$
TRFK 12/19	$0.79^{fghi} \pm 0.11$	TRFK 12/12	$0.96^{cdefg} \pm 0.01$
TRFK 11/52	$1.18^{bc} \pm 0.08$	TRFK 11/26	$1.04^{cde} \pm 0.08$
TRFK 11/4	$0.76^{ghij} \pm 0.01$	TRFK 7/9	$1.32^{ab} \pm 0.05$
TRFK 7/3	$1.00^{cdef} \pm 0.25$	TRFK 6/8	$0.54^{j} \pm 0.03$
TRFK 31/11	$0.63^{ij} \pm 0.01$	TRFK 100/5	$0.76^{ghij} \pm 0.04$
TRFK 108/82	$0.93^{defg} \pm 0.05$	TRFK 303/216	$0.68^{hij} \pm 0.05$
TRFK 303/178	$0.69^{hij} \pm 0.04$		

Appendix VI: Theanine levels in green tea during season two

Percentage theanine during season 2 (n=3, M ± SD)

TRFK 31/8	1.15^{bc} ± 0.02	TRFK 56/89	1.28^{ab} ± 0.05
TRFK 7/14	0.59^{j} ± 0.07	TRFK 54/40	0.90^{def} ± 0.09
TRFK 303/577	0.74^{ghi} ± 0.02	TRFK 303/259	1.10^{c} ± 0.03
TRFK 301/4	0.63^{ij} ± 0.08	TRFK 301/5	0.80^{efgh} ± 0.02
TRFK K-PURPLE	0.73^{ghij} ± 0.05	TRFCA SFS 150	1.34^{a} ± 0.12
TRFK 12/19	0.96^{d} ± 0.04	TRFK 12/12	1.11^{c} ± 0.10
TRFK 11/52	0.88^{def} ± 0.04	TRFK 11/26	0.79^{fgh} ± 0.03
TRFK 11/4	1.11^{c} ± 0.10	TRFK 7/9	1.28^{ab} ± 0.07
TRFK 7/3	0.85^{defg} ± 0.05	TRFK 6/8	0.82^{defgh} ± 0.05
TRFK 31/11	0.69^{hij} ± 0.04	TRFK 100/5	0.95^{de} ± 0.04
TRFK 108/82	0.92^{def} ± 0.07	TRFK 303/178	0.64^{ij} ± 0.08
TRFK 303/216	0.81^{defgh} ± 0.08		

Appendix VII: Theanine levels in green tea during season three

Percentage theanine during season 3 (n=3, M ± SD)

TRFK 31/8	$1.09^{a} \pm 0.23$	TRFK 56/89	$0.45^{g} \pm 0.03$
TRFK 7/14	$0.41^{gh} \pm 0.09$	TRFK 54/40	$0.52^{fg} \pm 0.03$
TRFK 303/577	$0.21^{ij} \pm 0.01$	TRFK 303/259	$0.42^{gh} \pm 0.09$
TRFK 301/4	$0.80^{bc} \pm 0.22$	TRFK 301/5	$0.69^{cd} \pm 0.08$
TRFK K-PURPLE	$0.48^{fg} \pm 0.06$	TRFCA SFS 150	$0.27^{ij} \pm 0.04$
TRFK 12/19	$0.48^{fg} \pm 0.02$	TRFK 12/12	$0.55^{efg} \pm 0.06$
TRFK 11/52	$0.45^{g} \pm 0.05$	TRFK 11/26	$0.68^{cde} \pm 0.07$
TRFK 11/4	$0.46^{fg} \pm 0.05$	TRFK 7/9	$0.52^{fg} \pm 0.11$
TRFK 7/3	$1.06^{a} \pm 0.06$	TRFK 6/8	$0.91^{b} \pm 0.22$
TRFK 31/11	$0.31^{hi} \pm 0.02$	TRFK 100/5	$0.61^{def} \pm 0.10$
TRFK 108/82	$0.23^{ij} \pm 0.01$	TRFK 303/216	$0.19^{ij} \pm 0.02$
TRFK 303/178	$0.14^{j} \pm 0.01$		

Appendix VIII: Theanine levels in black tea during season one

Percentage theanine during season 1 (n=3, M ± SD)

TRFK 31/8	$0.83^{bcd} \pm 0.02$	TRFK 56/89	$0.84^{bcd} \pm 0.01$
TRFK 7/14	$0.47^{fg} \pm 0.01$	TRFK 54/40	$0.88^{abcd} \pm 0.02$
TRFK 303/577	$0.49^{fg} \pm 0.02$	TRFK 303/259	$0.58^{ef} \pm 0.11$
TRFK 301/4	$0.76^{d} \pm 0.02$	TRFK 301/5	$0.85^{abcd} \pm 0.02$
TRFK K-PURPLE	$0.80^{cd} \pm 0.02$	TRFCA SFS 150	$0.42^{g} \pm 0.11$
TRFK 12/19	$0.58^{ef} \pm 0.04$	TRFK 12/12	$0.93^{abc} \pm 0.04$
TRFK 11/52	$0.88^{abcd} \pm 0.01$	TRFK 11/26	$0.87^{abcd} \pm 0.18$
TRFK 11/4	$0.63^{e} \pm 0.16$	TRFK 7/9	$0.96^{ab} \pm 0.02$
TRFK 7/3	$0.95^{ab} \pm 0.04$	TRFK 6/8	$0.47^{fg} \pm 0.02$
TRFK 31/11	$0.45^{g} \pm 0.08$	TRFK 100/5	$0.76^{d} \pm 0.02$
TRFK 108/82	$0.98^{a} \pm 0.23$	TRFK 303/216	$0.63^{e} \pm 0.14$
TRFK 303/178	$0.62^{e} \pm 0.16$		

Appendix IX: Theanine levels in black tea during season two

Percentage theanine during season 2 (n = 3, M ± SD)

TRFK 31/8	$1.08^{a} \pm 0.02$	TRFK 56/89	$1.09^{a} \pm 0.08$
TRFK 7/14	$0.55^{ijk} \pm 0.09$	TRFK 54/40	$0.85^{cde} \pm 0.09$
TRFK 303/577	$0.57^{hijk} \pm 0.03$	TRFK 303/259	$1.02^{ab} \pm 0.08$
TRFK 301/4	$0.48^{k} \pm 0.06$	TRFK 301/5	$0.70^{fgh} \pm 0.10$
TRFCA SFS 150	$1.11^{a} \pm 0.05$	TRFK 303/178	$0.51^{jk} \pm 0.07$
TRFK 12/19	$0.73^{efg} \pm 0.03$	TRFK 12/12	$1.01^{ab} \pm 0.14$
TRFK 11/52	$0.67^{ghi} \pm 0.12$	TRFK 11/26	$0.74^{efg} \pm 0.02$
TRFK 11/4	$0.95^{bc} \pm 0.13$	TRFK 7/9	$0.89^{cd} \pm 0.08$
TRFK 7/3	$0.63^{ghij} \pm 0.04$	TRFK 6/8	$0.65^{ghi} \pm 0.10$
TRFK 31/11	$0.64^{ghij} \pm 0.08$	TRFK 100/5	$0.82^{def} \pm 0.06$
TRFK 108/82	$0.73^{efg} \pm 0.04$	TRFK 303/216	$0.59^{hijk} \pm 0.07$
TRFK K-PURPLE	$0.35^{l} \pm 0.03$		

Appendix X: Theanine levels in black tea during season three

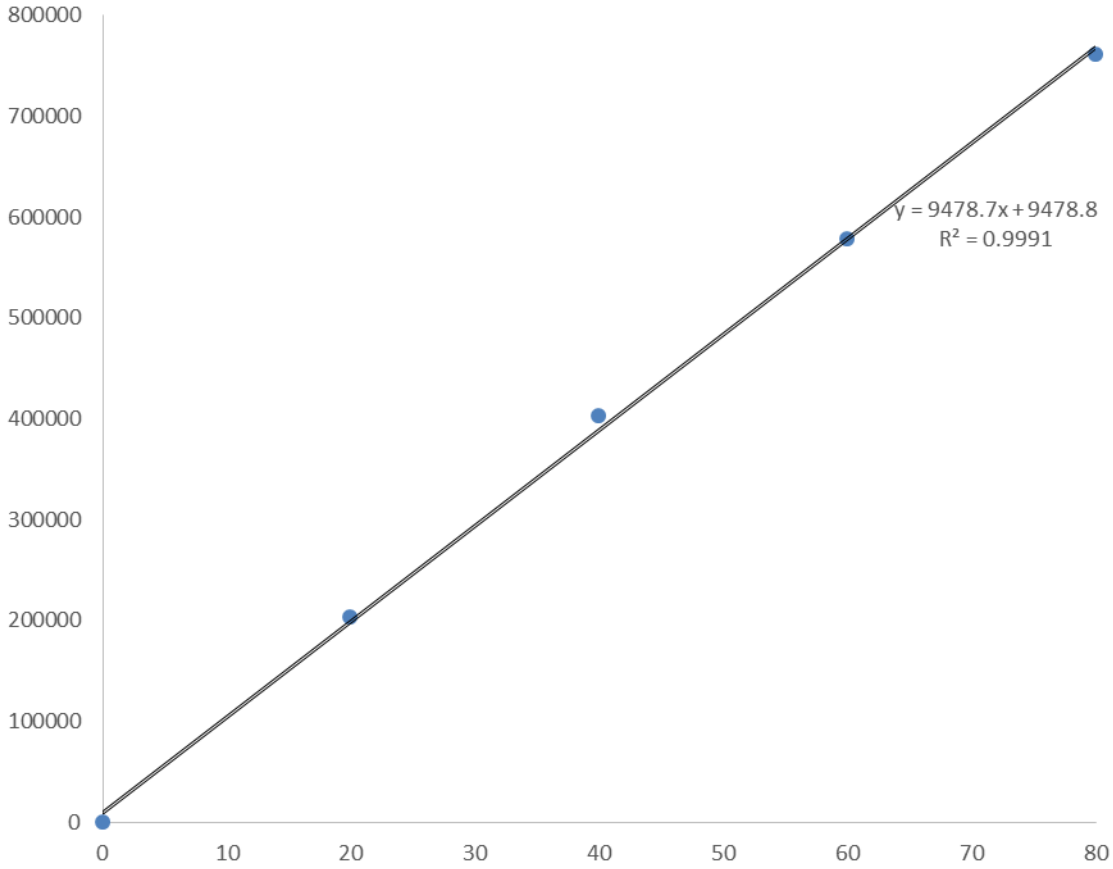
Percentage theanine during season 3 (n=3, M ± SD)

TRFK 31/8	$0.65^{bc} \pm 0.01$	TRFK 56/89	$0.98^{a} \pm 0.05$
TRFK 7/14	$0.26^{g} \pm 0.04$	TRFK 54/40	$1.00^{a} \pm 0.03$
TRFK 303/577	$0.35^{fg} \pm 0.01$	TRFK 303/259	$0.55^{cde} \pm 0.09$
TRFK 301/4	$0.45^{ef} \pm 0.08$	TRFK 301/5	$0.61^{bc} \pm 0.01$
TRFK K-PURPLE	$0.46^{def} \pm 0.03$	TRFCA SFS 150	$0.28^{g} \pm 0.02$
TRFK 12/19	$0.65^{bc} \pm 0.19$	TRFK 12/12	$0.62^{bc} \pm 0.03$
TRFK 11/52	$0.88^{a} \pm 0.16$	TRFK 11/26	$0.57^{bcde} \pm 0.09$
TRFK 11/4	$0.71^{b} \pm 0.03$	TRFK 7/9	$0.60^{bcde} \pm 0.11$
TRFK 7/3	$0.57^{bcde} \pm 0.01$	TRFK 6/8	$0.47^{def} \pm 0.01$
TRFK 31/11	$0.21^{g} \pm 0.01$	TRFK 100/5	$0.61^{bcd} \pm 0.01$
TRFK 108/82	$0.67^{bc} \pm 0.03$	TRFK 303/216	$0.66^{bc} \pm 0.06$
TRFK 303/178	$0.58^{bcde} \pm 0.16$		

Appendix XI: Theanine levels in made tea commercially available in the Kenyan market

Brand name	% Theanine (M ± SD)
Rwanda BT_1	$0.98^{cde} \pm 0.02$
Rwanda BT_2	$1.60^{a} \pm 0.02$
Rwanda GT_1	$1.19^{c} \pm 0.05$
Rwanda GT_2	$1.16^{c} \pm 0.02$
Uganda BT	<LOD
Uganda BT	$0.90^{ef} \pm 0.10$
Kenya BT	$1.02^{cde} \pm 0.03$
Kenya (SV)	<LOD
Kenya (GL)	<LOD
Kenya (LL)	<LOD
Kenya (GTP)	$0.12^{i} \pm 0.02$
Tanzania (LS)	<LOD
Tanzania (H)	<LOD
Tanzania (PP)	<LOD
Tanzania (OGT)	$1.53^{ab} \pm 0.04$
Tanzania (LB)	$1.09^{cd} \pm 0.03$
Cameroon BT	$0.48^{gh} \pm 0.03$
Sri Lanka BT	$0.52^{g} \pm 0.02$
Sri Lanka GT	$0.14^{i} \pm 0.02$

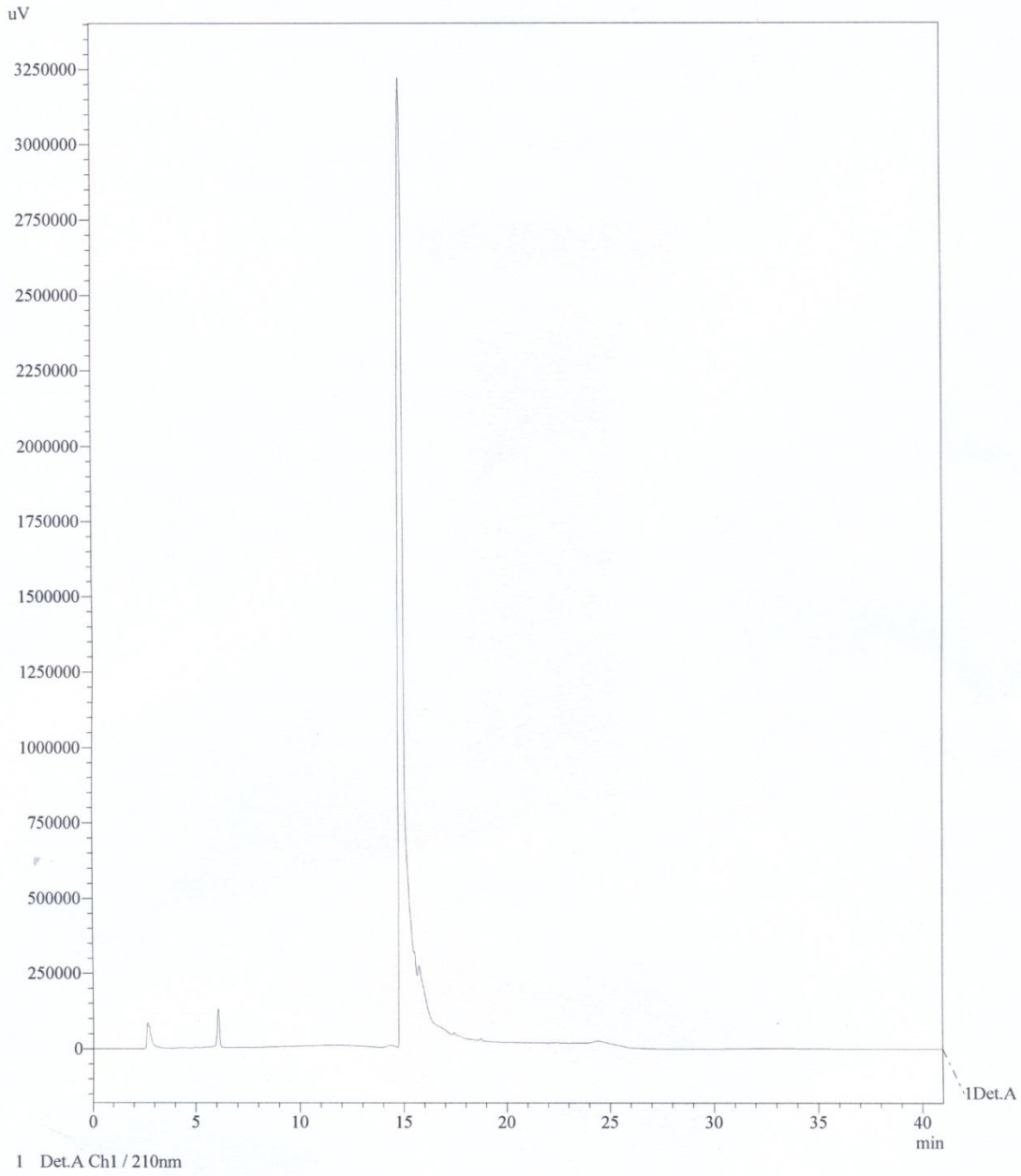
Appendix XII: A linear calibration curve for theanine working standards



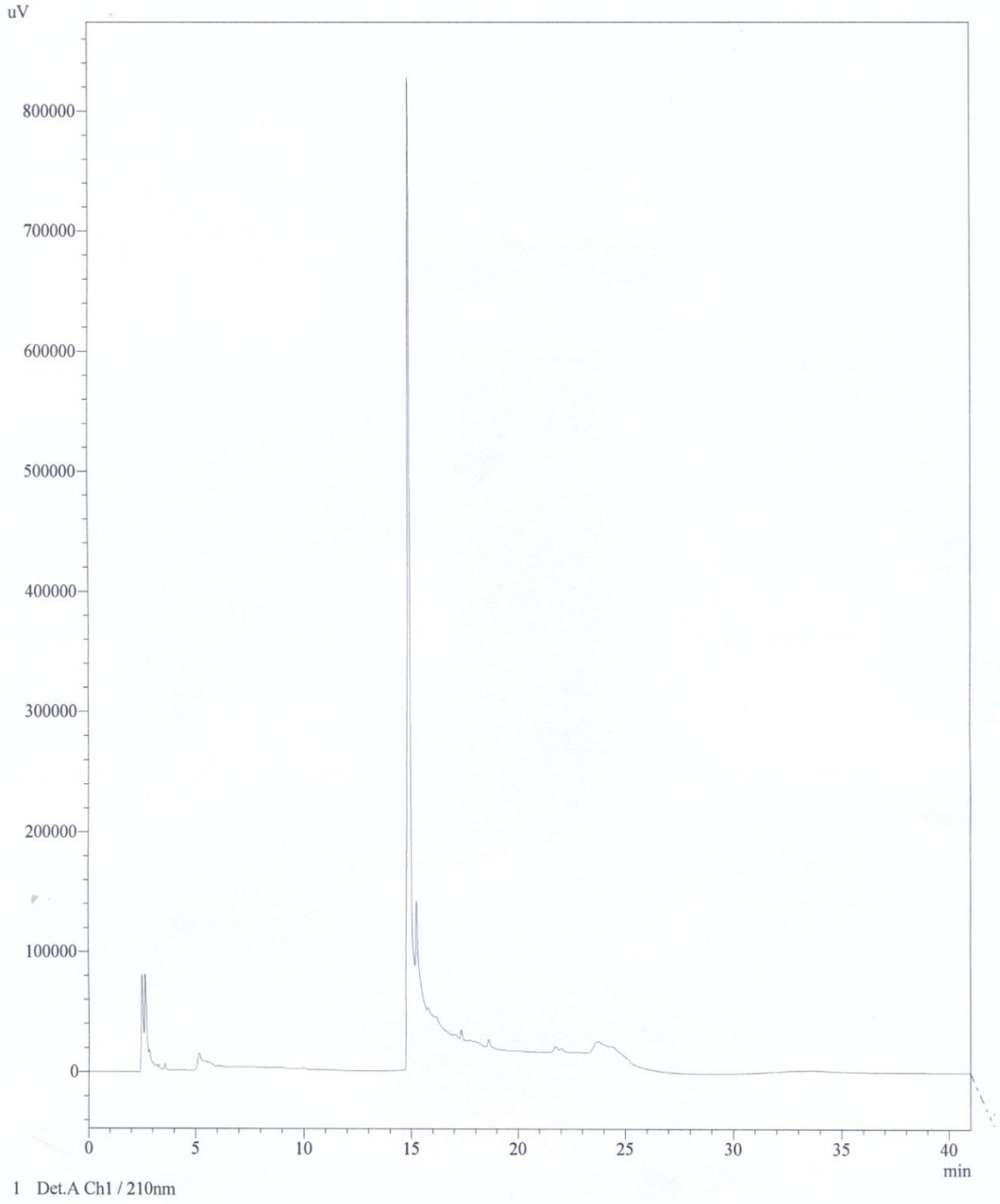
Key: X – Concentration of the working standards, µg/mL

Y – Intensity, µV

Appendix XIII: HPLC chromatogram for Rwanda (Green Tea)



Appendix XIV: HPLC Chromatogram for Kenya (Strawberry and Vanilla)



Appendix XV: HPLC Chromatogram for TRFK 301/4, green tea