DEVELOPMENT OF AN IMPROVED ISOCRATIC HPLC METHOD FOR THE DETERMINATION OF GALLIC ACID, CAFFEINE AND CATECHINS IN TEA

KINGORI MWANGI SIMON

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

EGERTON UNIVERSITY

NOVEMBER, 2018

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not, wholly or in parts, been presented in any other university for an academic award.

Kingori M. Simon

SM11/14416/15

Signature: ……. Date: …….

RECOMMENDATION

This thesis is the candidate's original work and has been prepared with our guidance and assistance and is being submitted with our approval as the University supervisors.

Dr. Peter O. Ongoma

Egerton University

Signature: …… Date: …….

Dr. Simon O. Ochanda

KALRO -Tea Research Institute

Signature: …… Date: …….

COPYRIGHT

DEDICATION

To my wonderful family and siblings

ACKNOWLEDGEMENTS

I give thanks to The Almighty God for His abundant grace that never fails. I am highly indebted to the Chemistry Department of Egerton University for giving me an opportunity to further my studies.

I sincerely thank my supervisors Dr. P.O. Ongoma and Dr. S.O. Ochanda for their wise counsel, reviews, suggestions and thoughtful criticism that helped me shape the project work. I particularly owe a lot to Dr. P.O. Ongoma for dedicating his valuable time to ensure the success of this project by inputting his vast knowledge in analytical applications.

I would like to appreciate Dr. J. Bore the Director of KALRO Tea Research Institute, Kericho, for allowing me to undertake this study in the institute's laboratories. I owe gratitude to the staff at the Tea Processing and Value Addition Program (TPVA) for their assistance in laboratory determinations. I am indebted to Miss E. Kipsura for her great contributions during HPLC data generation.

Finally, my appreciation also goes to my family and friends for their patience, understanding, financial and material support throughout the time of this study

ABSTRACT

Polyphenols are the primary compounds responsible for the health benefits of tea, including its antioxidant and anti-inflammatory properties. Caffeine contributes to tea's stimulant properties, while gallic acid and polyphenols contributes to its antioxidant properties. Most of the High Performance Liquid Chromatography (HPLC) methods used for the determination of tea biochemicals include gradient elution systems which involve expensive instrumentation. The aim of this study was to develop an improved sensitive, fast, cost effective and accurate isocratic HPLC method with photo diode array (PDA) detection for analysis of gallic acid, caffeine and catechins in tea (*Camellia sinensis*), using a suitable internal standard. The developed HPLC analytical method consisted of a C_6-phenyl column (4.6 x 150 x 5µm) and an isocratic elution system of water-acetonitrile-methanol-ortho phosphoric acid-ethyl acetate (77.5:18:2.0:0.5:2.0 v/v/v/v/v) at a flow rate of 1.0 mL/min. The detection was done using a PDA detector at a wavelength of 278 nm. This resulted in an excellent chromatographic separation of the catechins, gallic acid and caffeine in tea in the presence of guaiacol (2-methoxyphenol) internal standard, which minimized sample matrix effect and instrumental fluctuations. The total analysis time in the new method was 12.5 min, more than three times faster than the *ISO 14502-2:2005(E)* method. The analytical results obtained for gallic acid, caffeine and catechins in four tea types–green Cut, Tear and Curl (CTC), black CTC, green orthodox and black orthodox–using this developed method and *ISO 14502-2:2005(E)* method showed that there was no significant statistical difference between the two methods. In the CTC and orthodox green tea types the CV was in the range of 2.9 – 14.8 while in the CTC and orthodox black tea types the CV was in the range of 3.7 – 21.4 when comparing the individual catechins, gallic acid and caffeine determined by the two methods. The method was validated against the Food and Drug Administration (FDA, 2013) guidelines and proved that the method is efficient, simple and fast for qualitative and quantitative determination of the biomolecules of interest in tea samples. This method produced excellent accuracy and precision. Within run precision was less than 2.18 %. Therefore, the results generated by this developed method can be adequately used as complimentary alternative to the subjective organoleptic determinations mostly used in the tea industry to determine quality and prices of tea.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
AR	Analytical Reagent
+C	(+) Catechin
C_{18}	Octadecylsilane
C_6-phenyl	Hexylphenyl
CAFF.	Caffeine
CD4	Cluster of Differentiation 4
CTC	Cut, Tear and Curl
DM	Dry matter
EC	Epicatechin
ECD	Electron Capture Detector
ECG	Epicatechingallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FDA	Food and Drug Administration (USA)
GA	Gallic acid
GMP	Good Manufacturing Practice
HACCP	Hazard analysis and critical control points
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
ISO	International Organization for Standardization
KALRO	Kenya Agricultural and Livestock Research Organization
KTDA	Kenya Tea Development Agency
LC–MS	Liquid chromatography–Mass spectrometer
LOD	Limit of Detection
LOQ	Level of Quantitation
MC	Moisture Content
ODS	Octadecylsilyl silane
PDA	Photodiode Array Detector
PVC	Poly vinyl chloride
RSD	Relative Standard Deviation
SAS	Statistical Analysis System

TFs	Theaflavins
TRI	Tea Research Institute
TRs	Thearubigins
UV–Vis	Ultraviolet–Visible
NADH	Nicotinamide Adenine Dinucleotide-Hydrogen

CHAPTER ONE
INTRODUCTION

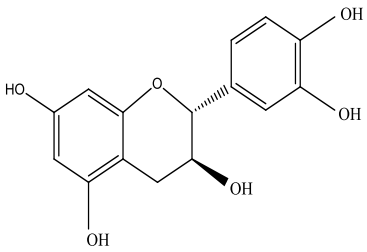
1.1 Background Information

Tea *(Camellia sinensis)* is a beverage consumed for both refreshment and health benefits since ancient times (Piljac *et al*., 2013). Tea has been consumed for various reasons including its antioxidant properties (Kuriyama *et al.,* 2006; Li *et al.,* 2006; Perumalla and Hettiarachychy 2011; Chacko *et al.,* 2010; Amerine *et al.,* 2013) sensory properties and potential health benefits. It is the second most consumed beverage in the world after water and is commonly served hot or iced (Martin, 2011). Tea is produced mostly from two tender leaves and a bud of the tea plant. Studies have shown that tea polyphenols provide several health benefits such as reduction of cholesterol, prevention of the development of obesity, protection against cardiovascular disease and cancer (Meydani and Hasan, 2010; Del Rio *et al*., 2013). Their other health benefits include: enhancing insulin activity (Anderson and Polansky, 2002), antimicrobial activities (Prabhakar *et al.,* 2010), immune-stimulatory activities (Meeran *et al.,* 2009), anti-inflammatory capacities (Tipoe *et al.,* 2007), protection against cardiovascular diseases (Tijburg *et al.,* 1997) and cerebral ischematic damage (Suzuki *et al.,* 2004). Furthermore,the catechins especially the epigallocatechin gallate (EGCG) found in a green tea catechin is reported to have anti - HIV activity when bound to cluster of differentiation (CD4) receptor (Kawai *et al.,* 2003).

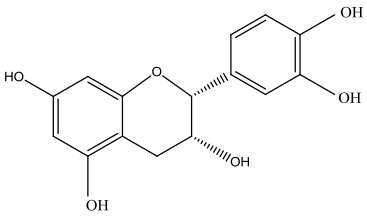
Tea is a unique beverage with biomolecules whose chemical compositions can be used as quality indicators (Farhat *et al*., 2014, Sliwinska *et al*., 2014). Levels of these chemicals are directly proportional to quality both in aerated and non-aerated teas. (Ochanda *et al*., 2015). Therefore, there is need to explore scientific techniques of determining quality parameters with an aim of complimenting the subjective organoleptic evaluation commonly used in the tea industry.

Catechins are the primary polyphenols in tea and account for 75-80% of the soluble ingredients (Rahim *et al*., 2014; Mutshekwa, 2017). They are powerful antioxidants that provide several health benefits (Nile and Park, 2014). Gallic acid and caffeine are also found in non-aerated and aerated curt, tear and curl (CTC) and orthodox teas (Koech *et al*., 2013; Mangenya *et al*., 2016). Caffeine is the major alkaloid in tea, present in the range of 1.0 to 4.0% (Rahim *et al.,* 2014). It is a diuretic and has the effect of delaying fatigue (Hoffman, 2010). Gallic acid has been shown to possess antimicrobial activity against human pathogens (Daglia, 2012), plant pathogens (Maddox *et al.,* 2010) and human pathogenic yeast

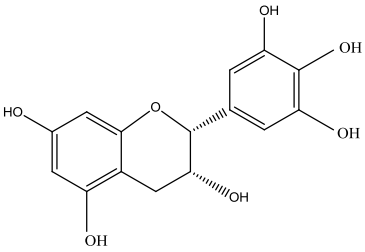
(Chanwitheesuk *et al.*, 2007). The structures below show the major tea catechins, gallic acid and caffeine.



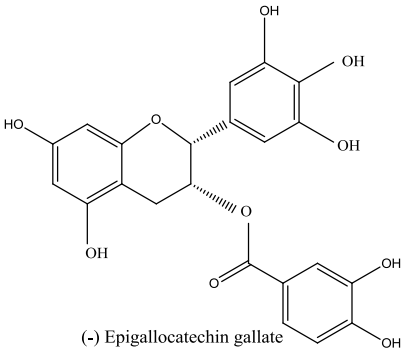
(+) Catechin



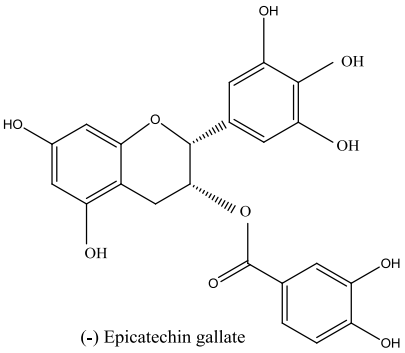
(-) Epicatechin,



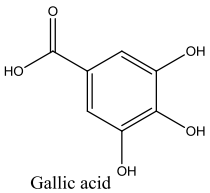
(-) Epigallocatechin



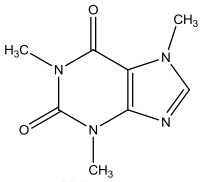
(-) Epigallocatechin gallate



(-) Epicatechin gallate



Gallic acid



Caffeine

High performance liquid chromatography (HPLC) methods have been developed to separate, identify and quantify gallic acid, caffeine and catechins present in tea. In separation components distributes between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction. The methods are mostly gradient elution systems (Ignat *et al*., 2011; Sang *et al*., 2011; Naldi *et al*., 2014). However, gradient elution as compared to isocratic systems requires expensive instrumentation and computerized processors. It is also often difficult to optimize the operating conditions and obtain reproducible results. Moreover, isocratic methods studied using C_{18} (ODS stationary phase)

column are also irreproducible, have poor resolution and low chromatographic efficiency, especially under methanol based mobile phases (Dalluge and Nelson, 2000; Saito *et al*., 2006) are also experienced.

Due to variability in the compositions of tea catechins, caffeine and gallic acid and their potential health benefits, it is important to establish a simple and reliable analytical method for their determination. The newly developed method is simple as it involves a less complex isocratic system and can be used in common laboratories having low cost HPLC machines. The method can be used to determine the quantities of catechins, gallic acid and caffeine in tea and value added tea products.

1.2 Statement of the Problem

Analysis of gallic acid, caffeine and catechins in tea or plant extracts has mostly been undertaken by using gradient elution high performance liquid chromatography (HPLC) with UV detection. This requires the use of expensive external standards for quantitation, long machine analysis time and high cost of chemicals/reagents since two or more mobile phases run during the analysis. In isocratic methods of HPLC analysis a single mobile phase is used which may constitute a single solvent or a combination of more than one mixable solvents in definite ratios. The methods are usually simple and fast as they do not require change of gradient elution during determinations. Presently, there are few studies that have used isocratic elution HPLC with diode array detection to analyze gallic acid, caffeine and catechins in tea extracts. Some published results of HPLC methods are not reliable since the result they generate are irreproducible. This emanates from incomplete resolution of the analytes and/or quality of the chromatograms, particularly when using conventional C_{18}-reverse phase column (ODS). In addition, complete separation of the catechins and other phenolics are column dependent. For these reasons there is a need to develop an improved isocratic HPLC method using C_6-phenyl column with diode array detection. The C_6-phenyl column offers additional polar and aromatic selectivity, which may not be possible with a traditional C_{18} column. Furthermore, in the tea industry, organoleptic analyses which serves as a primary screen to determine customer preference is commonly used. The measurements are more rapid, reliable and repeatable, but subjective which may lead to compromise in quality scores of tea leading to losses in income to the tea farmers. Sensitive, fast, cost effective and accurate instrumental methods of analysis will give results that are more reliable and more objective than those from sensory evaluations.

1.3 Objectives

1.3.1 General Objective

To develop an improved isocratic HPLC method for the simultaneous identification and quantitation of gallic acid, caffeine and catechins in tea with a short analysis time.

1.3.2 Specific Objectives

1. To determine a solvent system design for isocratic elution of gallic acid, caffeine and catechins in tea HPLC analytical method.
2. To identify a suitable internal standard (IS) for the isocratic HPLC method for quantitating gallic acid, caffeine and catechins in tea.
3. To validate the developed isocratic HPLC method for the simultaneous determination of gallic acid, caffeine and catechins in tea against the Food and Drug Administration, 2013 validating procedures.
4. To evaluate the performance of the newly developed isocratic HPLC method against that of *ISO 14502-2:2005(E)* method for analysis of catechins, gallic acid and caffeine in tea.
5. To carry out economic evaluation of the developed isocratic HPLC method and *ISO 14502-2:2005(E)* method

1.5 Justification

Tea researchers have made many attempts to explain tea quality and its quality attributes chemically and physically so as to develop an equipment to replace the experiential sensory assessment. Progresses have been made in this area, especially in black tea. The findings could offer information that can be used to develop techniques and equipment for assessing customer sensory preference.

Various analytical tools have been applied in estimation of tea quality in terms of colour, strength, briskness, astringency and other characteristics attributed to theaflavins (TF). The total content of polyphenols and caffeine are good indicators for assessment of the origin and the quality of tea. Advanced methods such as capillary electrophoresis, electronic tongue and lipid membrane taste sensors have been used in the estimations. However, these techniques are not widely used commercially the limiting factors being the high cost involved, requirement of skilled personnel, and laboratory set-up which is not the case with the subjective traditional methods commonly used. The aim of the present work was to develop a considerably simplified isocratic high performance liquid chromatograph (HPLC) method, which would be fast, cost effective and suitable for routine analysis. For this purpose,

various techniques of determination of catechins, gallic acid and caffeine in tea and tea value added products by high performance liquid chromatograph (HPLC) were studied that lead to useful information on performance of columns, mobile phases, detectors and use of standards for quantitation.

CHAPTER TWO
LITERATURE REVIEW

2.1 Kenyan tea

In Kenya tea was first planted in Limuru in 1903 (Wamalwa, 2014). Since then, commercial tea growing has been extended to cover wide regions of the Mau ranges, Kisii, Nandi Hills, the slopes of Mt. Kenya and Mt. Elgon. Recent expansion in the industry has seen Kenya grow to become the world's largest exporter of black CTC tea (Basu *et al*., 2010; Hicks, 2009). The tea industry earned the country Kenya shillings (Ksh.) 129 billion in foreign exchange in the year 2017. This becomes the highest export earning ever, marking an increase of 7.5 per cent from the Ksh120 billion recorded in 2016. Tea earnings from the domestic market stood at Ksh15 billion, which represents seven per cent of the total production (Sibhatu *et al*., 2018).

Tea farms in Kenya are not sprayed with chemicals except fertilizer application to replenish soil nutrients (Grigg, 2003; Mucheru *et al*., 2014). Tea growing areas receive long hours of sunlight daily and between 1,200–1,400 mm of rainfall spread throughout the year. This ensures that the supply of tea, both in quality and quantity is consistent throughout the year. There are about 50 varieties of tea in Kenya, which are developed to suit the seven growing regions (Blowfield and Dolan, 2010). With each new variety developed, chemical properties are enhanced, making Kenyan tea to be associated with health attributes (Marsh *et al*., 2014; Sharangi, 2009). Over 90 percent of Kenya's tea is handpicked (Kawooya *et al*., 2015; Mbui, 2016). Only the finest top two leaves and a bud are used for tea production and this contributes to the excellent aroma in the tea cup (Martin, 2011, Pang *et al*., 2012). Kenya's tea factories are certified with internationally acclaimed standards ISO 22,000; HACCP; Rain Forest Alliance, Fair Trade GMP) (Verhagen *et al*., 2014; Wijayasiri and Jayaratne, 2011).

2.2 Tea processing

Two leaves and a bud is recommended as raw material for processing quality tea (Plate 1). The recommended shoot composition for quality tea manufacture are, two or three leaves and a bud, single or two leaf banjis which must constitute at least 85% of a lot randomly sampled. Immature shoots should not exceed 5% in the given lot while coarse leaf should not be more than 10%. Over ten per cent of coarse leaf in the shoot composition affects the chemical composition of the shoots (Mewshaw, 2003).

Plate 4: Two leaves and a bud of green tea shoot

2.2.1 Black tea processing

Black tea processing steps involves plucking, sorting, withering, maceration, aeration, drying and packaging. Two leaves and a bud are plucked from the field and then taken to the factory for processing. After sorting the leaves are withered. During withering, diverse biochemical changes occur (Hur *et al*., 2014) and include changes in proteins, caffeine, sugars, organic acids, polyphenol oxidase activity, chlorophyll, minerals, volatile components, and permeability of cell membranes (Gaur and Agnihotri, 2014; Kumar *et al*., 2013). In maceration also referred to as rolling the process is accomplished by CTC machines where the cell structures are disrupted, exposing the cell contents to atmospheric oxygen (Gulati, 2013), bringing various enzymes into intimate contact with their substrates in this case the polyphenols. The chemical and biochemical reactions initiated during withering proceed at an accelerated rate during and after the rolling leading to formation of theaflavins and thearubigins that affect the final cup quality (Fellows, 2009; Kilel, 2013). Fermentation or aeration is an important reaction that occur where the development of colour, strength and quality of tea by the production of non-volatile compounds through the enzymatic oxidation of catechins and their gallates and the production of volatile compounds responsible for the

characteristic aroma of black tea (Corzo-Martınez *et al*., 2012; Darriet and Pons, 2017). These chemical and biochemical reactions make fermentation the most critical step in black tea manufacturing (Sang *et al*., 2011).

Drying/firing cause cessation of enzyme activity and reduce the moisture content to about 3% of the dry mass (KTDA, 2011; Holden *et al*., 2013). However, other changes other than removal of moisture that occur during this step include a significant loss of volatile compounds, an increase in the levels of amino acids, the binding of polyphenols to other tea components and an increase in carboxylic acids and maillard reactions (Panda, 2016; Saputro *et al*., 2017). In grading or sizing, the teas are sorted into primary and secondary grades (Moseti, 2013; Smith, 2016). Tea grading facilitates the international trade in tea and is an important tool for tea experts in making evaluations between the different manufactured grades (Paine and Paine 2012).

2.2.2 Green tea processing

In green tea processing fresh leaves are first steamed or roasted to deactivate the enzymes (Pérez–Jiménez et al., 2012; Senanayake, 2013). This is done for around 20 min in batches in the roaster. The hot roasted leaves are then spread evenly over perforated troughs to cool. The processing of green tea is beneficial, as it increases the shelf life of the catechins when enzymes responsible for oxidation are deactivated (Corollar *et al.*, 2013). Additionally, the moisture level is considerably brought down resulting in a stable product that can be stored without degradation in quality. Green tea acquires some desirable characteristics like flavour and colour during the drying process (Karori *et al*., 2007).

2.2.3 Orthodox tea

Orthodox tea is a loose leaf tea product prepared in traditional methods and hence, the name Orthodox (Sadowska-Rociek *et al*., 2014). The harvesting is done using traditional ways requiring long working hours and immense manpower. The processing is by use of hand to get the whole leaves in steps that includes withering, rolling of the withered leaves to obtain chemicals, oxidation to get the reddish brown color of the leaves and firing for getting the final dried Orthodox tea leaves (Smith, (2016).

The process of producing Orthodox teas is highly controlled by a trained professional to make sure that the best flavors are extracted from the leaves. As compared to CTC blends, Orthodox teas produce more authentic tea experience. Orthodox blends of all types – oolong, white, green or black – have a delicate flavor (Battle, 2017). On the other hand, CTC teas have a generic taste and produce dark strong liquors having a distinct astringent flavor. Since

Orthodox teas give an authentic flavor, they are highly prized in the beverage market across the world (Richardson,2014).

2.3 Biosynthesis of flavan 3-ols in tea

Flavan-3-ols in tea consists of a mixture of six different catechins, namely: (+) catechin, (+) gallocatechin, (–) epicatechin, (–) epicatechin gallate, (–) epigallocatechin, and (–) epigallocatechin gallate. The biosynthetic pathway for flavan-3-ols starts from flavanones as shown in the simplified biosynthetic pathway in Figure 1. The flavones are reduced by chalcone isomerase enzyme (F3H, flavanone-3-hydroxylase), which due its stereo-specificity leads to 2R stereochemistry followed by attachment of a hydroxyl group at position 3 to form 2,3-trans dihydroflavonol. The dihydroflavonols are then reduced by two NADPH dependent reductases, dihydroflavonol 4-reductase (DFR) and flavan 3,4-cis-diol reductase (FRD) to firstly a flavan-3,4-diol and finally to the flavan-3-ols.

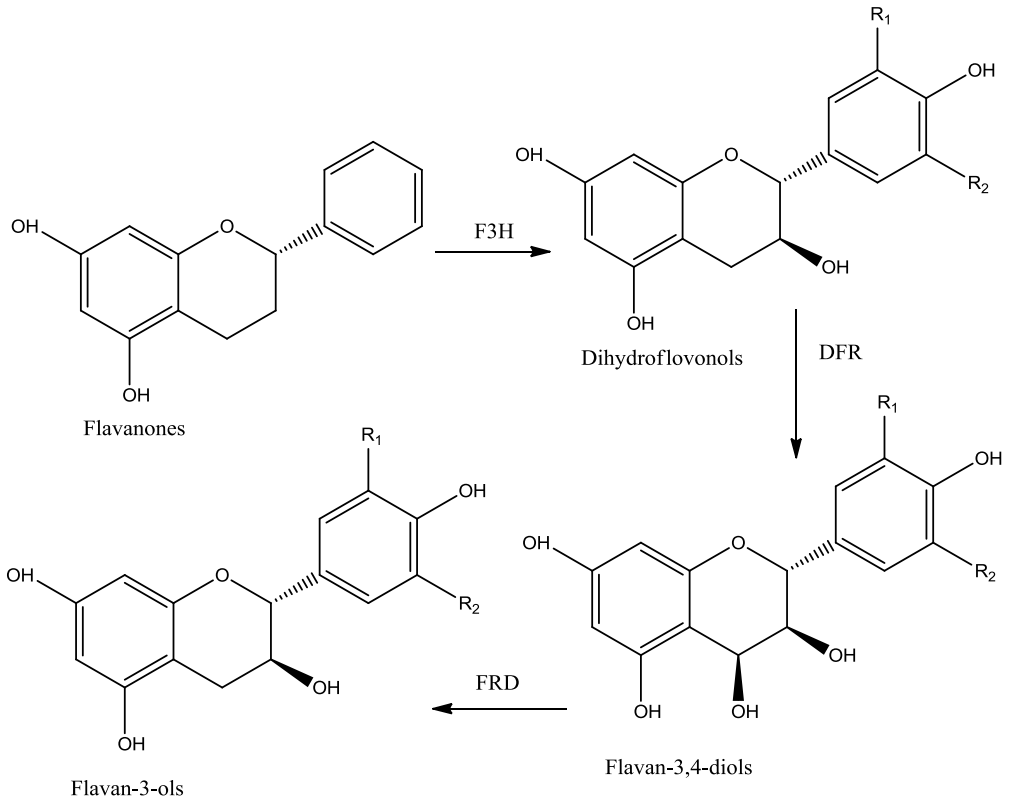


Figure 1: A simplified biosynthetic pathway of 2,3-trans flavan-3-ol where, R_1 and R_2 can be either H or OH. F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; FDR, flavan 3,4-cis-diol reductase are enzymes (Source: Wright, 2005).

Generally, the flavan-3-ols differ in the number of hydroxyl groups on their B-rings, stereochemistry of C3 and attachment of an extra galloyl group at the 3 hydroxyl group. The

tea flavan-3-ols also differ according to the degree of hydroxylation of their B-ring. Tea flavan-3-ols can therefore either have a dihydroxy B-ring as in C, EC and ECG or a trihydroxy B ring as observed in (+) GC, (-) EGC and (-) EGCG (Ozdal *et al*., 2016).

2.4 Tea chemistry

Tea plants are known for their polyphenols and methyl xanthines. The presence of these two groups of compounds give tea its popularity (Pagliosa *et al*., 2010). They are responsible for the unique taste of tea, in addition to various compounds associated with tea aroma (Dias, 2013). The Chemical composition of the tea shoot varies with agro - climatic conditions, season, cultural practice and the type of planting material (Owuor *et al*., 2011).

2.4.1 Tea catechins

Catechins, are a group of polyphenolic flavan-3-ol monomers and their gallate derivatives, are the primary compounds responsible for the health benefits of tea (Tsao, 2010; Cardona *et al*.,2013); including its antioxidant and anti-inflammatory properties (Shahidi and Ambigaipalan, 2015). The polyphenol catechins are found in the highest concentrations in fresh tender leaves, are located in the cytoplasmic vacuoles (Pandey and Rizvi 2009) and constitute about 30% of the dry weight in a tea shoot (Watson, 2014; Da *et al*., 2015). Catechins are the major compounds that are oxidisable in the tea leaf, with (-) epigallo catechin (EGC) and (-) epigallocatechin gallate (EGCG) being the most predominant (Su and Wang, 2013).

Generally, the content of soluble non-oxidized catechins is greater in green tea than in black tea and are effective chemotherapative agents (Zaveri, 2006; Zhang *et al*., 2011)). The catechins play a significant role during fermentation. As the leaves are heated, rolled and dried during processing, the catechins content decreases because the leaves undergo oxidation, hydrolysis, polymerization and biotransformation. The biochemical changes during processing result in a tea product that has at least 15% less polyphenol content than freshly plucked tea leaves (Ananingsih *et al.*, 2013;Yang *et al*., 2016). The total catechins content of various green tea types were found to vary more than 10 fold, from 21.38 to 228.20 mg/g of dry plant material for water infused extract (Vrhovsek *et al.*, 2012; Li *et al*., 2016). In addition, qualitative analysis of catechin showed that total polyphenols and the antioxidant activity of green tea from different producers had strikingly different quantities of catechins and antioxidant activity (Lavola *et al.*, 2012)

2.4.2 Caffeine, theanine and gallic acid in tea

Caffeine is a methyl xanthine alkaloid that contributes to green teas stimulant properties (Horžić *et al*.,2012), while theanine (gamma glutamylethylamide) is glutamic acid analog which contributes to green tea's relaxing properties (da Silva Pinto, 2013). Caffeine, is less sensitive to heat and does not undergo considerable reduction during tea processing (Chemat and Khan, 2011). Caffeine and theanine have a synergistic physiological effect in enhancing mental alertness (Paulus *et al*., 2015). They are secondary metabolites that serve as defence components in plants. They provide plants with resistance to pathogens and predators (Walters, 2011), reduce oxidative stress and protect them from other environmental variables (Iriti *et al*.,2010). Their amounts found naturally in plants vary with environmental conditions (Lee *et al.*, 2010). Gallic acid exists in plant material in the form of free acids, esters, catechin derivatives and from hydrolysable tannins (Garrido and Borges,2013; Macheix, 2017). This ubiquitous chemical is one of the most biologically active phenolic compounds of plant origin. The antioxidant activity of gallic acid and its derivatives have been reported in several studies (Cai *et al.*, 2004).

2.5 The role of polyphenol oxidase (PPO)

The enzyme polyphenol oxidase plays a key role in tea oxidation; it is present in chloroplasts (Tomás and Espín, 2001). Polyphenol oxidase (PPO) has remarkable specificity for the ortho - dihydroxy functional group of the tea catechins. In intact plant, the enzyme is physically separated from the substrates and flavanols (Gulati, 2013). During the oxidation process the enzyme and substrate are brought together in the presence of oxygen by rupturing the membrane in order for the polyphenols to diffuse into the cytoplasm (Magoma *et al*., 2017). As a first step during fermentation, the catechins are oxidized to highly reactive, transient orthoquinones by PPO (Badea and Antoce, 2015).

2.6 Theaflavins and thearubigins

The quinones, derived from a simple catechin and a gallocatechin, dimerise to produce theaflavins which are orange-red substances that contribute significantly to astringency, briskness, brightness and colour of tea beverage (Sujith, 2010). Theaflavins (TFs) comprise 0.3 to 2.0% of the dry weight of black tea (Bahorun *et al*., 2012). Theaflavins of black tea comprise a number of fractions namely theaflavin, theaflavin monogallate and digallate, epitheaflavic acid and isotheaflavin (Panda, 2016). As the gallation increases the astringency also increases. The proportions of theaflavin fractions present in black tea depend upon the method and conditions of manufacture (Wachira *et al*., 2013). Plain tea quality

parameters of black (aerated) tea usually change with the degree of wither and oxidation (Owuor and Obanda, 2007). Total TFs decrease as the degree of physical wither increases and increase with increased degree of oxidation (Obanda *et al*., 2004). Thearubigins are complete condensation products of oxidized catechins with theaflavins being responsible for the colour, mouth feel and body of the tea liquor (Ngure *et al*., 2009).

2.7 HPLC analysis of catechins

Polyphenols such as the catechins and gallic acid are unstable; hence their assays are relatively difficult (Mildner - Szkudlarz and Bajerska, 2016). Despite this their composition studies have continued to increase (Khan, and Mukhtar, 2007). Since many of the phenolic compounds are water soluble and aromatic in nature, reverse-phase HPLC with UV-detection has been used in their analysis (Guillarme *et al*., 2010; Ignat *et al*., 2011). However, polyphenols are structurally similar for this reason, their analysis requires high chromatographic selectivity and resolution (File///D: /AN-20583 © 2012 Thermo Fisher Scientific Inc.). Additionally, analysis of gallic acid, caffeine and catechins in tea has been undertaken using paper chromatography (Lin *et al.*, 1998), thin layer chromatography (Colon and Nerin, 2012), spectrophotometric methods (Khoddami *et al.*, 2013), Liquid chromatography coupled with mass spectrometry (LC–MS) and capillary electrophoresis (Bonoli *et al.*, 2003;Chen *et al.*, 2003; Atoui *et al.*, 2005; Spáčil *et al.*, 2008).

2.7.1 HPLC gradient and isocratic systems in the determination of catechins

Analysis of catechins and related phenolic compounds in tea has mostly used gradient elution techniques (Valls *et al*., 2009). The absorption regions occur at 280–370 nm for the determination of catechins. Isocratic elution using HPLC methods to separate catechins and gallic acid in tea have also been reported (Lin *et al*., 1998; Sharma *et al*., 2005). However, compared to isocratic systems gradient elution requires expensive instrumentation and computerized processors. It is also difficult to optimize the operating conditions and obtain reproducible results (Schlake *et al*., 2013). For gradient elution, total run time is higher compared with isocratic elution due to column equilibration that is essential at the end of the gradient (File///D: /AN-20583 © 2012 Thermo Fisher Scientific Inc.). Isocratic methods studied using C_{18} (ODS stationary phase) column are also irreproducible, have poor resolution and low chromatographic efficiency under methanol based mobile phase during the analysis of catechins (Dalluge *et a*l., 2000; Saito *et al*., 2006). Methanol, ethyl acetate and acetic acid mobile phase are known to give an improved chromatographic resolution and

eliminate peak tailing of less polar catechins under isocratic conditions (Jandera, 2011; Dhanani *et al*., 2017; Pascual-Maté *et al*., 2018).

2.7.2 Detection systems in liquid chromatography

Electrochemical detection (van der Burg-Koorevaar *et al.*, 2011) and post column derivatization using cinnamaldehyde with detection at absorption of 640 nm (Santagati *et al.*, 2008) has been reported. New techniques involving liquid chromatography coupled to with detectors such as diode array, electrochemical detection (ECD) and mass spectrometer have emerged in recent times (Marquet and Lachatre, 1999). In a study conducted by Guillarme *et al.*, 2010 it was reported that electrochemical detectors had 1000 times better sensitivity compared to a UV detector. In another related study, it has been shown that ECD to be 300 times more sensitive than UV detectors, especially when determining catechins (Mizukami *et al.*, 2007). Additionally, gas chromatography with mass spectrometry (GC-MS) and liquid chromatography with mass spectrometry (LC-MS) have come into wide use (Careri *et al.*, 2002). The non-volatile compounds in tea are best suited for liquid chromatography assay with amperometric detector (AD) or by liquid chromatography assay with photodiode array (PDA) and UV–Visible absorbance detectors (Magwaza and Opara, 2015).

2.7.3 HPLC columns used in the catechins assay

In a recent study the C_{18}-(pentafluorophenyl) HPLC column was used because it proved to be fast and efficient for the determination of catechins, gallic acid and caffeine under gradient elution conditions (File///D:/AN-20583 © 2012 Thermo Fisher Scientific Inc.). The introduction of the fluorine groups into the C_{18}-(pentafluorophenyl) stationary phase in the HPLC column led to significant analyte-stationary phase interactions (Nagy *et al*., 2018). This chemistry makes this type of column well suited for the elucidation of polar compounds containing hydroxyl, carboxyl, nitro and any other polar functional groups (Herrera-Herrera *et al*., 2012). The selectivity of the column becomes more evident when these functional groups are located on the aromatic ring (Caruso *et al*., 2017). This resulted in the C_{18}-(pentafluorophenyl) HPLC column ideal for the determination of polyphenols; including catechins, gallic acid and caffeine (Pérez-Rentero *et al*., 2013).

In this study, a C_6-phenyl HPLC column is incorporated for analysis of polyphenols: catechins, gallic acid and the coexisting caffeine in tea under isocratic conditions. The C_6-phenyl column combines low adsorption ability of the hexyl spacer for less polar groups and strong π-π interactions between the phenyl group and the substrate (polyphenols) via dipole-dipole and dipole-induced dipole forces to enhance its performance (Babu *et al*., 2014). The

hexyl spacer increases the width of the column resulting in greater selection to improve separation and also to reduce secondary interactions between metal impurities and silanol groups. Additionally, the column is stable in the pH range 2–7.5 (*http://www.cerij.or.jp/service/09_chromatography/L-column2_C_6-Phenyl_01.html accessed on 12/11/2016)*. These characteristics are appropriate to improved selectivity and resolution of the polyphenolic compounds being studied. The method will therefore be used to determine the quality of tea and characterization of Kenyan tea in terms of the region of origin. The structure of the C_6-phenyl column is shown in Figure 2.

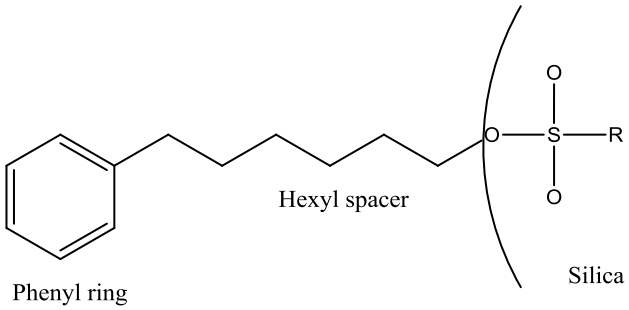


Figure 2: Structure of a C_6-phenyl column

2.8 Method Validation

2.8.1 Fundamental parameters in method validation

The base parameters for method validations include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications (Chen *et al*., 2012). When changes are made to a previously validated method, the analyst exercise judgment as to how much additional validation is needed. Full validation is important when developing and implementing a method for the first time (Broeders *et al*., 2014). Partial validations are modifications of already validated analytical methods. Partial validation can range from as little as one intra assay accuracy and precision determination to a nearly full validation (Sonawane *et al*., 2014). Cross-validation is a comparison of validation parameters when two or more analytical methods are used to generate data within the same study or across different studies (Silverman, 2018). An example of cross validation would be a situation where an original validated analytical method serves as the reference and the revised analytical

2.8.2 Accuracy of the method

Accuracy of a measured result indicate its closeness to the true value (Lobo *et al*., 2008). Accuracy differs from trueness a can be used to characterize an individual result while trueness always refers to the mean value of a large number of results. Because of that and because accuracy can characterize an individual result, accuracy involves also precision. Hence accuracy of a method which signifies the results delivered by the method is affected by systematic (bias) as well as random (precision) error components. Therefore, it is studied as two components of trueness and precision (Wilcox, 2010).

2.8.3 Precision (Reproducibility) of the method

Precision and accuracy are terms used to describe systems and methods that measure, estimate, or predict (Zhang *et al*., 2010). In all these cases the method provides a measured value, that is as close to the true value as possible. Precision and accuracy are ways of describing the error that can exist between these two values. Precision is how close a measurement comes to another measurement (Powers, 2011). Precision is determined by a statistical method called a standard deviation and this is how much, on average, measurements differ from each other. High standard deviations indicate low precision while low standard deviations indicate high precision. To determine if a value is precise the average of the data is found, then each measurement subtracted from it to get the deviations. Then deviations are always averaged to give values of uncertainty which is a plus or minus value that says how precise a measurement is (Almes *et al*., 2016).

2.8.4 Selectivity of the method

Most samples are mixtures of compounds and the analytical method must therefore be selective towards the analyte (Snyder *et al*., 2012). Selectivity is the extent to which other substances interfere with the determination of a substance according to a given procedure. The larger the interference the less selective is the procedures. Methods can be selective to different extent with those that are 100% selective being classified as specific. Analytical techniques are in general never specific or it is impossible to prove that they are. However, analytical methods can be specific within their scope of application, for example a given analyte in a given matrix in a given concentration range (Lehotay *et al*., 2010). An analytical method is regarded selective if its results are not affected by other sample components to any significant extent. Compounds, other than the analyte, which contribute to the analytical signal are called interfering compounds or interferents. Interfering compounds may suppress/enhance the signal. Additionally, may behave like the analyte and yield a signal

indistinguishable from that of the analyte (Kruve *et al*., 2015). In the case of chromatographic methods, it is very common to assess selectivity regarding chromatographic separation of compounds.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample collection

Samples of tea as processed non-aerated Curt, tear and curl (CTC and orthodox green teas, as well as aerated CTC and aerated orthodox black teas were sourced in triplicate from Kenya Tea Development Agency (KTDA) Kangaita Tea Factory of Kirinyaga County in Kenya (Plate 2). The Factory was preferred to supply the different tea type samples due its reputation of processing high quality specialty teas particularly the orthodox types.

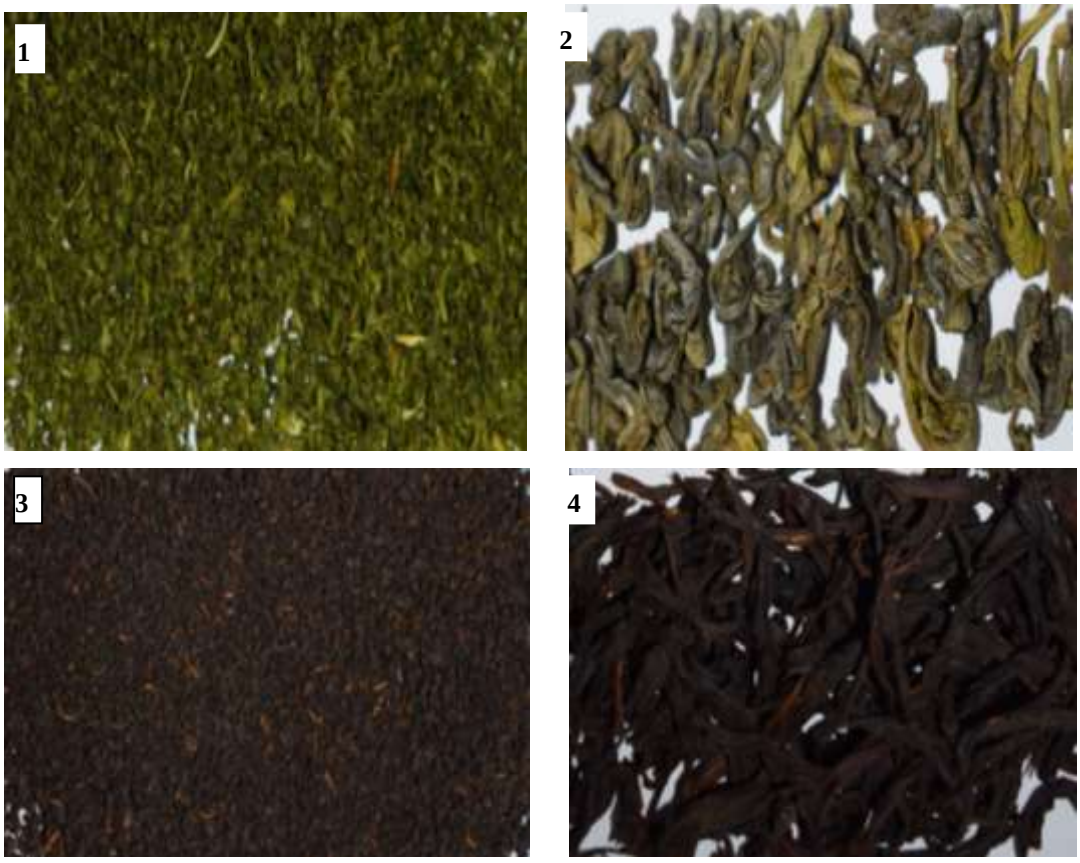


Plate 5: Teas used in this study **1.** Non-aerated (green) CTC tea **2.** Non-aerated (green) orthodox tea **3.** Aerated (black tea **4.** Aerated (black) orthodox tea

The samples were packed in well labelled aluminium lined bags and transferred to the laboratory for further treatment. The samples collected were handled with care to avoid contamination. To ensure homogeneity, the tea samples were ground in accordance with the procedures and stored away from light and moisture in well-sealed containers (ISO 1572,1980).

3.1.2 Reagent and Chemicals

All standards viz. gallic acid (GA), (-) epigallocatechin (EGC), caffeine(CAFF), (+) catechin (+C), (–) epigallocatechin (EC), (–) epigallocatechin gallate (EGCG), (–) epicatechin gallate (ECG), guaiacol (2-methoxyphenol), 3-fluorocatechol, 4-methylcatechol and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co., US, through Kobian suppliers. Acetonitrile, methanol, glacial acetic acid, ethyl acetate, methanoic acid, ortho-phosphoric acid and acetone were purchased from Finar India Ltd through Kobian suppliers. All the reagents and chemicals were of recommended analytical grade. The solvents were degassed and filtered through a 0.45µm filter (Millipore filter No. HAWP04700) before use.

3.2 Methods

3.2.1 Sample extraction using ISO 14502-2:2005(E) procedures

Finely milled tea test samples were weighed (0.200 ± 0.001) g into extraction tubes using analytical balance (BL-3200 HL, Shimadzu, Japan). The extraction tubes containing the sample were placed in a water bath set at 70°C and 5.0 mL of hot methanol/water (7:3 v/v) extraction mixture was dispensed. The extraction tubes were stoppered and mixing done with the help of a vortex mixer. The heating of the extraction tubes in the water bath continued for 10 min, mixing on the vortex mixer (MV-1000, Taiwan) after 5 min and 10 min. The extraction tubes from the water bath were removed, allowed to cool to room temperature and the stoppers removed. The tubes were placed in a centrifuge at 3500 rpm for 10 min after which the supernatant was carefully decanted into graduated tubes. The extraction steps were repeated resulting in two extracts. The two extracts were combined and made to 10 mL with cold methanol/water (7:3 v/v) extraction mixture. On mixing, the extract was allowed to attain room temperature (20–25°C) before carrying out the assay.

3.2.2 Determination of the sample percentage dry matter content

About 2.00 ± 0.01 g weight of representative portion of the tea material to be tested was weighed on a broad shallow pan. The pan containing the weighed material was transferred to an oven set at a temperature of 103 ± 2°C. The material was allowed to dry in the oven (Memmert) for a period of 5 hours after which the dried tea material was transferred to a desiccator and allowed to cool to the room conditions before taking the final weight of the material. It was necessary to determine moisture content since the quantitated levels of biomolecules of interest in the sample were to be expressed in percentage dry weight basis. Percent moisture content (M.C) was calculated using equation 1.

$$\text{Percent (\%) moisture content (M.C)} \quad \frac{\text{mass of moisture lost}}{\text{mass of sample}} \quad \text{X } 100 \qquad \textbf{1}$$

The percentage moisture content in the tea samples we re determined in triplicate with the mean percentage used to calculate the percentage dry matter (M.C).

3.2.3 Preparation of Stabilizing Solution

A solution of 25 ml of EDTA (10 mg/mL), 25 ml ascorbic (10mg/mL) acid solution and 50ml acetonitrile (HPLC grade) was transferred to a 500 mL one-mark volumetric flask then diluted to the mark with distilled water before mixing was done.

3.2.4 Preparation of standard solutions

Stock solutions of primary standards (1 mg/L) of analytes gallic acid, epigallocatechin (EGC), caffeine(CAFF.), (+) catechin (+C), (–) epicatechin (EC), (–) epigallocatechin gallate (EGCG) and (–) epicatechingallate (ECG), were prepared by dissolving in the stabilizing solution, gently warming if necessary (max. 40°C) and then cooled to room temperature (20–25°C). Mixed working standards from the stock solutions were serially diluted (1–1000 µg/mL) with appropriate volume of the stabilizing solution. A calibration curve for each of the standards was generated. The internal standards of fluorocatechol, guaiacol (2-methoxyphenol) and 4-methylcatechol were each tested along the mixed standards.

3.2.5 Development of the isocratic HPLC method

In this study a Gemini C_6-phenyl column was used based on its construction uniqueness. Different mobile phase compositions were investigated including water which is the base solvent in reverse phase chromatography and organic solvents especially methanol, acetonitrile and tetra hydrofuran (THF) commonly used as organic modifiers. The internal standards of 3-fluorocatechol, guaiacol (2-methoxyphenol) and 4-hydroxybenzaldehyde were each tested along the mixed standards. Relative response factors for each of the individual mixed standards were developed against the selected internal standard. Validation procedures were carried out and finally a comparative tea samples analysis study was undertaken using the developed method and *ISO 14502-2:2005(E)* method. Once separated, each component was depicted as a peak at the column outlet fitted with photo diode (PDA) detector.

(i) The mobile phase solvent composition

The analytical procedure adopted was optimized by performing an elution profile to determine the best solvent composition and elution conditions for isocratic HPLC by increasing the elutropic strengths of the trial solvent systems:

1: water: methanol: acetic acid: EDTA
2: water: methanol: ortho-phosphoric acid: EDTA
3: water: acetonitrile: methanol: acetic acid: ethyl acetate
4: water: acetonitrile: methanol: ortho-phosphoric acid: ethyl acetate
5: methanol: water: ethanoic acid
6: acetonitrile: water: ethanoic acid
7: acetonitrile: water: methanol: acetic acid
8: water: methanol: ethyl acetate
9: methanol: EDTA: water
10: acetic acid: acetone: water
11: water: acetonitrile: acetic acid: EDTA.

The mobile phase compositions were varied systematically starting with (0:100:0.1% v/v/v) and increasing stepwise by 10% until correct elution ratios were attained; for example, methanol: water: ethanoic acid (100:0:0.1% v/v/v).

(ii) HPLC Instrumentation and Chromatographic Conditions

A Shimadzu 20A series HPLC (Plate 3) was used in this study to perform all analytical determinations. Investigations were carried out using an isocratic mobile phase system with a reverse phase Gemini phenyl hexyl column as the column of choice. Detection was by a PDA detector at an absorbance wavelength of 278 nm. The PDA detector wavelength scans were performed at the wavelength ranges of 200–400 nm in order to determine the wavelength of maximum absorption by the biomolecules of interest in the study. Table 1 provides a list of both the HPLC instrumental and the chromatographic conditions that were used in the development of the method.

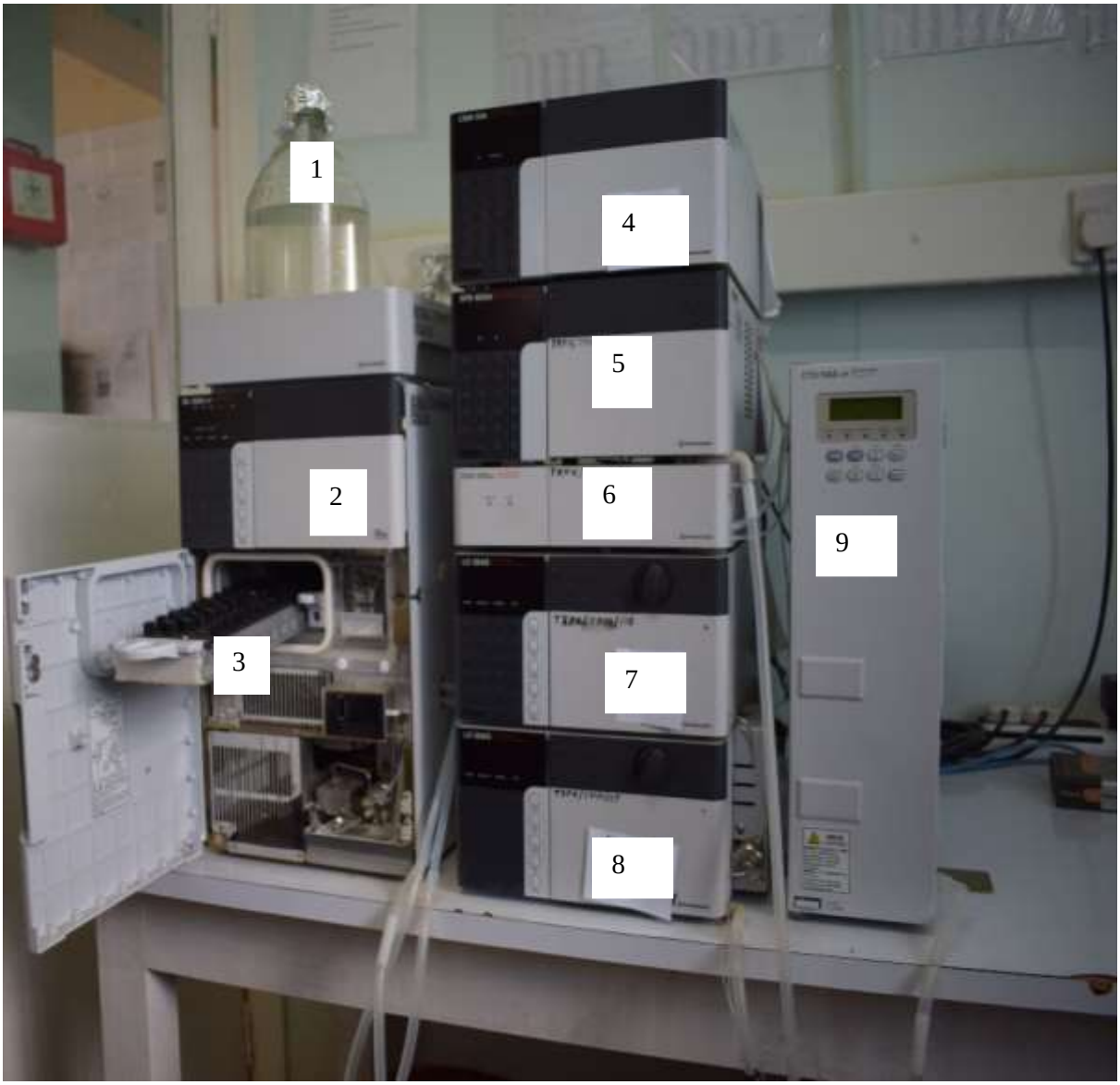


Plate 6: Shimadzu 20A series HPLC: **1**-mobile phase container; **2**-auto sampler unit; **3**-sample rack; **4**-control bus unit; **5**-PDA detector; **6**-degasser unit; **7** and **8**-analytical pumps; **9**-column oven.

Table 6: HPLC instrumental conditions for the developed method

Instrumentation	**Model**
HPLC	Shimadzu 20A series
Degasser	DGU-20A_{5R}
LC pump	20 AD
Column	C_6-phenyl (4.6 x 250 x 5µm particle size)
Column Temperature	30-40°C
Mobile phase	1; 2; 3; 4; 5; 6; 7; 8;9;10 and 11 (Section 3.5.1) above
Detector	PDA (SPD-20MA)
Auto sampler	SIL 20 AT_{HT}
Injection volume	1–20 µL
Run Time	10–30 minute
Guard column	C_6-phenyl (4 x 30 mm, 5 µm)–to prevent clogging and contamination of the main column.
Syringe flush	mobile phase
Auto-sampler purging	mobile phase

(iii) Optimization of HPLC for catechins, caffeine and gallic acid analysis

The analytical conditions were optimized on the basis of peak resolution, baseline, elution time, and mobile phase composition, flow rate, temperature and detection wavelength. Selectivity of the analytes was checked such that each peak of gallic acid, caffeine, catechins and internal standard had a stable chromatographic baseline and suitable resolution. The column temperature was maintained at 35 °C.

(iv) Development of internal standard relative response factors (RRF)

3-fluorocatechol, guaiacol (2-methoxyphenol), and 4-methylcatecol were tested to find their suitability as internal standards for selective separation and quantitation of the analytes under investigation. A stock solution of 100µg/mL of each internal standard was prepared using the stabilizing solution. A 1.0mL of mixed standard solution (100µg/mL) containing gallic acid, caffeine and the catechins was transferred into a graduated tube. A 1.0 ml of the internal standard solution was further added. The mixture was diluted five-fold using the stabilizing solution and thoroughly mixed in a vortex mixer. A volume of 20µL of the mixed standard of GA, (–) EGC, Caffeine, (+) C, (–) EC, (–) EGCG, (–) ECG and internal standard (2–1000µg/mL) was injected into the isocratic HPLC system. The results obtained were used to determine relative response factors (RRF) for gallic acid, caffeine and catechins using equation 2.

$$\text{RRF} = \frac{\text{(concentration of analyte) (peak area of internal standard)}}{\text{(concentration of internal standard) (peak area of analyte)}} \qquad \mathbf{2}$$

(v) Method validation

The isocratic HPLC method was validated according to Food and Drug Administration 2013 (FDA) guidelines. The test procedures for specificity, linearity, recovery, accuracy, precision (repeatability), limit of detection (LOD) and limit of quantitation (LOQ) were carried out. The linearity solutions for GA, (–) EGC, caffeine, (+) C, (–) EC, (–) EGCG, and (–) ECG were prepared by performing serial dilutions of a single stock solution in the range of 2.5–500 µg/mL and the resulting active response for each linearity solution plotted against the corresponding theoretical concentration. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation of products. Limit of detection (LOD) and limit of quantitation (LOQ) were obtained from the standard deviation (σ) of the blank response (n= 6) and slope (S) of calibration curves using the formula 3.3 σ/S and 10 σ/S, respectively. System suitability tests were performed on the HPLC system to determine the accuracy and precision of the system by injecting six times a solution of standard reference material having a well-known concentration. Reproducibility was determined using

calculated percent Relative Standard Deviation (RSD) of retention time and peak area for six injections. Sample stability was tested by monitoring changes in the levels of gallic acid, caffeine and catechins in tea at intervals of 5 h within 30 h of storage at room temperature conditions (20–25°C).

3.3 Analysis of tea samples

The tea samples were quantified for gallic acid, caffeine and the catechins by the developed isocratic reverse phase HPLC method and the *ISO 14502-2:2005(E)* method. The prepared tea sample was diluted fivefold with the stabilizing solution and the requisite amount of the internal standard added and analysed in triplicate.

3.4 Economic evaluation of the developed isocratic HPLC method and *ISO 14502-2:2005(E)* method

A cost effectiveness comparison analysis was done between the newly developed method and the *ISO 14502-2:2005(E)* method used for the determination of tea biochemicals at the Kenya Agricultural and Livestock Research Organization Tea Research Institute (KALRO-TRI) laboratories located in Kericho County, Kenya. The comparison was based on the cost of reagents, standards, sample analysis throughput and PDA detector lamp turn over.

3.5 Statistical analysis

All statistical analysis was carried out using SAS® V 9.1 (SAS.2002) for windows statistical software. Analysis of variance (ANOVA) was used to determine the means, coefficient of variation and any differences between the samples. Least Significance Difference (LSD) was used to separate means. The probability limit was set at $p \leq 0.05$ significant level. Results of the parameter determined were expressed as a mean of the triplicate determinations.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 The chromatographic column for the isocratic HPLC method

A simple, sensitive, fast, cost effective and accurate isocratic HPLC method with diode array detection for analysis of gallic acid, caffeine and catechins in tea using a suitable internal standard was developed from the fact that complete separation of the catechins and chromatographic quality are column- dependent (Ananingsih *et al*., 2013). Additionally, the mobile phase composition affects the sample matrix retention in the column and how well peaks are resolved. In this developed method a Gemini C_6-phenyl column, which is uniquely constructed to offer multiple interactions via a hexyl spacer for low adsorption of less polar groups and a phenyl group head for strong π-π interactions with the substrate (*http://www.cerij.or.jp/service/09_chromatography/L-column2_C6-Phenyl_01.html accessed on 12/11/2016)* was used (Figure 2,section 2.7.2).

The column's dual nature of both the alkyl and phenyl phases offers polar and aromatic selectivity, which makes it superior for the analysis compared to the traditional C_{18}-column. For instance, the column enabled high chromatographic resolution of structural isomer pairs of catechin and epicatechin, and also gave stable baseline.

4.2 Determination of mobile phase chromatographic conditions

4.2.1 Choice of suitable mobile phase solvent composition

A comprehensive study using eleven possible mobile phase combinations as described in section 3.2.5(i) was systematically carried out to determine the best mobile phase combination for the method. The matrix ratios (v/v) and total run time (min) for each of the mobile phase combinations were determined and are recorded in Table 2.

Table 7: Isocratic elution solvent systems for gallic acid, caffeine and catechins for HPLC assay

System	Matrix composition	Matrix ratio (v/v)	Run time (min)
1	Water: methanol: acetic acid: EDTA	80:19.3:0.5:0.2	60
2	Water: methanol: Ortho phosphoric acid: EDTA	79.3:20:0.5:0.2	60
3	Water: acetonitrile: methanol: acetic acid: ethyl acetate	77.5:18:2.0:0.5:2.0	9.7
4	Water: acetonitrile: methanol: Ortho phosphoric acid (50%) : ethyl acetate	77.5:18:2.0:0.5:2.0	9.7
5	Water: methanol: methanoic acid	79.5:20:2.0:0.5	60
6	Water: acetonitrile: methanoic acid	79.5:20:0.5	50
7	Water: acetonitrile: methanol: acetic acid	79.5 :18:2.0:0.5	14.5
8	Water: methanol: ethyl acetate	85:10: 5	40
9	Water: methanol: EDTA (20µg/ml)	79:20: 1	60
10	Water: acetic acid: acetone	None	None
11	Water: acetonitrile: acetic acid: EDTA	86.3:13:0.5:0.5:0.2	45

The chromatograms for the mobile phase combinations (1-11) are shown in Figure 2 and in Appendix I. Combinations 1, 2; 3 and 4, respectively, gave similar chromatographic finger prints for the mixed standards while peak 1 (GA) was not detected in combinations 5 and 11. Additionally, mobile phase combination 10 exhibited poor resolution for the mixed standards accompanied by a drifting base line. The results indicated that the best overall resolution, with the shortest run time, was obtained using the combination 4 mobile phase solvent composition of water-acetonitrile-methanol-ortho-phosphoric acid-ethyl acetate (77.5:18:2.0:0.5:2.0j) as shown by the mixed standards chromatogram in Figure 3.

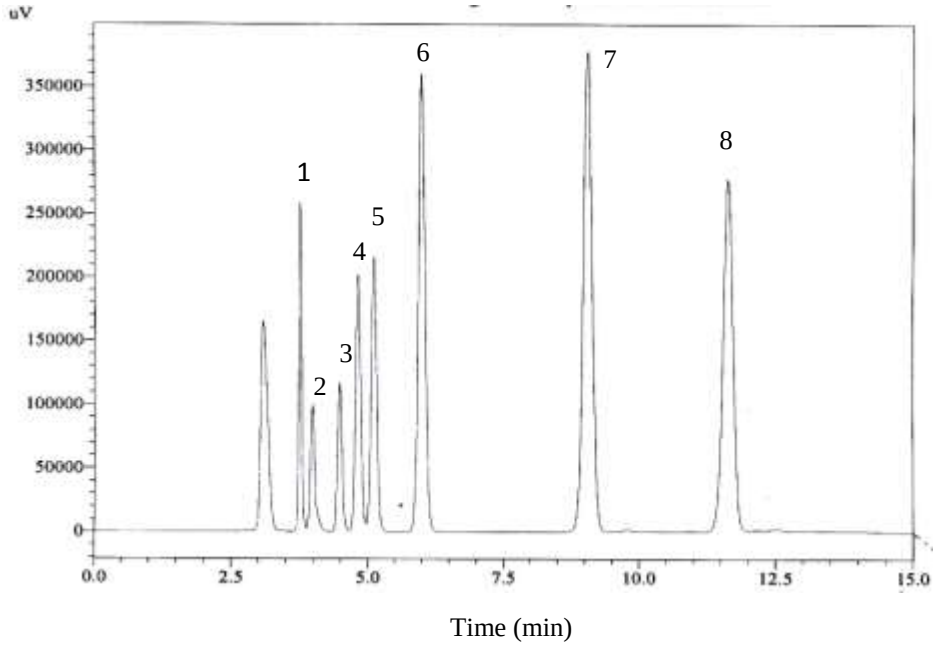


Figure 3: HPLC chromatogram of mixed standards: **1** gallic acid (GA); **2** (–) epigallocatechin (EGC); **3** caffeine (CAFF), **4** (+) catechin (+C), **5** (–) epicatechin (EC); **6** (–) epigallocatechin gallate (EGCG); **7** (–) epicatechin gallate (ECG), **8** internal standard guaiacol (2-methoxyphenol)

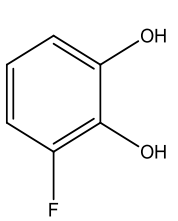
As seen from Figure 3, the above solvent composition gave well resolved peaks and an analysis time of 9.7 min for the mixed standards and 12.5 min when the internal standard was incorporated. Under the conditions of the method being investigated, these compounds were eluted in the order of gallic acid, (–) EGC, caffeine, (+) C, (–) EC, (–) EGCG, (–) ECG and guaiacol internal standard, respectively. This combination equally gave good resolutions for the peaks of interest in both non-aerated and aerated tea extracts as observed in Figures (6a) and (6b) in section 4.3, indicating that gallic acid, caffeine and catechins were all present in the teas analysed. In the presence of guaiacol (2-methoxyphenol) internal standard the total run time was achieved within 12.5 min both in the mixed standards and extracted tea samples Figures 3, (6a) and (6b) and with high detection sensitivity. These compounds were eluted at 3.776, 4.084, 4.516, 4.959, 5.192, 6.151, 9.679 and 12.062 min, respectively (Table 5 in section 4.5.3 and Appendix II). These final conditions were used to analyse the tea samples in complimentary to the other optimized conditions of the mobile phase composition flow rate of 1.0mL/min, sample injection volume of 20µL and photo diode array (PDA) detector operated at the range of 200–400nm with the wavelength of 278nm that gave maximum absorption for gallic acid, (–) EGC, caffeine, (+) C, (–) EC, (–) EGCG and (–) ECG as well as the internal standard guaiacol (2-methoxyphenol) used for detection.

The mobile phase composition of the developed method used low pH (2–4), which is necessary as catechins are unstable in basic solutions (Herrera-Herrera *et al*., 2012; Ananingsih *et al*., 2013) and can bind various metals especially calcium, magnesium, iron, zinc as well as trace levels of other minerals that would react with the catechins (Karschner,2010). Addition of very small amount of ethyl acetate increased resolution efficiency, especially for the closely eluting peaks of caffeine and EGC (Joubert and de Beer, 2011; Chen *et al*., 2015). Also, being a chelating agent helped to prevent decomposition of the catechins by binding with trace ions in the chromatographic system (Qin *et al*., 2015; Adam, 2017). The presence of acid is necessary to maintain the pH of the mobile phase low (2–4) so as to stabilize the polyphenols, modify separations of ionisable solutes and eliminate peak tailing. Methanol and ethyl acetate enhanced separation selectivity of charged analytes by increasing retention on hydrophobic bonding phases via formation of hydrogen bonds with the hydroxyl groups and oxygen ring atoms of the catechins (Jandera, 2011, Pauli *et al*., 2014).

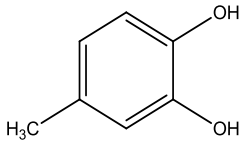
The developed method compares reasonably well with the method used by Saito *et a*l., (2006). In their method the presence of acetic acid equally gave good chromatographic separation of all the compounds studied. However, under isocratic conditions, the analysis took a total run time of 37 min. But when they introduced flow rate gradient all the components eluted within 27 min. Additionally, in their case, only caffeine and three catechins (EGCG, EC and +C) were reportedly determined. Another difference is that they used a Lichrosorb RP-C_{18} column, whereas in the new method the Gemini C_6-phenyl column used gave excellent resolution in a short time of less than 10 min for gallic acid, caffeine and all the five catechins, and both qualitative and quantitative determinations of gallic acid, caffeine and the catechins in tea yielded excellent results.

4.3 Suitable internal standard for the developed isocratic method

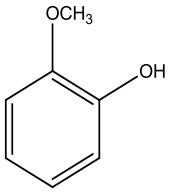
The choice of a suitable internal standard for this method was investigated amongst 3-fluorocatechol, guaiacol (2-methoxyphenol), and 4-methylcatechol since they have the phenol structure as shown below. Resveratrol (3,5,4'-trihydroxystilbene) a potential internal standard was not tested in the study.



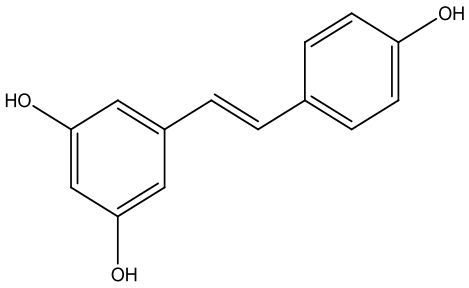
3-fluorocatechol



4-methylcatechol



guaiacol(2-methoxyphenol)



resveratrol

Therefore, the available internal standards including 3-fluorocatechol, guaiacol (2-methoxyphenol), and 4-methylcatechol were tested using HPLC with the isocratic mobile phase solvent of water–acetonitrile–methanol–ortho–phosphoric–acid–ethyl acetate (77.5:18:2.0:0.5:2.0 v/v/v/v/v) on a Gemini RP C_6-phenyl (4.6 x 250 x 5µm particle size) column. Out of the three internal standards, 3-fluorocatechol eluted the fastest with a retention time of 7.970 min followed by 4-methylcatechol at 9.613 min while guaiacol (2-methoxyphenol) came out at retention time of 12.062 min (Appendix III). The 3-fluorocatechol internal standard did not co-elute with any of the biomolecules in the mixed standards as shown in Figure 4 (a).

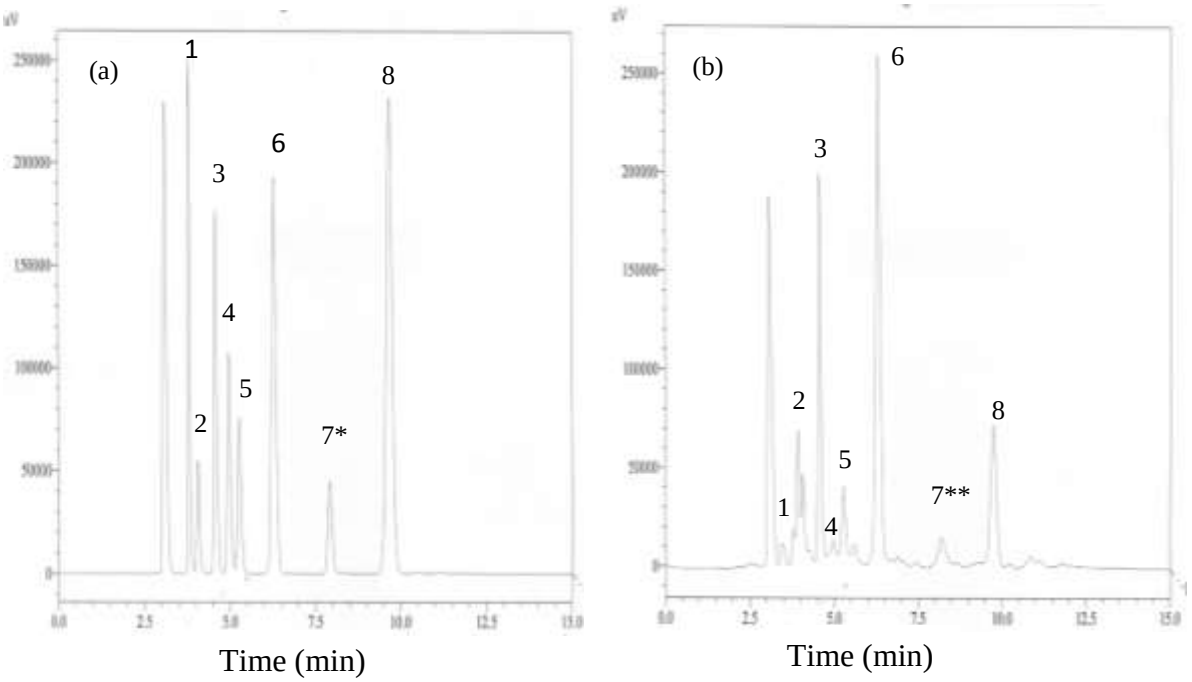


Figure 4: HPLC chromatograms of (a) mixed catechin standards spiked with 3-fluorocatechol and (b) Unspiked non- aerated (green) tea sample **1** gallic acid (GA); **2** (–) epigallocatechin (EGC); **3** caffeine (CAFF), **4** (+) catechin (+C), **5** (–) epicatechin (EC); **6** (–) epigallocatechin gallate (EGCG); **7*** 3-fluorocatechol or **7**** unknown peak and **8** (–) epicatechin gallate (ECG)

When an extracted sample of either non-aerated (green) or aerated (black) tea was run, an unknown peak (**7****) appeared at a retention time closer to that of 3-fluorocatechol peak as clearly demonstrated in Figure 4 (b) for the extract of non-aerated (green) tea. This would definitely be a source of interference, due to sample matrix effect and can affect the determined results of the tea samples. Hence, this renders 3-fluorocatechol ineffective as an internal standard for the analysis of tea samples. As earlier observed tea matrices have a large number of naturally occurring beneficial chemicals besides those targeted in the study.

On the other hand, under the specified analytical conditions of the developed method, 4-methylcatechol as internal standard eluted at a retention time of 9.613 min which was very close to that of (–) epicatechin gallate (ECG) at 9.698 min (Table 5, Appendix II and Appendix III). When 4-methylcatechol was spiked in the mixed standard solution of GA, (–) EGC, Caffeine, +C, (–) EC, (–) EGCG and (–) ECG, it co-eluted with (–) ECG as shown in Figure 5 (b), giving a fortified peak of (–) ECG standard. This is clearly seen on a closer comparison of the peak height behaviour for the catechin mixed standards for HPLC chromatogram in Figures 5(a) and 5(b) where the same mixed catechin standard was run before and after spiking with 4-methylcatechol. On spiking with 4-methylcatechol all the peaks of GA, (–) EGC, Caffeine, +C, (–) EC, and (–) EGCG diminished while that of (–)

ECG remained relatively large Figure 5 (b). The visualization of the of peaks on the chromatograms was done relative to the large peak. In this case, large peaks caused the smaller peaks to appear diminished and hence the peaks behaviour of Figure 5(b) can only be as a result of (–) ECG being fortified by the addition of the 4-methylcatechol. This made the other peaks to appear small in contrast to the observations in Figure 5 (a). This is coelution and therefore limits 4-methylcatechol as an internal standard of choice in the analysis of tea catechins. Additionally, because 3-fluorocatechol and 4-methylcatechol eluted in-between the standards, they were rendered inacceptable as good internal standards. Consequently, in a complex sample matrix, rich in biomolecules like natural tea, they would exert interferences and at the same time fail to distinguish instrumental condition which would cause interferences that might affect analytes that elute after them (Oroian and Escriche, 2015).

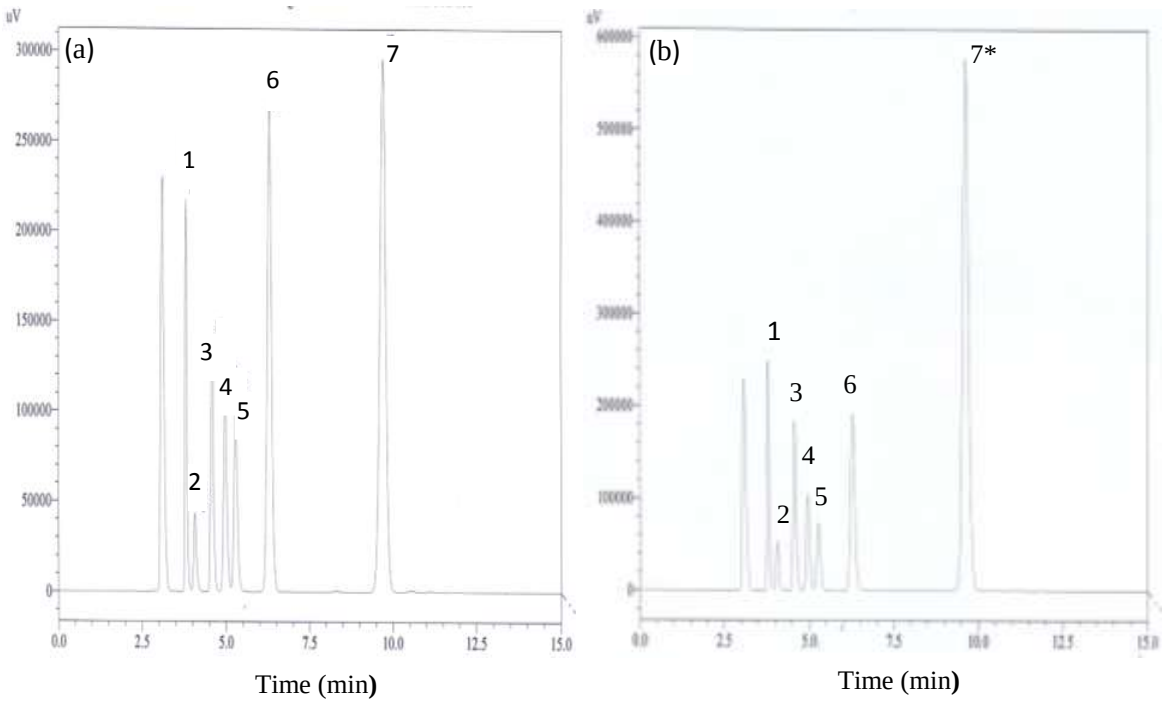
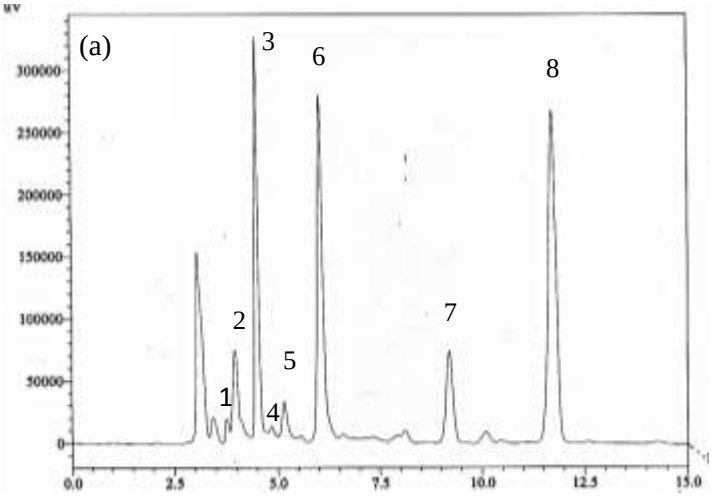


Figure 5: HPLC chromatogram: (a) Catechins mixed standards (b) Catechins mixed standards spiked with 4-methylcatechol: **1** gallic acid (GA); **2** (–) epigallocatechin (EGC); **3** caffeine (CAFF), **4** (+) catechin (+C), **5** (–) epicatechin (EC); **6** (–) epigallocatechin gallate (EGCG), **7** (–) epicatechin gallate (ECG) and **7*** (–) epicatechin gallate (ECG) fortified/or overlapped with 4-methylcatechol.

Guaiacol (2-methoxyphenol) was the preferred internal standard for the developed method since it did not co-elute with the mixed standards and/or components of the tea sample matrices, including analytes of interest in this study; Figures 3, 6 (a) and 6 (b). With a retention time of 12.062 min, guaiacol (2-methoxyphenol) is unlikely to co-elute with the components in the tea sample matrix which can cause interferences for by this time all

components in injected tea extract will have come out of the column. This was evident in all the tea types, whether non-aerated (green) or aerated black tea; Figures 7 (a) and 7 (b) section 4.5.1. Hence, guaiacol was used in all experimental analyses and gave good quantities of gallic acid, caffeine and the major catechins.



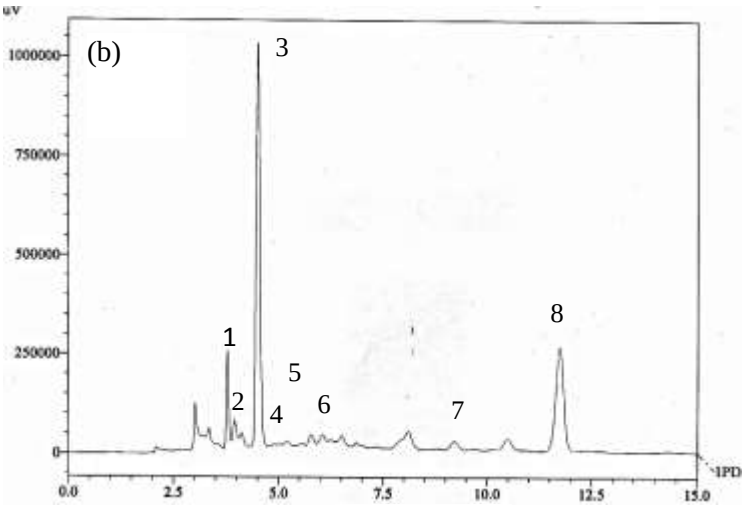


Figure 6: HPLC chromatogram: (a) non-aerated (green), (b) aerated (black) teas: **1** gallic acid (GA); **2** (–) epigallocatechin (EGC); **3** caffeine (CAFF), **4** (+) catechin (+C), **5** (–) epicatechin (EC); **6** (–) epigallocatechin gallate (EGCG); **7** (–) epicatechin gallate (ECG) and **8** guaiacol (2-methoxyphenol) internal standard

4.4 Relative response factors (RRF)

In chromatography, a response factor is defined as the ratio between the concentration of a compound being analyzed and the response of the detector to that compound (Jennings, 2012). A chromatogram will show a response from a detector as a peak that will aid in the

calculation of the response for each analyte. The individual analyte response is then used to establish the RRF between two analytes

The relative response factor results obtained for GA, caffeine and the catechins relative to the internal standard guaiacol (2-methoxyphenol) are provided in Table 3. The RRFS were subsequently used for quantitation purposes and gave satisfactory levels of the studied biomolecules in tea. From the findings of the study, the RRF can be reliably used in quantitation of GA, (–) EGC, Caffeine, (+) C, (–) EC, (–) EGCG and (–) ECG in different types of tea and tea based products.

Table 8: Relative response factors (RRF) for GA, caffeine and the catechins in relation to guaiacol (2-methoxyphenol) internal standard

Component	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean
GA	0.29	0.28	0.29	0.28	0.28	0.29
EGC	3.72	3.49	3.53	3.41	3.49	3.56
(+) C	1.85	1.82	1.85	1.81	1.82	1.83
CAFF	0.35	0.36	0.37	0.35	0.36	0.36
EC	1.55	1.55	1.56	1.52	1.54	1.55
EGCG	0.82	0.81	0.82	0.80	0.80	0.81
ECG	0.64	0.61	0.61	0.60	0.61	0.62

GA- gallic acid; EGC- epigallocatechin; (+) C- (+) catechin; CAFF- Caffeine; EC- epicatechin; EGCG-epigallocatechin gallate

4.5 Validation of the Method

The performance characteristics in this study were specificity, linearity, precision, accuracy, LOD and LOQ. Further, a method comparison study was done in order to ascertain effectiveness of the method developed (Taverniers *et a*l., 2010).

4.5.1 Specificity of the method

The specificity of the method was investigated by injecting a mixed standard for GA, caffeine and the catechins prepared according to the procedure in section 3.2.4 into the HPLC system. Further, extracted placebos for both non-aerated (green) and aerated (black) tea extracts were analysed to demonstrate the absence of coelution of analyte and other constituents of the sample. The analytical conditions gave good specificity as the method was selective in the analysis of the mixed standard as was demonstrated in Figure 3. The

chromatographic peaks for the non-aerated (green) and aerated (black) tea extracts also showed good specificity and reliability in achieving the intended purpose of discriminating the analytes of interest from other substances in the test materials; Figures 7 (a) and 7 (b). The potential interferants in the validation of selectivity was the presence of closely related structural isomers especially (+) C and (-) EC.

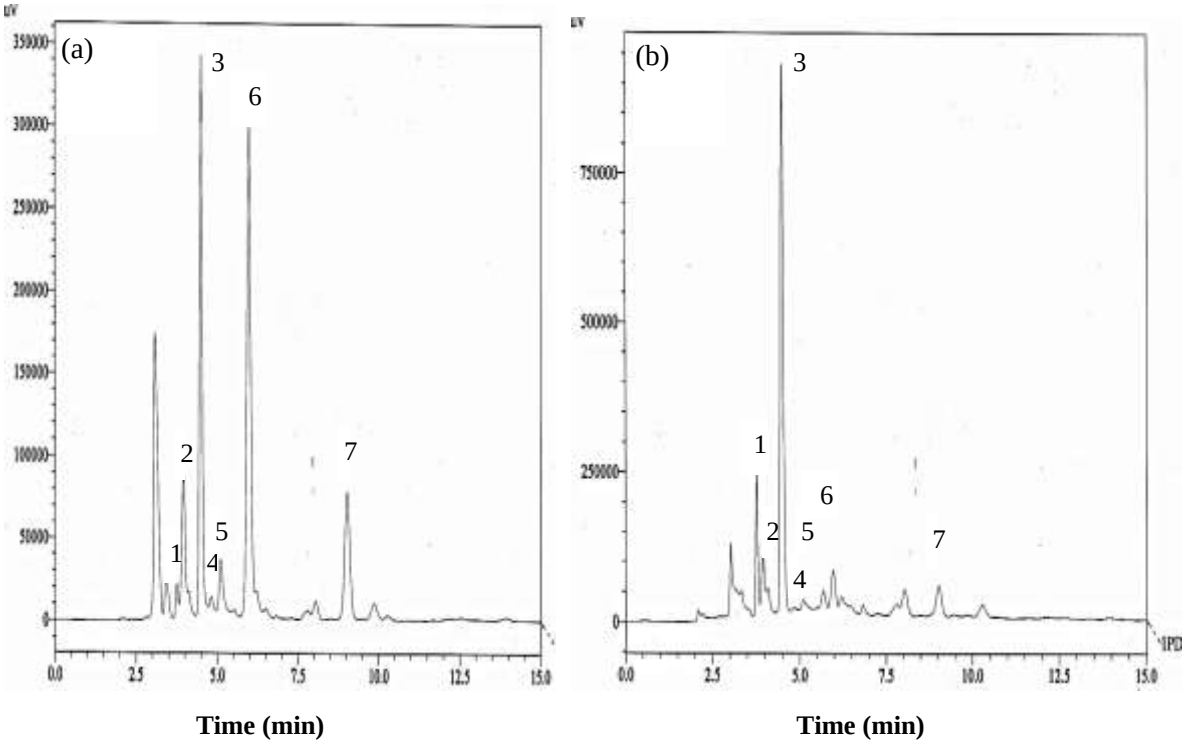


Figure 7: HPLC chromatogram (a) non- aerated (green) tea, (b) aerated (black) tea: **1** gallic acid (GA); **2** (–) epigallocatechin (EGC); **3** caffeine (CA), **4** (+) catechin (+C), **5** (–) epicatechin (EC); **6** (–) epigallocatechin gallate (EGCG) and **7** (–) epicatechin gallate (ECG).

4.5.2 System suitability: Linearity, level of detection (LOD) and quantitation (LOQ) of the method

The linearity, LOD and LOQ were evaluated to determine the suitability of the developed method in the analysis of gallic acid, caffeine and the catechins in tea.

(i) Linearity of the method

The standard solutions were prepared at six concentrations in appropriate concentration ranges of 2.5–500 µg/ml. Each of the individual standards GA, (–) EGC, Caffeine, (+) C, (–) EC, (–) EGCG and (–) ECG was prepared in triplicate at each concentration. The mean peak area of the three injections and their corresponding signal levels were used to generate equations for the regression line and correlation coefficient (R^2)

for each of the standards. The curves were plotted for peak area (PA) ratios against concentration (µg/ml). Peak area (PA) ratio was taken to be area of a given concentration of the individual standard divided by area of added quantity of internal standard.

All calibration curves were linear over a wide concentration range with correlation coefficient greater (>) than 0.997 (Figure 8 and Appendix IV). The equations for the calibration curves are provided in Table 4. This showed that the method met the requirements of validation based on this parameter and hence fit for application during analysis.

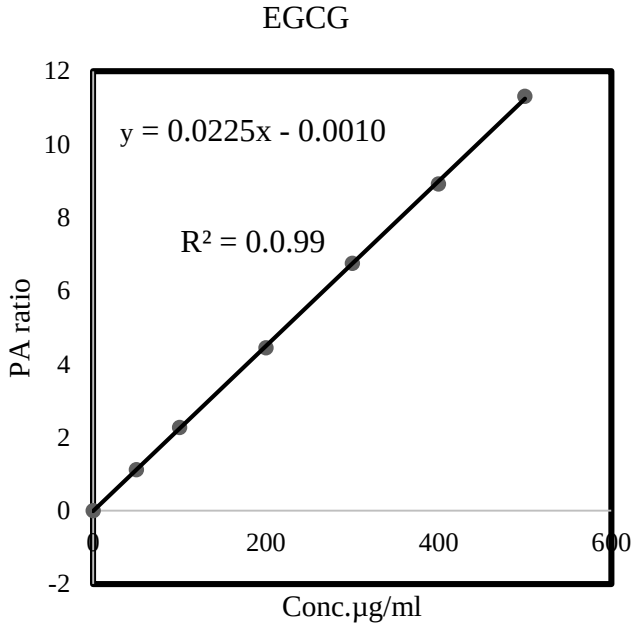


Figure 8: Standard calibration curve for (–) Epigallocatechin gallate (EGCG)

Table 9: Linearity, correlation coefficient (R^2), limit of detection (LOD) and limit of Quantitation (LOQ) of the studied biomolecules.

Component	Linear range (µg/mL)	Regression line equation	Correlation coefficient(R^2)	LOD (µg/mL)	LOQ (µg/mL)
EGC	25–400	y = 0.0003 x – 0.0015	0.9995	0.06	0.18
+C	25–250	y = 0.0110 x – 0.0162	0.9969	0.09	0.27
EC	25–400	y = 0.0136 x – 0.0270	0.9970	0.02	0.18
EGCG	25–250	y = 0.0225 x – 0.0010	0.9999	0.16	0.48
ECG	25–250	y = 0.0031 x + 0.0420	0.9969	0.11	0.33
GA	2.5–30	y = 0.0480 x – 0.0183	0.9998	0.04	0.12
Caffeine	2.5–30	y = 0.0441 x – 0.0058	0.9990	0.07	0.21

GA- gallic acid; EGC- epigallocatechin; (+) C- (+) catechin; CAFF- Caffeine; EC- epicatechin; EGCG-epigallocatechin gallate

(ii) The detection limits: LOD and LOQ

Limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value whereas the quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD found was 0.06, 0.09, 0.02, 0.16, 0.11, 0.04 and 0.02 (µg/mL), whereas, the LOQ was 0.18, 0.27, 0.18, 0.48, 0.33, 0.12 and 0.21 (µg/mL) for (–) EGC, (+) C, (–) EC, (–) EGCG (–) ECG, GA and Caffeine, respectively as in Table 4 above. The low levels indicate that the method is capable of detecting significantly low levels of gallic acid, caffeine and catechins in tea matrices and tea based products. Moreover, the conditions set in the developed method can be used in making decisions in tea related product formulations which would otherwise require very low levels of tea lacing to enhance flavour and strength. This is in line with the Food and Drug Administration (FDA) method as has been used in other developed methods (Spellman, 2016).

4.5.3 Reproducibility (Precision) of the method

. The precision of the method was investigated by preparing one sample solution containing the target level of analyte. Ten replicates of this sample solution were analysed with the retention time and peak area being recorded. The mean, standard deviation and relative standard deviation (RSD %) found are summarized in Table 5. The within run

precision (RSD %) ranged from 0.241–2.175 % for all the compounds studied. This demonstrates that the present method is sufficiently precise for all samples tested and can be used to get good predictive results.

Table 10: Precision test for GA, (–) EGC, (+) C, CAFF, (–) EC, (–) EGCG and (–) ECG

Component	**Parameter**	**Mean**	**SD**	**RSD%**
GA - (2.5µg/ml)	RT	3.776	0.029	0.768
	PA	113606	2471	2
EGC - (50µg/ml)	RT	4.084	0.031	0.759
	PA	197057	474	< 1
CAFF - (2.5 µg/ml)	RT	4.516	0.054	1.196
	PA	133484	785	1
+ C - (25 µg/ml)	RT	4.90	0.055	1.122
	PA	326029	75975	2
EC - (25 µg/ml)	RT	5.192	0.050	0.963
	PA	430567	2541	1
EGCG - (25 µg/ml)	RT	6.121	0.054	0.898
	PA	627129	5796	1
ECG - (25 µg/ml)	RT	9.659	0.087	0.954
	PA	114368	574	1

GA- gallic acid; EGC- epigallocatechin; (+) C- (+) catechin; CAFF- Caffeine; EC- epicatechin; EGCG-epigallocatechin gallate; RT- retention time; PA peak area

4.5.4 Accuracy of the method

Accuracy refers to the deviation of a measurement from a standard or true value of the quantity being measured (Avery and Burkhart, 2015). Accuracy of the analytical method was determined by preparing quality control (QC) materials of GA, (–) ECG, (+) C, caffeine, (–) EC, (–) EGCG, and (–) EGC in a similar way to unknown samples at four predetermined concentration levels. The predetermined concentration levels targeted low, medium and high concentration levels of the catechins, gallic acid and caffeine in tea types. The QC materials

were repeatedly measured to determine the accuracy of the determinations. The mean recoveries for the catechins ranged from 99.2–105.5%, while the recovery for gallic acid was 99.9% and that for caffeine was 105.3% and are summarized in Table 6. These results demonstrated that the method is satisfactory for the intended purpose and adequate for routine analysis. The more than 100% recovery rates could be explained in terms of the standards acquired for quality control material. The percentage purity for the standards were in the range 95-99.9%. It was not possible to immediately determine the effect the impurities would have on the sample absorbance values as this would require more specialized equipment which were not available during the analysis period. The other factors that would affect the result were the challenges encountered in handling the small size standard sample supplied (most often $\leq$ 1.00mg), difficulties in obtaining moisture free and the instability of the sample standard material especially the catectins to atmospheric conditions as they easily oxidize forming other oxidative products. According to the Food and Drug Administration (FDA) standard used for validation purpose in this study in its specific recommendations for method validation, it is stated that accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). It is further stated that the mean value of the determination should be within ±15% of the theoretical value for the method to be considered validated in accuracy and precision.

Table 6: Accuracy test for GA, (–) EGC, (+) C, CAFF, (–) EC, (–) EGCG and (–) ECG

Tea Component	Concentration (µg/ml)	Accuracy (µg/ml)	Recovery (%)
Gallic acid	2	2.10 ± 0.50	105.0
	5	4.84 ± 0.50	96.8
	10	9.79 ± 0.50	97.9
	15	14.66 ± 0.50	97.7
Epigallocatechin	25	24.32 ± 0.90	97.3
	50	50.89 ± 0.20	101.8
	100	98.70 ± 0.60	98.7
	150	148.79 ± 0.80	99.2
+ Catechin	25	26.81 ± 0.50	107.2
	50	50.15 ± 0.50	100.2
	100	99.39 ± 0.50	99.4
	150	149.02 ± 0.50	99.3
Caffeine	2	2.04 ± 0.90	102.0
	5	5.55 ± 0.70	111.0
	10	10.35 ± 0.50	103.5
	15	14.95 ± 0.90	99.7
Epicatechin	25	26.53 ± 0.90	106.1
	50	48.98 ± 0.80	98.0
	100	102.35 ± 0.70	102.4
	150	148.83 ± 0.90	99.2
Epigallocatechin	25	25.23 ± 0.40	100.9
gallate	50	50.10 ± 0.90	100.2
	100	103.71 ± 0.82	103.7
	150	151.52 ± 0.60	101.0
Epicatechin	25	25.32 ± 0.50	101.3
gallate	50	49.37 ± 0.90	98.7
	100	100.97 ± 0.70	101.0
	150	152.39 ± 0.70	101.6

4.6 Analysis of tea samples by the developed method

In this study, processed non-aerated (green) CTC, non-aerated (green) orthodox, aerated (black) CTC and aerated (black) orthodox teas from Kangaita tea factory of Kirinyaga County in Kenya, were extracted for gallic acid, caffeine and catechins using the *ISO 14502-2:2005(E)* procedures and analysed in triplicate by the developed method. All the components (gallic acid, caffeine and the five catechins (GA, (–) EGC, (+) C, (–) EC, (–) EGCG and (–) ECG) were found in all the tea types Figure 6 (a) and 6 (b). The results are shown in Table 7. The *ISO 14502-2:2005(E)* method was applied for comparison purposes and statistical analysis carried out using SAS® V 9.1 for windows statistical software (SAS, 2002). ANOVA was used to determine the means, coefficient of variation and Least Significance Difference (LSD) was used to separate means. The probability limit was set at $p \leq 0.05$ significant level and the standard deviation (SD) done using the student t-test. The percent moisture and dry matter contents were determined as described in section 3.2.2. Table 7 shows a characteristic data recorded for percent moisture and dry matter contents of the tea types used in this study**.** The moisture in the different green and black tea samples were in the range 5–6 % M.C and 94–95% DM, respectively.

Table7: Percent moisture (% M.C) and dry matter (%DM) contents for the different tea grades

Tea type	**% Moisture content**				**% dry matter content**
	1	**2**	**3**	**Mean**	
Non-aerated green CTC	6.0	5.0	5.0	5.0	95
Non-aerated green orthodox	6.0	6.0	5.0	6.0	94
Aerated CTC	6.0	5.0	5.0	5.0	95
Aerated orthodox	6.0	5.0	6.	6.0	94

As seen in Table 8, the major catechin in non-aerated (green) CTC and non-aerated (green) orthodox teas was (–) EGCG which registered 8.86 ± 1.27% and 9.09 ± 0.56%, while in aerated (black) CTC and aerated (black) orthodox teas it registered (–) EGC at 2.45 ± 0.08% and 1.74 ± 0.01%, respectively. The order of catechin quantities in the green teas was EGCG > EGC > ECG > EC > +C, whereas the order was EGC > EGCG ≈ ECG > EC ≥ +C for the Black tea quantities. The amount of EGCG observed in the extracted samples of catechins is supported by findings of different groups of scientists who reported that in HPLC

analysed green tea samples EGCG was higher in quantity than other catechins (Ahmad *et al.,* 2014).

In general, both non-aerated (green) CTC and non-aerated (green) orthodox teas contained significantly higher concentrations of catechins than the black tea samples, whereas the contents of gallic acid and caffeine remained constant in all the teas. The differences can be attributed to the manufacture process where catechins combine with the biological enzyme polyphenol oxidase(PPO), and with the help of the atmospheric oxygen, oxidize into theaflavins and thearubigins. Gallic acid and caffeine are affected minimally by the oxidative process of manufacture, and hence, remain relatively constant during the oxidative degradation of the catechins and other polyphenols.

Looking at the individual types of tea, the total catechins percentages found by the new developed method were 19.46% and 19.88% for non-aerated CTC and orthodox teas, 3.65% and 3.37% for aerated CTC and Orthodox teas. In this regard, the results for green types of tea obtained by this developed method were comparable with those of Anesini *et al*., (2008), which were highlighted to fall in the range of 14.32 to 21.02 % for total polyphenol catechins in green tea. Overall, the total catechins found by the developed method were on average higher than those of the *ISO 14502-2:2005(E)* method. Conversely, the t-test proved that the results obtained by the two methods did not differ significantly at the 95% level. This is not surprising given that the same solvent mixture, methanol-water (7: 3 v/v) was used to extract the biomolecules from the teas. As a result, equal amounts of GA, Caffeine, (–) EGC, (+) C, (–) EC, (–) EGCG and (–) ECG) were found by the two methods. Furthermore, due to closer polar proximity of the catechins with methanol, methanol can be expected to form hydrogen bonds with the hydroxyl groups and the oxygen ring atoms of catechins leading to the enhanced extraction, whilst the water assists the tea leaves in spreading matrix structure. The main advantage of the developed method over the *ISO 14502-2:2005(E)* method is that it takes <10 minutes to complete the analysis, which is approximately five times faster compared to the *ISO 14502-2:2005(E)* method (run time = 40–45 minutes under gradient elution).

Therefore, the developed isocratic HPLC–PAD method in this study is economical and less complicated. And with the modifications, optimizations and validation incorporated in this method, it can be applied to analyze gallic acid, caffeine and catechins in tea in an ordinary laboratory in 12.5 min.

Table 8: Method comparison for levels of GA, Caffeine and Catechins

Tea type	Component	mean ± SD (n= 3)		ANOVA	
		New method	**ISO Method**	**CV**	**LSD**
Green CTC	GA	0.60[a]* ± 0.03	0.63[a]* ± 0.20	**12.8**	**0.28**
	EGC	5.76[a]* ± 0.61	5.86[a]* ± 0.50	**5.8**	**1.10**
	Caffeine	3.52[a]* ± 0.24	3.73[a]* ± 0.21	**3.8**	**0.49**
	+C	0.56[a]* ± 0.03	0.53[a]* ± 0.03	**7.3**	**0.14**
	EC	1.97[a]* ± 0.24	1.36[a]* ± 0.19	**14.8**	**0.86**
	EGCG	8.86[a]* ± 1.29	8.71[a]* ± 0.43	**13.8**	**4.24**
	ECG	2.31[a]* ± 0.37	2.29[a]* ± 0.19	**8.7**	**0.70**
	Total catechin	19.46[a]* ± 1.27	18.74[a]* ± 1.02	**8.4**	**5.63**
Green	GA	0.72[a]* ± 0.05	0.68[a]* ± 0.04	**7.9**	**0.19**
Orthodox	EGC	6.20[a]* ± 0.21	5.92[a]* ± 0.06	**2.9**	**0.62**
	Caffeine	3.28[a]* ± 0.46	3.82[a]* ± 0.20	**7.5**	**0.94**
	+C	0.50[a]* ± 0.03	0.50[a]* ± 0.02	**3.4**	**0.06**
	EC	1.80[a]* ± 0.09	1.55[a]* ± 0.13	**5.9**	**0.35**
	EGCG	9.09[a]* ± 0.56	9.37[a]* ± 0.53	**8.2**	**2.67**
	ECG	2.30[a]* ± 0.08	2.65[a]* ± 0.21	**6.2**	**0.54**
	Total catechin	19.88[a]* ± 0.50	20.03[a]* ± 0.36	**2.9**	**2.00**
Black CTC	GA	0.40[a]* ± 0.04	0.48[a]* ± 0.06	**8.8**	**0.14**
	EGC	2.45[a]* ± 0.08	2.49[a]* ± 0.06	**3.7**	**0.32**
	Caffeine	3.52[a]* ± 0.42	3.70[a]* ± 0.37	**6.9**	**0.87**
	+C	0.19[a]* ± 0.02	0.20[a]* ± 0.02	**12.6**	**0.09**
	EC	0.16[a]* ± 0.04	0.14[a]* ± 0.05	**9.2**	**0.05**
	EGCG	0.48[a]* ± 0.04	0.36[a]* ± 0.03	**12.2**	**0.18**
	ECG	0.47[a]* ± 0.05	0.44[a]* ± 0.04	**7.1**	**0.11**
	Total catechin	3.65[a]* ± 0.04	3.63[a]* ± 0.08	**8.8**	**0.21**
Black	GA	0.52[a]* ± 0.02	0.54[a]* ± 0.04	**6.7**	**0.13**
Orthodox	EGC	1.74[a]* ± 0.01	1.57[a]* ± 0.51	**21.4**	**1.25**
	Caffeine	3.07[a]* ± 0.05	3.46[a]* ± 0.18	**4.9**	**0.56**
	+C	0.20[a]* ± 0.04	0.20[a]* ± 0.05	**13.9**	**0.10**
	EC	0.30[a]* ± 0.05	0.22[a]* ± 0.02	**12.2**	**0.11**
	EGCG	0.43[a]* ± 0.02	0.58[a]* ± 0.10	**14.8**	**0.26**
	ECG	0.69[a]* ± 0.06	0.71[a]* ± 0.04	**8.4**	**0.21**
	Total catechin	3.37[a]* ± 0.07	3.29[a]* ± 0.56	**11.2**	**1.31**

[a]* Data expressed as $p \leq 0.05$ compared to ISO method

4.7 Cost analysis comparison between the developed method and ISO 14502-2: 2005 (E)

Sample preparation and extraction in both the newly developed isocratic and the ISO 14502-2: 2005 (E) methods were handled in the same manner. The two methods also used the same analytical column, pump systems with a mobile flow rate of 1.0 ml/min and PDA detection system operated at 278 nm. In the ISO 14502-2: 2005 (E) method gradient elution system was used requiring the use of two mobile phases simply called mobile phase A and B (Table 9). The elution system for the developed HPLC method was kept constant throughout the entire analysis period at a composition ratio of water-acetonitrile-methanol-ortho-phosphoric acid-ethyl acetate (77.5:18:2.0:0.5:2.0 v/v/v/v/v) as earlier demonstrated. The cost for each of the reagents used in the generation of mobile phases in the two methods was determined and is shown in Table 9.

4.7.1 Cost dynamics of one litre mobile phase in ISO 14502-2: 2005 (E) and the newly developed HPLC methods

The combined cost of making one litre mobile phase each of A -Water: acetonitrile: acetic acid (88: 9: 2 v/v/v) and B-Water: acetonitrile: acetic acid (18: 80: 2 v/v/v) in the ISO 14502-2: 2005 (E) method was Ksh. 2,377 while one litre of the new HPLC isocratic method was Ksh. 781.5 (Table 9). The cost difference is atleast three times more in favour of the new method. Table 10 shows the sample throughput in the two methods for a one litre mobile phase and the associated income value based on the service charter analysis cost of Ksh 3,500 currently charged to profile a tea sample at the KALRO-TRI. The ISO 14502-2: 2005 (E) method uses a total run time of 42 min to profile a single sample. The single sample run time for new HPLC isocratic method is 12.5 min with internal standard guaiacol (2-methoxyphenol) incorporated for quantitation purposes. At a mobile flow rate of 1.0 mL/min one sample would require 42 mL for complete profiling using the ISO method while in the new developed method only 12.5 mL would be required. Based on the dynamics of the mobile phase alone, assuming the other analysis factors were held constant, it can be demonstrated that the ISO method can generate Ksh. 81,623 only for one litre of mobile phase used while the developed method can generate Ksh. 279,218.5 from a client charged on the basis of one litre mobile phase consumed. The cost difference is substantial and hence there is need to develop analytical methods with short analysis run times. The findings clearly demonstrate that the newly developed isocratic HPLC method is superior to the ISO 14502-2: 2005 (E) method in terms of sample throughput at 80 and 24 samples per litre mobile phase consumed respectively, effective in cost reduction and income generation.

Table 9: Cost of making one litre of mobile phase for ISO 14502-2: 2005 (E) and newly developed HPLC isocratic methods

Mobile phase	Reagent	Quantity (mL)	Cost (Ksh.)
ISO 14502-2: 2005 (E)	HPLC water	880	445
(mobile phase A)	Acetonitrile	90	162
	Acetic acid	20	120
	Sub-Total	**1,000**	**727**
ISO 14502-2: 2005 (E)	HPLC water	180	90
(mobile phase B)	Acetonitrile	800	1,440
	Acetic acid	20	120
	Sub-Total	**1,000**	**1,650**
	Grand Total	**2,000**	**2,377**
New HPLC Isocratic method	HPLC water	775	387.5
	Acetonitrile	180	324
	Methanol	20	20
	Ortho-phosphoric acid	5	30
	Ethyl acetate	20	20
	Grand Total	**1,000**	**781.5**

Cost of reagents (Ksh): HPLC water (1L) = 500; Acetonitrile (2.5L) = 4,500; Acetic acid (1L) = 6000; Methanol (2.5L) = 2,500; Ortho-phosphoric acid (1L) = 6000; Ethyl acetate (2.5L) = 2,500.

Table 10: Sample throughput analysis based on one litre mobile phase each of ISO 14502-2: 2005 (E) and the new isocratic HPLC methods

Item	**ISO 14502-2: 2005 (E) method**	**New isocratic HPLC method**
Cost (Ksh.) of mobile phase	2,377 (A and B)	781.5
Total analysis time (min)	42 min	12.5 min
Mobile phase volume	42 mL	12.5 mL
Sample throughput 1litre mobile phase	24	80
Income generated per litre (Ksh.)	**81,623**	**279,218.5**

4.7.2 Cost effectiveness based on standards and mobile phase used

The ISO method uses external standards of caffeine to quantify the analytes where three concentration levels of calibration curve standards must be run for every set of 20 samples analysed per day in the ISO 14502-2: 2005 (E) method. On the other hand, the new method makes use of an internal standard, guaiacol (2-methoxyphenol) and hence does not require a run time for standards. Table 11 shows cost analysis based on standards used in the two methods.

Table 11: Cost of standards in analysis of 20 samples using ISO 14502-2: 2005 (E) and newly developed Isocratic HPLC methods

Method	**Item**	**Quantity**	**Cost (KShs)**
ISO method	Sample analyzed	20	
	External standard used	5,10,15µg/mL equivalent to 300µg caffeine*	6
	Internal Standard	-	-
	Cost of mobile phase	1000Ml	2,377
	Total cost ISO method		**2,389**
New isocratic method	Sample analysed	20	
	External Standard used	-	
	Internal standard	30µg/mL spiked in each sample equivalent to $2.7x10^{-6}$µL guaiachol**	0.0016
	Cost of mobile phase	1000	781.5
	Total cost New isocratic method		**781.502**

where,

1. *100µg caffeine was weighed in 500mL distilled water. A total of 3mL of the stock solution was used to make the three concentrations of (5, 10, 15µg/mL).
2. **54µL guaiacol (IS) was taken into 10mL methanol to make stock. Further 10µL of the stock was standardized to 2mL.

From the findings of Tables 11 above it is more than six times more expensive to use the caffeine calibration curve of the ISO 14502-2: 2005 (E) method compared to guaiachol (IS) used in the newly developed isocratic HPLC method. Furthermore, the calibration curve method is time consuming as the standards have to be analysed before the samples. This leads to the use of extra mobile phase leading to increase in analysis cost and the overall analysis period. It is equally evident that the cost of mobile phase used is three times more expensive for the ISO 14502-2: 2005 (E) method than the newly developed method.

4.7.3 Cost effectiveness based on detector lamps

The PDA detector system uses both a UV–vis and tungsten filament lamps. The two lamps complement each other during wavelength scanning in the range of 190–800 nm. The lamps have a very specific life time of 2000 h, beyond which the reliability of data obtained is highly compromised. Table 12 provides the cost saving dynamics of the new method based on the detector operating time.

Table 12: PDA detector analysis cost dynamics between ISO 14502-2: 2005(E) and new Isocratic HPLC method

Item	ISO 14502-2: 2005(E) method	New Isocratic HPLC method
Detector	PDA	PDA
Lamp	Uv-vis/tungsten filament	Uv-vis/tungsten filament
Lamp life time	2000h	2000h
Sample analyzed(2000hr)	2,900	9,600
Cost of sample profiled	10,150,000	33,600,000

It is clearly seen that the new method can analyze three times more samples in the life time of the detector lamps compared to the ISO 14502-2: 2005(E) method. From the findings in Table 12 and proceedings elsewhere in this documentation, it is quite evident that the new method will immensely cut down the cost of doing analysis and greatly improve the speed of doing analysis.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Tea is a popular beverage and currently is consumed in many parts of the world. Tea value added products, specialty (orthodox and oolong), green, white and purple teas are some of the products of tea which are become popular in the world market. Characterization of these products based on their biochemical composition for ease of identification and traceability of their origin has become of great concern. To complement the current methods in use, there was need to develop a sensitive, fast, cost effective and accurate HPLC method. From the findings in this study, it can be concluded that:

1. An improved isocratic HPLC method using a Gemini C_6-phenyl column and water-acetonitrile-methanol-ortho-phosphoric acid-ethyl acetate (77.5:18:2.0:0.5:2.0 v/v/v/v/v) mobile phase solvent has been developed and can qualitatively and quantitatively determine gallic acid, caffeine, (–) EGC, (+) C, (−) EC, (−) EGCG and (−) ECG in tea and tea related products.
2. Using this method, with guaiacol (2- methoxyphenol) as an internal standard, gallic acid, caffeine and the catechins include (–) EGC, (+) C, (–) EC, (–) EGCG and (–) ECG) were all rapidly separated with excellent chromatographic resolutions within 12.5 min sample run time.
3. The method validation parameters of specificity, accuracy and precision (repeatability) prove that the method is reliable, simple and adequate for the quantitative determination of tea catechins, gallic acid and caffeine in tea samples because of good performance of the mobile phase solvent composition, guaiacol (2-methoxycatechol) internal standard and the column.
4. The results obtained by the new developed method for values of gallic acid, caffeine and the catechins were 19.46% and 19.88% for non-aerated CTC and orthodox teas whereas aerated CTC and Orthodox teas had 3.65% and 3.37%, respectively. These results also indicated no significant statistical difference betwen the developed method and the ISO 14502-2:2005(E) method.

5.2 Recommendations

1. The developed relative response factors (RRF) for gallic acid, caffeine, (−) EGC, (+) C, (−) EC, (−) EGCG and (−) ECG alongside the internal standard guaiacol (2-methoxyphenol) be subjected to intensive inter-laboratory use, with the aim of their adoption as standard parameters for laboratories dealing with analysis of tea biomolecules in different types of teas and tea value added products.
2. Resveratrol should be investigated as an internal standard be investigated in further studies since it is a stronger reducing agent than guaiacol (2- methoxyphenol).
3. This work be extended to the determination of other tea quality parameters like the levels of anthocyanins in purple tea varieties, whose determination is known to be expensive and requires long analysis time.

REFERENCES

Adam, A. A. E. M. (2017). *Isolation, Characterization and Biological Activity of Flavonoids from Cordia Sinensis and Acacia Orefota* (Doctoral dissertation, Sudan University of Science and Technology).

Ahmad, R. S., Butt, M. S., Huma, N., Sultan, M. T., Arshad, M. U., Mushtaq, Z. and Farhan Saeed, F. (2014). Quantitative and Qualitative Portrait of Green Tea Catechins (Gtc) Through HPLC, *International Journal of Food Properties*, **17**(7), 1626-1636.

Almes, G., Kalidindi, S., Zekauskas, M., and Morton, A. (2016). *A one-way delay metric for IP performance metrics (IPPM)* (No. RFC 7679).

Amerine, M. A., Pangborn, R. M., and Roessler, E. B. (2013). Principles of sensory evaluation of food. Elsevier.

Anderson, R. A., and Polansky, M. M. (2002). Tea enhances insulin activity. *Journal of agricultural and food chemistry*, **50**(24), 7182-7186.

Ananingsih, V. K., Sharma, A., and Zhou, W. (2013). Green tea catechins during food processing and storage: a review on stability and detection. *Food Research International*, **50**(2), 469-479.

Anesini, C.; Ferraro, G.E.; Filip, R. (2008). Total polyphenol content and antioxidant capacity of commercially available tea (Camellia sinensis) in Argentina. *Journal of Agriculture and Food Chemistry*, **56** (19), 9225–9229.

Atoui, A. K., Mansouri, A., Boskou, G., and Kefalas, P. (2005). Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food chemistry*, **89**(1), 27-36.

Avery, T. E., and Burkhart, H. E. (2015). *Forest measurements*. Waveland Press.

Babu, Sukumaran Santhosh, Vakayil K. Praveen, and Ayyappanpillai Ajayaghosh (2014). "Functional π-gelators and their applications." *Chemical reviews* **114** (4): 1973-2129.

Badea, G. A., and Antoce, A. O. (2015). Glutathione as a possible replacement of sulphur dioxide in wine making technologies: a review. *Scientific Papers Series B, Horticulture*, **59**, 123-40.

Bahorun, T., Luximon - Ramma, A., Neergheen - Bhujun, V. S., Gunness, T. K., Googoolye, K., Auger, C., and Aruoma, O. I. (2012). The effect of black tea on risk factors of cardiovascular disease in a normal population. *Preventive medicine*, **54**, S98-S102.

Basu- Majumder, A., Bera, B., and Rajan, A. (2010). Tea statistics: global scenario. *Inc. Journal of Tea Science*, **8**(1), 121-124.

Battle, W. (2017). *The World Tea Encyclopaedia: The world of tea explored and explained from bush to brew*. Troubador Publishing Ltd.

Blowfield, M. E., and Dolan, C. (2010). Fair trade facts and fancies: What Kenyan Fair trade tea tells us about business' role as development agent. *Journal of Business Ethics*, **93**(2), 143-162.

Bonoli, M., Colabufalo, P., Pelillo, M., Gallina Toschi, T., and Lercker, G. (2003). Fast determination of catechins and xanthines in tea beverages by micellar electrokinetic chromatography. *Journal of agricultural and food chemistry*, **51**(5), 1141-1147.

Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., and Morisset, (2014). Guidelines for validation of qualitative real-time PCR methods. *Trends in Food Science and Technology*, **37**(2), 115-126.

Cai, Y., Luo, Q., Sun, M., and Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life sciences*, **74**(17), 2157-2184.

Cardona, F., Andrés - Lacueva, C., Tulipani, S., Tinahones, F. J., and Queipo - Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. *The Journal of nutritional biochemistry*, **24**(8), 1415-1422.

Careri, M., Bianchi, F., and Corradini, C. (2002). Recent advances in the application of mass spectrometry in food - related analysis. *Journal of Chromatography A*, **970**(1), 3 - 64.

Carloni, P., Tiano, L., Padella, L., Bacchetti, T., Customu, C., Kay, A., and Damiani, E.(2013) Antioxidant activity of white, green and black tea obtained from the same tea cultivar. *Food research international*, **53**(2), 900-908.

Caruso, E., Gariboldi, M., Sangion, A., Gramatica, P., and Banfi, S. (2017). Synthesis, photodynamic activity, and quantitative structure-activity relationship modelling of a series of BODIPYs. *Journal of Photochemistry and Photobiology Series B: Biology*, **167**, 269-281.

Chacko, S. M., Thambi, P. T., Kuttan, R., and Nishigaki, I. (2010). Beneficial effects of green tea: a literature review. *Chinese medicine*, **5**(1), 1.

Chanwitheesuk, A., Teerawutgulrag, A., Kilburn, J. D., and Rakariyatham, N. (2007). Antimicrobial gallic acid from Caesalpinia mimosoides Lamk. *Food chemistry*, **100**(3), 1044-1048.

Chemat, F., and Khan, M. K. (2011). Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrasonics sonochemistry*, **18**(4), 813-835.

Chen, B., Mei, J., Kalman, L., Shenanigan, S., Williams, I., Gagnon, M., and Zehnbauer, B. screening (2012). Good laboratory practices for biochemical genetic testing and newborn for inherited metabolic disorders. *Morbidity and Mortality Weekly Report:*

Recommendations and Reports, **61**(2), 1-44.

Chen, C.N., Liang, C.M., Lai, J.R., Tsai, Y.J., Tsay, J.S., and Lin, J.K. (2003). Capillary electrophoretic determination of theanine, caffeine, and catechins in fresh tea leaves and oolong tea and their effects on rat neurosphere adhesion and migration. *Journal of agricultural and food chemistry*, **51**(25), 7495-7503.

Chen, W. B., Li, S. Q., Chen, L. J., Fang, M. J., Chen, Q. C., Wu, Z., and Qiu, Y. K. (2015). Online polar two phase countercurrent chromatography× high performance liquid chromatography for preparative isolation of polar polyphenols from tea extract in a single step. *Journal of Chromatography series B*, **997**, 179-186.

Colon, M., and Nerin, C. (2012). Role of catechins in the antioxidant capacity of an active film containing green tea, green coffee, and grape fruit extracts. *Journal of agricultural and food chemistry*, **60**(39), 9842-9849.

Corollar, D., Singh, P., Khan, S., and Chaudhary, C. (2013). Asian Journal of Medicine.

Corzo-Martınez, M., Corzo, N., Villamiel, M., and Del Castillo, M. D. (2012). Browning reactions. *Food biochemistry and food processing*, 56

da Silva Pinto, M. (2013). Tea: A new perspective on health benefits. *Food Research International*, **53**(2), 558-567.

Da, H., Gu, X. J., and Xiao, P. G. (2015). *Medicinal plants: Chemistry, biology and omics*. Woodhead Publishing, pp.105-130.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current opinion in biotechnology*, **23**(2), 174-181.

Dalluge, J. J., and Nelson, B. C. (2000). Determination of tea catechins. *Journal of Chromatography series A*, **881**(1), 411-424.

Darriet, P., and Pons, A. (2017). Wine. In *Springer Handbook of Odour* (pp. 25-26). Springer, Cham.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P., Tognolini, M., Borges, G., and Crozier, A. (2013). Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants and redox signaling* **18**(14): 1818-1892.

Dhanani, T., Singh, R., and Kumar, S. (2017). Extraction optimization of gallic acid, (+) - catechin, procyanidin B_2, (–) epicatechin, (–) epigallocatechin gallate, and (–) epicatechingallate: their simultaneous identification and quantification in Saraca asoca. *Journal of food and drug analysis*, **25**(3), 691-698.

Dias, T. R. (2013). White Tea (Camellia sinensis (L.)): An-tioxidant Properties and beneficial

Health Effects. *International Journal of Food Science and Nutritional Diet*, **2**(2), 19 - 26.

Farhat, G., Drummond, S., Fyfe, L., and Al-Dujaili, E. A. (2014). Dark chocolate: an obesity paradox or a culprit for weight gain? *Phytotherapy research* **28**(6): 791-797.

Fellows, P. J. (2009). *Food processing technology: principles and practice*. Elsevier.

File///D:/AN-20583 © 2012 Thermo Fisher Scientific Inc.

Food and Drug administration, Guidance for industry: bioanalytical method validation 2001 https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf. Accessed 23rd march 2018.

Garrido, J., and Borges, F. (2013). Wine and grape polyphenols—A chemical perspective. *Food research international*, *54*(2), 1844-1858

Gaur, S., and Agnihotri, R. (2014). Green tea: A novel functional food for the oral health of older adults. *Geriatrics and gerontology international*, **14**(2), 238-250.

Grigg, D. (2003). *An introduction to agricultural geography*: Routledge.

Gulati, A. (2013). TEA MANUFACTURE. *Science of Tea Technology*, **401**.

Guillarme, D., Casetta, C., Bicchi, C., and Veuthey, J.L. (2010). High throughput qualitative analysis of polyphenols in tea samples by ultra-high pressure liquid chromatography coupled to UV and mass spectrometry detectors. *Journal of Chromatography A*, **1217**(44), 6882-6890.

http: // www . ceri j. or. jp / service / 09 chromatography / L – column 2_C_6 - Phenyl_01 . html accessed 12/11/2016

Herrera - Herrera, A. V., González - Curbelo, M. Á., Hernández - Borges, J., and Rodríguez Delgado, M. Á. (2012). Carbon nanotubes applications in separation science: a review Analytica *Chimica Acta*, **734**, 1-30.

Hicks, A. (2009). Current status and future development of global tea production and tea products. *Au J* 12.

Hoffman, J. R. (2010). Caffeine and energy drinks. *Strength and Conditioning Journal*, **32**(1), 15-20.

Holden, S. R., Gutierrez, A., and Treseder, K. K. (2013). Changes in soil fungal communities, extracellular enzyme activities, and litter decomposition across a fire chronosequence in Alaskan boreal forests. *Ecosystems*, **16**(1), 34-46

Horžić, D., Jambrak, A. R., Belščak - Cvitanović, A., Komes, D., and Lelas, V. (2012).

Comparison of conventional and ultrasound assisted extraction techniques of yellow tea and bioactive composition of obtained extracts. *Food and Bioprocess Technology*, **5**(7), 2858-2870.

Hur, S. J., Lee, S. Y., Kim, Y. C., Choi, I., and Kim, G. B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food chemistry*, **160**, 346-356.

Ignat, I., Volf, I., and Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food chemistry* **126**(4): 1821-1835.

Iriti, M., Vitalini, S., Fico, G., and Faoro, F. (2010). Neuroprotective herbs and foods from different traditional medicines and diets. *Molecules*, **15**(5), 3517-3555.

ISO. 1572:1980. Tea - Preparation of ground sample of known dry matter content

ISO. 14502-2:2005. Determination of substances characteristic of green and black tea Part 2: Content of catechins in green tea: Method using high-performance liquid chromatography.

Jandera, P. (2011). Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Analytica chimica acta*, **692**(1), 1-25.

Jennings, W. (2012). Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography. Elsevier.

Joubert, E., and de Beer, D. (2011). Rooibos (Aspalathus linearis) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany* **77**(4): 869-886.

Karori, S. M., Wachira, F. N., Wanyoko, J. K., and Ngure, R. M. (2007) Antioxidant capacity of different types of tea products. *African Journal of Biotechnology*, **6**(19).

Karschner, E. L. (2010). *Pharmacodynamic and Pharmacokinetic Characterization of Sativex and Oral THC* (Doctoral dissertation, University of Maryland, Baltimore).

Kawai, K., Tsuno, N. H., Kitayama, J., Okaji, Y., Yazawa, K., Asakage, M., Hori, N., Watanabe, T., Takahashi, K., and Nagawa, H. (2003). Epigallocatechin gallate, the main component of tea polyphenol, binds to CD4 and interferes with gp120 binding. *Journal of Allergy and Clinical Immunology*, **112**(5), 951-957.

Kawooya, R., Tumwine, V., Namutebi, V., Mugisa, C., Naluugo, R., and Kajobe, R. (2015). Tea research in the year 2015 in Uganda: An overview. *African Journal of Agricultural Science and Technology (AJAST)*, **3**(12), 505-513

Khan, N., and Mukhtar, H. (2007). Tea polyphenols for health promotion. *Life sciences*,**81**(7) 519-533.

Khoddami, A., Wilkes, M. A., and Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules*, **18**(2), 2328-2375.

Kilel, E. C. (2013). *Chemical and sensory quality evaluation of newly developed tea clones in Kenya* (Masters dissertation).

KTDA(2011), From bushes to cup (Tea life cycle). *Kenya Tea Development Agency Limited*. Retrieved on April 5, 2018 from htt://www,ktdateas.com/index.php/the-tea/from-bush-to-cup.

Koech, K., Wachira, F., Ngure, R., Wanyoko, J., Bii, C., and Karori, S. (2013). Antibacterial and Synergistic Activity of Different Tea Crude Extracts Against Antibiotic Resistant *S. aureus, E. coli* and a Clinical Isolate of *S. typhi. Science Journal of Microbiology.*

Kruve, A., Rebane, R., Kipper, K., Oldekop, M. L., Evard, H., Herodes, K.; Leito, I. (2015). Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I. *Analytica chimica acta*, **870**, 29-44.

Kumar, Ramsey Shanmugasundaram Senthil, Subramanian Murugesan, Govindasamy Kottur , and Daniel Gyamfi (2013). "Black tea: The plants, processing/manufacturing and production." In *Tea in health and disease prevention*, pp. 41-57.

Kuriyama, S., Shimazu, T., Ohmori, K., Kikuchi, N., Nakaya, N., Nishino, Y. and Tsuji, I. (2006). Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. *Jama*, **296**(10): 1255-1265.

Lavola, A., Karjalainen, R., and Julkunen -Tiitto, R. (2012). Bioactive polyphenols in leaves, stems, and berries of Saskatoon *(Amelanchier alnifolia Nutt.)* cultivars. *Journal of agricultural and food chemistry*, **60**(4), 1020-1027.

Lee, J.E., Lee, B.J., Chung, J.O., Hwang, J.A., Lee, S.J., Lee, C.H., and Hong, Y.S. (2010). Geographical and climatic dependencies of green tea (Camellia sinensis) metabolites: a 1H NMR - based metabolomics study. *Journal of agricultural and food chemistry*, **58**(19), 10582-10589.

Lehotay, S. J., Son, K. A., Kwon, H., Koesukwiwat, U., Fu, W., Mastovska, K.; analysis of Leepipatpiboon, N. (2010). Comparison of QuEChERS sample preparation methods for the pesticide residues in fruits and vegetables. *Journal of Chromatography A*, **1217**(16), 2548-2560.

Li, D. W., Zhu, M., Shao, Y. D., Shen, Z., Weng, C. C., and Yan, W. D. (2016). Determination and quality evaluation of green tea extracts through qualitative and quantitative analysis of multi-components by single marker (QAMS). *Food chemistry* **197:** 11-20.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., and Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food chemistry* **96**(2): 254-260.

Lin, J.K., Lin, C.L., Liang, Y.C., Lin-Shiau, S.Y., and Juan, I.M. (1998). Survey of catechins, gallic acid, and methylxanthines in green, oolong, pu-erh, and black teas. *Journal of agricultural and food chemistry*, **46**(9), 3635-3642.

Lobo, J. M., Jiménez - Valverde, A., and Real, R. (2008). AUC: a misleading measure of the. performance of predictive distribution models. *Global ecology and Biogeography*, **17**(2), 145-151

Macheix, J. J. (2017). *Fruit Phenolics:* pp. 85-90.

Maddox, C. E., Laur, L. M., and Tian, L. (2010). Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa. Current microbiology,* **60**(1), 53-58.

Magoma, G. N., Kariuki, D. W., Kinyua, J. K., and Obwoge, J. O. (2017). Phytochemical screening and antimicrobial studies of green, orthodox and black Kenyan tea *(JKUAT Institutional Repository).*

Magwaza, L. S., and Opara, U. L. (2015). Analytical methods for determination of sugars and sweetness of horticultural products—A review. *Scientia Horticulturae*, **184**, 179-192.

Mangenya, T. M. (2016). *Assessment of Catechins and other Polyphenols as Parameters of Tea Quality in selected Ecological Zones in Kenya* (Doctoral dissertation, Jomo Kenyatta University of Agriculture and Technology).

Marquet, P., and Lachatre, G. (1999). Liquid chromatography–mass spectrometry: potential in forensic and clinical toxicology. *Journal of Chromatography series B: Biomedical Sciences and Applications*, **733**(1), 93-118.

Marsh, A. J., Hill, C., Ross, R. P., and Cotter, P. D. (2014). Fermented beverages with health-promoting potential: past and future perspectives. *Trends in Food Science and Technology*, **38**(2), 113-124.

Martin, L. C. (2011). Tea: the drink that changed the world. *Tuttle Publishing*.

Mbui, C. K. (2016). *Effect of strategic management practices on export value addition in the tea subsector in Kenya* (Doctoral dissertation, COHRED, JKUAT).

Meeran, S. M., Akhtar, S., and Katiyar, S. K. (2009). Inhibition of UVB - induced skin tumor development by drinking green tea polyphenols is mediated through DNA repair and subsequent inhibition of inflammation. *Journal of Investigative Dermatology*, **129**(5), 1258-1270.

Mewshaw, M. (2003). Do I Owe You Something? *A Memoir of the Literary Life*. LSU Press.

Meydani, M., and Hasan, S. T. (2010). Dietary polyphenols and obesity. *Nutrients*, **2**(7), 737-751.

Mildner-Szkudlarz, and Bajerska, J. (2016). Phytochemicals as functional bread compounds Physiological effects. *Bread and its Fortification, CRC Press, Boca Raton*, 79-101.

Miller, W. G., Jones, G. R., Horowitz, G. L., and Weykamp, C. (2011). Proficiency testing/ external quality assessment: current challenges and future directions. *Clinical chemistry*, clinchem-2011.

Mizukami, Y., Sawai, Y., and Yamaguchi, Y. (2007). Simultaneous analysis of catechins, gallic acid, strictinin, and purine alkaloids in green tea by using catechol as an internal standard. *Journal of agricultural and food chemistry*, **55**(13), 4957-49643.

Moseti O Kelvin. (2013). *Levels of selected heavy metals and fluoride in tea (Camellia sinensis) grown, processed and marketed in Kenya* (Doctoral dissertation, Egerton University).

Mucheru- Muna., Mugendi, D., Pypers, P., Mugwe, J., Kung'u, J., Vanlauwe, B., and Merckx, R. (2014). Enhancing maize productivity and profitability using organic inputs and mineral fertilizer in central Kenya small-hold farms. *Experimental Agriculture*, **50**(2), 250-269.

Mutshekwa, N. (2017). *Effect of time-based hot air drying method on chemical composition of jatropha zeyheir tea* (Doctoral dissertation, University of Limpopo).

Nagy, G., Peng, T., and Pohl, N. L. (2018). Recent liquid chromatographic approaches and developments for the separation and purification of carbohydrates. *Analytical Methods*, **9**(24), 3579-3593.

Naldi, M., Fiori, J., Gotti, R., Périat, A.,Veuthey, J. L., Guillarme, D., and Andrisano, V. (2014). UHPLC determination of catechins for the quality control of green tea. *Journal of pharmaceutical and biomedical analysis* **88:** 307-314.

Ngure, F. M., Wanyoko, J. K. Mahungu, S. M., and Shitandi, A. A. (2009) catechins patterns in relation to theaflavin and thearubigins formation. *Food chemistry,* **115**(1), 8-14.

Nile, S. H., and Park, S. W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition,* **30**(2), 134-144.

Obanda, M., Owuor, P. O., Mang'oka, R., and Kavoi, M. M. (2004). Changes in thearubigin fractions and theaflavin levels due to variations in processing conditions and their influence on black tea liquor brightness and total colour. *Food chemistry,* **85**(2), 163-173.

Ochanda, S. O., Wanyoko, J. K., Faraj, A. K., Onyango, C. A., and Ruto, H. K. (2015). Effects of Tea (*Camellia sinensis*) Phytochemicals on the Yoghurt Cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) During Development and Storage of Tea Fortified Yoghurts. *Journal of Food Research* **4**(4): 59.

Oroian, M., and Escrich, I. (2015). Antioxidants: Characterization, natural sources, extraction and analysis. *Food Research International*, **74**, 10-36.

Owuor, P. O., and Obanda, M. (2007). The use of green tea (Camellia sinensis) leaf flavan-3-ol composition in predicting plain black tea quality potential. *Food chemistry*, **100**(3), 873-884.

Owuor, P. O., Kamau, D. M., Kamunya, S. M., Msomba, S. W., Uwimana, M. A., Okal, A.W and Kwach, B. O. (2011). Effects of genotype, environment and management on yields and quality of black tea. In *Genetics, Biofuels and Local Farming Systems* (pp. 277-307). Springer, Dordrecht.

Ozdal, T., Sela, D. A., Xiao, J., Boyacioglu, D., Chen, F., and Capanoglu, E. (2016). The reciprocal interactions between polyphenols and gut microbiota and effects on bio accessibility. *Nutrients*, **8**(2), 78.

Pagliosa, C. M., Vieira, M. A., Podestá, R., Maraschin, M., Zeni, A. L. B., Amante, E. R.and, Amboni, R. D. D. M. C. (2010). Methylxanthines, phenolic composition, and antioxidant activity of bark from residues from mate tree harvesting (Ilex paraguariensis A St. Hil.). *Food Chemistry*, **122**(1), 173-178.

Paine, F. A., and Paine, H. Y. (2012). *A handbook of food packaging.* Springer Science and Business Media.

Panda, H. (2016). The complete book on cultivation and manufacture of tea. Asia Pacific Business Press Inc.

Pandey, K. B., and Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, **2**(5), 270-278.

Pang, X., Qin, Z., Zhao, L., Cheng, H., Hu, X., Song, H., and Wu, J. (2012). Development of regression model to differentiate quality of black tea (Dianhong): correlate aroma properties with instrumental data using multiple linear regression analysis. *International Journal of Food Science and Technology*, **47**(11), 2372-2379.

Pascual - Maté., Osés, S. M., Fernández - Muiño, M. A., and Sancho, M. T. (2018). Analysis of polyphenols in honey: Extraction, separation and quantification procedures. *Separation and Purification Reviews*, **47**(2), 142-158.

Pauli, E.D., Malta, G.B., Sanchez, P.M., Moreira, I.C. and Scarminio, L.S (2014). Mixture

design analysis of solvent extractor effects on epicatechin, epigallocatechin gallate, epigallocatechin and antioxidant activities of the *Camellia sinensis* L. leaves. Analytical Chemistry Research **2**, 23- 29

Paulus, R., Roth, A., Titus, L., Chen, R., Bridges, M. C., and Woodyard, S. (2015). Impact of various caffeine vehicles on mood and cognitive, neurological and physiological functions over five hours. *The Ohio Journal of Science*, **115**(2), 12-23.

Pérez -Jiménez, A., Peres, H., Rubio, V. C., and Oliva -Teles, A. (2012). The effect of dietary methionine and white tea on oxidative status of gilthead sea bream *(Sparus aurata). British Journal of Nutrition*, **108**(7), 1202-1209.

Pérez-Rentero, S., Somoza, A., Grijalvo, S., Janoušek, J., Bělohradský, M., Stará, I. G., and Eritja, R. (2013). Biophysical and RNA Interference inhibitory properties of oligonucleotides carrying tetrathiafulvalene groups at terminal positions. *Journal of Chemistry, 2013*.

Perumalla, A. V. S., and Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—Potential applications in food safety and quality. *Food Research International* **44**(4): 827-839.

Piljac-Žegarac, J., Šamec, D., and Piljac, A. (2013). Herbal teas: A focus on antioxidant properties. In *Tea in Health and Disease Prevention* (pp. 129-140).

Powers, D. M. (2011). Evaluation: from precision, recall; F-measure to ROC, informedness, markedness and correlation.

Prabhakar, J., Senthilkumar, M., Priya, M., Mahalakshmi, K., Sehgal, P., and Sukumaran, V. (2010). Evaluation of antimicrobial efficacy of herbal alternatives (Triphala and green tea polyphenols), MTAD, and 5% sodium hypochlorite against *Enterococcus faecalis* biofilm formed on tooth substrate: an in vitro study. *Journal of Endodontics*, **36**(1), 83-86.

Qin, Y., Song, F., Ai, Z., Zhang, P., and Zhang, L. (2015). Protocatechuic acid promoted alachlor degradation in Fe (III) / H_2O_2 Fenton system. *Environmental Science and Technology*, **49**(13), 7948-7956.

Rahim, A., Nofrizal, S., and Saad, B. (2014). Rapid tea catechins and caffeine determination by HPLC using microwave-assisted extraction and silica monolithic column. *Food chemistry*, **147**, 262-268.

Richardson, L. B. (2014). *Modern tea: A fresh look at an ancient beverage*. pp 26-30 Chronicle Books.

Sadowska-Rociek, A., Surma, M.; Cieślik, E. (2014). Comparison of different modifications

on QuEChERS sample preparation method for PAHs determination in black, green, red and white tea. *Environmental Science and Pollution Research*, **21**(2), 1326-1338.

Saito, S. T., Welzel, A., Suyenaga, E. S., and Bueno, F. (2006). A method for fast determination of epigallocatechin gallate (EGCG), epicatechin (EC), catechin(C) and caffeine (CAF) in green tea using HPLC. *Food Science and Technology (Campinas)*, **26**(2), 394-400.

Sang, S., Lambert, J. D., Ho, C.T., and Yang, C. S. (2011). The chemistry and biotransformation of tea constituents. *Pharmacological research*, **64**(2), 87-99.

Santagati, N. A., Salerno, L., Attaguile, G., Savoca, F., and Ronsisvalle, G. (2008). Simultaneous determination of catechins, rutin, and gallic acid in cistus species extracts by HPLC with diode array detection. *Journal of chromatographic science*, **46**(2), 150-156.

Saputro., Arifin Dwi., Davy Van de Walle., Roger P, A., Michael Amoafo Mensah., Claudia Delbaere., Nathalie De Clercq., Jim Van Durme., and Koen Dewettinck. (2017) "Quality attributes of dark chocolates formulated with palm sap-based sugar as nutritious and natural alternative sweetener." *European Food Research and Technology* **243**(2): 177-191.

Statistical Analysis System SAS, (2002). version 9.1.

Schlake, R., Stefkovich, J., Schlegel, E., Fetner, M., and Kaziunas, A. (2013). *U.S. Patent Application No. 13/532,051*.

Senanayake, S. N. (2013). Green tea extract: Chemistry, antioxidant properties and food applications – A review. *Journal of Functional Foods*, **5**(4), 1529-1541.

Shahidi, F., and Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. *Journal of functional foods*, **18**, 820-897.

Sharangi, A. (2009). Medicinal and therapeutic potentialities of tea –A review. *Food Research International*, **42**(5), 529-535.

Sharma, V., Gulati, A., Ravindranath, S. D., and Kumar, V. (2005). A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *Journal of Food Composition and Analysis*, **18**(6), 583-594.

Sibhatu, K. T., Krishna, V. V., and Qaim, M. (2018). Production diversity and dietary diversity in smallholder farm households. *Proceedings of the National Academy of Sciences*, **112**(34), 10657-10662.

Silverman, B. W. (2018). *Density estimation for statistics and data analysis*. Routledge.

Sliwinska, M., Wisniewska, P., Dymerski, T., Namiesnik, J., and Wardencki, W. (2014). Food analysis using artificial senses. *Journal of agricultural and food chemistry* **62**(7): 1423-1448.

Smith, K. (2016). *World Atlas of Tea: From the leaf to the cup, the world's teas explored and enjoyed*. Hachette UK.

Snyder, L. R., Kirkland, J. J., and Glajch, J. L. (2012). *Practical HPLC method development*. John Wiley and Sons.

Sonawane, L. V., Poul, B. N., Usnale, S. V., Waghmare, P. V., and Surwase, L. H. (2014). method validation and its pharmaceutical application-A review. *Pharm Anal Acta*,**5**(288)

Spáčil, Z., Nováková, L., and Solich, P. (2008). Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta*, **76**(1), 189-199.

Spellman, F. R. (2016). *Food supply protection and homeland security*. Bernan Press.

Su, G., and Wang, K. (2013). Methodological aspects to the determination of phenolic compounds in tea. *Current Analytical Chemistry*, **9**(3), 374-381.

Sujith Kumar, P. V. (2010). *Role of Specific Enzymes in Biosensor for Tea Analysis* (Doctoral Dissertation, University of Mysore).

Suzuki, M., Tabuchi, M., Ikeda, M., Umegaki, K., and Tomita, T. (2004). Protective effects of green tea catechins on cerebral ischemic damage. *Medical Science Monitor*, **10**(6), BR166-BR174.

Taverniers, I., Van Bockstaele, E., and De Loose, M. (2010). Analytical method validation and quality assurance. *Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development and Manufacturing*, 1-48.

Tijburg, L., Mattern, T., Folts, J., Weisgerber, U., and Katan, M. (1997). Tea flavonoids and cardiovascular diseases: a review. *Critical Reviews in Food Science and Nutrition*, **37**(8), 771-785.

Tipoe, G. L., Leung, T.- M., Hung, M.-W., and Fung, M.- L. (2007). Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. *Cardiovascular and Haematological Disorders-Drug Targets (Formerly Current Drug Targets-Cardiovascular and Hematological Disorders)*, **7**(2), 135-144.

Tomás - Barberán, F. A., and Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, **81**(9), 853-876.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231-1246.

Valls, J., Millán, S., Martí, M. P., Borràs, E., and Arola, L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A*, **1216**(43), 7143-7172.

van der Burg-Koorevaar, M. C., Miret, S., and Duchateau, G. S. (2011). Effect of milk and brewing method on black tea catechin bioaccessibility. *Journal of Agricultural and Food Chemistry*, **59**(14), 7752-7758.

Verhagen, A., Hengsdijk, H., Bezlepkina, I., Groenestein, K., and van't Klooster, K. (2013). Linking good agricultural practices and climate smart agriculture**.** Wageningen University research **(536)**

Vrhovsek, U., Masuero, D., Gasperotti, M., Franceschi, P., Caputi, L., Viola, R., and Mattivi, F. (2012). A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *Journal of agricultural and food chemistry*, **60**(36), 8831-8840.

Wachira, F. N., Karori, S., Kerio, L. C., and Wanyoko, J. K. (2013). Cultivar Type and Antioxidant Potency of Tea Product. In *Tea in Health and Disease Prevention* (pp. 91-102).

Walters, D. (2011). *Plant defense: warding off attack by pathogens, herbivores and parasitic plants*: John Wiley and Sons.

Wamalwa, B. P. (2014). Sustainable Supply Chain Management as a Strategic Tool for Competitive Advantage in Tea Industry in Kenya. *Journal of Management and Sustainability*, **4**(3), 157.

Watson, R. R. (2014). Polyphenols in plants: isolation, purification and extract preparation. Academic Press.

Wilcox, R. R. (2010). *Fundamentals of modern statistical methods: Substantially improving power and accuracy*. Springer.

Wijayasiri, J., and Jayaratne, S. (2011). Implications of agriculture food standards for Sri Lanka: Case studies of tea and fisheries export industries. *Art net working paper series*, **104**, pp18-35

Wright, L. P. (2005). Biochemical analysis for identification of quality in black tea *(Camellia sinensis)* Philosophiae *Doctor (Biochemistry) Thesis*, 29

Yang, C. S., Zhang, J., Zhang, L., Huang, J., and Wang, Y. (2016). Mechanisms of body

weight reduction and metabolic syndrome alleviation by tea. *Molecular nutrition and food research*, **60**(1), 160-174.

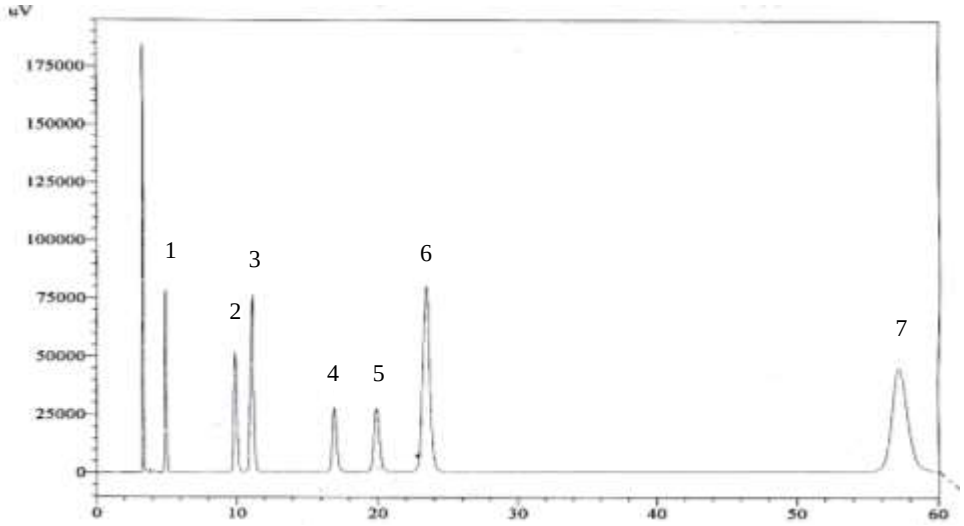
Zhang, L., Tiwana, B., Qian, Z., Wang, Z., Dick, R. P., Mao, Z. M., and Yang, L. (2010, October). Accurate online power estimation and automatic battery behavior based power model generation for smartphones. In *Proceedings of the eighth IEEE/ACM/IFIP international conference on Hardware/software codesign and system synthesis* (pp. 105-114). ACM.

Zaveri, N. T. (2006). Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life sciences*, **78**(18), 2073-2080.

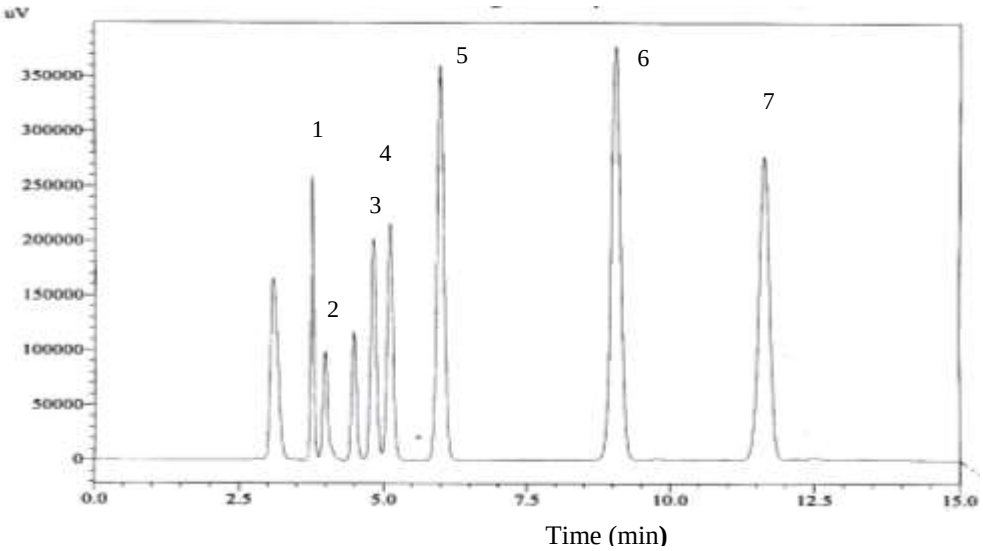
Zhang, L., Li, N., Ma, Z. Z., and Tu, P. F. (2011). Comparison of the chemical constituents of aged pu-erh tea, ripened pu-erh tea, and other teas using HPLC-DAD-ESI-MS -. *Journal of agricultural and food chemistry*, **59**(16), 8754-8760.

APPENDICES

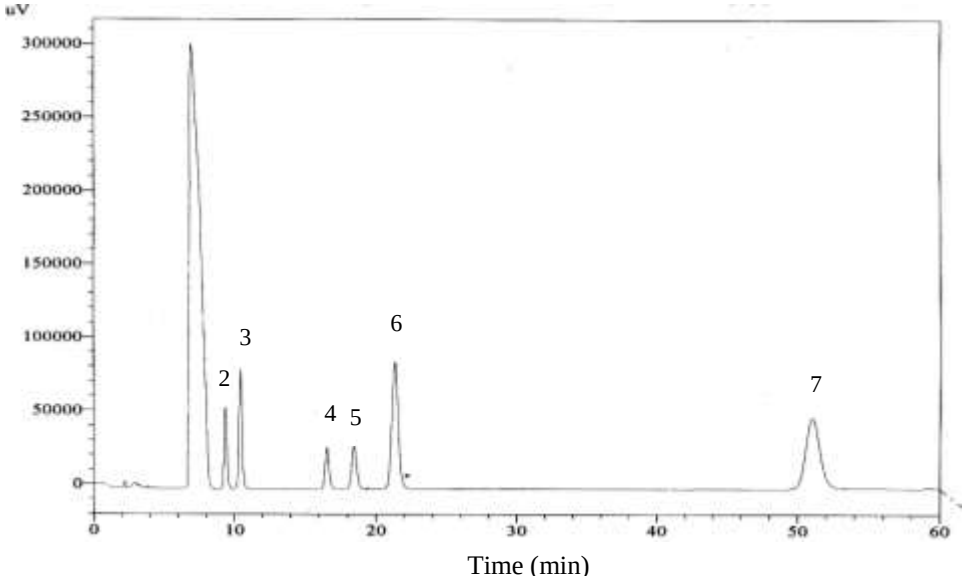
Appendix I: Chromatograms for the mixed standards using mobile phase solvent combinations 1-11 excluding solvent system number 4



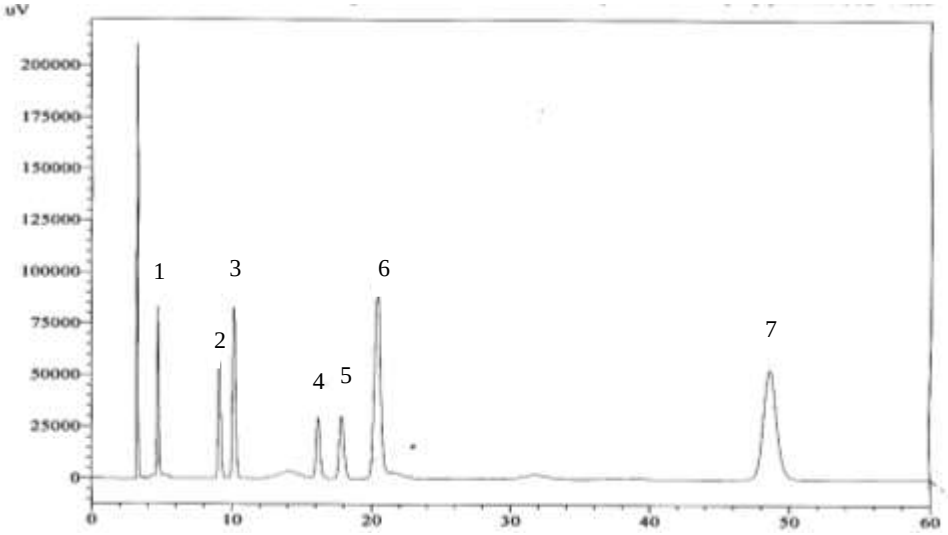
Mobile phase combination 1 and 2



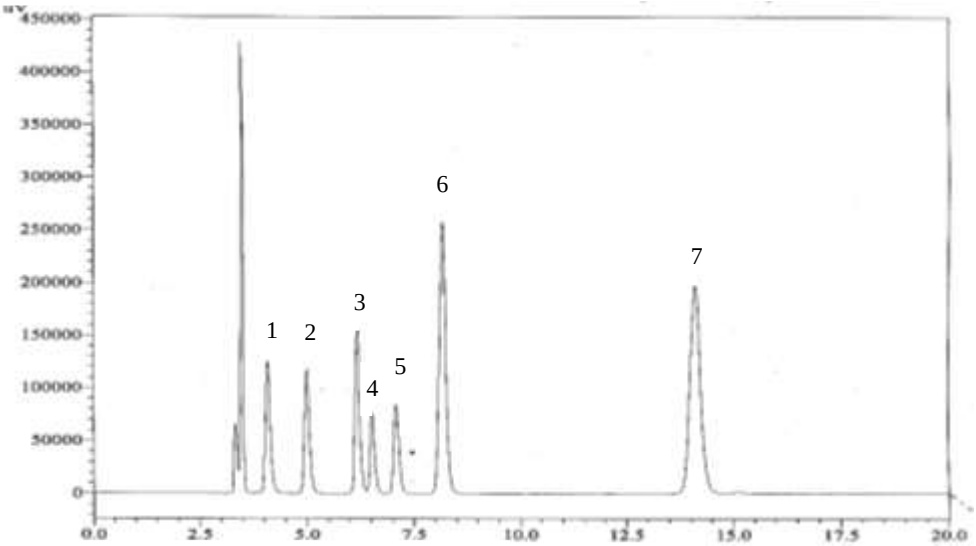
Mobile phase combination 3



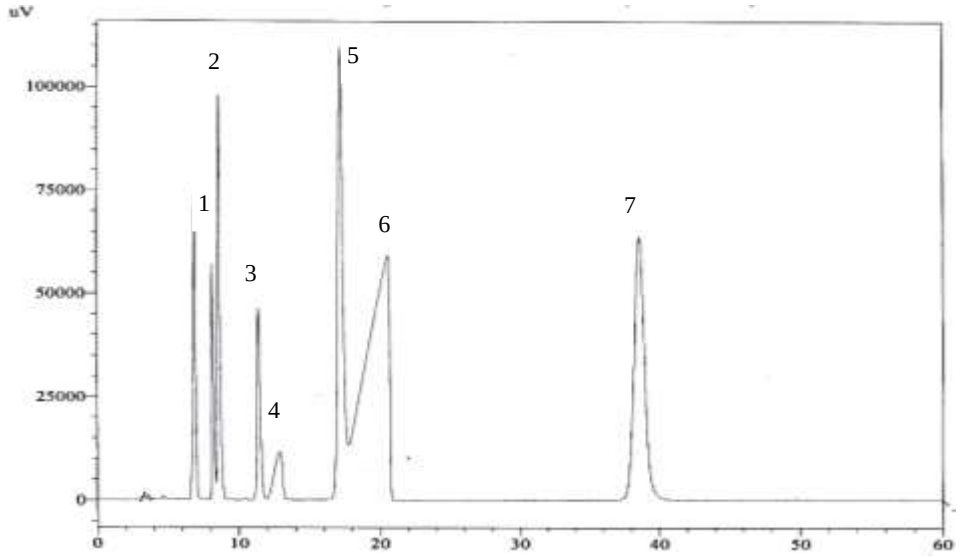
Mobile phase combination 5



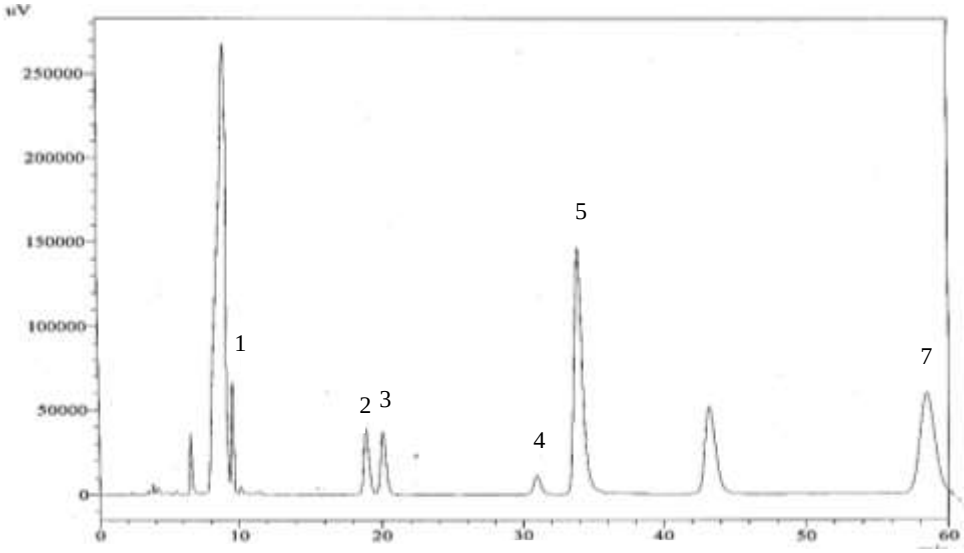
Mobile phase combination 6



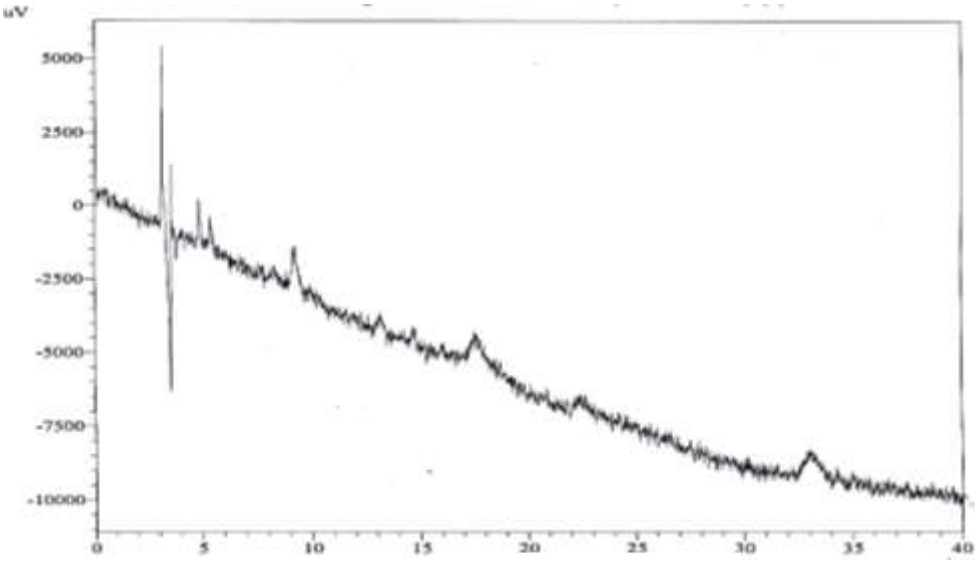
Mobile phase combination 7



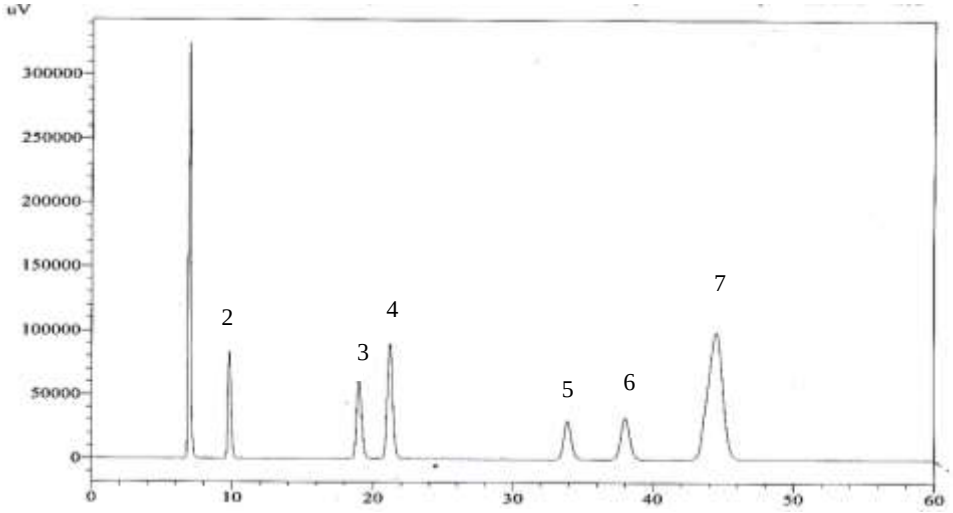
Mobile phase combination 8 Time (min)



Mobile phase combination 9 Time (min**)**

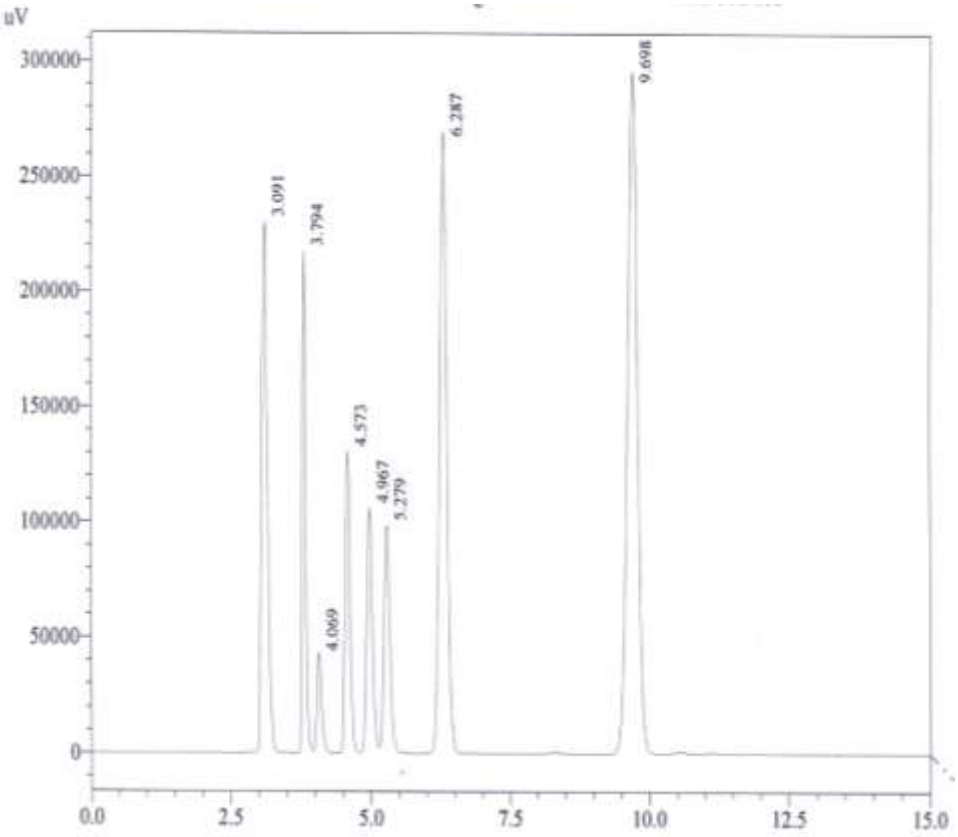


Mobile phase combination 10 **Time (min)**

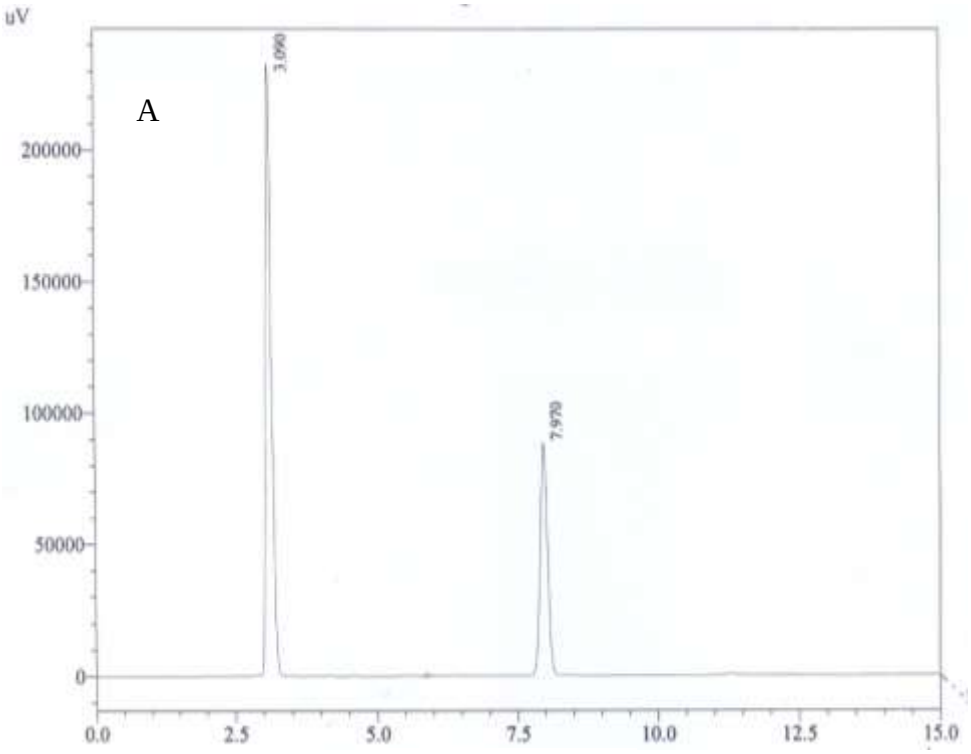


Mobile phase combination 11

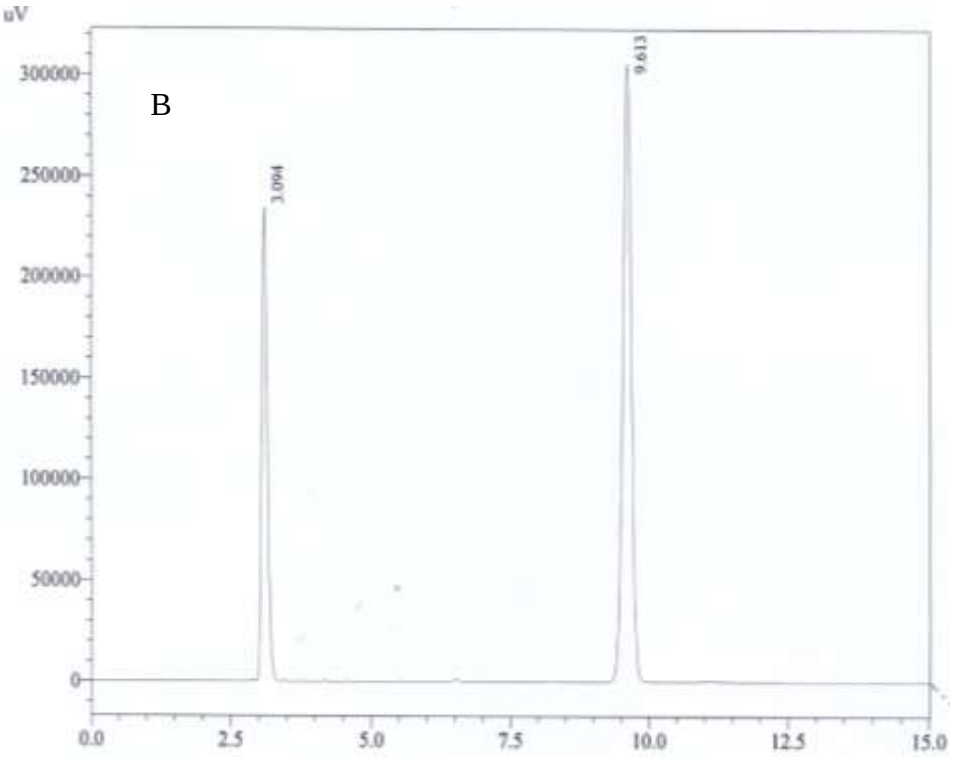
Appendix II: A HPLC chromatogram for the mixed standards of GA, (-) EGC, caffeine, (+) C, (-) EC, (-) EGCG and (-) ECG showing retention times in minutes



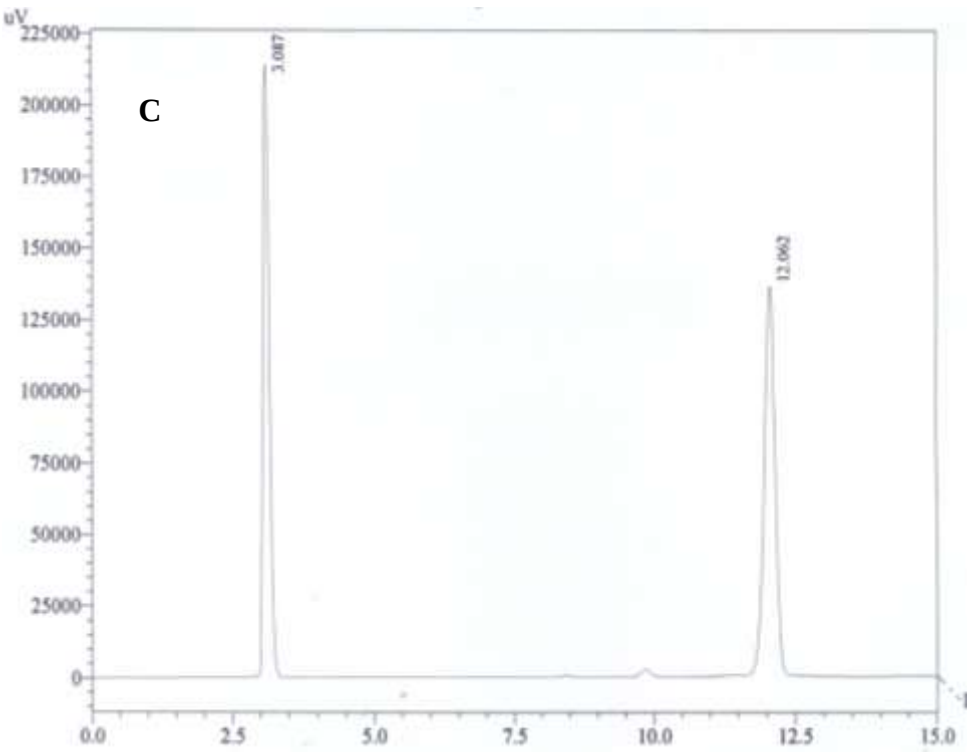
Appendix III: Chromatograms for A: 3-fluorocatechol, B: guaiacol (2-methoxyphenol) and C: 4-methylcatecol, depicting their retention times (RT) in min.



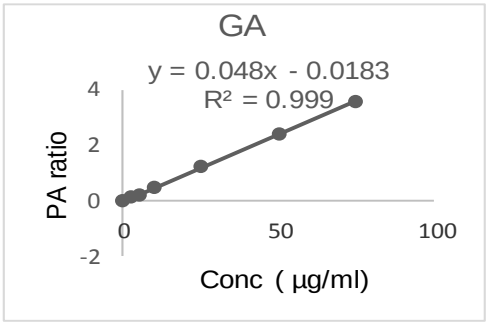
Time (min)

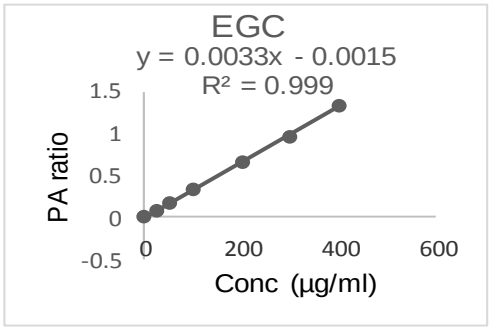


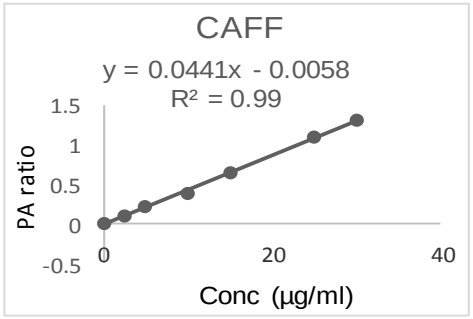
Time (min)

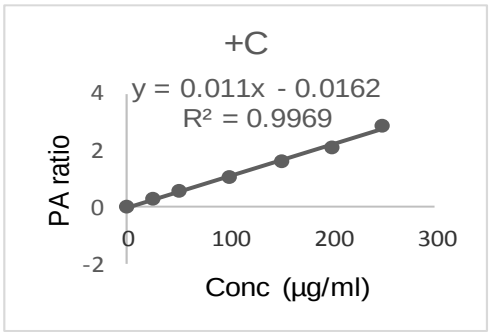

C
uV
225000
200000
175000
150000
125000
100000
75000
50000
25000
0
3.087
12.062
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (min)

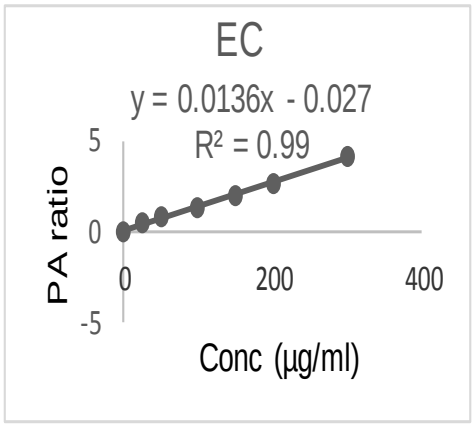
Appendix IV: Linearity test graphs for standards used in the study

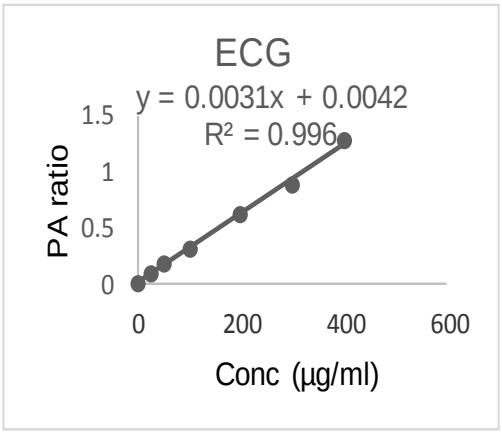












Appendix V: Terms from the FDA validation guidelines glossary applied in the developed method

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed *trueness*.

Analyte: A specific chemical moiety being measured, which can be intact drug, biomolecule or its derivative, metabolite, and/or degradation product in a biologic matrix.

Analytical run (or batch): A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

Calibration standard: A matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations analytes in QCs and in unknown study samples are determined.

Internal standard: Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Limit of detection (LOD): The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

Lower limit of quantification (LLOQ): The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

Matrix effect: The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A comprehensive description of all procedures used in sample analysis.

Precision: The closeness of agreement (*degree of scatter*) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Processed: The final extract (prior to instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Quantification range: The range of concentration, including ULOQ and LLOQ, which can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reproducibility: The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

Sample: A generic term encompassing controls, blanks, unknowns, and processed samples, as described below:

Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Quality control sample (QC): A spiked sample used to monitor the performance of a method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Unknown: A sample that is the subject of the analysis.

Selectivity: The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants, or matrix components.

Stability: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Standard curve: The relationship between the experimental response value and the analytical concentration (also called a *calibration curve*).

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

Upper limit of quantification (ULOQ): The highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.

Validation:

Full validation: Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.

Partial validation: Modification of validated bioanalytical methods that do not necessarily call for full revalidation.

Cross-validation: Comparison validation parameters of two bioanalytical methods.