ASSESSMENT OF THE LEVELS OF OCHRATOXIN-A IN COFFEE BEANS FROM THE COFFEE GROWING REGION OF KIAMBU COUNTY, KENYA

PETER MACHARIA KARURI

MASTER OF SCIENCE

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JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

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Assessment of the Levels of Ochratoxin-A in Coffee Beans from the Coffee Growing Region of Kiambu County, Kenya

Peter Macharia Karuri

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DECLARATION

This thesis is my original work and has not been presented in any other University for the award of a degree.

Signature ………………………………… Date …………………………..

Peter Macharia Karuri

The thesis has been submitted for examination with our approval as university supervisors.

Signature ………………………………. Date ………………………………...

Dr. Jackson Kiptoo,

JKUAT, Kenya

Signature ……………………………….. Date …………………………………..

Dr. Leonard Gitu

JKUAT, Kenya

DEDICATION

This work is dedicated to my parents Mrs. Ann Wangeci Karuri and Mr. Dedan Karuri whose guiding wisdom and selfless sacrifice ensured that I got an education that has not only been invaluable to my everyday life, but has enabled me under take this task.

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS AND ACRONYMS

Acetyl CoA   Acetyl Co-enzyme A

ACS   American Chemical Society

BDL   Below detection limit.

CBD   Coffee Berry diseases

ELISA   Enzyme linked immunosorbent assay

EU   European Union

FCS   Farmers' Co-operative Society

GC   Gas chromatography

HPLC   High performance liquid chromatography

HPLC –FD   High Performance Liquid Chromatography coupled with fluorescence detector

HPLC-ESI   High-performance liquid chromatography/electrospray ionization

HPLC-MS/MS Liquid chromatography-tandem mass spectrometry

IAC   Immuno Affinity Column

IPCS   International Programme of Chemical safety

LOD   Limit of detection

LOQ   Limit of quantification

MCOh   Absorption wavelength of Methanol

MS   Mass Spectrometer

OTA   Ochratoxin A

PBS   Phosphate buffered solution

Pka   Potential acid dissociation constant

TLC   Thin layer Chromatography

UV   Utra violet

ABSTRACT

Coffee is one of the major foreign exchange earners in Kenya. The presence of mycotoxins in the processed coffee is one of the greatest emerging threats to coffee quality. Mycotoxins are toxic, carcinogenic and mutagenic metabolites of certain moulds and are some of the compounds encountered in the food and beverage industry. There are two main classes of mycotoxins found in stored crop produce, namely afflatoxins common in grains, and Ochratoxins being the most predominant in fruits. Of the Ochratoxins, Ochratoxin-A (OTA) is the most predominant and an emerging problem in coffee production over the world. The objective of this study was to assess the levels of OTA in Kenyan coffee. Samples were collected from all the coffee growing areas of Kiambu County, a major source of Kenyan coffee and consisting of areas with varying climatic conditions using a stratified sampling design. OTA in green and roasted coffee was determined using high performance liquid chromatography coupled with fluorescence detector after sample clean up by use of immuno affinity columns. HPLC –FD analysis on standards showed that the method could detect below the acceptable levels of OTA on all considered limits. To test whether, roasting causes significant reduction or increment of OTA t-test and Mann Whitney u-test were used. HPLC analysis indicated non- appreciable levels of OTA in premium coffee types AA and AB while acceptable levels were detected in *Mbuni*. Test of the efficiency of the method used was carried out by spiking the samples with standard solutions. Spiking of green coffee indicated a recovery level of 79.56%±1.31%, while that of roast coffee gave a recovery level of 50.62%±2.77%. The recovery studies showed that appreciable levels of OTA are

lost during extraction and clean up procedures while roasting causes appreciable decline to OTA levels and so is the processing of coffee. TLC analysis could detect up to a minimum of 150 µg/Kg of OTA and it is therefore unreliable in analysis of the low limits of OTA allowed in coffee. The result of this study provide baseline data as to the levels of OTA contamination in the Kenyan coffee industry and suggests possible control measures.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background to the Study

1.1.0 History of Coffee and its Importance

Coffee is believed to have originated from the horn of Africa in the Ethiopian province of Kaffa. According to records, the fruits of the plant known as cherries were eaten by slaves taken by Arabs from the present day Sudan. The Arabs from Yemen are believed to be among the first people to brew coffee as a beverage. The coffee plants were later grown by Europeans in their colonies and the brew became a major drink in Europe. Today not withstanding its history, coffee is one of the major contributors to world economy (Ukers & William, 1935).

Coffee is a beverage made from beans of plants referred to as coffee plant. Because coffee is drunk for pleasure, its flavor is the most important quality criterion. The above notwithstanding, the globalization of its trade and extensive use of agricultural chemicals as well as discovery of biologically induced toxins produced during storage and processing, have made other quality parameters to become important (Kaye, 1986).

1.2 Coffee Industry in Kenya

Coffee is one of the major foreign exchange earners for Kenya, and Kenya is ranked 21st largest producer in the world. In terms of quality, Kenyan coffee is amongst the best in aroma. Most of the Kenyan coffee is grown in the highlands around Mt. Kenya and Nairobi as well as areas near Mt. Elgon to the border with Uganda. Most of the

Kenyan coffee is grown in small farms and milling is done through the co-operative system. *Coffea arabica* variety is the most widely grown as opposed to *Coffea robusta* grown in neighboring countries (Kenneth, 2001).

Kenyan coffee is usually medium bodied and is considered to have a winey acidity taste. The Coffee Board of Kenya is the body assigned with the duty of promoting, marketing and grading the coffee produce in Kenya. In grading, coffee beans are assigned grades according to bean size and large beans are considered better quality. It is important to note that specialized standards are being developed for grading coffee based on far reaching and modern parameters (Leopold, 2008).

Plate 1-1: Photo of green coffee beans.

1.2.1 Coffee Quality Parameters

Following the awareness of the fact that despite food products, whether taken for health necessity or pleasure carry with them adulterants which have serious health implications, Food analytical parameters have radically changed so that palatability has become just a minor requirement for quality enforcers. Organoleptic tests are quickly being replaced with physically quantifiable techniques. Though different markets may have varying quality limits the measurable parameters are now more or less the same. The parameters can be classified as those that touch on:

(i)   Taste and aroma

(ii)   Purity (absence of adulterating products)

(iii)   Safety (absence of non-intentional contaminants such as pesticides and mycotoxins)

(iv)   General food quality e.g. moisture content (Oosterveld *et al.*, 2003).

In recent years environmental (Green issues) such as organic farming, fair trade or sustainable coffee have also become criteria of choice to consumers. These new requirements though non-quantifiable can bring benefits for the planters, who receive a guaranteed minimum price or a bonus for above quality by selling under special labels issued by environmental management bodies. Hundreds of compounds formed at roasting by chemical interaction between carbohydrates, chlorogenic acids, amino acids and other reactive compounds present in the green beans have been identified.

1.2.2 Aroma

Aroma contributes to the organoleptic (Analysis based on senses) quality of a cup of coffee. Modern analysis is replacing the traditional tasting procedures by identifying the active smelling compounds in coffee, and quantatively measuring them through

physical and chemical procedures. The above has been achieved by sniffing out all the components coming out of column outlet of a gas chromatography at brewing conditions and quantifying them. They have further been classified as in flavors (leading to improved flavors) and off flavors (leading to poor flavor) (Pittet *et al.*, 1996).

1.2.3 Purity

Findings showing precise carbohydrates fingerprints for pure soluble coffee, led to the establishment of an iso-analytical procedure and standards (Anon, 1997 b) and of various national standards in Europe. The standards indicate maximum acceptable levels of certain carbohydrates, and have helped in reducing import of adulterated products to the European Union.

Table 1-1; Maximum acceptable levels of carbohydrates as per the EU specifications (Anon, 1997).

	Indicator Carbohydrate	Maximum content in pure coffee (%)	Control limit (%)
1.	Total glucose	2.1	2.6
2.	Total xylose	0.4	0.6
3.	Free fructose	0.6	1.0

1.2.4 Safety

The possible presence of contaminants such as pesticides and polycyclic hydrocarbons formed when roasting, have at various times alerted authorities.

Mycotoxins, particularly of the ochratoxin type formed by the action of fungi during cherry processing have been of significant interest due to their acute toxicity at significantly low levels which would otherwise be considered insignificant for ordinary toxins. Ochratoxin-A is considered as particularly important and levels as low as 5 µg/Kg are considered significant (Pittet *et al.*, 1996).

1.2.5 Pesticide Residues

Coffee tree is prone to attack by many pests and diseases and this results in reduced yields and poor quality produce. Robusta is noted for its resistance to pests and diseases, though its beans are of poorer quality than those of Arabica. Kiambu County produces mostly Arabica coffee which is more prone to attack. The control of disease is done through chemical spray. Coffee diseases are caused by micro fungi, bacteria and some viruses. Coffee diseases include, Coffee Berries Disease (CBD), Coffee Leaf Rust, Sooty Mould, Berry Blight Extra. Among these diseases, CBD is most predominant (Waller & Duncan, 1987). Coffee being a capital intensive crop has to be protected against these pests and diseases throughout the year by use of pesticides. The pesticides may be classified as contact or systematic. Contact pesticides are deposited on the plant surface and kill the pest on contact, while systematic pesticides penetrate the plant cuticle and move up the plant vesicular system (Hassal, 1990). Though systematic pesticides are more effective, they bring in the danger of phytotoxicity when pesticide levels accumulate in plant (Waller & Duncan, 1987). Copper based fungicides are most commonly used in Kenya, while insecticides are mostly organophosphates. Use of organochlorides has been discontinued due to

environmental concerns. Contamination of coffee pulp with copper has been shown through research study (Kiptoo, 2001).

1.3 Mycotoxins

Mycotoxins are the toxic /carcinogenic/mutagenic metabolites of certain moulds, products of biosynthetic process during growth. Mycotoxin compounds are extremely stable and also dangerous in minute quantities. Once formed, mycotoxins cannot be removed from the commodity concerned by normal processing or removal of visible moulds, neither will they be destroyed by low temperature cooking, as is the case with other natural plant poisons or microorganisms. Mycotoxins are capable of causing diseases or even death in humans and other animals. Because of their pharmacological activities some mycotoxins or mycotoxin derivatives have found use as antibiotics , growth promotants and other kinds of drugs , still others have been implicated as chemical warfare agents. The most important of known mycotoxins associated with human and veterinary diseases include aflatoxins, ergot alkaloids, fumonisns, ochratoxin-A, putulin, tricothecines, and zearalenone (Bennet, 1999).

1.3.1 Ochratoxin - A

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the aspergillus or penicillium families and are weak organic acids consisting of a derivative of an isocoumarin.Three types of Ochratoxins have been isolated i.e. Ochratoxin-A, B and C which closely related molecular structure. Among the Ochratoxins, Ochratoxin-A is most prevalent and consequently most important.

Qualitative tests via TLC have indicated presence of OTA in Kenyan coffee (Kathurima, 2004). Ochratoxins are 3,4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L-β-phenylalanine. Ochratoxin A, the most important compound of this class, has fluorescent properties and is a secondary metabolite of *Aspergillus ochraceus* and *Penicillium verrucosum.* The molecular weight of Ochratoxin A is 403.82. The biosynthetic pathway of OTA has not been determined completely, but in principle it refers to a shikimate pathway (from shikimic acid) and a pentaketide pathway, which describe the formation of the phenylalanine and the dihydroisocoumarin parts, respectively. The first step of the pentaketide pathway represents the condensation between one acetate unit (acetyl-CoA) and four malonate units. Recently, it has been proved that a polyketide synthase is necessary for this condensation reaction (Dana-Maria, 2010). Ochratoxin A is a white, crystalline substance, with high solubility in polar solvents, It is slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. When recrystallized from benzene (containing 1 mol benzene/mol) or xylene, respectively . OTA exhibits UV adsorption: λMeOHmax (nm; ε) = 333. The fluorescence emission maximum is at 467 nm in 96% ethanol and 428 nm in absolute ethanol. OTA has weak acidic properties. The pKa values are in the ranges 4.2–4.4 and 7.0–7.3, respectively. Figure 1-1 shows the structures of OTA.
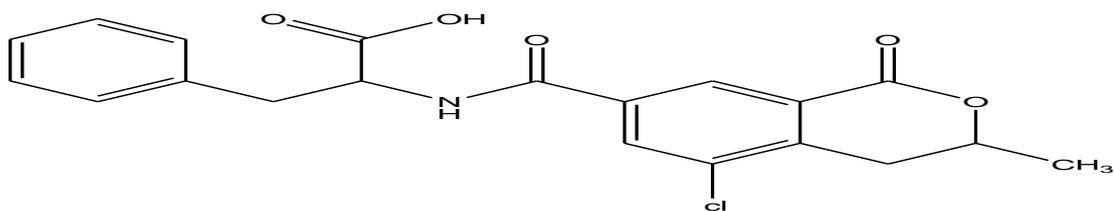
Ochratoxin-A

N{[(3R)-5-Chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-Isochromen-7-yl]carbonyl}-L-phenylalanine.
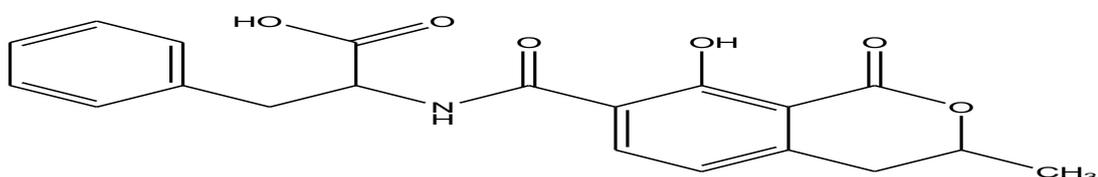
Ochratoxin-B

N{(8-hydroxy-3-methyl-1-oxo-7-isochromanyl)Carbonyl}-3-phenyl-L-alanine
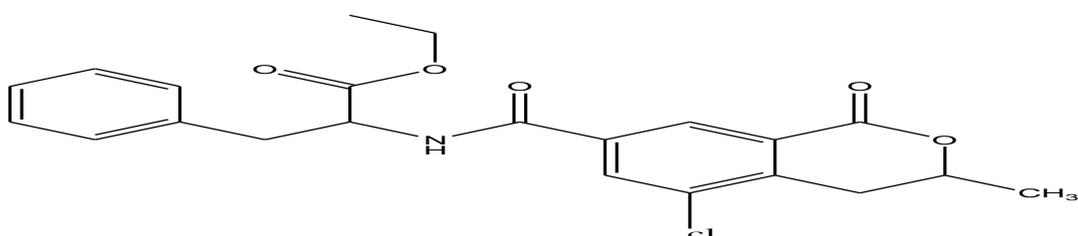
Ochratoxin-C

N{[(3R)-5-chloro-3'4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl]Carbonyl}-L-(phenyl-d5) alanine ethyl ether

Figure 1-1: The structures of Ochratoxins   (Source: Vian *et al.*, 1997)

1.3.2 Toxicity of Ochratoxin-A and Health Implications

Ochratoxin compounds are extremely stable and also dangerous in minute quantities, and a few parts per billion are of concern. Exposure to Ochratoxins through diet can

have an acute toxicity to mammalian kidneys and may be carcinogenic. Ochratoxin-A is absorbed from the gastro intestinal tract. In most species Ochratoxin-A is absorbed in the stomach as a result of its acidic properties. Absorption also takes place in the small intestine particularly in the proximal jejunum. In non-ruminant animal's species and where most research has been done, such as pigs, chickens, rabbits and rats, around half of the ingested Ochratoxin-A may be absorbed. The absorbed Ochratoxin-A is distributed via blood, mainly to the kidneys, and at lower concentrations to the liver, muscle and fat, with a proportion metabolized into the non- toxic metabolite Ochratoxin alpha and other less toxic minor metabolites (Waller & Duncan, 1989). Ochratoxin-A has a long serum half- life in humans on the basis of its strong binding to serum micro molecules (Ringot et al., 2006).

The sub-chronic and chronic effects of Ochratoxin-A are of greatest concern. Ochratoxin-A has been shown to poison the kidney (nephrotoxic), cause liver damage (hepatoxic), harm development of embryo (teratogenic) and affect the functioning of immune system (immunotoxic) to several species of animals and carcinogenic in mice and rats causing tumors of the kidney and liver. Ochratoxin-A has been shown to induce DNA damage, DNA repair and chromosomal aberrations in mammalian cells and mice treated in vitro and in vivo respectively (Binder et al., 2007) .

Human exposure as demonstrated by concentrations of Ochratoxin-A in blood and human milk has been observed in various countries in Europe (Nehad et al., 2007). Ochratoxin-A was found more frequently and in high concentrations in blood samples obtained from people living in regions where the fatal human kidney disease, Balkan endemic nephropathy has been observed (Albassan et al., 1987).

1.3.3 Ochratoxin-A Limits

In the European Union (EU), the Commission Regulation (EC) No 1881/2006 of 19 December 2006, as amended lays down specific maximum limits for ochratoxin A (OTA) for foodstuffs such as unprocessed cereals and products derived from unprocessed cereals, dried vine fruit, coffee beans and soluble coffee, wine, grape juice, spices, liquorice and products for infants (EU Regularitia, 2006). The lowest maximum limit other than in products for infants is established for wine and grape juice at 2.0 µg/kg, while the highest maximum limits is set at 80 µg/kg for liquorice extract for use in foods. Additional national maximum limits for OTA are established in EU Member States such as Denmark, Hungary, Italy and Germany in their national legislation.

Egypt, Bosnia and Herzegovina refer to the Commission Regulation for setting up their national maximum limits for OTA. Although, the limits established are not identical to those in the EU, they are very similar.

In Russia, OTA limits are established for several cereals and cereal products, as well as various products for children. The limit in wheat, barley, rye, oat and rice cereals and cereal products is set at 0.005 mg/kg while the limit for specific products for children is set at 0.5 µg/kg. In China a limit of 5.0 µg/kg for OTA is set for cereals, milled products from cereals, legumes and pulses. In the Codex Alimentarius Standard, an OTA limit of 5µg/kg is set for raw wheat, barley and rye, only. These limits are adopted in Gulf Cooperation countries, Nigeria and Kenya. In India, a maximum limit for OTA is established for the same foodstuffs as in Codex; however the limit is set at 20 µg/kg. No specific limits for OTA in foodstuffs are set in USA, Canada, Australia and New Zealand, Japan, Mexico and South Africa (Mariko, 2009).

1.3.4 Effects of Ochratoxin-A and Geographical Distribution.

Two fungal species are associated with OTA production i.e. *Penicillium verrucosum* and *Aspergillus ochraceus* (IPCS, 2011).The frequency of occurrence of these species differs according to climatic and geographic regions as well as commodities affected. *P. verrucosum* is a common storage fungus and is a source of Ochratoxin A in crops in the cool temperate region such as Canada, Eastern and North Western Europe and parts of South America. It grows only at temperatures below 30 degrees Celcius and low water activity. It may produce Ochratoxin at temperatures as low as 5 degrees Celsius (Nagoya, 2001). *A. ochraceus* on the contrally to penicillium species is limited to conditions of high humidity and temperature,growing in the tropical and subtropical climates and is the source of contamination for coffee and cocoa beans spice dried vice fruit, grape juice and wine.

A. ochraceus which grows in moderate temperature and high water activity is a significant source of Ochratoxin-A and infects coffee beans during sun drying causing contamination in green coffee. Other minor aspergillus species such as *A. carbonarius* and *A. niger* also contribute mildly to OTA production (Hanak *et al*., 2002).In Kenyan coffee *Cladosporim ssp* was found to be most significant in a study done on Kiambu coffee, yeast, *Penicillium ssp*, *Fusarium ssp* and *Aspergillus ssp* were also found. (Kathurima, 2004).

1.3.5 Effects of Processing Coffee Beans

Ochratoxin-A is a moderately stable molecule and is able to survive most food processing to some extent and may thus occur in consumer products (Vizthum, 1998).

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For coffee, roasting or fermentation is the most influencing to Ochratoxins. The degree to which Ochratoxin are destroyed will depend on other parameters such as pH, temperature and other ingredients present. Roasting has been reported to reduce toxin content by a mere 20%, however physical treatment of grain such as scouring while cleaning causes reduction of Ochratoxin-A contamination by more than 50%, (Stegen *et al*., 1997).

A study done by the Nagoya City Health Research Institute in conjunction with Natural Institute of Hygienic Sciences of Nagaja, Japan showed that heat treatment (roasting) of coffee beans at 200 degrees Celsius for 10 or 20 minutes reduced the levels of Ochratoxin-A by only $0 - 12\%$ in the dried whole beans. Almost all the Ochratoxin A was infused into the coffee decoction when roasted samples were ground and extracted with boiling water. Reduction of Ochratoxin-A concentration of contaminated coffee beans by roasting under these conditions is ineffective (Nagoya, 2001).

1.4 Methods of OTA Determination

The methods of OTA determination have to take into consideration the nature of OTA extraction which is based on soaking the Ochratoxin from the solid matrix of the coffee bean, followed by removal of interfering substances before a chromatographic separation is done (Nakojima *et al*., 1997). In the extraction matrix it is important to carry out adequate clean up to remove substances such as lipids and pigments that could interfere in the analytical techniques. Liquid liquid extraction with water, organic solvents and a mix of salts and acids are used, Followed by solid phase extraction with immune-affinity columns (Stegen *et al.,* 1997).

In the analytical phase the extract is passed through column liquid chromatograph using reversed phase columns because the OTA extract is usually in a polar phase. Different analytical methods have been developed for mycotoxin analysis in food products and feeds. Thin-layer chromatography is a chromatographic technique that is useful for the separation, purity assessment and identification of organic compounds. Because of the simplicity and rapidity of TLC, it is also used to monitor the progress of organic reactions.

TLC has found widespread use in mycotoxin determination but has now largely been replaced by other chromatographic methods e.g. High Performance Liquid Chromatography and Gas Chromatography, because of superior analytical performance. In countries, which do not have expensive equipment at their disposal but which often produce and export agricultural commodities, TLC remains important. It is also in use for screening purposes prior to labour intensive and time consuming HPLC and GC methods.

HPLC and ELISA have proven to be highly efficient for mycotoxin determination in a wide variety of matrices. GC has also been used, mostly with MS detector. In association with HPLC, Tandem Mass Spectrometry provides high selectivity and sensitivity. Flame ionisation detection (in GC) and fluorescence detection (in HPLC) have also been used.

A very important aspect concerning mycotoxin analysis is sample preparation and cleanup.

Cleanup steps are essential for any analytical method in order to eliminate the interferences. The accuracy of a certain analytical method critically depends on the type of cleanup applied. Immuno-affinity cleanup is an efficient sample purification

method, particularly in the case of HPLC-flourescence/MS. In order to reduce analysis time, simultaneous analysis of different mycotoxin types is usually desired. The use of a cleanup column with different types of antibodies, corresponding to each toxin category, can be a solution to this problem.

The most important advantages of immune affinity column cleanup are listed below:

(i) Provision of clean extracts due to the specificity of the antibody;

(ii) Applicability to complex matrices;

(iii) Good precision, accuracy and sensitivity of analytical methods;

(iv) Rapid clean up;

(v) Limited use of organic solvents.

The need for derivatization is a disadvantage of some methods such as gas Chromatography due to the extremely low concentrations. Cross-reactivity may occur in ELISA trials. In some cases, ELISA results are confirmed by HPLC-MS/MS, but in other cases there is the possibility of false positives. For this reason, HPLC-MS/MS has to be used for confirmation purposes. As a general conclusion of the literature survey HPLC-ESI-MS/MS together with an efficient sample cleanup step seems to the method of choice for mycotoxin analysis in food samples. The choice of HPLC-ESI-MS/MS with immune affinity cleanup can be explained in terms of method accuracy and sensitivity. Low LOD values can be obtained, due to Mass Spectrometry sensitive detection, enhanced by concentration steps during sample preparation. The linear range, recoveries and standard deviations obtained with HPLC-ESI-MS/MS, as well as immune affinity column specificity, are also reasons for choosing this method (Dana-Maria, 2010).

HPLC is a preferred form of chromatographic separation. The detection procedure must take into consideration the low maximum limits of Ochratoxin-A and thus must be very sensitive. Mass Spectrometry through election spray inlet is widely accepted (Nehad *et al*., 2007). However, a more specific detection is based on the unique property of Ochratoxin ability to fluoresce, therefore increasing the selectivity of detection system. HPLC coupled with fluorescence detector set at an excitation wavelength of 330nm and an emission wavelength of 460nm is a preferred analytical technique (Kaiji *et al*., 2004).

1.4.1 Principles of high performance liquid chromatography

1.4.1.1 Principles

High Performance Liquid Chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive (Karger *et al*., 1973).

1.4.1.2 The Column and the Solvent

There are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase.

1.4.1.2.1 Normal Phase High Performance Liquid Chromatography

Although it is described as "normal", it is not the most commonly used form of HPLC. The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column (Skoog *et al*., 2007).

1.4.1.2.2 Reversed Phase High Performance Liquid Chromatography

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There will not be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to be attracted to the non-polar column through van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. This means that the polar molecules that will travel through the column more quickly. Reversed Phase HPLC is the most commonly used form of HPLC.
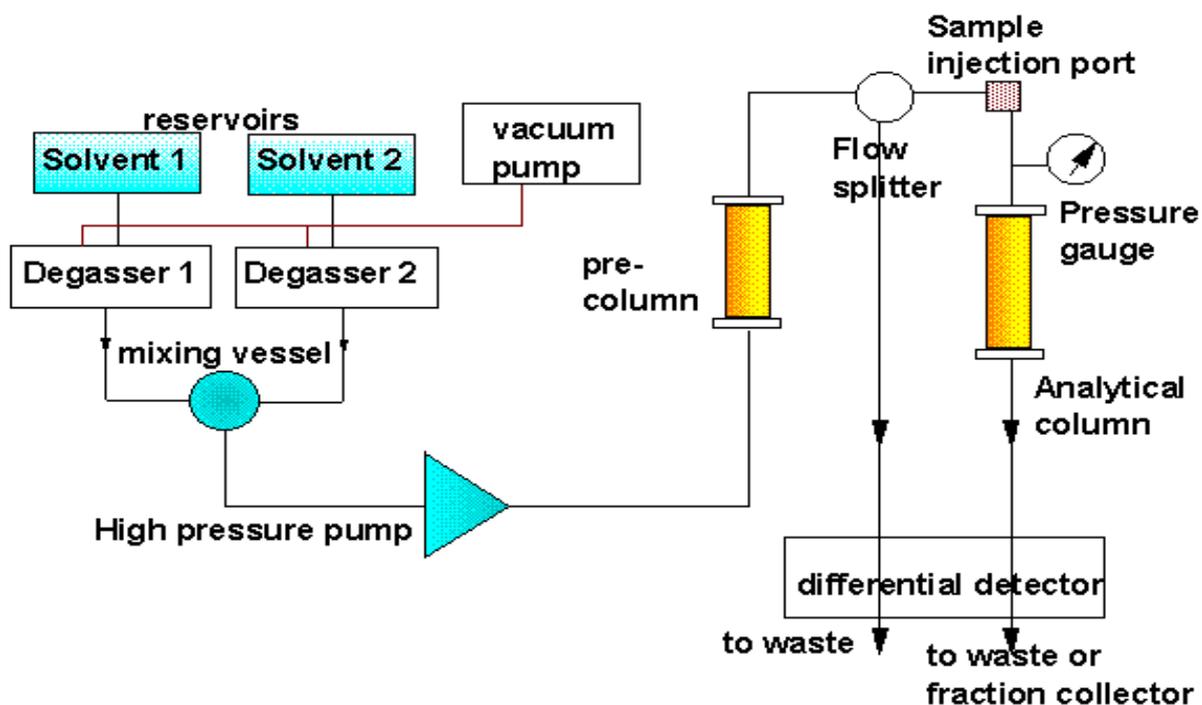
Figure 1- 2: A flow scheme for HPLC

1.4.1.3 Retention Time

The time taken for a particular compound to travel through the column to the detector is known as its *retention time*. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. For a particular compound, the retention time will vary depending on: the pressure used (because that affects the flow rate of the solvent); the nature of the stationary phase (not only what material it is made of, but also particle size); the exact composition of the solvent and the temperature of the column. This means that conditions have to be carefully controlled if retention time is used as a way of identifying compounds (Willard *et al*., 1988).

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1.4.1.4 The Detector

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption. Many organic compounds absorb UV light of various wavelengths. If a beam of UV light shines through the stream of liquid coming out of the column, and a UV detector is placed on the opposite side of the stream a direct reading of how much of the light is absorbed is obtained. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time (Skoog *et al*., 2007).

1.4.2 Principle of Immuno-affinity Column

The stationary phase is typically a gel matrix, often of agarose, a linear sugar molecule derived from algae. Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well-known and defined property, and can be exploited during the affinity purification process. The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium. The other molecules in the mobile phase will not become trapped as they do not possess this property. The stationary phase can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution. Possibly the most common use of affinity chromatography is for the purification of recombinant proteins(Nakajima *et al*., 1997).

The working principle of immuno affinity column is illustrated in figure 1-3.
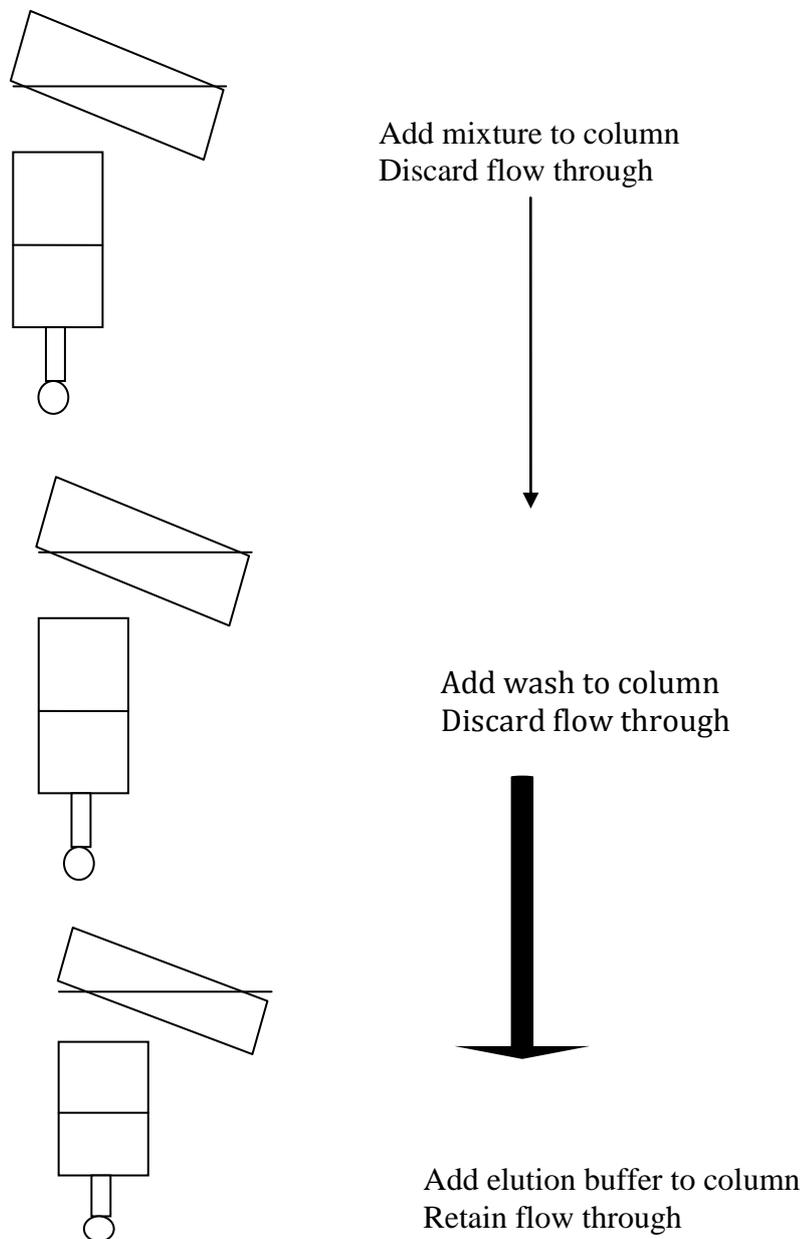
Figure 1- 3: Immuno Affinity Column Chromatography (Nakajima *et al*., 1997)

1.4.2.1 Column setup

Binding to the solid phase may be achieved by column chromatography whereby the

solid medium is packed onto a column, the initial mixture run through the column to

allow setting, a wash buffer run through the column and the elution buffer

subsequently applied to the column and collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using a batch treatment, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the eluate.

Sometimes a hybrid method is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

A third method, expanded bed adsorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensure that the solid phase does not exit the column with the liquid phase.

Affinity columns can be eluted by changing salt concentrations, pH, PI, charge and ionic strength directly or through a gradient to resolve the particles of interest (Uhlen, 2008).

Affinity chromatography can be used in a number of applications, including mycotoxins, nucleic acid purification, protein purification from cell free extracts, and purification from blood (Uhlen, 2008).

1.4.3 Principle of Fluorescence Detector

Molecules have various states referred to as energy levels. Fluorescence Spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an

excited electronic state of higher energy. Within each of these electronic states are various vibrational states (Holler, 2006).

In Fluorescence Spectroscopy, the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. This process is often visualized with a Jablonski diagram. Figure 1-4 displays a Jablonski diagram.

The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined (Rendel and Mowthorpe, 1987).

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in fluorescence excitation measurement, the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An emission map is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity as a function of excitation and emission wavelengths, and is typically depicted as a contour map (Eisinger *et al*., 1979).
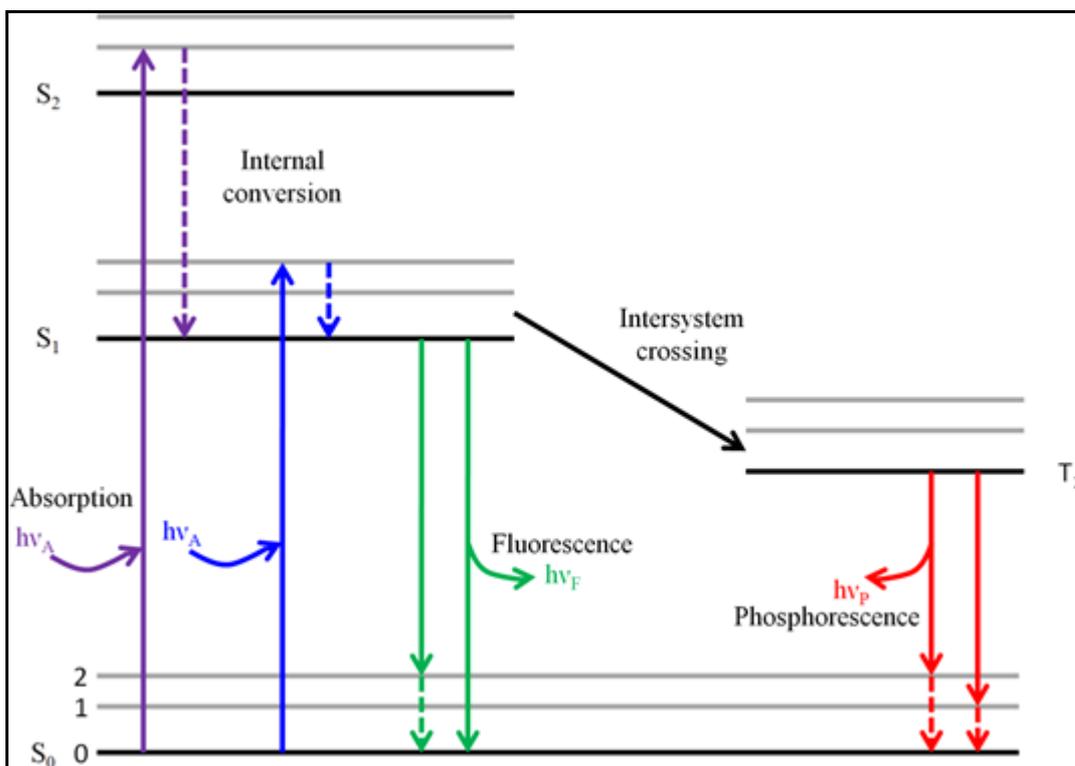
Figure 1- 4: Jablonski diagram

1.4.3.1 Instrumentation

Two general types of instruments exist:

- Filter fluorometers use filters to isolate the incident light and fluorescent light.

- Spectrofluorometers use diffraction grating monochromators to isolate the incident light and fluorescent light.

Both types use the following scheme: The incident light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.

22
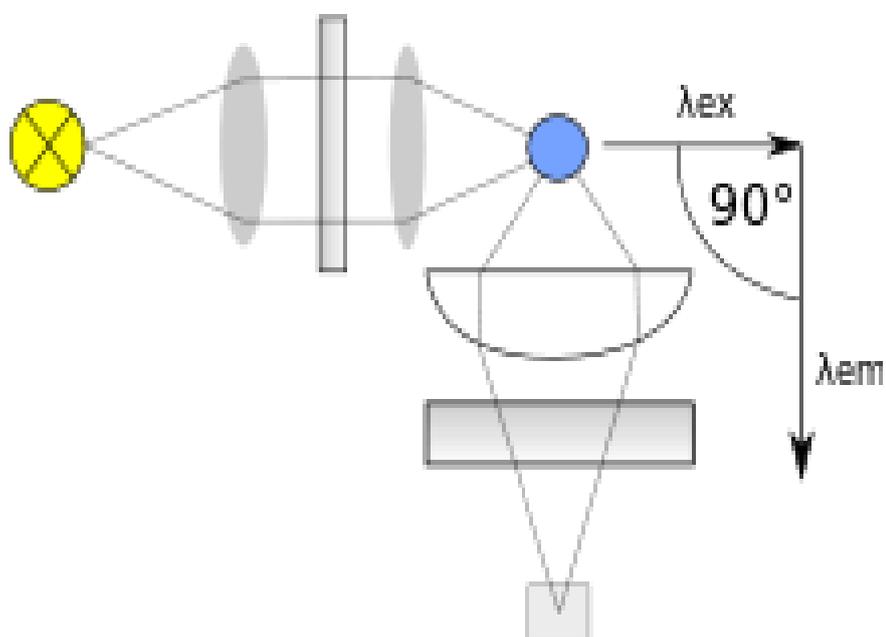
Figure 1-5: A simplistic design of the components of a fluorimeter (Sharma & Schulman, 1999)

Various light sources may be used as excitation sources, including lasers, light emitting diodes, and lamps; xenon arcs and mercury-vapor lamps in particular. A laser only emits light of high irradiance at a very narrow wavelength interval, typically under 0.01 nm, which makes an excitation monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be changed by much. A mercury vapor lamp is a line lamp, meaning it emits light near peak wavelengths. By contrast, a xenon arc has a continuous emission spectrum with nearly constant intensity in the range from 300-800 nm and a sufficient irradiance for measurements down to just above 200 nm.

As mentioned before, the fluorescence is most often measured at a 90° angle relative to the excitation light. This geometry is used instead of placing the sensor at the line of the excitation light at a 180° angle in order to avoid interference of the transmitted excitation light. No monochromator is perfect and it will transmit some stray light, that is, light with other wavelengths than the targeted. An ideal monochromator would only transmit light in the specified range and have a high wavelength-independent transmission. When measuring at a 90° angle, only the light scattered by the sample causes stray light. This results in a better signal-to-noise ratio, and lowers the detection limit by approximately a factor 10000, when compared to the 180° geometry. Furthermore, the fluorescence can also be measured from the front, which is often done for turbid or opaque samples.

The detector can either be single-channeled or multi-channeled. The single-channeled detector can only detect the intensity of one wavelength at a time, while the multi-channeled detects the intensity of all wavelengths simultaneously, making the emission monochromator or filter unnecessary. The different types of detectors have both advantages and disadvantages.

The most versatile fluorimeters with dual monochromators and a continuous excitation light source can record both an excitation spectrum and a fluorescence spectrum. When measuring fluorescence spectra, the wavelength of the excitation light is kept constant, preferably at a wavelength of high absorption, and the emission monochromator scans the spectrum. For measuring excitation spectra, the wavelength passing though the emission filter or monochromator is kept constant and the excitation monochromator is scanning. The excitation spectrum generally is identical

to the absorption spectrum as the fluorescence intensity is proportional to the absorption (Sharma & Schulman, 1999).

1.4.3.2 Analysis of Data

At low concentrations the fluorescence intensity will generally be proportional to the concentration of the fluorophore. Unlike in UV/visible spectroscopy, 'standard', device independent spectra are not easily attained. Several factors influence and distort the spectra, and corrections are necessary to attain 'true', i.e. machine-independent, spectra. The different types of distortions will here be classified as being either instrument- or sample-related. Firstly, the distortion arising from the instrument is discussed. As a start, the light source intensity and wavelength characteristics varies over time during each experiment and between each experiment. Furthermore, no lamp has a constant intensity at all wavelengths. To correct this, a beam splitter can be applied after the excitation monochromator or filter to direct a portion of the light to a reference detector.

Additionally, the transmission efficiency of monochromators and filters must be taken into account. These may also change over time. The transmission efficiency of the monochromator also varies depending on wavelength. This is the reason that an optional reference detector should be placed after the excitation monochromator or filter. The percentage of the fluorescence picked up by the detector is also dependent upon the system. Furthermore, the detector quantum efficiency, that is, the percentage of photons detected, varies between different detectors, with wavelength and with time, as the detector inevitably deteriorates.

Two other topics that must be considered include the optics used to direct the radiation and the means of holding or containing the sample material (called a cuvette or cell). For most UV, visible, and near infra-red measurements the use of precision quartz cuvettes is necessary. In both cases, it is important to select materials that have relatively little absorption in the wavelength range of interest. Quartz is ideal because it transmits from 200 nm-2500 nm; higher grade quartz can even transmit up to 3500 nm, whereas the absorption properties of other materials can mask the fluorescence from the sample.

Correction of all these instrumental factors for getting a 'standard' spectrum is a tedious process, which is only applied in practice when it is strictly necessary. This is the case when measuring the quantum yield or when finding the wavelength with the highest emission intensity for instance.

As mentioned earlier, distortions arise from the sample as well. Therefore some aspects of the sample must be taken into account too. Firstly, photodecomposition may decrease the intensity of fluorescence over time. Scattering of light must also be taken into account. The most significant types of scattering in this context are Rayleigh and Raman scattering. Light scattered by Rayleigh scattering has the same wavelength as the incident light, whereas in Raman scattering the scattered light changes wavelength usually to longer wavelengths. Raman scattering is the result of a virtual electronic state induced by the excitation light. From this virtual state, the molecules may relax back to a vibrational level other than the vibrational ground state. In fluorescence spectra, it is always seen at a constant wave number difference relative to the excitation wave number e.g. the peak appears at a wave number 3600 cm^{-1} lower than the excitation light in water.

Other aspects to consider are the inner filter effects. These include re-absorption. Re-absorption happens because another molecule or part of a macromolecule absorbs at the wavelengths at which the fluorophore emits radiation. If this is the case, some or all of the photons emitted by the fluorophore may be absorbed again. Another inner filter effect occurs because of high concentrations of absorbing molecules, including the fluorophore. The result is that the intensity of the excitation light is not constant throughout the solution and as a result only a small percentage of the excitation light reaches the fluorophores that are visible for the detection system. The inner filter effects change the spectrum and intensity of the emitted light and they must therefore be considered when analysing the emission spectrum of fluorescent light (Lakowicz, 1999).

Fluorescence spectroscopy is used in, among others, biochemical, medical, and chemical research fields for analyzing organic compounds. There has also been a report of its use in differentiating malignant, bashful skin tumors from non-malignant ones.

1.5 Statement of the Problem

For a medium sized economy like Kenya with a surging population, and diminishing arable land that is heavily dependent on the coffee industry for employment, income and foreign exchange earnings, responsiveness to importing countries norms on Ochratoxin levels in coffee is a necessity. As market competition increases and global warming makes areas such as South Europe which were originally unsuitable for coffee growing suitable for the same, pressure on quality demand for coffee will shift from aroma to safety. Ochratoxin-A is an emerging problem in coffee production

worldwide. In the past decade, coffee importing companies and researchers have taken a great interest in the mycological quality of green coffee beans to estimate the risk to humans posed by this mycotoxin.

1.6 Justification

Kenyan coffee is exported worldwide to countries such as U.S.A and European Union countries such as Great Britain, German, and France, which are embracing quality assurance measures as opposed to quality control. Such countries will in future prefer to import coffee from countries where research on quality parameters will include OTA levels which must meet their market regulations. Competitors in the coffee market mainly from South America and surrounding regions that include countries such as Brazil, Guatemala and Vietnam have done a great deal of research in this area. Egypt which imports Robusta coffee from neighbouring countries has done extensive research on the same and acts as a research centre for those countries. There is little research on OTA in Kenya. It is therefore imperative that research in this area should be emphasized so that proper records can be maintained by interested bodies and corrective measures taken either during harvesting, storage or processing if some of the coffee produced is found not to meet market demands. It is hoped that the study of OTA in coffee produced in Kiambu County will form a nucleus for further research in other Kenyan counties. Kiambu County has one of the widest climatic variations within the coffee producing regions ranging from very low temperatures and high rainfall in areas near Lari and Limuru to high temperatures and low rainfall in Juja and areas bordering Machakos County. Kiambu County is thus very representative of most coffee producing regions in Kenya.

1.7 Hypothesis

The Kenyan coffee from Kiambu County does not contain significant levels of OTA.

1.8   Objectives

1.8.1   General objective

To assess the levels of Ochratoxin-A contamination in coffee beans from Kiambu County, Kenya.

1.8.2 Specific objectives

1. To determine levels of Ochratoxin-A in green coffee beans, roasted coffee beans and in brewed coffee from Kiambu County, Kenya.

2. To investigate the effect of coffee processing on OTA levels.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Area

The study area includes the entire Kiambu County (Figure 2-1). The county is in the

Central Kenya region and bordering the Capital city Nairobi, Kenya.
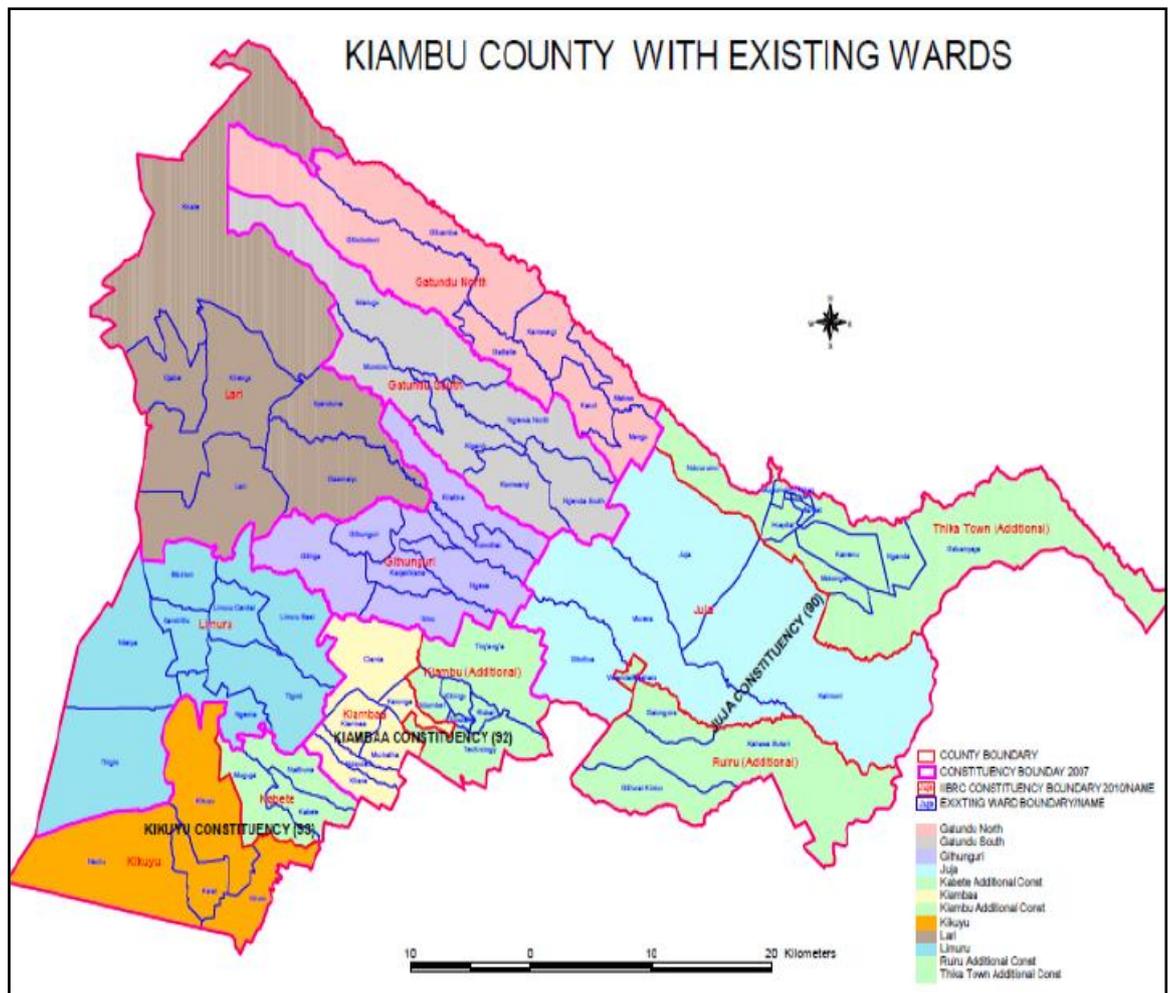
Figure 2-1: Map of Kiambu County (Source: IEBC, 2011).

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2.2 Sample Collection and Sampling Frame

Out of approximately ninety registered coffee estates and farmers' cooperative societies (FCs), eighteen were selected through a stratified sampling design. The samples represent approximately twenty percent of registered trading institutions in Kiambu County. The research relied on stratified sampling procedure to increase on representation. One Kilogram samples were taken each from approximately 20% of co-operative societies in each constituency and certified private coffee estates. The cooperative societies were randomly selected from list of existing societies. The total for each constituency formed the bulk sample which was thoroughly mixed, from where the analytical samples were obtained. Eight analytical samples from each of the eight constituencies were obtained and each treated as a separate sample.

Table 2-1: List of sampled places.

S/No.	Estates Sampled	S/No.	Cooperatives Sampled
1	Yadini	10	Komothai
2	Oakland	11	Gathage
3	Barikongo	12	Gatitu
4	Mahuti	13	Thiririka
5	Gitare	14	New Gatukuyu
6	Gethumbuini	15	Mirimaini
7	Handege	16	Ndumberi
8	Mitaro	17	Kiambi
9	Machure	18	Ichaweri

2.3 Reagents and Chemicals

The reagents and chemicals of analar grade were used in this study. Ochratoxin-A standard solution (Analytical grade St. Louis U.S.A) (99.7%), Sodium bicarbonate(99%), Potassium dihydrogen phosphate (99.5%), Potassium chloride (99.5%), Potassium dichromate, (97%), Sulphuric Acid (99.9%), HPLC grade methanol(99.8%), Acetone (pesticide grade) (99.5%), Benzene(99.9%), Glacial acetic acid (99.8%), Acetone (A.C.S grade) (95%), Ethyl alcohol(12%), Sodium hypochlorite solution and Pure water conductivity under point five six (0.56) MScm,[-1](Kaiji *et al.,* 2006).

2.4 Apparatus

Extraction was carried out by use of general glassware. Buffered aliquots were cleansed of contaminants by passing them through an immuno affinity column. Separation was carried out using High Performance Liquid Chromatography instrument, interfaced with fluorescence detector.

2.5 Extraction of OTA

25 g of green and roasted coffee were weighed into 250 ml glass flasks and extracted with 100 mL of methanol and 100 mL of 3% aqueous sodium bicarbonate solution. The suspension was shaken for 30 minutes. The suspension was homogenized in a blender for 5 minutes and filtered through 0.45 mm membrane paper. After the filtration 4 mL aliquots of filtered extract was transferred to a flask and made to 100 mL with phosphate buffered solution and homogenized. The resulting solution was labeled solution I.

Plate 2-1: Extracted sample solutions of green and roasted coffee.

2.5.1Thin-Layer Chromatography Analysis

The eluent consisted of Acetonitrile/Water/Acetic acid (500:500:1). The standard OTA was spotted under UV lamp at 366 nm. OTA appears as a distinct blue spot under UV illumination on ordinary TLC plates. The R_F value for each spot was calculated. The R_F values were used for identification. Thin layer chromatography was generally applied in qualitative identification and determination of limits of detection.

2.6 Cleanup Process and HPLC Analysis

2.6.1 Immuno-affinity Columns.

The Immuno-affinity columns (plate 2) were set at room temperature, connected to vacuum system and attached to a 60 ml reservoir. Solutions were passed through this system at a flow rate of 2 to 3 mL/minute. Each column was washed with 10 mL of milli-Q water at same flow rate. The column was dried by applying slight vacuum for 30 seconds, 4 mL of methanol were applied to the IA and given three minutes to allow solvent to permeate the gel before elution. The eluent was evaporated to dryness under a stream of nitrogen gas at 40 degrees Celsius and reconstituted with 1mL of mobile phase. The solutions were transferred to plastic centrifuge tubes (volume 1.5 mL) and centrifuged at 5.00xg for 5 minutes to obtain a supernatant. 50 µl of the solution was injected to the HPLC. Four samples spiked with standard OTA solution (between 1-5 µg/Kg) were subjected to similar clean up to test the efficiency of the clean-up and recovery process.

Plate 2-2: Immuno affinity columns

2.6.2 Measurement Conditions

Each sample was analyzed in triplicate. The total concentration of Ochratoxin-A was calculated by considering the sample concentration and number of dilutions. The fluorescent detector was set at excitation wavelength of 330 nm and fluorescent wavelength 460 nm (Tsubouchi *et al.,* 1992). Octa-decyl Silyl silica gel column 4.6 nm in inner diameter, 250 mm in length with particle size of 50 μm was used. Eluent consisted of Acetonitrile/water/acetic acid (500:500:1) Alkalization solution consisted of 0.1M sodium hydroxide solution. Eluent flow rate: 1.0 mL/min, alkalization solution flow rate: 0.3 ml/min. Column oven temperature was set at 40 ˚C. Standard calibration curves were prepared by plotting graphs of peak areas against OTA concentrations. The whole process starting from sample collection, sample preparation, and the analytical process is summarized in a single flow diagram. Figure 2-2 shows a flow diagram of sampling and analytical procedures.

2.6.3 Recovery and Confirmation Studies.

Recovery studies were carried out by spiking the sample with 1.5 to 50 ng/g of OTA and calculating percent recovery after taking them through the normal experimental procedure. The spiked samples were allowed to equilibrate overnight at 20 ºC with care being taken to ensure that all of the standard solution has been absorbed.
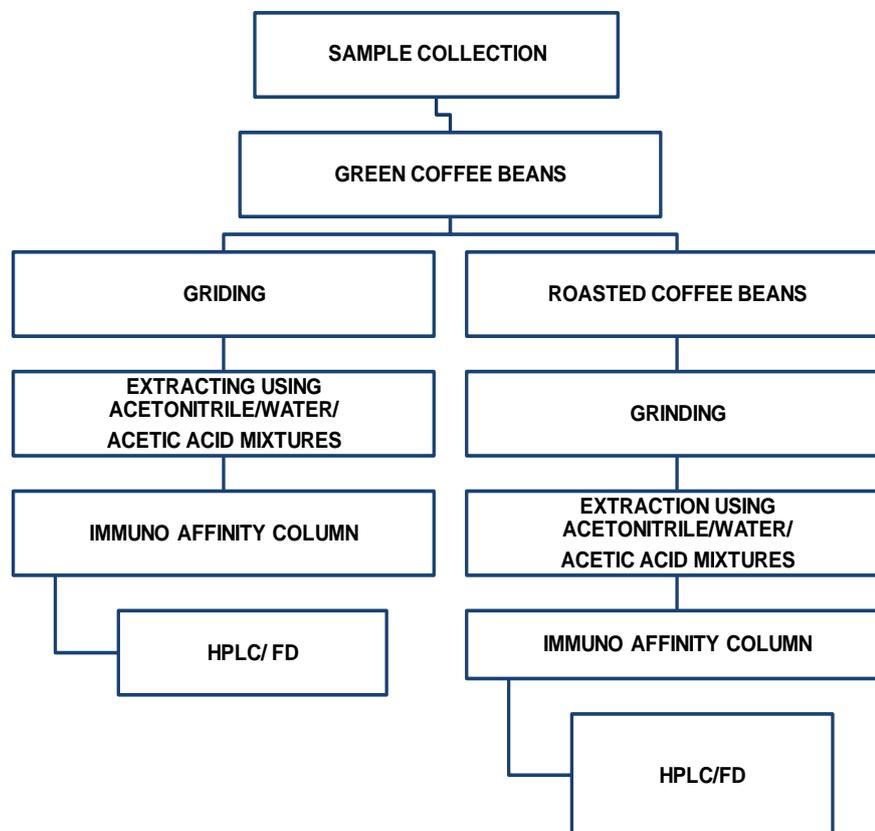
Figure 2-2: Flow diagram of sampling and analytical procedures.

2.6.4 Data analysis

The data was analyzed by aid of excel statistical package. Mean and Standard Deviation were used to assess extent of central tendency and variation. Paired t-test was used to test whether significant difference exists between samples from different sources and those from same source that have undergone varied processing procedures. Mann Whitney u-test was used to ascertain whether, roasting causes significant reduction or increment of OTA because the distribution may not be normal.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Results of TLC Analysis.

Standard OTA solutions were tested through TLC for R_F values, the following values were obtained. Table 3-1 shows the calibration data, while plate 3-1 shows the chromatogram from which the data was obtained.

3.1.1 TLC Calibration data.

Table 3-1; Standard solutions R_F values for TLC.

Trial	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average R_F	Standard Deviation
R_F	0.403	0.419	0.411	0.411	0.411	0.413	0.0067
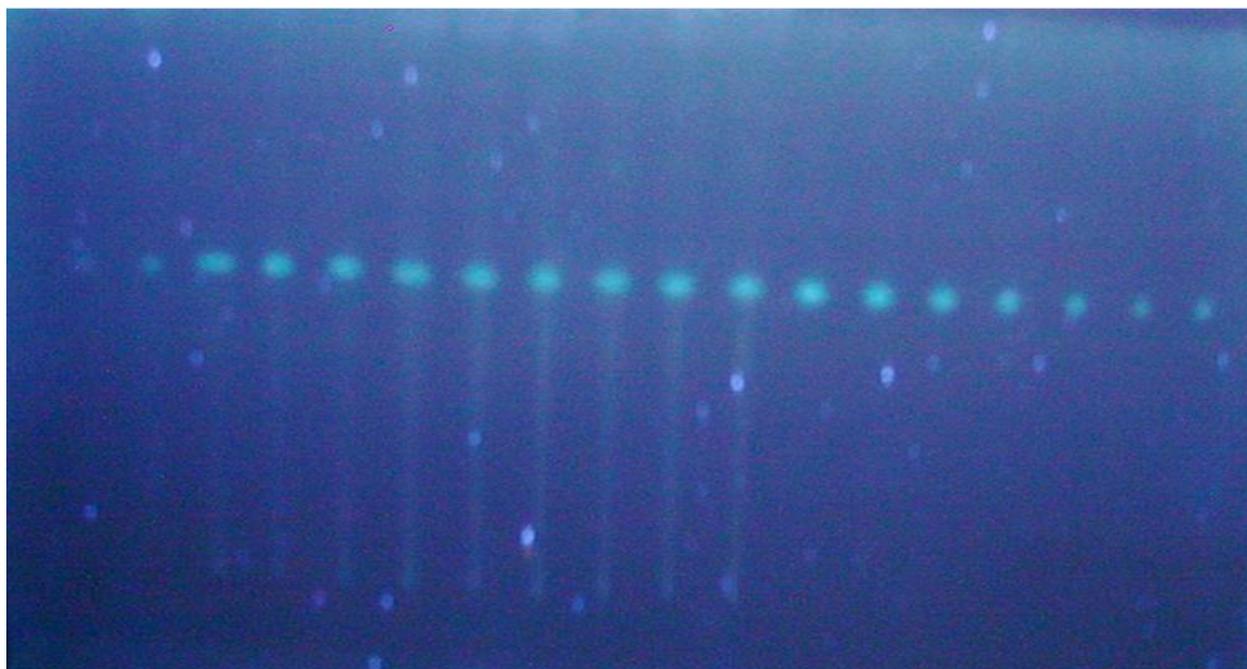
Plate 3-1: TLC chromatograms for OTA standards under UV lamp.

3.1.2. Minimum Detectable Limits with TLC

Standard solutions of OTA were gradually diluted and spotted. TLC could detect up to a minimum of 150 µg/Kg standard solution based on three times standard deviation of the blank. It was therefore found to be unsuitable for analysis of samples that may be expected to have much lower levels that would be considered significant. The analysis on samples yielded negative results but this was found to be inconclusive due to the low detection limit of the method and heavy masking by other ingredients for example caffeine as indicated by figure3-2. The conclusion was to proceed with clean up by use of immuno affinity column and analysis by the more sensitive HPLC technique. Figure 3-2 shows a sample of the TLC chromatograms. Solutions 8 and 9 are from roast samples from Machure and Komothai while 11 and 12 are the respective green samples.
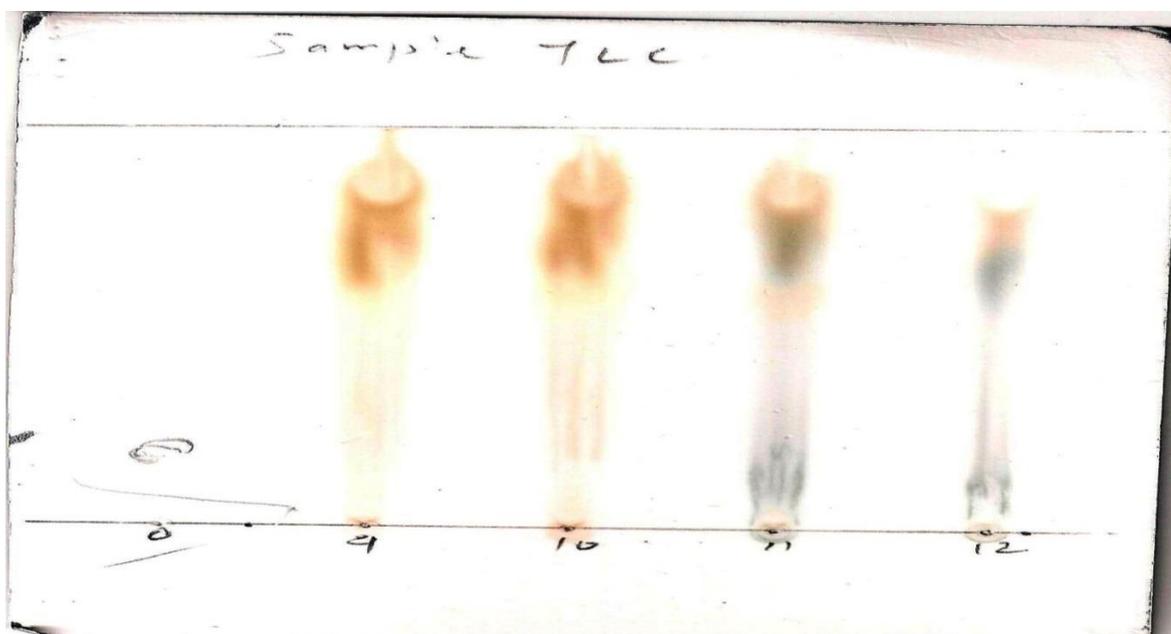
Figure 3-1: TLC chromatograms for sample solutions with marked sample numbers.

3.2. High Performance Liquid Chromatography Analysis.

HPLC is a more elaborate method than TLC. Figure3-3 shows a sample of standard solution chromatogram of HPLC coupled with fluorescence detector. When the method was used with standard OTA solutions, a limit of detection of about 2 µg/Kg was obtained and a linear calibration curve at 2-25 µg/Kg was obtained as shown by the following results.

3.2.1. Chromatograms of standard solutions of OTA
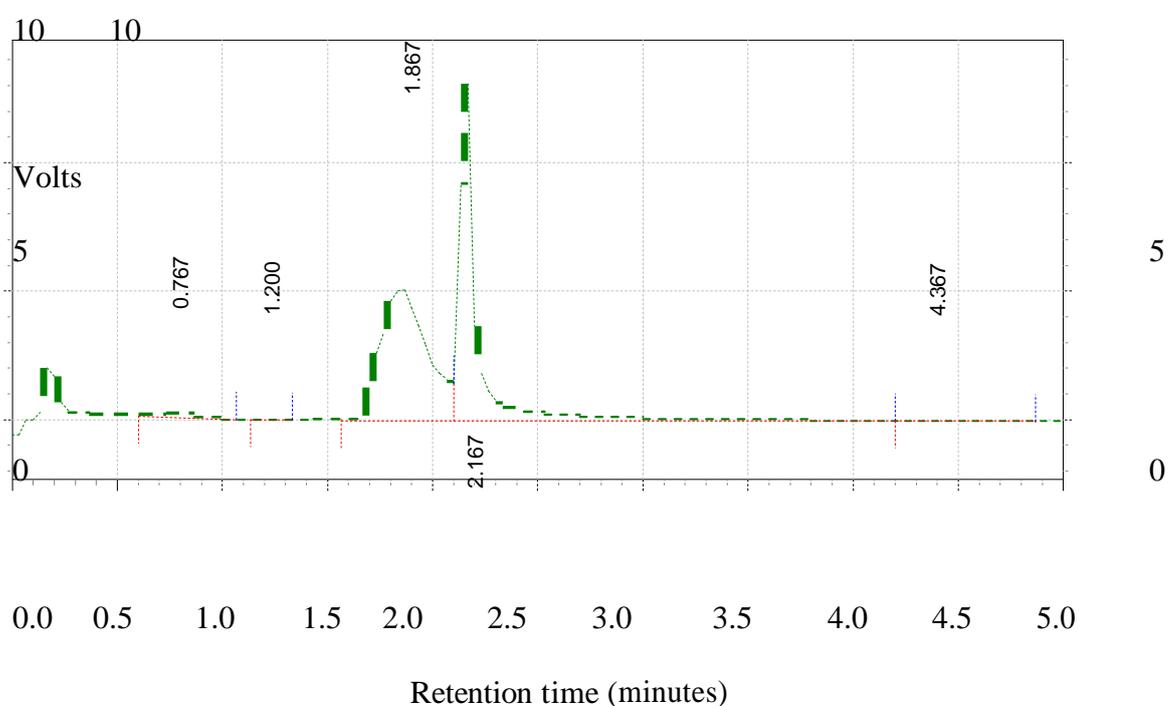
Figure 3-2: Chromatogram for 1.25 µg/Kg OTA standard solution

.Tables 3-2 and 3-3 shows the average retention times, peak areas and peak heights for the various standard solutions used in preparation of the calibration curve. The peaks for OTA standards were detected at a retention time of between 2.1 and 2.2 minutes.

Table 3-2; Results From Standard Solution Chromatograms for OTA

Concentration	Retention Time	Area	Area%	Height	Height%
1.25 µg/Kg	2.167	76457	47.85	13121	70.75
3.125 µg/Kg	2.133	108392	65.12	23432	84.05
6.25 µg	2.133	177378	79.50	34183	90.69
12.5 µg/Kg	2.133	347057	85.76	93509	95.13
12.5 µg/Kg	2.145	604137	95.62	234425	97.22

3.2.2. HPLC Calibration Data

Table 3-3 shows a summary of the peak areas that were used to preparation of the calibration data for the construction of calibration curve, while Figure 3-4 is the calibration curve resulting from the data with the resulting regression equation.

Table 3-3; Peak Areas for Standard solutions of OTA

Concentration µg/Kg	Peak area
1.25	76457
3.125	108392
6.25	177378
12.5	347057
25	604137
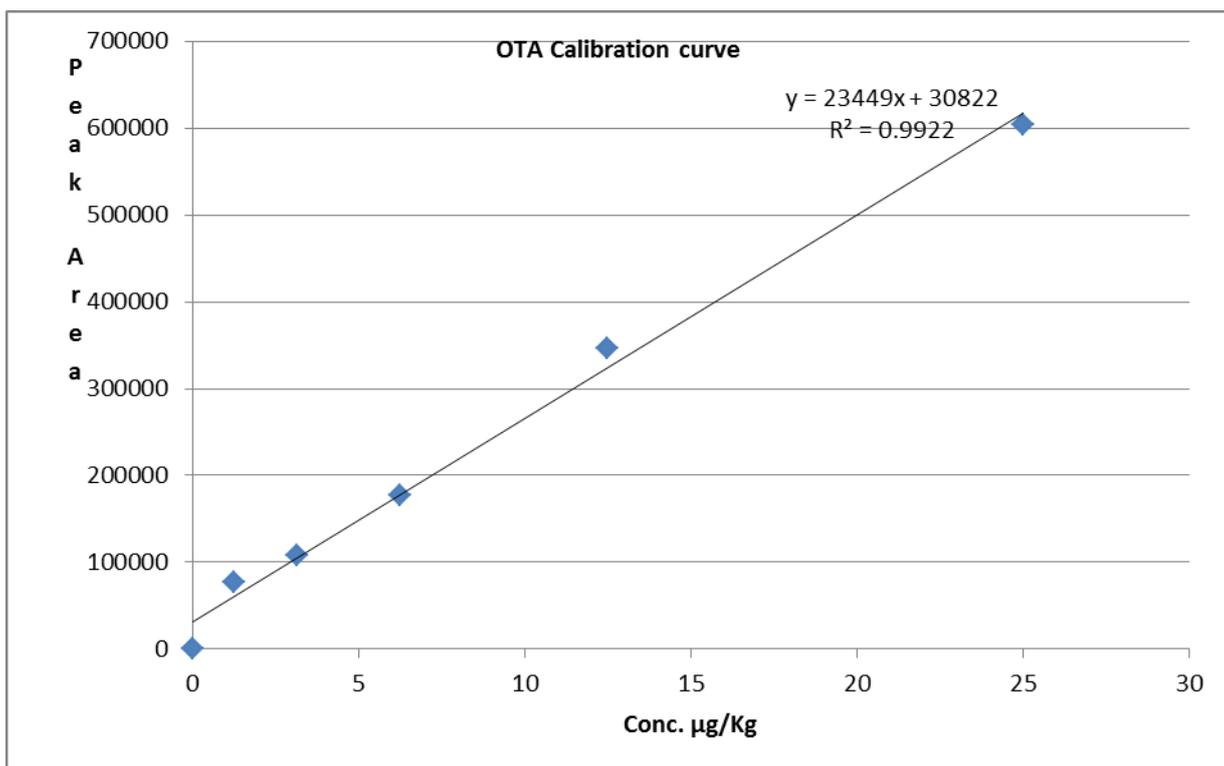
Figure 3-3: Calibration curve for HPLC analysis

From the above calibration curve, Limit of detection (LOD) is calculated at 2.05±0.036 μg/Kg. The Limit of quantification (LOQ) is thus calculated at 2.41μg/Kg of OTA.

3.3. Recovery Studies

A blend of the samples earlier analyzed was spiked with low concentrations of OTA standard solutions to assess the efficiency of the HPLC method used. The recovery level from green non-roasted coffee was on average 79.56 ± 1.31 while the recovery level for roasted coffee beans spiked before roasting was 50.62 ± 2.77 which is approximately 29% lower than in green coffee. Tables 3-4 and 3-5 shows the data on recovery studies, while Figure 3-5 shows a graph of comparison of percentage recovery between the two processes.

Table 3-4; Recovery Percentages of Spiked Raw Samples

Sample Type	Spike conc. µg/Kg	Recovered Conc. µg/Kg	% Recovery
Green 1	3.25	2.54	77.99
Green 2	3.25	2.52	77.6
Green 3	3.25	2.56	78.9
Green 4	5	4.03	80.59
Green 5	5	4.03	80.72
Green 6	5	4.0	79.98
		Average	79.558
		Standard deviation	1.309588

Table 3-5; Recovery Percentages of Spiked Roast Coffee

Sample Type	Spike conc. µg/Kg	Recovered Conc. µg/Kg	% Recovery
Roast 1	3.25	1.67	51.34
Roast 2	3.25	1.51	46.57
Roast 3	3.25	1.72	52.84
Roast 4	5	2.60	51.94
Roast 5	5	2.59	51.72
Roast 6	5	2.59	51.78
	Average		50.6175
	Standard deviation		2.77244
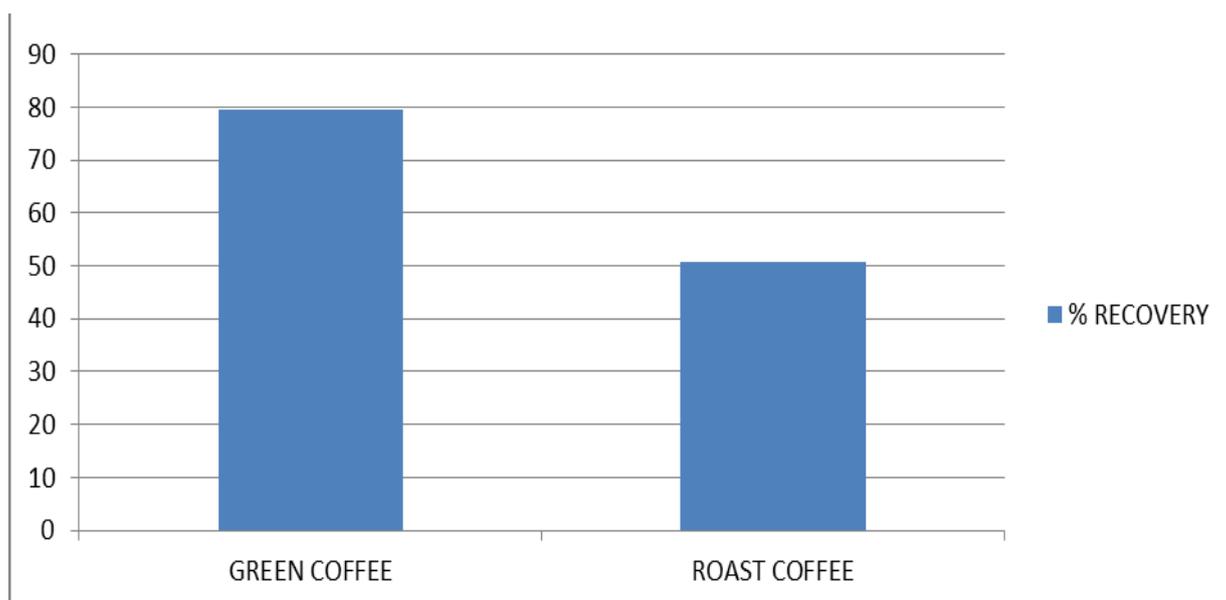
Figure 3-4: Chart comparing percent recoveries of OTA in green and roast coffee

3.3.1. Comparing Percent Recoveries of OTA in Green and Roast Coffee

Percentage recoveries of OTA in green and roast coffee were compared by use of both parametric and non-parametric methods in form of t-test and U-test respectively. The parametric method gave a t value of 39.25 which is way above the tabulated value of 3.36 at ninety percent confidence limit as shown in Table 3-6. The non-parametric test accepts a higher calculated value, however the calculated value of zero is below the tabulated value of 5 as shown in Table 3-7. Both tests indicate strongly there is significant difference in levels of OTA recoveries in coffee beans that have undergone the varied processing procedures.

Table 3-6; T-test on Recovery Samples.

TRIAL	1	2	3	4	5	6	AVERAGE	STDEV
Conc.	3.25	3.25	3.25	3.25	3.25	3.25		
Green beans % Recovery(x1)	77.99	77.6	78.9	80.59	80.72	79.98		
Roast beans % recovery (x2)	51.34	46.57	52.84	51.94	51.72	51.78		
x1-x2	26.65	31.02	26.06	28.65	29	28.2	28.26	1.775

t cal=39.25

t tab=3.36

t cal=39.25 > t tab meaning that there is significant difference in OTA between roasted and green coffee.

Table 3-7; U-test rankings

Category	R1	R1	R1	R1	R1	R1	R2	R2	R2	R2	R2	R2
%Recovery	**77.99**	**77.6**	**78.9**	80.59	**80.72**	79.98	51.34	46.57	52.84	51.94	51.72	51.78
Rank	8	7	9	11	12	10	2	1	6	5	3	4

U1= 57 – (6*7)0.5 =36

U2 = 21 – (6*7) 0.5 = 0

U CAL =0 (Smaller value)

U tab = 5

U CAL <U tab meaning that there is significant difference in OTA between roasted coffee and green coffee.

3.4 Levels of OTA.

3.4.1 Levels of OTA in coffee beans

Green and roasted coffee samples were subjected to OTA analysis by aid of HPLC-FD instrument after a clean-up process to remove interfering matrix. The samples were from Yadini, Oakland, Mahuti, Barikongo, Gitare, Gathumbuini, Handege,

Mitaro and Machure estates and Komothai, Gathage, Gatitu, Thiririka, New Gatukuyu, Mirimaini, Ndumberi, Kiambi and Ichaweri farmers' Co-operative Societies.

The analysis of grades AA and AB from all the samples gave negative OTA presence below detection limits in reference to the calibration curve.

3.4.2. Control sample analysis

Analysis of OTA on coffee beans that had dried in the farm and therefore had not undergone similar processing procedure was carried out on a sample from Gachororo in Juja Constituency, the warmest region in Kiambu County as control sample.

Analysis of the unroasted control sample indicated a signal within the OTA retention time, with a peak area whose average was 69487 ± 82 indicating an experimental concentration of 1.5±0.006 µg/Kg and a theoretical concentration of 2.027 ± 0.0075 based on recovery studies adjustment factor. The recorded value is way below the limit of quantification. Table 3-8 shows the results of analysis of green *mbuni* samples Roasting of the control samples resulted in loss of signal detection with resulting peak areas being below the calibration curve y intercept.

Table 3-8; Green *Mbuni* control sample analysis, Gachororo, Juja.

	Experimental conc. µg/Kg	Theoretical conc. µg/Kg
AV	1.547 ± 0.006	2.027 ± 0.008

Average observed concentration of 1.5 ± 0.006 µg/Kg and a theoretical concentration of 2.027 ± 0.008 µg/Kg meant that OTA was detectable but below LOQ.

3.4.3 Roast *mbuni* Control Sample Analysis-Gachororo, Juja.

Roast *mbuni* analysis gave an average peak area of 28752 which was below 30822 the value where the calibration curve cuts the y-axis. The above effectively means that the results were below limit of detection and quantification. The observation also gives credit to the argument that physical means can be applied to eradicate or reduce mycotoxins to low levels.

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

The focus of this study was to compare the levels of OTA in coffee samples collected from the coffee growing regions of Kiambu County, Kenya. The study also aimed at observing the effects of processing to the levels of OTA. The efficiency of analytical methods used was also studied by comparing detection limits and carrying out recovery studies. The mean concentration of OTA detected by TLC was found to be a minimum of 150 µg/Kg. The analysis showed that it would be difficult to rely on TLC to detect and quantify the low concentrations that are critical in OTA determination. Masking by the intensely colored ingredients of coffee as can be seen from chromatograms also makes it difficult to identify low concentrations of OTA without carrying out a clean-up process. The analysis of sample concentration of OTA was found to be below detection and therefore quantification limits for all the twenty samples of grade AA and AB standard coffee from the study region. The Mbuni control sample concentration of OTA though qualitatively detected was found to be below the quantifiable detection limit. Recovery studies indicated a recovery level of 79.56% ± 1.31% for green coffee beans and 50.62% ± 2.77% for roasted coffee. The observations can be summarized as follows:

Normal processed coffee of grade AA and AB does not contain appreciable levels of OTA.

Mbuni (Dry berry rejects) contain appreciable levels of OTA but below the recommended limit of 5ppb.Recovery studies indicate that appreciable levels of OTA

are lost during extraction and clean up processes (up to 25%) and any reasonable calculation must factor in the same

Coffee roasting causes appreciable decline in OTA levels of between 20% and 30%, as observed by the difference in recovery levels of green and roast coffee whose averages were 79.56% and 50.56% respectively.

Coffee from Kiambu County is within acceptable levels of OTA of all important importing countries that have set their own standards. The above observations indicate that current growth, storage and processing conditions are suitable for premium coffee. The above observation also tends to confirm that the organoleptic quality levels of Kenyan coffee by extension go with quantifiable parameters particularly in terms of mycotoxins. The professional handling of coffee in most FCS and estate factories is paying dividends and should be encouraged and enhanced. The qualitative detection of OTA in the *Mbuni* control sample indicates that though OTA may be absent from sampled coffee it is none the less a real threat that should be avoided. The fact that OTA is detected in a sample of coffee that has not undergone normal professional storage and processing conditions is an indicator that OTA is most likely to be synthesized during storage. The observation that there is appreciable reduction in OTA through roasting indicates that it is advisable for the country to look into ways of exporting processed coffee because this is not only likely to fetch more income for the country but can safe guard the loss of market in case of presence of marginal levels of OTA.

4.2 Recommendations

4.2.1 Recommendations Arising From This Study

The facts from current observations lead to the recommendation that current cherry processing procedures should be upheld and sustained. Factories whether owned by co- operative societies or private estates should be encouraged to document processing procedures during de-husking of berries and drying of coffee beans so that any deviation from the norm can be noted and arrested. Modern management procedures such as ISO procedures should be encouraged by governing bodies as a way of encouraging quality assurance to promote market confidence. Coffee farmers should be sensitized on storage of non-premium coffee such as the berries affected by coffee berries disease which is ultimately sold as *Mbuni* so that it does not achieve moisture levels that are conducive to growth of fungus that cause formation of mycotoxins. Coffee beans that have undergone different post-harvest procedures should be marketed separately so that short falls on one procedure do not lead to devaluation of entire coffee produce.

4.2.2 Recommendations for Further Study

This study used Kiambu County as source of base line data in the study of OTA levels in Kenyan coffee. Though Kiambu is quite representative of most coffee growing regions in the country due to its varied climatic conditions, it none the less cannot conclusively give a good account of OTA levels in Kenya and similar studies in other major producer locations such as Nyeri and Murang'a counties need to be carried out.

The storage conditions of non-premium coffee such as 'mbuni' that does not undergo normal processing procedure needs to be thoroughly studied and improvements recommended because this is where appreciable levels of OTA may be found and could be the beginning of the failure in terms of quality of the Kenyan coffee.

The root cause of OTA is fungal biosynthesis. The understanding of the fungus present in the county and the country that can cause formation of OTA and other mycotoxins and the identification of methods of containing their spread would be a valuable course by biologists.

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APPENDICES

APPENDIX A.

a.   OTA Standard Overlays Using HPLC-FD Method.
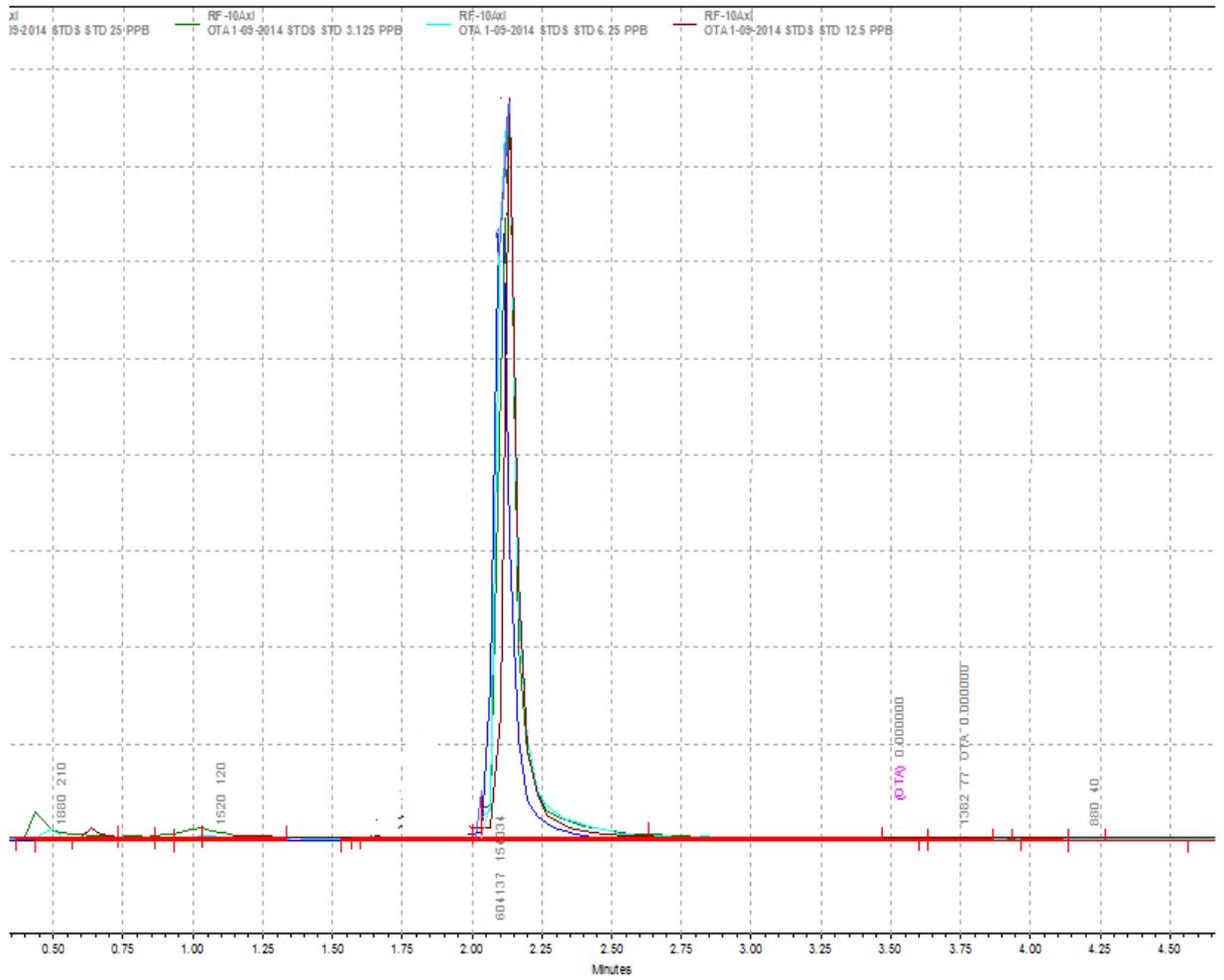
b. HPLC Chromatograms of Green and Roast Coffee
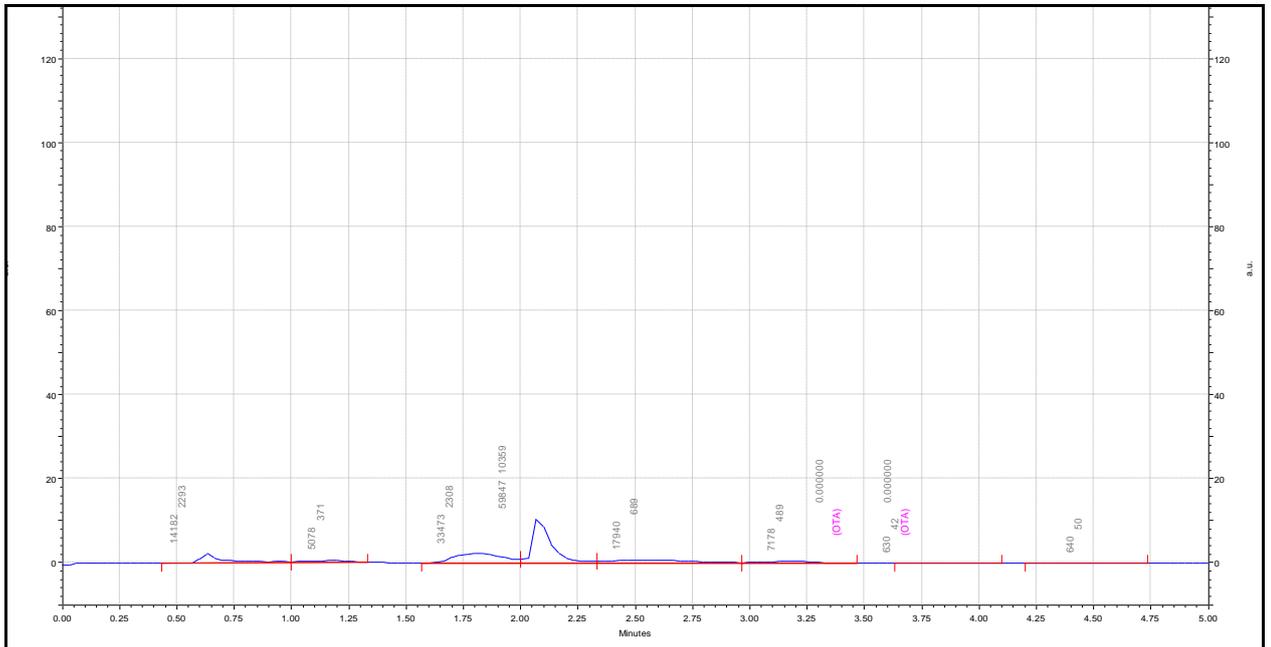
Figure Ab-1: Green coffee from Oakland estate sample chromatogram.
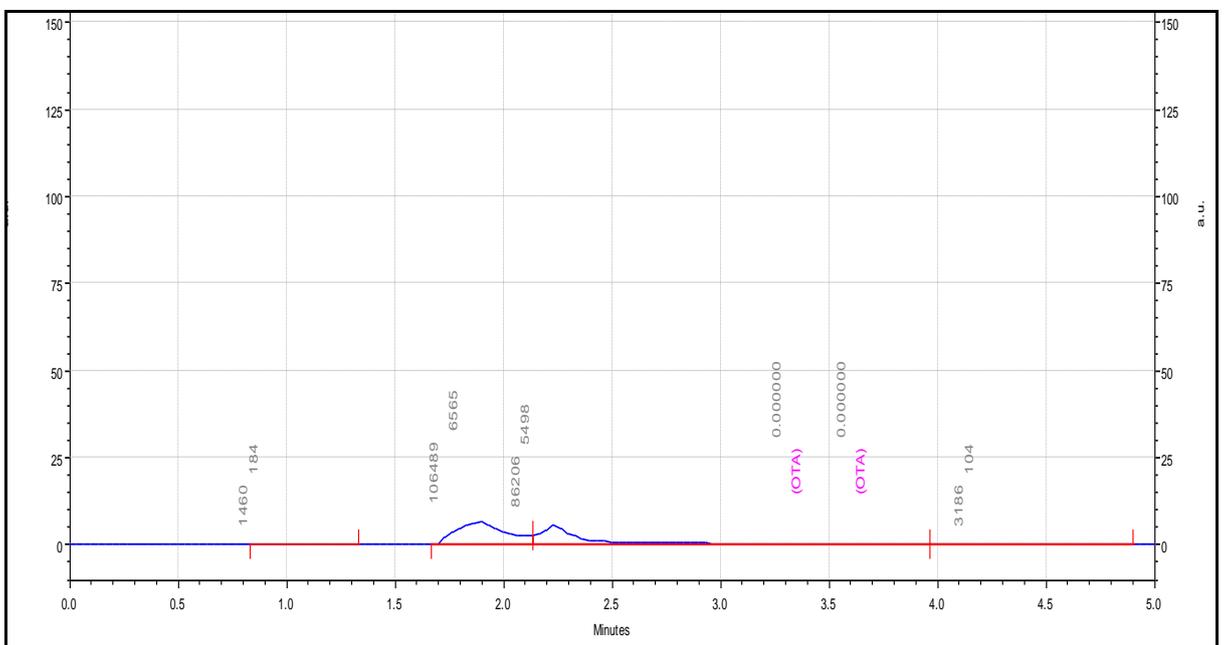
Figure Ab-2: Roast coffee from Oakland estate sample chromatogram.
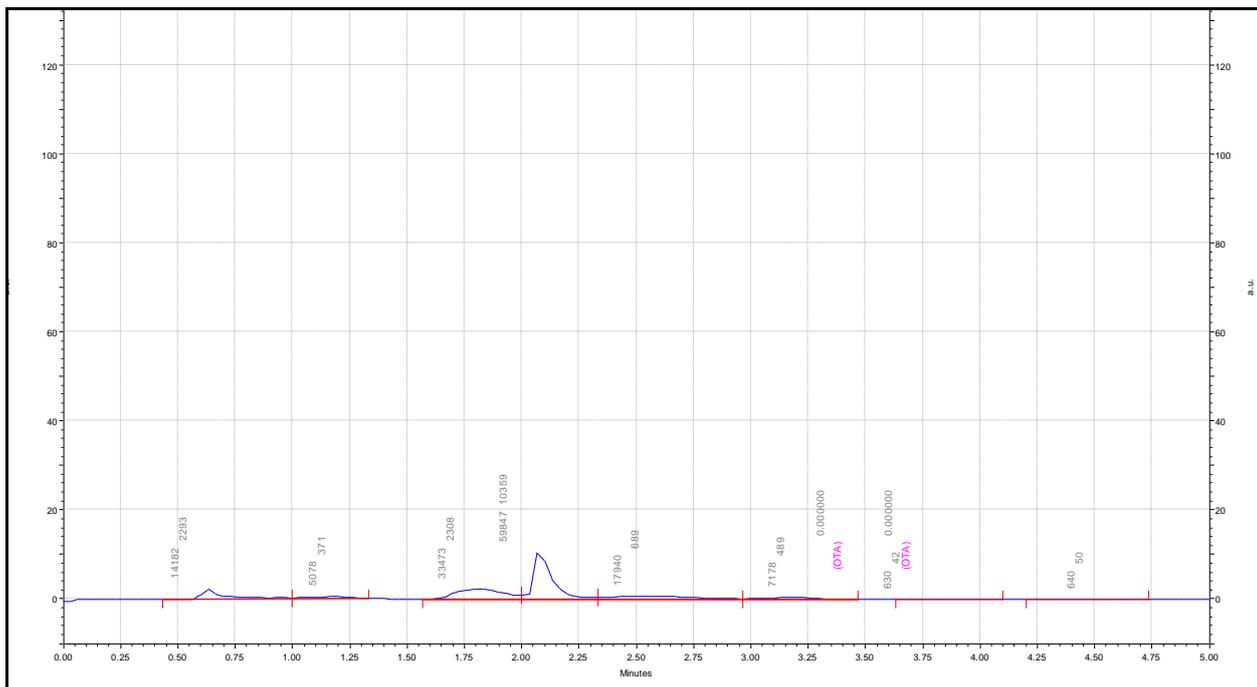
Figure Ab-3: Green coffee from Yadini estate sample chromatogram.
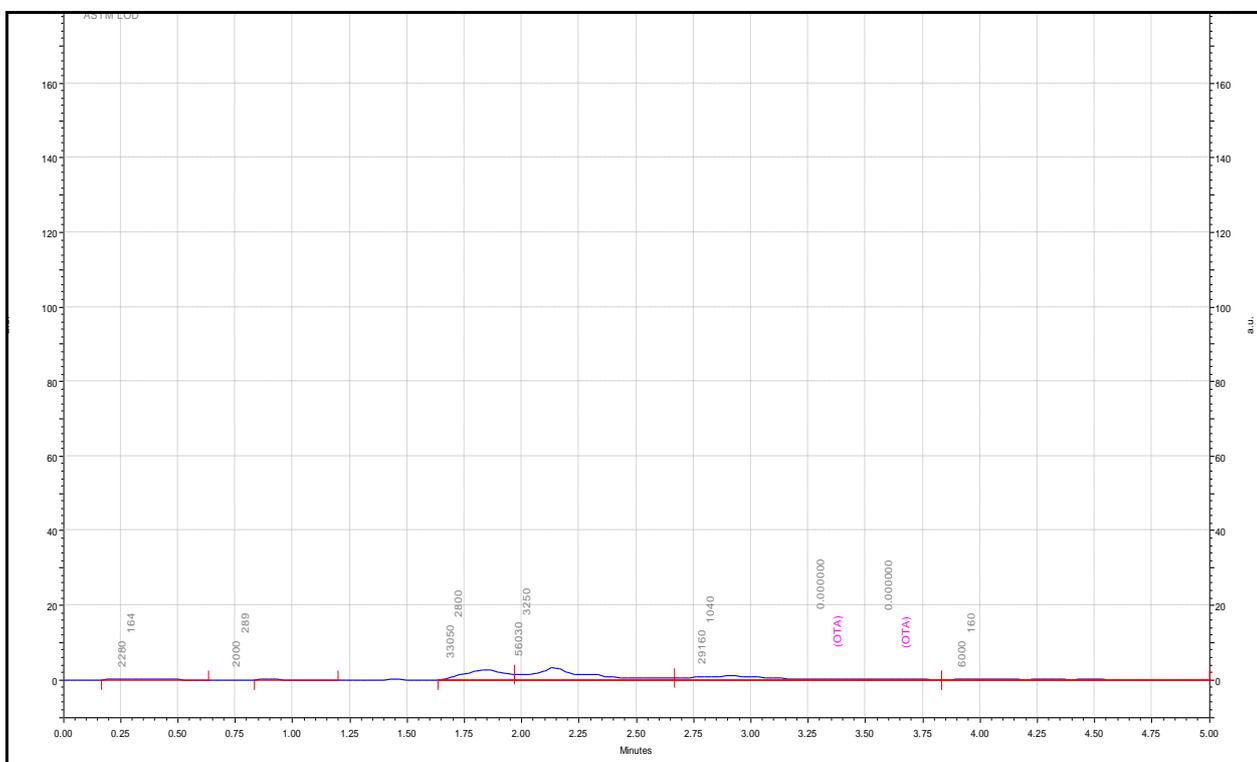
Figure Ab-4: Roast coffee from Yadini estate sample chromatogram.
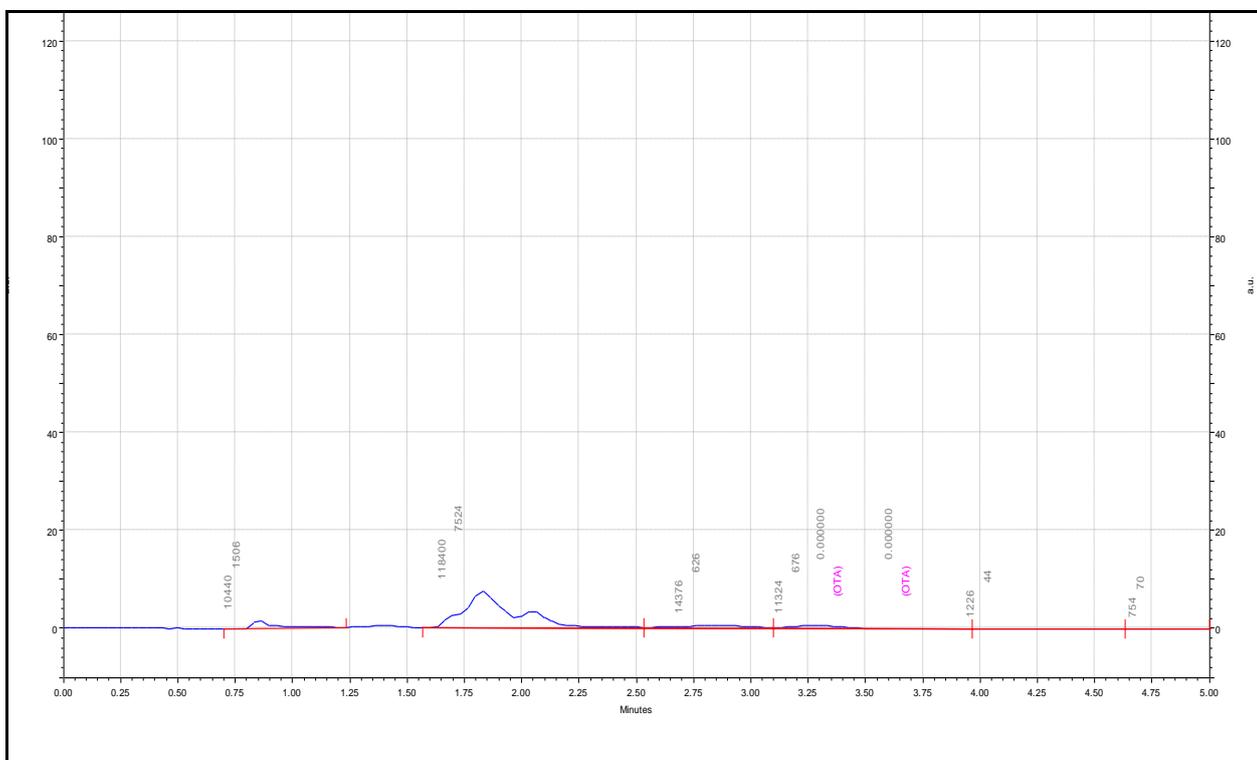
Figure Ab-5: Green coffee from Thiririka FCS sample chromatogram.
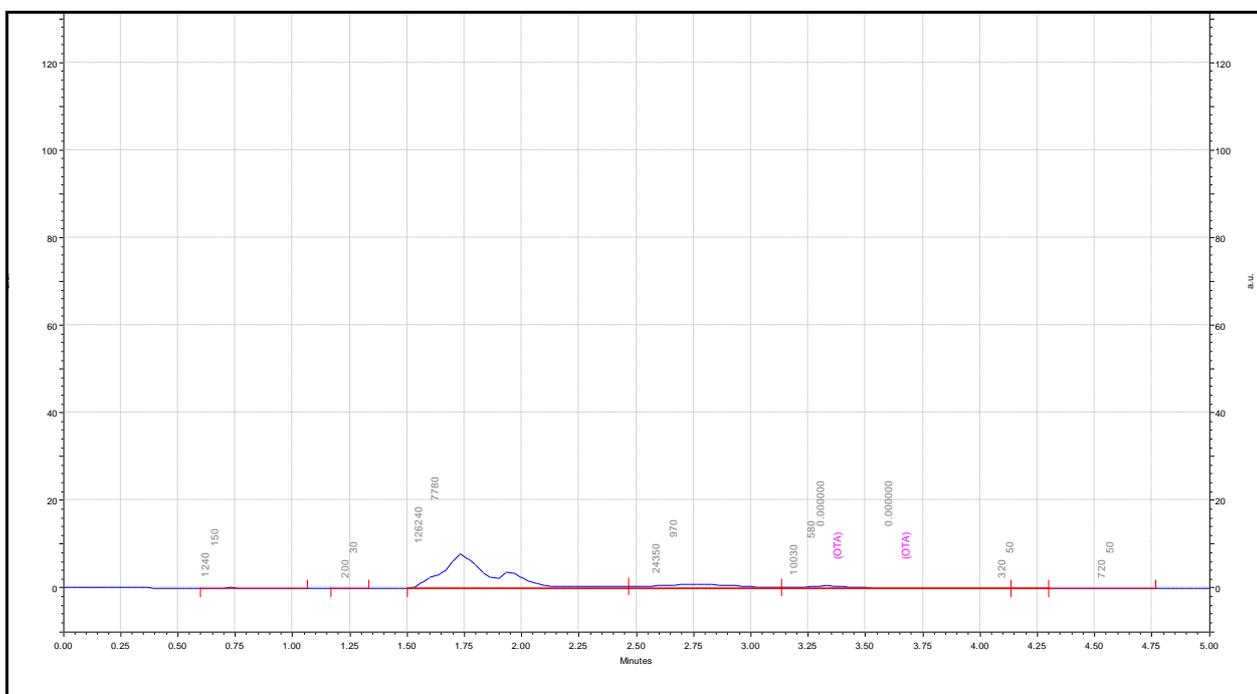
Figure Ab-6: Roast coffee from Thiririka FCS sample chromatogram.

APPENDIX B.

1.   **TLC Chromatograms of Green and Roast Coffee**
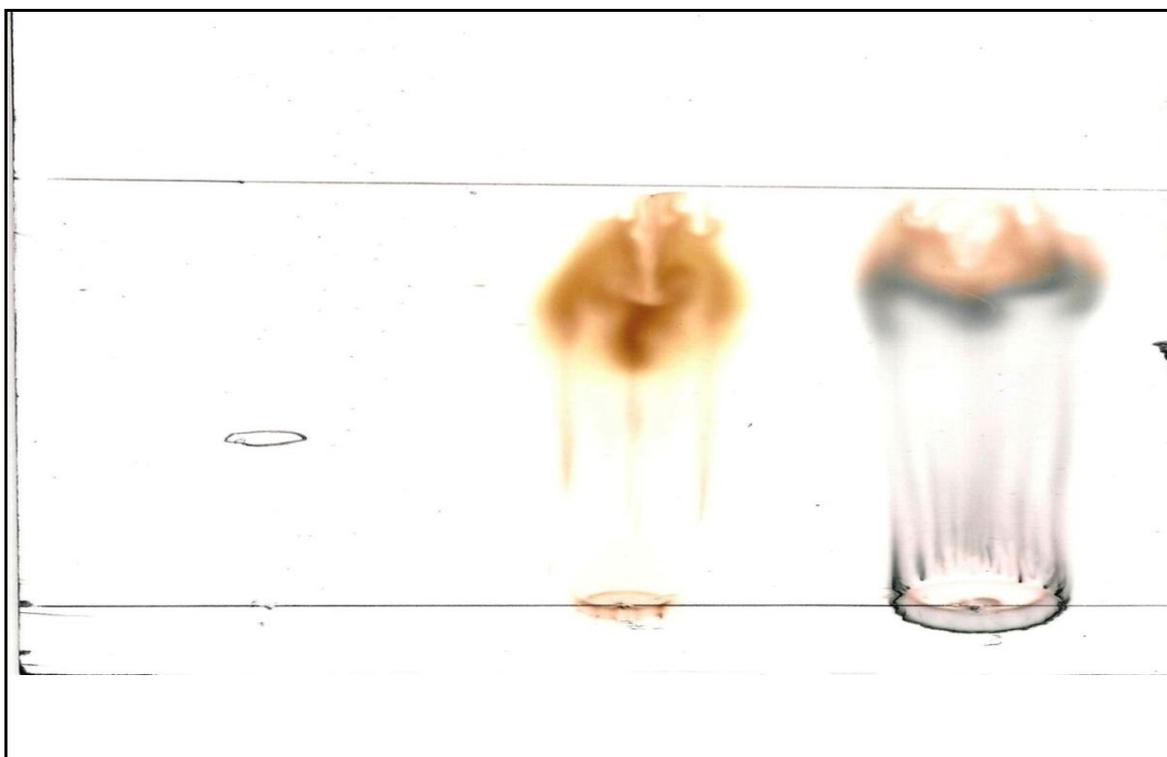
Figure B-1: TLC chromatograms for green and roast coffee from Kamuchege FCS
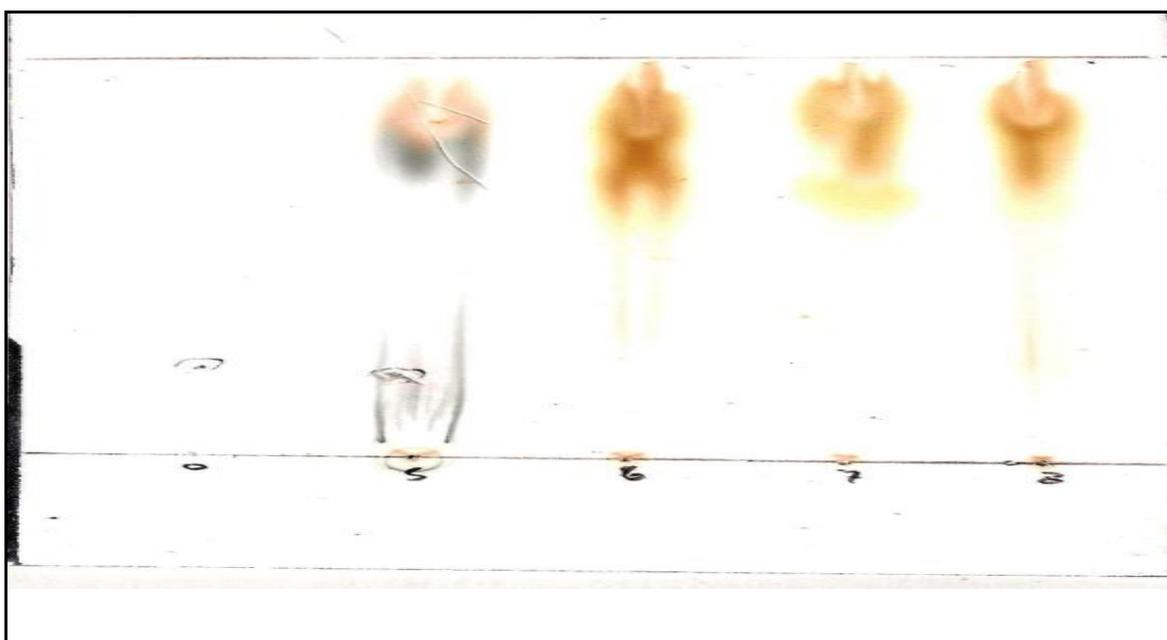
Figure B-2: TLC chromatograms for green and roast coffee from Handege estate.

Figure B-2: TLC chromatograms for green and roast coffee from Ichaweri FCS.

APPENDIX C.

1.   U-test table

Alpha = .005 (two-tailed)

n1\n2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2																			
3											0	0	0	1	1	1	1	1	2
4						0	0	1	1	2	2	3	3	4	4	5	5	5
5				0	0	1	2	3	3	4	5	6	6	7	8	9	9	10
6				0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
7				0	2	3	4	5	7	8	9	11	12	13	15	16	18	19	20
8			0	1	3	4	6	7	9	11	12	14	16	17	19	21	22	24	26
9			0	2	4	5	7	9	11	13	15	17	19	21	23	25	27	29	31
10			1	3	5	7	9	11	13	16	18	20	23	25	27	30	32	35	37
11			1	3	6	8	11	13	16	18	21	24	26	29	32	35	37	40	43
12		0	2	4	7	9	12	15	18	21	24	27	30	33	36	39	43	46	49
13		0	2	5	8	11	14	17	20	24	27	30	34	37	41	44	48	51	55
14		0	3	6	9	12	16	19	23	26	30	34	38	41	45	49	53	57	61
15		1	3	6	10	13	17	21	25	29	33	37	41	46	50	54	58	62	67
16		1	4	7	11	15	19	23	27	32	36	41	45	50	54	59	64	68	73
17		1	4	8	12	16	21	25	30	35	39	44	49	54	59	64	69	74	79
18		1	5	9	13	18	22	27	32	37	43	48	53	58	64	69	74	80	85
19		1	5	9	14	19	24	29	35	40	46	51	57	62	68	74	80	85	91
20		2	5	10	15	20	26	31	37	43	49	55	61	67	73	79	85	91	97

Alpha = .01 (two-tailed)

n1\n2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2																		0	0
3								0	0	0	1	1	1	2	2	2	2	3	3
4				0	0	1	1	2	2	3	3	4	5	5	6	6	7	8	8
5				0	1	1	2	3	4	5	6	7	7	8	9	10	11	12	13
6			0	1	2	3	4	5	6	7	9	10	11	12	13	15	16	17	18
7			0	1	3	4	6	7	9	10	12	13	15	16	18	19	21	22	24
8			1	2	4	6	7	9	11	13	15	17	18	20	22	24	26	28	30
9		0	1	3	5	7	9	11	13	16	18	20	22	24	27	29	31	33	36
10		0	2	4	6	9	11	13	16	18	21	24	26	29	31	34	37	39	42
11		0	2	5	7	10	13	16	18	21	24	27	30	33	36	39	42	45	46
12		1	3	6	9	12	15	18	21	24	27	31	34	37	41	44	47	51	54
13		1	3	7	10	13	17	20	24	27	31	34	38	42	45	49	53	56	60
14		1	4	7	11	15	18	22	26	30	34	38	42	46	50	54	58	63	67
15		2	5	8	12	16	20	24	29	33	37	42	46	51	55	60	64	69	73
16		2	5	9	13	18	22	27	31	36	41	45	50	55	60	65	70	74	79
17		2	6	10	15	19	24	29	34	39	44	49	54	60	65	70	75	81	86
18		2	6	11	16	21	26	31	37	42	47	53	58	64	70	75	81	87	92
19	0	3	7	12	17	22	28	33	39	45	51	56	63	69	74	81	87	93	99
20	0	3	8	13	18	24	30	36	42	46	54	60	67	73	79	86	92	99	105

Alpha = .02 (two-tailed)

n1\n2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2																			
3		--	0	0	0	0	0	1	1	1	2	2	2	3	3	4	4	4	5
4		--	--	0	1	1	2	3	3	4	5	5	6	7	7	8	9	9	10
5		--	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6		--	1	2	3	4	6	7	8	9	11	12	13	15	16	18	19	20	22
7		0	1	3	4	6	7	9	11	12	14	16	17	19	21	23	24	26	28
8		0	2	4	6	7	9	11	13	15	17	20	22	24	26	28	30	32	34
9		1	3	5	7	9	11	14	16	18	21	23	26	28	31	33	36	38	40
10		1	3	6	8	11	13	16	19	22	24	27	30	33	36	38	41	44	47
11		1	4	7	9	12	15	18	22	25	28	31	34	37	41	44	47	50	53
12		2	5	8	11	14	17	21	24	28	31	35	38	42	46	49	53	56	60
13		2	5	9	12	16	20	23	27	31	35	39	43	47	51	55	59	63	67
14		2	6	10	13	17	22	26	30	34	38	43	47	51	56	60	65	69	73
15		3	7	11	15	19	24	28	33	37	42	47	51	56	61	66	70	75	80
16		3	7	12	16	21	26	31	36	41	46	51	56	61	66	71	76	82	87
17		4	8	13	18	23	28	33	38	44	49	55	60	66	71	77	82	88	93
18		4	9	14	19	24	30	36	41	47	53	59	65	70	76	82	88	94	100
19		4	9	15	20	26	32	38	44	50	56	63	69	75	82	88	94	101	107
20		5	10	16	22	28	34	40	47	53	60	67	73	80	87	93	100	107	114

n1\n2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2							0	0	0	0	1	1	1	1	1	2	2	2	2
3				0	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8
4			0	1	2	3	4	4	5	6	7	8	9	10	11	11	12	13	14
5		0	1	2	3	5	6	7	8	9	11	12	13	14	15	17	18	19	20
6		1	2	3	5	6	7	10	11	13	14	16	17	19	21	22	24	25	27
7		1	3	5	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34
8	0	2	4	6	7	10	13	15	17	19	22	24	26	29	31	34	36	38	41
9	0	2	4	7	10	12	15	17	20	23	26	28	31	34	37	39	42	45	48
10	0	3	5	8	11	14	17	20	23	26	29	33	36	39	42	45	48	52	55
11	0	3	6	9	13	16	19	23	26	30	33	37	40	44	47	51	55	58	62
12	1	4	7	11	14	18	22	26	29	33	37	41	45	49	53	57	61	65	69
13	1	4	8	12	16	20	24	28	33	37	41	45	50	54	59	63	67	72	76
14	1	5	9	13	17	22	26	31	36	40	45	50	55	59	64	67	74	78	83
15	1	5	10	14	19	24	29	34	39	44	49	54	59	64	70	75	80	85	90
16	1	6	11	15	21	26	31	37	42	47	53	59	64	70	75	81	86	92	98
17	2	6	11	17	22	28	34	39	45	51	57	63	67	75	81	87	93	99	105
18	2	7	12	18	24	30	36	42	48	55	61	67	74	80	86	93	99	106	112
19	2	7	13	19	25	32	38	45	52	58	65	72	78	85	92	99	106	113	119
20	2	8	13	20	27	34	41	48	55	62	69	76	83	90	98	105	112	119	127

Alpha = .05 (two-tailed)

Alpha = .10 (two-tailed)

n1\n2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2																			
3		0	0	1	2	2	3	4	4	5	5	6	7	7	8	9	9	10	11
4		0	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18
5		1	2	4	5	6	8	9	11	12	13	15	16	18	19	20	22	23	25
6		2	3	5	7	8	10	12	14	16	17	19	21	23	25	26	28	30	32
7		2	4	6	8	11	13	15	17	19	21	24	26	28	30	33	35	37	39
8		3	5	8	10	13	15	18	20	23	26	28	31	33	36	39	41	44	47
9		4	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54
10		4	7	11	14	17	20	24	27	31	34	37	41	44	48	51	55	58	62
11		5	8	12	16	19	23	27	31	34	38	42	46	50	54	57	61	65	69
12		5	9	13	17	21	26	30	34	38	42	47	51	55	60	64	68	72	77
13		6	10	15	19	24	28	33	37	42	47	51	56	61	65	70	75	80	84
14		7	11	16	21	26	31	36	41	46	51	56	61	66	71	77	82	87	92
15		7	12	18	23	28	33	39	44	50	55	61	66	72	77	83	88	94	100
16		8	14	19	25	30	36	42	48	54	60	65	71	77	83	89	95	101	107
17		9	15	20	26	33	39	45	51	57	64	70	77	83	89	96	102	109	115
18		9	16	22	28	35	41	48	55	61	68	75	82	88	95	102	109	116	123
19		10	17	23	30	37	44	51	58	65	72	80	87	94	101	109	116	123	130
20		11	18	25	32	39	47	54	62	69	77	84	92	100	107	115	123	130	138

2.   T-Test TABLE

Table with right tail probabilities

$t_{(p,df)}$
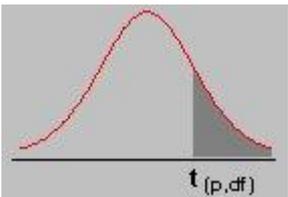
df\p	0.40	0.25	0.10	0.05	0.025	0.01	0.005	0.0005
1	0.324920	1.000000	3.077684	6.313752	12.70620	31.82052	63.65674	636.6192
2	0.288675	0.816497	1.885618	2.919986	4.30265	6.96456	9.92484	31.5991
3	0.276671	0.764892	1.637744	2.353363	3.18245	4.54070	5.84091	12.9240
4	0.270722	0.740697	1.533206	2.131847	2.77645	3.74695	4.60409	8.6103
5	0.267181	0.726687	1.475884	2.015048	2.57058	3.36493	4.03214	6.8688

6	0.264835	0.717558	1.439756	1.943180	2.44691	3.14267	3.70743	5.9588
7	0.263167	0.711142	1.414924	1.894579	2.36462	2.99795	3.49948	5.4079
8	0.261921	0.706387	1.396815	1.859548	2.30600	2.89646	3.35539	5.0413
9	0.260955	0.702722	1.383029	1.833113	2.26216	2.82144	3.24984	4.7809
10	0.260185	0.699812	1.372184	1.812461	2.22814	2.76377	3.16927	4.5869
11	0.259556	0.697445	1.363430	1.795885	2.20099	2.71808	3.10581	4.4370
12	0.259033	0.695483	1.356217	1.782288	2.17881	2.68100	3.05454	4.3178
13	0.258591	0.693829	1.350171	1.770933	2.16037	2.65031	3.01228	4.2208
14	0.258213	0.692417	1.345030	1.761310	2.14479	2.62449	2.97684	4.1405
15	0.257885	0.691197	1.340606	1.753050	2.13145	2.60248	2.94671	4.0728
16	0.257599	0.690132	1.336757	1.745884	2.11991	2.58349	2.92078	4.0150
17	0.257347	0.689195	1.333379	1.739607	2.10982	2.56693	2.89823	3.9651
18	0.257123	0.688364	1.330391	1.734064	2.10092	2.55238	2.87844	3.9216
19	0.256923	0.687621	1.327728	1.729133	2.09302	2.53948	2.86093	3.8834
20	0.256743	0.686954	1.325341	1.724718	2.08596	2.52798	2.84534	3.8495
21	0.256580	0.686352	1.323188	1.720743	2.07961	2.51765	2.83136	3.8193
22	0.256432	0.685805	1.321237	1.717144	2.07387	2.50832	2.81876	3.7921
23	0.256297	0.685306	1.319460	1.713872	2.06866	2.49987	2.80734	3.7676
24	0.256173	0.684850	1.317836	1.710882	2.06390	2.49216	2.79694	3.7454
25	0.256060	0.684430	1.316345	1.708141	2.05954	2.48511	2.78744	3.7251

APPENDIX D.

1. Sample mean

$$A = \frac{1}{n} * \sum_{i=1}^{n} x_i$$

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….D-1

A=average (or arithmetic mean),

n = the number of terms (e.g., the number of items or numbers being averaged)

x_1 = the value of each individual item in the list of numbers being averaged

2. Sample variance and standard deviation

Formula for variance

$$s^2 = \frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2.$$

………………………………………………………………D-2

This estimator is unbiased if the variance exists and the sample values are drawn independently with replacement. $N-1$ corresponds to the number of degrees of freedom in the vector of residuals, $(x_1 - \bar{x}, ..., x_n - \bar{x})$.

Taking square roots reintroduces bias, and yields the corrected sample standard deviation, denoted by *s:*

$$s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2}.$$

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While s^2 is an unbiased estimator for the population variance, *s* is a biased estimator for the population standard deviation, though markedly less biased than the

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uncorrected sample standard deviation. The bias is still significant for small samples (*n* less than 10), and also drops off as $1/n$ as sample size increases. This estimator is commonly used, and generally known simply as the "sample standard deviation".

3.   Linear correlation coefficient

The quantity *r*, called the *linear correlation coefficient*, measures the strength and the direction of a linear relationship between two variables. The linear correlation coefficient is sometimes referred to as the *Pearson product moment correlation coefficient* in honor of its developer Karl Pearson.

- The mathematical formula for computing *r* is:

$$r = \frac{n\sum xy - \left(\sum x\right)\left(\sum y\right)}{\sqrt{n\left(\sum x^2\right) - \left(\sum x\right)^2}\sqrt{n\left(\sum y^2\right) - \left(\sum y\right)^2}}$$
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………………………………………………….D-4

where *n* is the number of pairs of data.

- The value of *r* is such that $-1 \leq r \leq +1$. The + and − signs are used for positive linear correlations and negative linear correlations, respectively.
- Positive correlation: If *x* and *y* have a strong positive linear correlation, *r* is close to +1. An *r* value of exactly +1 indicates a perfect positive fit. Positive values indicate a relationship between *x* and *y* variables such that as values for *x* increases,

values for *y* also increase.

- Negative correlation: If x and y have a strong negative linear correlation, r is close to -1. An r value of exactly -1 indicates a perfect negative fit.   Negative values indicate a relationship between x and y such that as values for x increase, values for y decrease.

- No correlation: If there is no linear correlation or a weak linear correlation, r is close to 0. A value near zero means that there is a random, nonlinear relationship between the two variables.

- Note that r is a dimensionless quantity; that is, it does not depend on the units employed.

- A perfect correlation of ± 1 occurs only when the data points all lie exactly on a straight line. If $r = +1$, the slope of this line is positive. If $r = -1$, the slope of this line is negative.

- A correlation greater than 0.8 is generally described as *strong*, whereas a correlation less than 0.5 is generally described as *weak*. These values can vary based upon the "type" of data being examined. A study utilizing scientific data may require a stronger correlation than a study using social science data.

- The *coefficient of determination, r^2*, is useful because it gives the proportion of the variance (fluctuation) of one variable that is predictable from the other variable. It is a measure that allows us to determine how certain one can be in making predictions from a certain model/graph.

- The *coefficient of determination* is the ratio of the explained variation to the total variation.

- The *coefficient of determination* is such that $0 \leq r^2 \leq 1$, and denotes the strength of the linear association between x and y.

✦   The *coefficient of determination* represents the percent of the data that is the closest to the line of best fit.

The *coefficient of determination* is a measure of how well the regression line represents the data. If the regression line passes exactly through every point on the scatter plot, it would be able to explain all of the variation. The further the line is away from the points, the less it is able to explain.

4.   Hypothesis test

T-test involves calculating a t-value from averages of OTA recovered from coffee beans that have undergone the varied processing procedures and comparing the calculated value with tabulated values. If the tabulated value is less than calculated, then there is no significant difference between the processes as far as OTA is concerned, otherwise there is significant difference.

Formula for t-test

$$t = \frac{\bar{x} - \Delta}{\frac{s}{\sqrt{n}}}$$ ………………………………………………………………………………………

…D-5

Where $\bar{x}$ is the mean of the change scores, Δ is the hypothesized difference (0 if testing for equal means), s is the sample standard deviation of the differences, and n is the sample size. The number of degrees of freedom for the problem is $n - 1$.

Wilcoxon-Mann-Whitney U Test is the non-parametric equivalent of the two sample t-test. The test is sometimes known as the Wilcoxon 2-sample test, the Mann-Whitney test or just the U-test. For small samples a direct method is recommended. It is very quick, and gives an insight into the meaning of the statistic.

U is then given by the following formula.

$$U_1 = R_1 - \frac{n_1(n_1 + 1)}{2}$$ ....

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where n_1 is the sample size for sample 1, and R_1 is the sum of the ranks in sample 1. Note that there is no specification as to which sample is considered sample 1. An equally valid formula for U is

$$U_2 = R_2 - \frac{n_2(n_2 + 1)}{2}.$$ ....

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The smaller value of U_1 and U_2 is the one used when consulting significance tables.

5. Beer-Lambert law

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as:

A = a(λ) * b * c
....D-8

where A is the measured absorbance, a(λ) is a wavelength-dependent absorptivity coefficient, **b** is the path length, and **c** is the analyte concentration. When working in concentration units of molarity, the *Beer-Lambert law* is written as:

A = ε * b * c
....D-9

Where ε is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}. Data are frequently reported in percent transmission ($I/I_0 * 100$) or in absorbance [$A = \log(I/I_0)$]. The latter is particularly convenient.