

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

Peter Karuri

Chemistry department, Jomo Kenyatta University of Agriculture and Technology PO box 62000 Nairobi, Kenya

Abstract

Coffee is one of the major foreign exchange earners in Kenya. The quality of the coffee beans determines the sustainability of the market for the same. 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 compounds encountered in the food and beverage industry. There are two main classes of mycotoxins found in stored crop produce, namely aflatoxins common in grains, and Ochratoxins being the most predominant, common in fruits. Of the Ochratoxins, Ochratoxin-A is most predominant and an emerging problem in coffee production over the world. In the present study, the presence of OTA in raw and processed coffee samples from Kiambu County, Kenya was investigated. The objective of this study was to assess the levels of OTA in Kenyan coffee. Sample clean-up was carried out using OTA immuno-affinity columns. 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 was determined using high performance liquid chromatography coupled with fluorescence detector after sample clean by use of immuno affinity columns. Analysis of Ochratoxin-A was on green and roasted beans to help in comparative studies. Whether, roasting causes significant reduction or increment of OTA was tested using t-test and Mann Whitney u-test. The result of this study provides baseline data as to the levels of OTA contamination in the Kenyan coffee industry and suggests possible control measures. HPLC analysis indicated non- appreciable levels of OTA in premium coffee types AA and AB while acceptable levels were detected in *Mbuni*. The calibration curve of OTA standards by HPLC-fluorescence procedure gave an LOD value of 2.05 µg/Kg and LOQ value of 2.441 µg/Kg. Test of the efficiency of 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 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.

1. Introduction

1.1 BACKGROUND TO THE STUDY

1.1.1 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 notwithstanding its history, coffee is one of the major contributors to world economy. (Vian, 1998).

Coffee is a beverage maker obtained from beans of plants referred to as coffee plant. The beverage made from the same is referred to as coffee. 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, other quality parameters have become important.

1.2.1 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 co-operative system. *Coffea arabica* variety is the most widely grown as opposed to *Coffea robusta* grown in neighboring countries.

Kenyan coffee is usually medium bodied (beans) and is considered to have a winy 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.

1.2.2 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 (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.

1.2.3 Aroma

This contributes to the organoleptic quality of a cup of coffee. Modern analysis is replacing the traditional tasting procedures by identifying the active smelling compounds in coffee, and quantitatively 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).

1.2.4 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 various national standards in Europe. The standards indicate maximum acceptable levels to certain carbohydrates, and have helped in reducing import of adulterated products to the European Union.

Table1; Maximum acceptable levels of carbohydrates as per the EU specifications. (Annon, 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

1.2.5 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 bio-action of fungus 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).

1.2.6 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 (Waller *et al*, 1987). 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 (Waller *et al*, 1987). Among the disease, CBD is most predominant (Waller *et al*, 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 *et al*, 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).

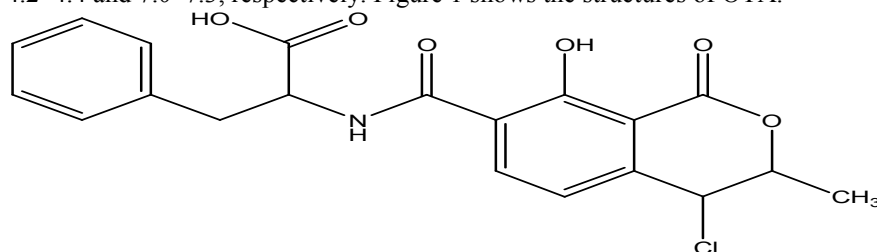
1.3.1 Mycotoxins

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, fumonisins, ochratoxin-A, putulin, tricothecines, and zearalenone (Bennet, 1999).

1.3.1.1 Ochratoxin - A

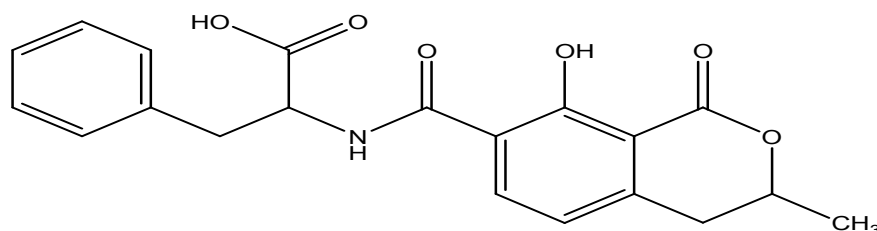
Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the aspergillus or penicillium families and are weak organic acids consisting of a derivative of an isocoumarin. Three types of Ochratoxins have been isolated i.e. Ochratoxin-A, B and C with closely related molecular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

Figure 1 shows the structures of common Ochratoxins found in coffee beans. 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: λ_{MeOHmax} (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 shows the structures of OTA.



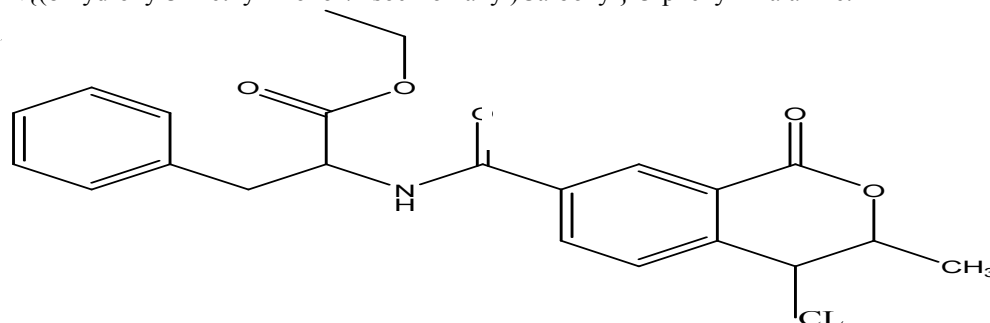
Ochratoxin-A

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

Figure1: The structures of Ochratoxins (Source: Vian, 1997)

1.3.1.2 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 and Duncan, 1989). Ochratoxin-A has a long serum half-life in humans. On the basis of its strong binding to serum micro molecules.

The sub-chronic and chronic effects of Ochratoxin-A are of greatest concern. Ochratoxin-A has been shown to be nephrotoxic, hepatotoxic, teratogenic and 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.

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).

1.3.2 Ochratoxin-A limits

In the 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. 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 and 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 5 µg/kg is set for raw wheat, barley and rye, only. These limits are adopted in GCC, 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).

1.3.3.1 Effects of Ochratoxin-A and geographical distribution.

Two fungal species are associated with OTA production i.e. *Penicillium verrucosum* and *Aspergillus ochraceus* (International Programme of Chemical Safety, 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 on temperatures below 30 degrees Celsius and low water activity. It may produce Ochratoxin at temperatures as low as 5 degrees Celsius.

A. ochraceus on the contrary 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 vine 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 *Cladosporium 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).

1.3.3.2 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). 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 analysis 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 analysis method in order to eliminate the interferences. The accuracy of a certain analysis method critically depends on the type of cleanup applied. Immuno-affinity cleanup is an efficient sample purification method, particularly in the case of HPLC-fluorescence/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 HPLC-FD and gas chromatography. 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 be 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.*, 1997). 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 Instrumentation

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

This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't 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 won't 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 form attractions with the hydrocarbon groups because of 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 now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

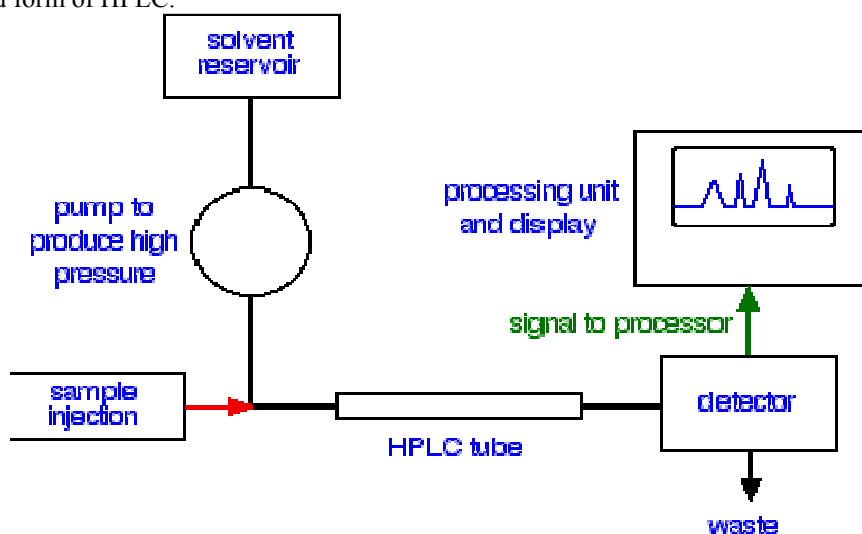


Figure 2. A flow scheme for HPLC

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 the temperature of the column

That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds (Willard *et al.*, 1981).

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 you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed.

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).

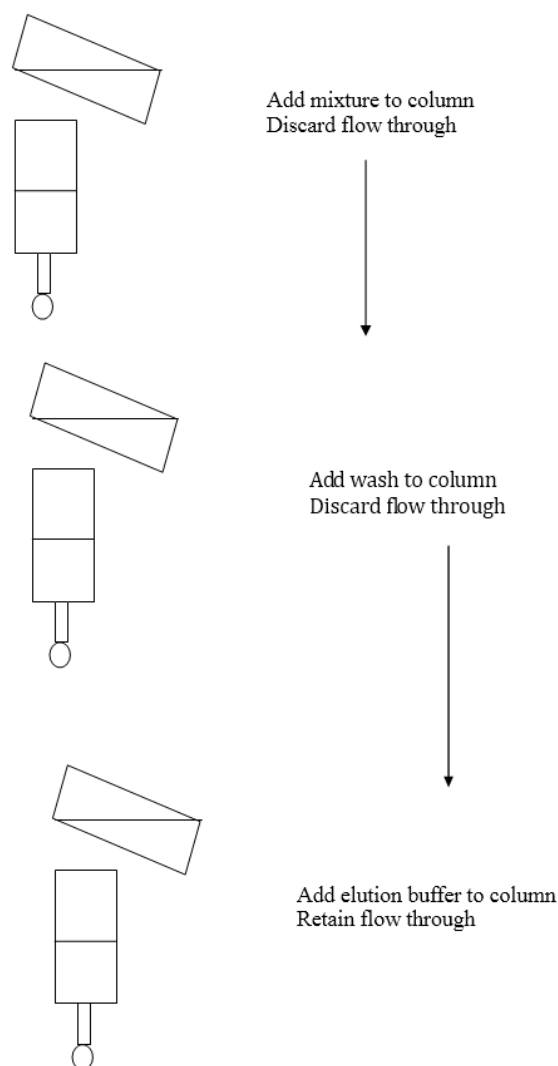


Figure 3: Immuno Affinity Column Chromatography (Nakajima *et al.*, 1997)

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).

1.4.2.2 Specific uses

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.

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.

For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well (Rendel., 1987).

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a 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).

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 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.

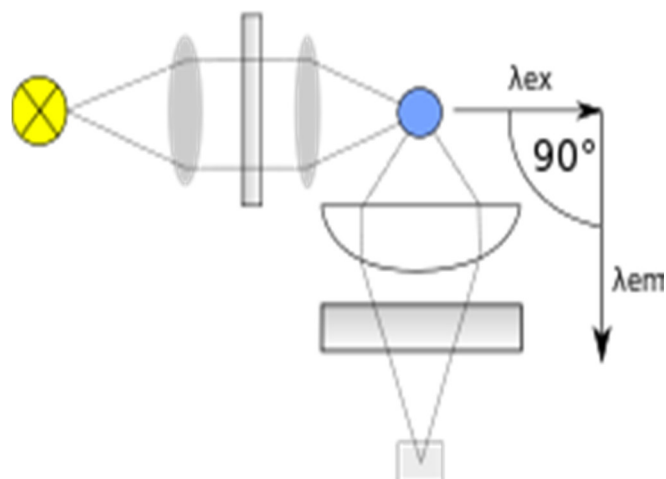


Figure 4. A simplistic design of the components of a fluorimeter (Sharma and Schulman 1999)

Various light sources may be used as excitation sources, including lasers, LED, 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.

Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance. The most common type of monochromator utilizes a diffraction grating, that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements the addition of two polarization filters are necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

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 through 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 and 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 NIR 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 wavenumber difference relative to the excitation wavenumber e.g. the peak appears at a wavenumber 3600 cm^{-1} lower than the excitation light in water.

Other aspects to consider are the inner filter effects. These include reabsorption. Reabsorption 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. Resultingly, 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 benign. Atomic Fluorescence Spectroscopy (AFS) techniques are useful in other kinds of analysis/measurement of a compound present in air or water, or other media, which is used for heavy metals detection, such as mercury. Fluorescence can also be used to redirect photons. Additionally, Fluorescence spectroscopy can be adapted to the microscopic level using microfluorimetry. In analytical chemistry, fluorescence detectors are used with HPLC (Holler *et al.*, 2006)

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, importer coffee 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. The research hopes to use the study of OTA in coffee produced in Kiambu County as 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 and 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 from Kiambu County, Kenya.

1.8.2 Specific objectives

- i. To determine levels of Ochratoxin-A in green coffee beans, roasted coffee beans and in brewed coffee.
- ii. To investigate the effect of coffee processing to OTA concentration.

2 MATERIALS AND METHODS

2.1 Study Area

The study area includes the entire Kiambu County (Figure 6). The county is in the Central Kenya region and bordering the Capital city Nairobi.

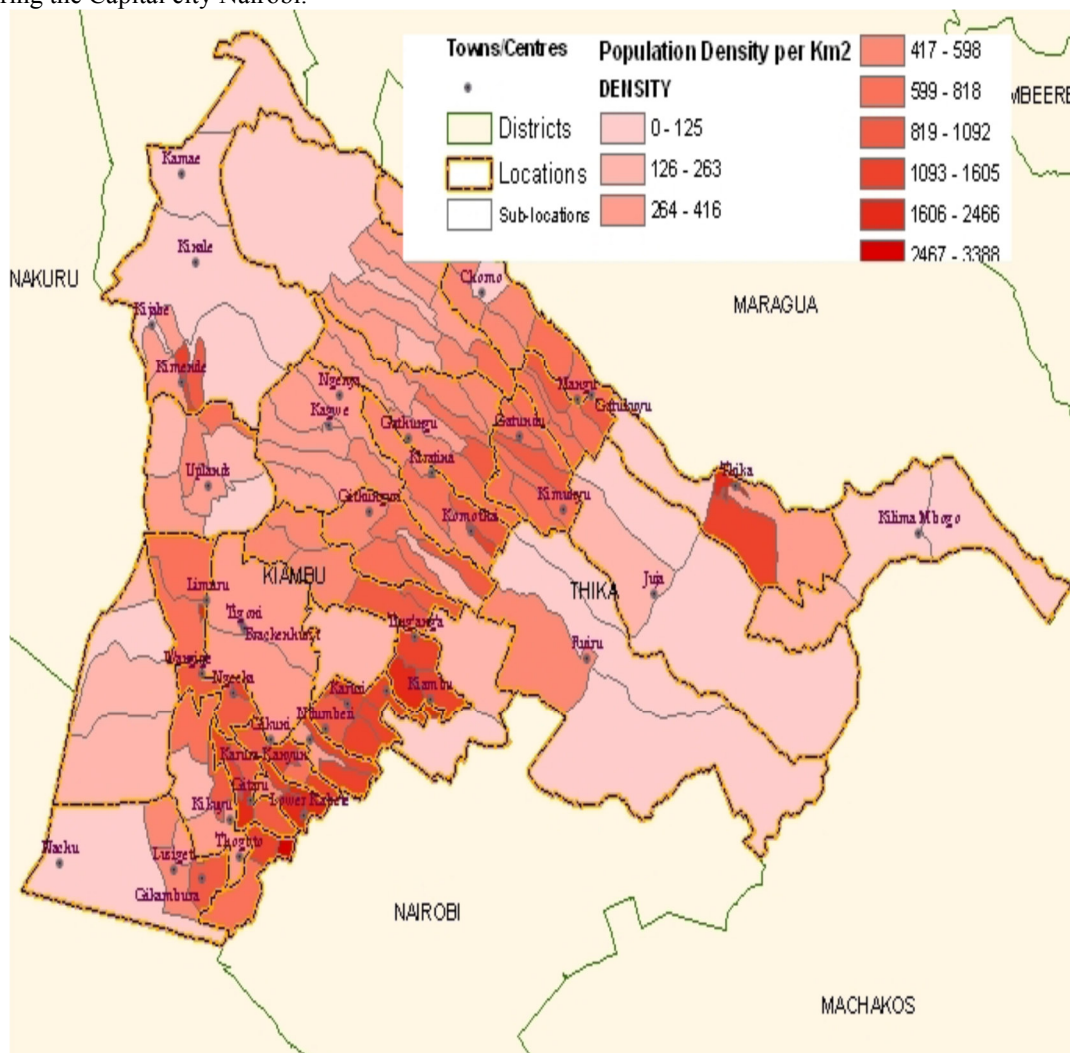


Figure 5: Map of Kiambu County (Source: Brand Kenya, 2011).

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 were 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.

Table2: 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

2.2.1 Research Design

The research used former constituencies as the principal sampling areas with cooperative societies as sampling points in a stratified sampling design.

2.3 Experimental

2.3.1 Reagents and Chemicals

The reagents and chemicals of high quantity grades 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, Pure water conductivity under point five six (0.56) MScm⁻¹ (Kaiji *et al*, 2006).

2.3.2 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.

2.4 Extraction

25 g of green and roasted coffee were weighed in a 250 ml glass flasks and extracted with 100ml of methanol and 100 ml of 3% aqueous sodium bicarbonate solution. The suspension was shaken for 30 minutes. The suspension was mixed on a blender for 5 minutes and filtered through 0.45 ml membrane paper .After the filtration 4ml 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.



Plate1: Extracted sample solutions of green and roasted coffee.

2.5 Thin-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. The R_F value for each spot was calculated. R_F stands for "ratio of fronts" and is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates. Hence, known R_F values of standards can be compared to those of (unknown) samples for identification purposes.

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 1 ml of mobile phase. The solutions were transferred to plastic centrifuge tubes (volume 1.5ml) and centrifuged at 5.00xg for 5 minutes to obtain a supernatant as sample solution which was subjected to liquid chromatography similarly to standard solutions i.e. by injecting 50 μ l to the HPLC. Four samples spiked with standard solution (between one and five μ g/Kg) were subjected to similar clean up to test the efficiency of the clean-up and recovery process.



Plate2: Immuno affinity columns

2.6.2 Measurement conditions

Each sample was analyzed thrice. The total concentration of Ochratoxin-A was calculated by considering the sample concentration and number of dilutions. The instrument was set under the condition of experiment at a retention time of 2.1 minutes as was expected. The fluorescent detector was set at excitation wavelength of 330 nm and fluorescent wavelength 460nm (Tsubouchi *et al.*, 1997).

Octa-decyl Silyl silica gel column 4.6mm in inner diameter, 250mm in length with particle size of 50 μ m was used. Eluent consisted of Acetonitrile/water/acetic acid (500:500:1) Alkalization solution consisted of Sodium hydroxide solution 0.1mol/lit. Eluent flow rate: 1.0 ml/min. Alkalization solution flow rate: 0.3ml/min. Column oven temperature was set at forty (40) degree Celsius. Standard curve was obtained from peak areas of resulting chromatograms by preparing a calibration curve, and plotting a graphs of peak area against Ochratoxin-A concentration for standard solutions (Ahmed *et al.*, 2007).

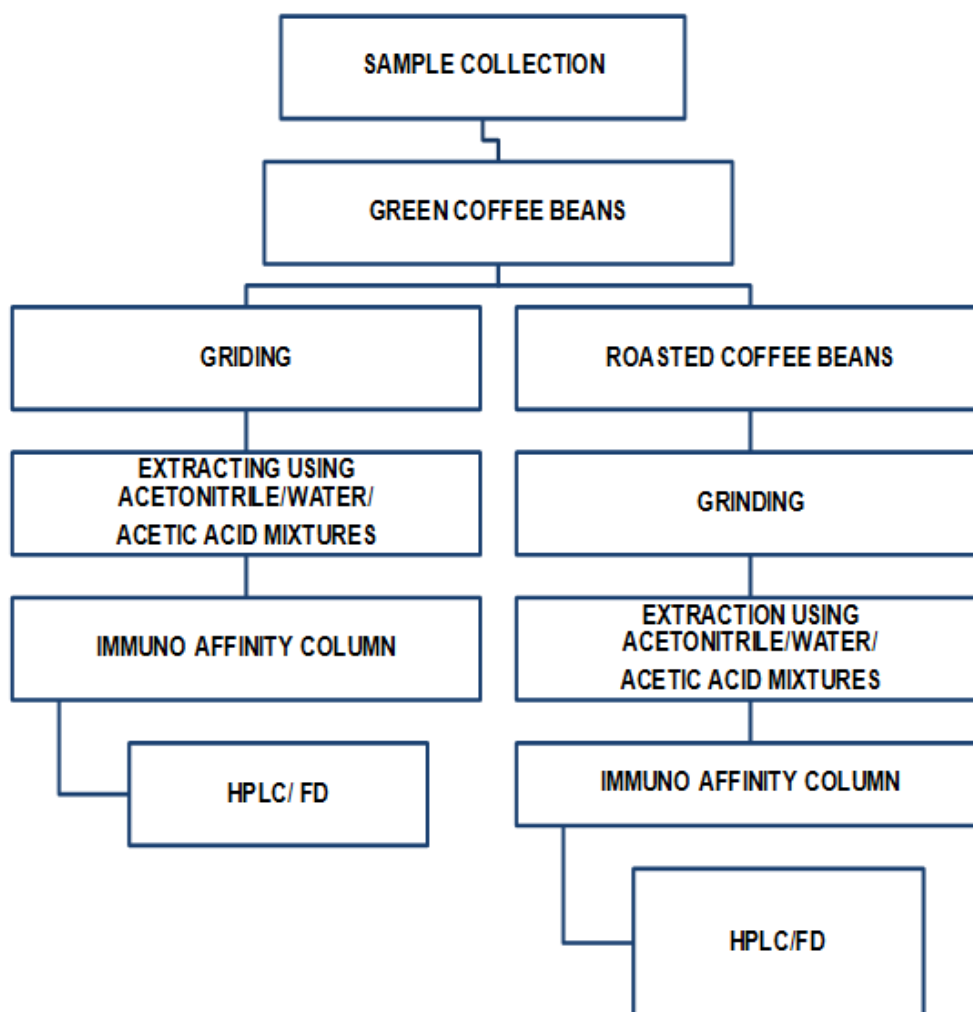


Figure 6: Flow diagram of sampling and analytical procedures.

2.6.3 Recovery and confirmation studies.

Recovery studies were carried out to one of the determined samples by spiking the sample with 1.5 to 50 ng/g of OTA and observing percent recovery after taking them through the normal experimental procedure. (Kawamura *et al.*, 1989). The test samples were soaked overnight into an equivalent weight of standard OTA solution at twenty degrees centigrade, with care being taken to ensure that all of the standard solution has been absorbed.

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. Whether, roasting causes significant reduction or increment of OTA was tested using Mann Whitney u-test because the distribution may not be normal.

2.6.4.1 Hypothesis test

Mann Whitney u-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.

t-test Formula:

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

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 $n - 1$.

2.6.4.2 Wilcoxon-Mann-Whitney U Test

This 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.

Steps

1. Choose the sample for which the ranks seem to be smaller (The only reason to do this is to make computation easier). Call this "sample 1," and call the other sample "sample 2."
2. Taking each observation in sample 1, count the number of observations in sample 2 that are smaller than it (count a half for any that are equal to it).
3. The total of these counts is U .

For larger samples, a formula can be used:

1. Arrange all the observations into a single ranked series. That is, rank all the observations without regard to which sample they are in.
2. Add up the ranks for the observations which came from sample 1. The sum of ranks in sample 2 follows by calculation, since the sum of all the ranks equals $\frac{N(N+1)}{2}$ where N is the total number of observations.

3. U is then given by:

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

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

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

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

3.0 RESULTS AND DISCUSSION.

3.1. Results of TLC analysis.

When standard OTA solutions were tested through TLC for RF values, the following values were obtained. Table 3 shows the calibration data, while figure 8 shows the chromatogram from which the data was obtained.

3.1.1 Calibration data.

Table3; Standard solutions RF values for TLC.

Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average RF	Standard Deviation
0.403	0.419	0.411	0.411	0.411	0.413	0.0067

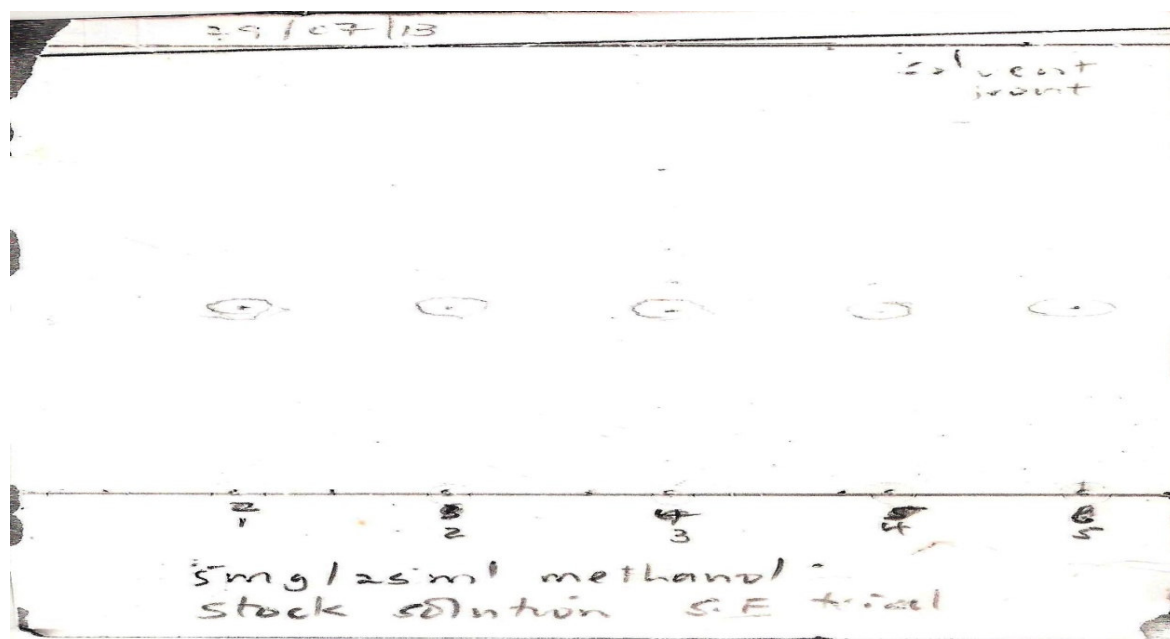


Figure 7: TLC chromatograms for OTA standards.

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 one hundred and fifty micrograms per Kilogram (150 ppbs) standard solution. 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 in gradients for example caffeine as indicated by attached chromatograms. The conclusion was to proceed with clean up by use of immuno affinity column and analysis by the more sensitive HPLC technique.

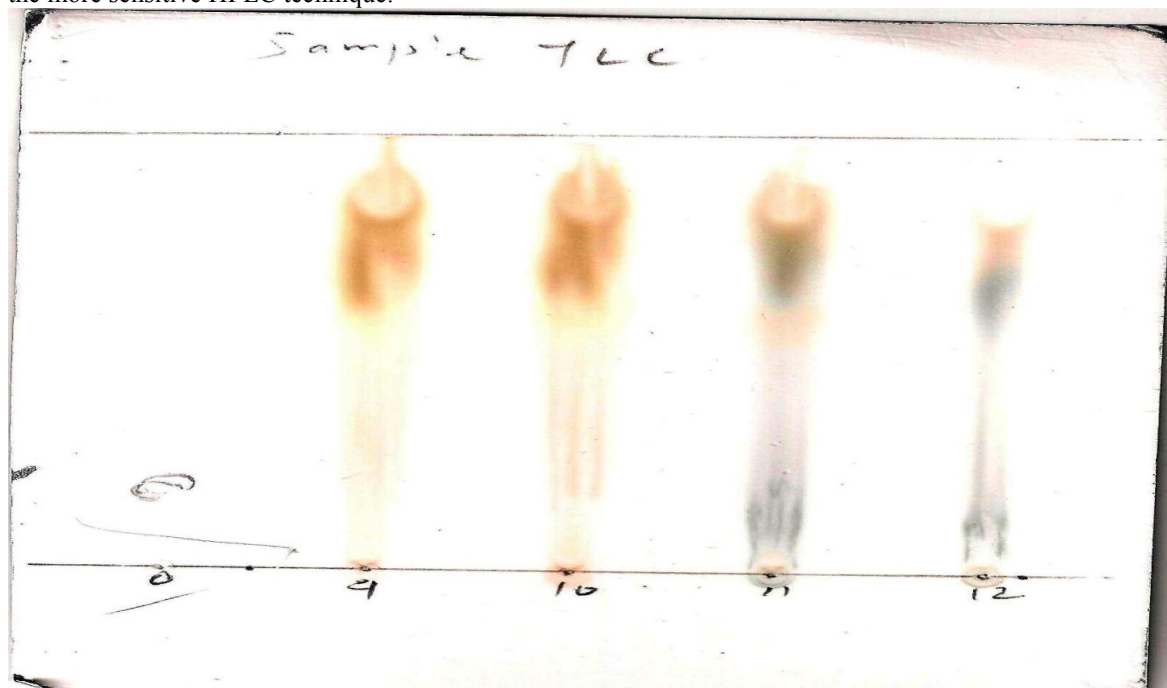


Figure 8: TLC chromatograms for sample solutions.

3.2. High performance liquid chromatography analysis.

HPLC is a more elaborate method than TLC. When HPLC coupled with fluorescence detector was used with standard OTA solutions, a limit of detection of about 2 µg/Kg was noted and a linear calibration curve at above 2 µg/Kg was obtained as shown by the following results. Figure 10 shows a sample of standard solution chromatogram.

3.2.1. Chromatograms of standard solutions of OTA

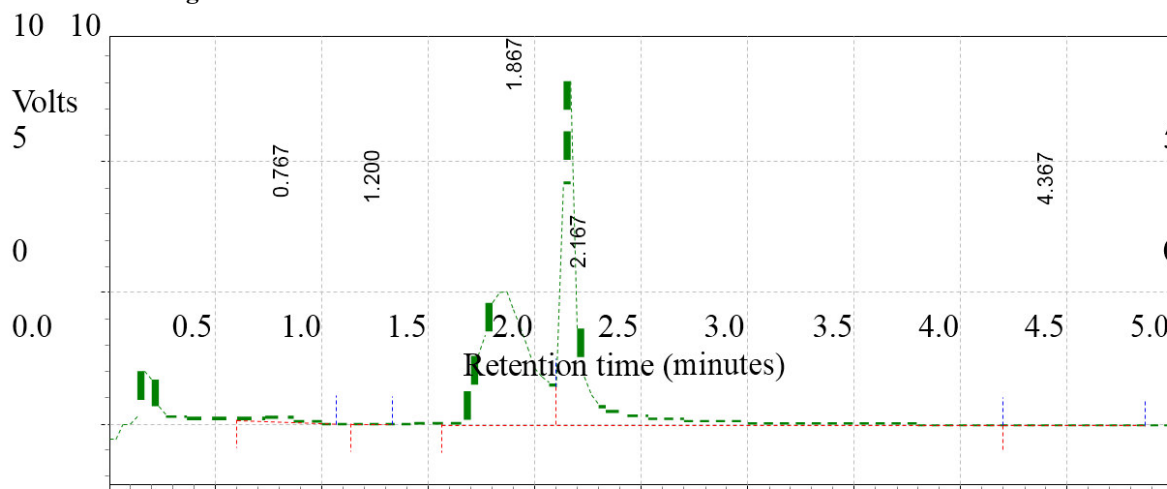


Figure 9: 1.25 µg/Kg standard solution chromatogram

Tables 4, 5, 6 and 7 shows the average retention times, peak areas and peak heights for the various standard solutions used in preparation of the calibration curve of standard solutions. The peaks for OTA standards

were detected at a retention time of between 2.1 and 2.2 minutes.

Table 4; Results from 1.25 µg/Kg standard solution chromatogram

RetentionTime	Area	Area%	Height	Height%
0.767	2790	1.75	266	1.43
1.200	310	0.19	53	0.29
1.867	79155	49.53	5065	27.31
2.167	76457	47.85	13121	70.75
4.367	1088	0.68	41	0.22
Totals	159800	100.00	18546	100.00

Table 5; Results from 3.125 µg/Kg standard solution chromatogram

RetentionTime	Area	Area%	Height	Height%
0.433	4892	2.94	849	3.05
1.033	4038	2.43	293	1.05
1.800	48274	29.00	3214	11.53
2.133	108392	65.12	23432	84.05
3.733	263	0.16	27	0.10
4.067	320	0.19	33	0.12
4.733	260	0.16	30	0.11
Totals	166439	100.00	27878	100.00

Table 6; Results from 6.25 µg/Kg standard solution chromatogram

RetentionTime	Area	Area%	Height	Height%
0.500	2952	1.24	419	1.11
1.067	1988	0.83	163	0.43
1.800	43926	18.43	2926	7.76
2.133	189484	79.50	34183	90.69
Totals	238350	100.00	37691	100.00

Table 7; Results 12.5 µg/Kg standard solution chromatogram

RetentionTime	Area	Area%	Height	Height%
0.633	5200	1.28	1086	1.10
1.200	1440	0.36	178	0.18
1.833	50983	12.60	3524	3.59
2.133	347057	85.76	93509	95.13
Totals	404680	100.00	98297	100.00

3.2.2. HPLC Calibration Data

Table 8 shows a summary of the peak areas that were used to preparation of the calibration data for the construction of calibration curve, While figure 10 is the calibration curve resulting from the data with the resulting regression equation.

Table 8; Standard solutions analysis results

CONC- µg/Kg	PEAK AREA
1.25	76457
3.125	108392
12.5	347057
25	604137

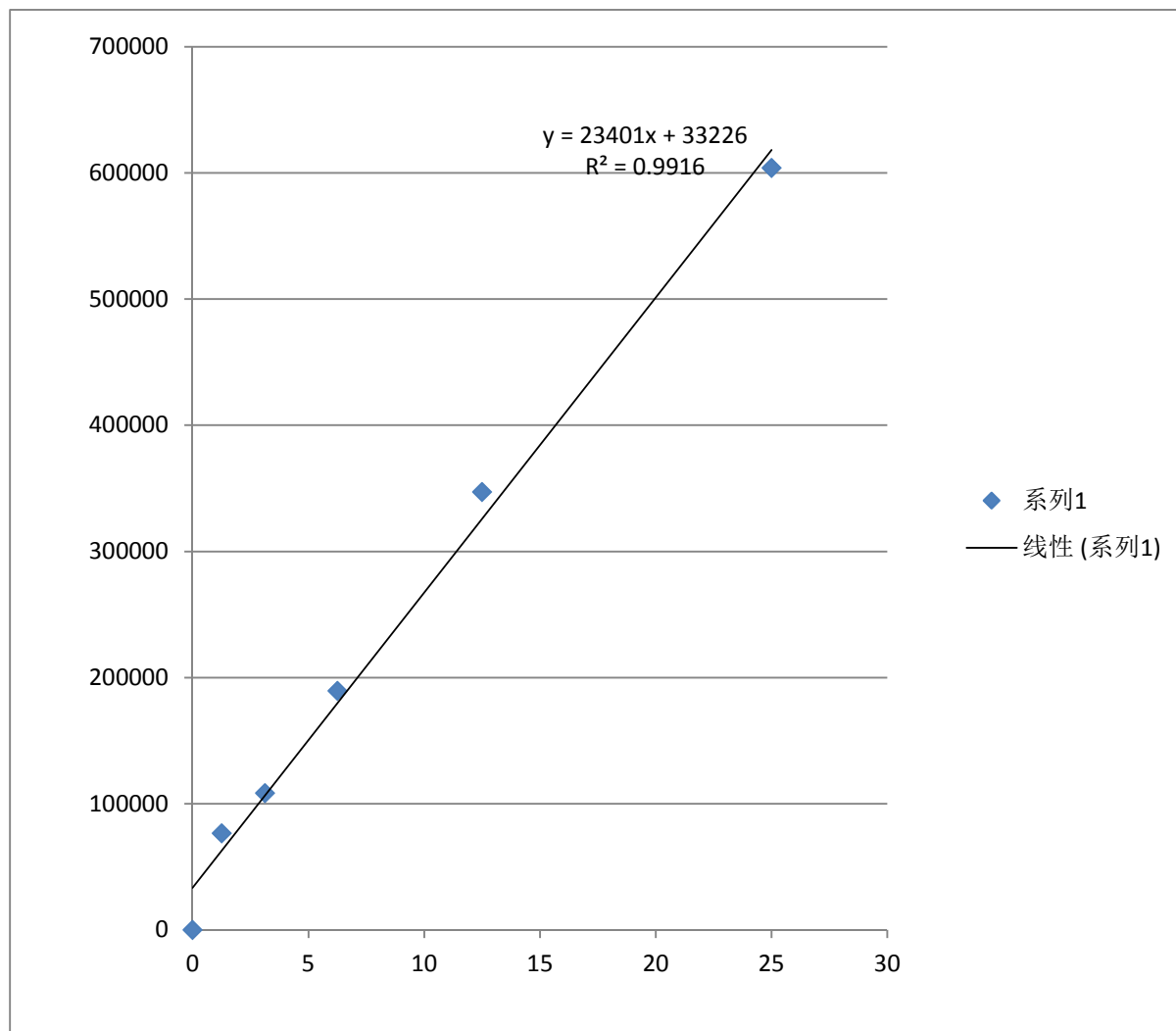


Figure 10: Calibration curve for HPLC analysis.

3.2.3. Calculations

Limit of detection (LOD) = 2.05ppbs.

The standard deviation of blank S = 0.036 ppb.

Limit of quantification (LOQ) = 2.05 + 10S

LOQ = 2.41 µg/Kg OTA.

3.3. Recovery studies

A blend of the samples earlier analysed was spiked with low concentrations of OTA standard solutions to assess the efficiency of the 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 9 and 10 shows the data on recovery studies while figure 11 shows a graph of comparison on percentage recovery between the two processes.

Table 9; Spiked raw samples

Sample Type	Peak Area	Expected Peak Area	Spike Conc µg/Kg	% Recovery
GREEN 1	85230	109279.25	3.25	77.99
GREEN 2	84791	109279.25	3.25	77.6
GREEN 3	86206	109279.25	3.25	78.9
GREEN 4	121072	150231	5	80.59
GREEN 5	121273	150231	5	80.72
GREEN 6	120163	150231	5	79.98
			AV	79.558
			STDEV	1.309588

Table10; Spiked roast coffee

SAMPLE TYPE	PEAK AREA	EXPECTED PEAK AREA	SPIKE CONC PPB	% RECOVERY
ROAST 1	56110	109279.25	3.25	51.34
ROAST 2	50877	109279.25	3.25	46.57
ROAST 3	57220	109279.25	3.25	52.84
ROAST 4	78032	150231	5	51.94
ROAST 5	77704	150231	5	51.72
ROAST 6	77801	150231	5	51.78
			AV	50.6175
			STDEV	2.77244

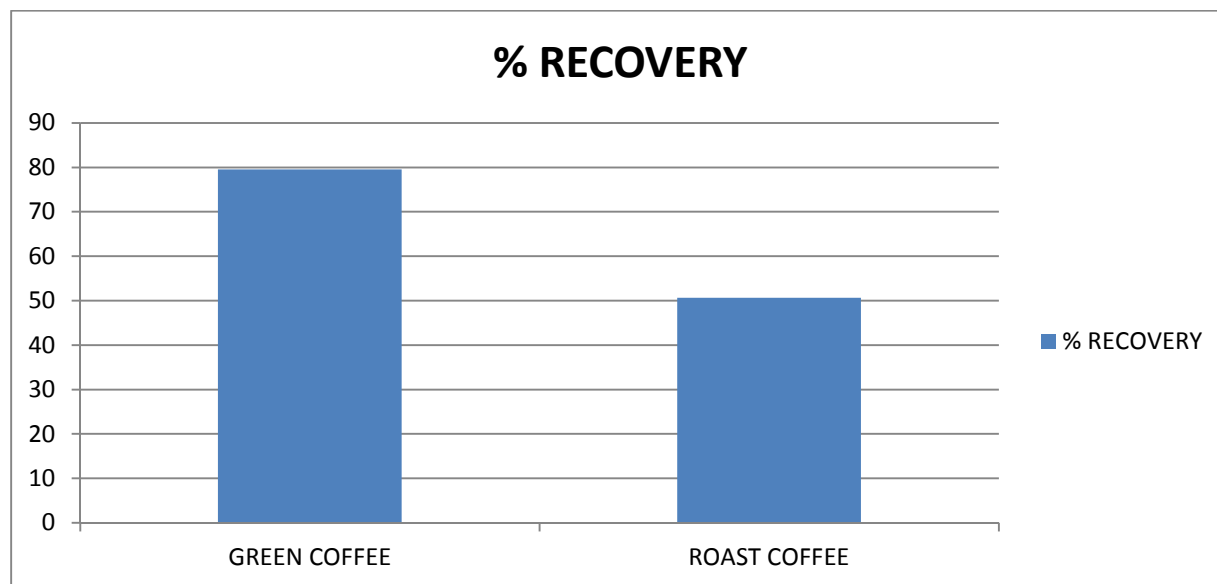


Figure11: Chart comparing percent recoveries of OTA in green and roast coffee

3.3. .1. Comparing percent recoveries of OTA in green and roast coffee

Percentage recoveries of OTA in green and roast 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 11. 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 12. Both test indicate strongly there is significant difference in levels of OTA recoveries in coffee beans that have undergone the varied processing procedures.

Table11; t-test

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 i.e There is significant difference between OTA in roasted and green coffee.

Table12; u-test

CATEGORY	R1	R1	R1	R1	R1	R1	R2	R2	R2	R2	R2	R2
%RECOVER	77.9	77.	78.	80.5	80.7	79.9	51.3	46.5	52.8	51.9	51.7	51.7
Y	9	6	9	9	2	8	4	7	4	4	2	8
RANK	8	7	9	11	12	10	2	1	5	6	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 i.e There is significant difference in OTA between roasted coffee and green coffee.

3.4. Sample analysis

On establishing that HPLC-FD could be relied upon to detect low OTA levels. Green and roasted coffee samples were subjected to OTA analysis after a clean-up process to remove interfering matrix. The analysis of grades AA and AB samples gave negative OTA presence as shown in table 13.

3.4.1. Unspiked samples

Table 13; Sample analysis grade AA and AB

SAMPLE	GREEN PEAK AREA	ROASTPEAK AREA	OTA CONC
YADINI EST	BDL	BDL	BDL
OAKLAND EST	BDL	BDL	BDL
MAHUTI EST	BDL	BDL	BDL
BARIKONGO EST	BDL	BDL	BDL
GITARE EST	BDL	BDL	BDL
GATHUMBUINI EST	BDL	BDL	BDL
HANDEGE EST	BDL	BDL	BDL
MITARO EST	BDL	BDL	BDL
MACHURE EST	BDL	BDL	BDL
KOMOTHAI FCS	BDL	BDL	BDL
GATHAGE FCS	BDL	BDL	BDL
GATITU FCS	BDL	BDL	BDL
THIRIRIKA FCS	BDL	BDL	BDL
NEW GATUKUYU FCS	BDL	BDL	BDL
MIRIMAINI FCS	BDL	BDL	BDL
NDUMBERI FCS	BDL	BDL	BDL
KIAMBI FCS	BDL	BDL	BDL
ICHAWERI FCS	BDL	BDL	BDL

3.4.2. Control sample analysis

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

The unroasted control sample indicated a signal in the OTA retention time, but the peak area whose average is 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 which is way below the limit of quantification. Table 14 shows the results of analysis of green *mbuni* samples

Roasting of the control sample resulted in loss of signal detection in the region above calibration curves y intercept. Table 15 shows the results of roast *mbuni* analysis

Table 14; Green *Mbuni* control sample analysis-Gachororo

	Peak Area	2x Peak Area	Experimental conc. µg/Kg	Theoretical conc. µg/Kg
AV	34743.335	69486.67	1.547	2.027
STDEV	41.00405	82.0081	0.00577	0.00750

Average concentration of 1.5±0.006

Table15; Roast *mbuni* control sample analysis-Gachororo

	Peak Area	2x Peak Area	Calculated µg/Kg	Equivalent Conc µg/Kg
AVERAGE	14376	28752	BELOW LOD	BELOW LOD

4. Conclusions

4.0 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 were 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 ppb. 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 coloured 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 $79.56\% \pm 1.31\%$ for green coffee beans and $50.62\% \pm 2.77\%$ for roasted coffee. The observations can be summarized as below:

1. Normal processed coffee of grade AA and AB does not contain appreciable levels of OTA.
2. Mbuni (Dry berry rejects) contain appreciable levels of OTA but below the recommended limit of 5ppb.
3. 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
4. Coffee roasting causes appreciable decline in OTA levels of between 20% to 30% , this is observed by the difference in recovery levels of green and roast coffee whose averages are 79.56% and 50.56% respectively.

4.2 Recommendations

4.2.1 Recommendations arising from this study

The study in general observes that levels of OTA in ordinary level export grade coffee from Kiambu county is within acceptable levels of all important importing countries that have set their own standards. The above observations indicates 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 goes 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 non 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 margin levels of OTA.

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 non the less can't conclusively give a good account of OTA levels in Kenya and similar studies in other major producers 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 methods of containing their spread would a valuable course by biologists.

5 References

- Ahmed, N.C., Farag, M.M., Saliman, K.M., Samed, A. K. M. and Nagulb, D.K.M., (2007). Evaluation of methods used to determine Ochratoxin-A in coffee beans. *Journal of Agriculture Food Chemistry*. 55 (23): 9576 – 9580.
- Albassam, M.A., Yong, S.I., Bharnagar, R., Sharma, A.K. and Prior, M.G., (1987). Histo pathological and electron microscopic studies on acute toxicity of Ochratoxin A in rats. IARC Scientific Publication, 427– 435.
- Anon. (1997), *Standard Contracts European Coffee Federation*, Brussels.
- Bennett, J.W. and Bentley, R. (1990). Pride and prejudice, *Perspective Biological medicine*, 42 : 333 - 355.
- Brand Kenya. (2011) "Kiambu-county". Available at <http://www.guide2kenya.com>. Accessed on (19/8/2011)

- Bucheli, P., Pittet., Meyer and Viani, R. (1998). Industrial storage of green roast coffee under tropical conditions and impact on Ochratoxin-A. *Journal of Agriculture and Food Chemistry* 46: 4507 - 4511.
- Davis D. J.W Searcy and U.L Diener, (1969) production of Ochratoxin A by aspergillus Ochra cells in a semi synthetic medium *App. Micro.*, 17:2-744.
- Debjani Saha, debopam Acharyal, Dipital Roy, Dilip Shrestha, Tarun K. (2007). Simultaneous enzyme immune essay for screening of aflotoxin B1 & Ochratoxin A in chilli samples. *Analytical chemical acta* 584 pp 343- 349.
- European commission, commission regularitia- (E.C) No. 472/2002. (2001). Official Journal of European Communities. L75, 18 - 20. Available at <http://www.Coffee Board of Kenya.org> (Retrieved on 10th August 2011).
- Fijamer Holler, Douglas A. Skoog & Stanley, R. Crouch. (2006) Principles of instrumental analysis 6th Edition, Brooks Cole publishers 700-750
- Forja, S., Dallasta and Marchelli C. E. (2006). Recent advances in mycotoxin determination in food and feed by hyphenated chromatographic technique 26: 54 – 70.
- Hamide Z. Senyuva, and John Gilbert. (2010). Immuno affinity column- clean-up techniques in food analysis. *Journal of chromatography B*. 978 pp 115- 132.
- Hanak, E., Boutrif, E., Fabre, and P., Pineiroi .M. (2002). Food Safety Management. European Commission Scientific edition. 11 – 13.
- Hassala, K., (1990). The biochemistry and uses of pesticides, 2nd edition, Macmillan publishers pp27 – 29
- Irina Yu. Goryachella, Sarah SE Saoger Barbara Delmulle Mariene Labeau, Sergei A Eremin, Indiko Barnavetro, and Carlos Van Petrghen. (2007). Simultaneous non-instrumental detection of aflatoxin B1 and Ochratoxin A using clean up tandem Immunoassay Column. *Analytical Chemica. Acta* 590 pp 118-124.
- JECFA,(2001). “Provisional weekly intake of OTA (PTWI)”. Available from <http://www.inchem.org/documents>. (Retrieved on August 10.2011).
- Kaiji Aoyama., Kaor., Marifuj.I. and Elichi Ishiguro. (2004) *Research Report of Animal Feed* , 5: 180 – 240.
- Kaiji, Aoyama., Elichi Ishiguro., Naoki., Naoki Kanemaru and Masatoshi Minamisawa; (2006). Research Report of Animal Feed *Journal of Agricultural Research* 5: 180 – 240.
- Kathurima, C. W. (2004). Assessment of Fungal Species Distribution Within the Kenyan Coffee industry. Masters Thesis. Jomo Kenyatta University of Agriculture and technology, Nairobi, Kenya.
- Kawamura, O., Sato S., Kajii, H., Nagayama, S., Ohtani, K., Chiba J. and Ueno Y. (1991). Use of antibodies, enzyme-linked immunosorbent assay and immune affinity column chromatography to determine Ochratoxin-A. *Antibodies Toxicon*, 27: 887 – 897.
- Kenya Coffee Traders Association Directory 2012
- Kiptoo, J. (2001). Assessment of the levels of some toxic and anti-nutritional substances in Kenyan coffee pulp. Masters Thesis. Kenyatta University, Nairobi, Kenya.
- Lakowicz, J. R. (1999). *Principles of Flourescence Spectroscopy*, Kluwer Academic and Plenum Publishers (1) 15-25.
- Latlanjio, V. M. Solfrijjo, S. Powers A. Visonts, (2007). Simultaneous determination of Ochratoxin A and fusarium toxins in maize by liquid chromatography/tandom mass spectrometry after multitoxin immune affinity clean up. *Rapid commun. Mass spectrometry* 21 pp 3253-3261.
- Lyon. (1993), I.A.R.C monographs of the evaluation of carcinogenic risks to human by world health organization; 56.
- Mislevic, B.P. R.V, Bruce and R. Gibson. (1983). Incidence of toxigenic and other mould in green coffee beans. *Journal of food production*, 46: 969-973.
- Moss, M. O. (1996). Mode of formation of Ochratoxin A. *Food Additives and Contaminants Journal*, 13: 5 – 9.
- Nakojima, M. H., Tsubouchi, M., Miyabe and Ueno, Y. (1997). Survey of Aflatoxin-B and Ochratoxins by liquid chromatography. *Food and Agriculture, Immuno assay* 9: 77 – 83.
- Nehad, E.A., Farog M.M., Kauther, M.S., Abdel, A.K.M and Khayria Nagulb. (2007). An exposure and intake assessment of Ochratoxin-A from imported coffee beans in Egypt. *World Journal of Agricultural sciences* ISSN 18A – 30LA: 285 – 294.
- O Flitenborg. J.C Frisvald U. Thrane, Moulds in food storage international *Journal of food microbiology* 33 91996) 85-102.
- Pitt, J. I. and Hocking, A.D. (1985). Methods for Isolation, enumeration and spoilage identification in Fungi and foods Academic Pres, Sydney Ap. pp 29-62
- Pittet A., Tornare., Hugget and Viani, R. (1996). Liquid chromatography using an immuno affinity column clean up procedure. *Journal of Agriculture and Food Chemistry*, 44: 3564 – 3569.
- Raquel, Drartedacc. and Bondeira, (2008). This Matsue Uekare *Carolina Passos da Cunha, Debora Andrade Costa*; Institute of Nation de Matologia .
- Rendel, D and Mowthorpe D, (1987). *Flourescence & Phosphorescence Chromatography*. 1st edition ACOL

- publishers 50-100.
- Sharma A. and Schulman S.G (1999) Introduction to fluorescence Spectroscopy Willey Interscience.
- Singer, E. J and Flores, J (1979). Front face fluorimetry of liquid samples. *Analytical biochemistry* 94 (1) 15-21
- Stegen G., Van G. der M., Jorissen A., Pittet, M., Saccon, W., Stenner. and Later, C. (1997). Screening of European Coffee final product (OTA) food additives and contaminants. *Journal of Agriculture and Food Chemistry* 14: 211 – 216.
- Sugita – Konish; Y, Tanaka. T, Nakijima, M., Fujita, H., Noriyuku, N. Mochisuki, and Takatori, K.(2006). The Comparison of two clean up procedures, multi-functional column for HPLC determination of Ochratoxin A in cereals, raisins and green coffee beans. *Talanta* 69 pp 650 – 655.
- Uhlén, M (2008). Affinity as a tool in life science. *Biotechniques* 44 (5): 649–54.
- Valenta, H. (1998) Chromatographic Methods for determination of Ochratoxin A in animal and human tissues and fluid. *Journal of chromatography A* 815 pp 75-92
- Vasanthi, S. and R.V, Baht, (2000). Ochratoxin in Indian coffee and its implications to International Trade, *Journal of Nutrition* 21:718-721
- Viscount, A; Pasale. G. Centonje. (1999). Determination of Ochratoxin A in wine by means of immune affinity column. Clean up and high performance liquid chromatography. *Journal of Chromatography. A*, 864 (1999) 89 – 101.
- Vizthum, O. G. (1998). *Thirty years of Coffee Chemistry Research*. Annual meeting of the ACS, Boston, MA, U.S.A.
- Waller, A. R and Duncan, B.D. (1987). A Bayer for the Symmetrimultiple comparison problem. *Journal of American Statistics Society*. 64: 1484 – 1503.
- World Health Organization. (1990). International programme of chemical safety; selected mycotoxins, ochratoxins, tricothecenes, ergot Environmental health criteria: Article 105 Geneva. Available at <http://www.inchem.org/documents>(retrieved on August 2011).